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01. Animal models for biomedical research

0100

Effectiveness of exogenous surfactant treatment to preterm newborn lambs

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The acute respiratory distress syndrome is the main cause of high mortality in preterm infants. Treatment with exogenous surfactant may prevent alveolar collapse and decrease alveolar surface tension. However, research has shown no significant improvement in mortality with exogenous surfactant treatment. Thus, the aim of this study was to compare the effectiveness of three different protocols of surfactant administration to premature lambs. The trial was constituted of 22 preterm (135 days of gestation) lambs born vaginally after induced labor. Immediately after birth, newborns were intubated and ventilated with a self-reinflating bag for 2 h, during which lambs were randomly allocated into four groups according to the surfactant replacement protocol: control (C, n = 5), single (S, n = 5), double (D; n = 6) or triple (T; n = 6) endotracheal administration of 100 mg/kg porcine surfactant (Butantan Institute®). In groups D and T, the total surfactant volume was divided in equal doses of instillation in a 20 or 30 min-interval, respectively. Regarding survival rate, only two C-lambs and one D-lamb survived throughout the experimental period. In addition, the C group presented a significant longer lifetime. In respect to clinical outcome, C-lambs showed more satisfactory lung auscultation, determined through respiratory rate and respiration effort score, and higher Apgar scores (8 ± 0.2) than S (5 ± 0.2), D (7 ± 0.1) and T (6 ± 0.1) groups. Moreover, S-lambs had statistically higher blood lactate levels than C-group after 2 and 4 h of life (S: 12.8 ± 4.1 mM; 18.7 ± 0 mM and C: 5.8 ± 2.3 mM; 6.1 ± 1.4 mM, respectively). Concerning the hemogasometric parameters, S-group presented acidemia (7.09 ± 0.07) and lower blood pH than C-group (7.28 ± 0.02). All lambs developed hypoxemia, which was more severe in S-group. However, pulse oximetry was within physiological range for all groups, but statistically lower for S-group (71 ± 1.6%). Furthermore, all neonates presented hypercapnia and low bicarbonate levels. A negative correlation between blood lactate and Apgar score ($r = -0.639$; $p < 0.0001$), pulse oximetry ($r = -0.345$; $p < 0.008$), blood pH ($r = -0.509$; $p < 0.0001$) and lifetime ($r = -0.558$; $p < 0.013$) was verified, while a positive correlation was determined among lung auscultation and blood pH ($r = 0.344$; $p < 0.003$), as well as for Apgar score and arterial partial oxygen pressure ($r = 0.455$; $p < 0.0001$). In conclusion, exogenous surfactant therapy did not improve the survival of premature lambs, regardless of the treatment regime applied. We believe that the commercial surfactant utilized here contributed to pulmonary edema leading to severe neonatal respiratory failure. The lack of surfactant absorption impaired the physiologic decrease in local surface tension. However, important neonatal knowledge was achieved, which will support further studies on premature newborn assistance.

Key Words: Preterm lamb, surfactant, lung, respiratory function, neonatal assistance

0101

Synthesis of testosterone and sulfonated steroids in the porcine testicular-epididymal compartment: need for concept revision?

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Sulfonated steroids (sSt) are traditionally regarded as inactive metabolites but have recently gained increasing interest as a potential pool of precursors which may enter the synthesis of active free steroids (fSt) by the activity of steroid sulfatase (StS). Due to its remarkably high production of sulfonated estrone (E1S) and dehydroepiandrosterone (DHEAS), the porcine testicular-epididymal compartment (pTEC) may be a useful model to gain further insight in the metabolism, transport and function of sSt. Expression of relevant genes was measured by real-time RT-PCR in five sexually mature boars at the following localizations: testis (TE), epididymal head (proximal and distal segment, EH1-2), epididymal body (four segments, EB1-4), epididymal tail (ET1-2) and deferent duct (DD). Expression of the steroidogenic enzymes CYP11A1, CYP17, CYP19 and HSD17B3 was virtually restricted to TE. Expression of HSD3B was only low in TE (median: 11.9 relative units) and EH1, increased gradually from EH2 to ET1 and was highest in ET2 (median: 244 R.U.). Substantial StS expression was detected from TE to DD with the exception of EB1-4. A similar expression pattern was found for the sSt transporter candidate SLC10A6. The sulfotransferases (SULTs) 1A1 (phenol preferring) and 1E1 (estrogen preferring) were predominantly expressed in EH1-2 and DD, whereas only minimal expression was detected in TE. Different from SULT2A1 (DHEA preferring), which was highly expressed in TE, the cholesterol and pregnenolone preferring SULT2B1 was predominantly localized in ET2 and DD. Expression patterns of StS and of the SULTs 1E1, 2A1 and 2B1 were basically confirmed on the protein level by immunohistochemistry. Despite low RGE values for HSD3B in TE, testosterone (T) concentrations in testicular superficial veins (tSV) as measured by liquid chromatography-tandem mass spectrometry were 20 to 30-fold higher compared to concentrations in testicular artery and systemic circulation confirming a significant T output from TE. Although gene expression data suggest that the epididymis is the major site of HSD3B expression in the pTEC and thus may provide 3-keto-4-ene precursors for T synthesis in Leydig cells, corresponding measurements showed a substantial efflux of androstenedione in tSV indicating testicular origin. Despite the virtual absence of SULT1E1 in TE, also a high efflux of E1S was measured in tSV challenging the role of this SULT as the major enzyme catalyzing the sulfonation of testicular estrogens in boars. In conclusion, the results suggest that in boars the epididymis and DD exhibit a significant steroid metabolic activity and challenge the current concept of the testis as the sole organ encompassing the whole machinery for T and E1S synthesis in boars.

Key Words: Boar, testis, epididymis, sulfatase pathway, sulfotransferase

0102

Expression pattern of transgene in cloned and recloned transgenic dogs

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The nature of having identical genomic background makes cloned animals extremely valuable for discerning genetic predisposition from environmental causes or vice versa for a trait or phenotype that an

animal develops. Limitation, however, has been imposed to the use of the cloned dogs due to the difficulty of reproducing the clones. Reproduction and expanding clonal size will waste time which could be overcome only by recloning the cloned animals. Here, we evaluated if the recloning technique can produce absolutely identical transgenic recloned dog with transgenic original one and if it can affect the expression status of transgene in recloned dog. Two cloned dogs were used, a transgenic cloned dog 'Ruppy1' (Hong et al., 2009 *Genesis*) that has a red fluorescent protein (RFP) gene insertion and 'Magic' (2011, Oh et al., *Theriogenology*) recloned using Ruppy1' adipose derived stem cell. To investigate transgene insertion copy number, Ruppy1 and Magic were screened by Southern blot analysis using DNA extracted from skin biopsies. The result showed that both cloned dogs carried single copy of the RFP gene. Although an obvious fluorescence was observed in the whole body of Ruppy1 and Magic at birth, we performed Western blot analysis to quantify transgene protein levels using tail tissues of both transgenic dogs. The relative quantitation of RFP/β-actin was determined by imageJ software and found no significant difference in the relative RFP expression between Ruppy1 and Magic. In the last experiment, DNAWalking Speed Up™ Premix Kit was used for determining the site of integration of the transgene. Transgene specific primers were used; TSP 1, TSP2 and TSP3. The products of third round walking PCR were sequenced and analyzed by homology search using BLASTn for site of integration. In agreement with our previous results, DNAWalking analysis revealed that the location of the transgene in Ruppy1 and Magic was in the same region of chromosome 25. In conclusion, recloning technique can provide a novel way of reproducing animal clones with the use of donor cell for unlimited supply of identical nuclei without time wasting. This study was Supported by MKE (#10033839-2011-13), IPET (#311011-05-1-SB010/#311062-04-1-SB010), Research institute for veterinary science and TS Corporation.

Key Words: Transgenic dog, recloning, adipose-derived mesenchymal stem cells, Ruppy1, magic

0103

Evaluation of placental vascularization using 3D Power Doppler angiography and stereology in a rabbit model of IUGR

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Inadequate remodeling of the uterine vascular network inducing decreased blood flow to the placenta plays a role in the pathophysiology of human preeclampsia and intrauterine growth restriction (IUGR). 3D Power Doppler angiography (3D PDA) is a non-invasive, safe way to study blood flow within an organ. The objective of this study was to evaluate 3D PDA and actual placental vascular development in an experimental rabbit model of IUGR. Placental restriction and IUGR was induced in 14 pregnant rabbits by a pharmacological treatment with 31.5–250 mg/kg/day L-NAME, a nitric oxide synthase (NOS) inhibitor, from day 24 to day 28 of gestation. Eight pregnant rabbits were used as controls. On day 28, 3D power Doppler indices were quantified in each uteroplacental unit under general anesthesia, using a Voluson E8 (GE Medical Systems®) ultrasound machine with a transabdominal multifrequency probe (RNA 5–9 MHz) enabling automatic 3D volume and power Doppler acquisitions. A total of 180 live fetuses were obtained, 108 from the L-NAME group and 72 from the control group. G28 fetal weight was significantly lower in the L-NAME groups (all treatment regimens) vs. controls (27.40 ± 0.55 g vs. 33.14 ± 0.62 g, $p < 0.0001$). In the L-NAME groups, the vascularization index (VI), flow index (FI) and vascularization flow index (VFI) were significantly lower than in controls [2.6 (1.4; 6.0) vs. 7.6 (3.5; 12.6), $p < 0.05$; 28.7 (26.5; 31.3) vs. 32.9 (28.3; 38.1), $p < 0.05$; 0.8 (0.4; 1.8) vs. 2.5 (1.1; 4.1), $p < 0.05$, for VI, FI and VFI, respectively]. The number of fetuses considered as small for gestational age (SGA; weight < 10th centile) was significantly higher in the L-NAME groups than in the control group (47/108 vs. 7/72, $p < 0.0001$). Stereological analyses showed that the volumic fraction of fetal capillaries was significantly reduced and that of the maternal vascular spaces increased in the L NAME groups (0.228 ± 0.063% vs. 0.296 ± 0.068%, $p < 0.05$ and 0.389 ± 0.144% vs. 0.286 ± 0.106%, $p < 0.05$, for fetal maternal fraction, respectively), whereas the percentage of trophoblastic, junctional and labyrinthic zones were not different between the two groups. These results demonstrate for the first time *in vivo* the sensitivity of 3D PDA in detecting placental hypo-perfusion, as demonstrated by stereology, in an animal model of vascular IUGR. This technique is currently evaluated for the early detection of placental pathologies in humans.

Key Words: Placenta, 3D Doppler angiography, IUGR, rabbit, stereology

0104

The intake of green tea does not interfere in the number of corpora lutea in superovulated rats

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The green tea originated from *Camellia sinensis* plant is considered a healthy drink, because it has been associated with prevention of cancer, cardiovascular diseases and osteoporosis, and its catechins possess antioxidative, antibacterial and antitumoral activities. Despite many potential benefits of green tea consumption, the catechins present in green tea can significantly inhibit proliferation, steroidogenesis and VEGF production by swine granulosa cells. It is therefore important to get additional insights on the possible reproductive-related consequences. Thus, the aim of this work was to verify the green tea influence on the ovulation rate and ovary weight in superovulated rats. Wistar rats were divided in two groups: the control group (n = 30) drinking water *ad libitum*; and the treated group (n = 30) drinking only green tea at 2.5% *ad libitum*. The study was approved by the ethics committee of the university. The experiment lasted for three months, and in the end of each month, 10 animals of each group were superovulated with 150 UI/kg of eCG (Folligon®; Intervet Schering-Plough) and 150 UI/kg of hCG (Vetecor®; Hertape Calier) and then sacrificed. Ovaries were collected, weighted and corpora lutea were counted. Data were analyzed using the unpaired *t*-test with Welch's correction and the effect of time was assessed by analysis of variance. Statistical differences were considered when $p \leq 0.05$. No differences were observed between groups in the different times. Mean values for ovaries weight and corpora lutea number were, respectively: first month (control = 0.30 ± 0.10, 61.10 ± 10.93 and treated = 0.35 ± 0.09, 67.80 ± 23.32); second month (control = 0.18 ± 0.05, 64.90 ± 27.77 and treated = 0.20 ± 0.06, 57.20 ± 25.41); and third month (control = 0.21 ± 0.04, 62.30 ± 20.17 and treated = 0.22 ± 0.06, 55.20 ± 26.13). The consumption of green tea for three months did not interfere with the ovulation and the weight of the ovaries in superovulated rats. Financial support was provided by FAPESP 2010/20583-2.

Key Words: Ovulation, corpus luteum, reproduction, green tea, ovary

0150

Production of transgenic pig harboring an endothelial cell-specific human CD73 expression cassette by nuclear transfer

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Nucleotide metabolism in the endothelium varies between different species. Recent studies demonstrated that this variability could contribute to tissue rejection following pig-to-nonhuman primate transplantation, even when organs from the alpha 1,3-galactosyltransferase gene knockout pigs were transplanted. Ecto-5'-nucelotidase (CD73) is an enzyme that catalyzes the hydrolysis of adenosine triphosphate to adenosine, which plays a role on anti-platelet aggregation. CD73 activity in pig endothelial cells is several-fold lower than in human endothelial cells. In this study, we report the generation of transgenic pigs harboring a human CD73 expression cassette. A pig endothelial cell specific promoter (Icam2) was used to prepare the human CD73 expression cassette. The pIcam2-hCD73 cassette was then nucleofected into porcine fibroblasts and aortic endothelial cells. Western blot and flow cytometric analyses showed human CD73 gene expression in endothelial cells. For the generation of nuclear donor cells, the pIcam2-hCD73 cassette was nucleofected into passage 3 fibroblasts and cell selection was performed using 400 μg/ml of hygromycin. Forty-three cell colonies harboring the pIcam2-hCD73 cassette were obtained as confirmed by PCR analysis. Nuclear transfer was performed using cells of eighteen colonies and *in vitro* matured porcine oocytes, and embryos were transferred into eighteen surrogate sows. Pregnancy was confirmed in seven sows, of which one gave birth to two piglets. PCR analysis revealed that both piglets harbored the pIcam2-hCD73 cassette.

Key Words: CD73, xenotransplantation, nuclear transfer, transgenic pig

0151

DNA methylation changes in oocytes and embryos during mouse aging

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This experiment was designed to compare DNA methylation levels in oocytes and early embryos during mouse aging and to assess if DNA methylation levels affect the developmental potential of oocytes in old and young mice. A total of 120 females of the Kunming white strain were divided into two groups. The first group was used in the experiment at the age of 6–8 weeks and the second group was maintained until the age of 35–40 weeks. DNA methylation levels in oocytes and embryos were assessed by immunoflourescence staining using anti-5-MeC primary-antibodies (Epigentek Group Inc.) and confocal microscopy. Fluorescence intensities were quantified using the EZ-C1 Free Viewer software. The fluorescence pixel values were measured within a constant area from ten different regions of chromosomes and ten different regions in the cytoplasm, and then the average cytoplasmic value was subtracted from the average chromosomal value. Each group was repeated at least three times, and a minimum of 20 oocytes or embryos were used in each group. The developmental potential of the oocytes was determined by *in vitro* fertilization and natural mating methods. All data were analyzed using the Student's *t*-test and the SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA), and the statistical significance was set at $p < 0.05$. Results indicated that DNA methylation levels in oocytes and early embryos were significantly decreased during mouse aging (Table 1). Cleavage and blastocyst development rates were significantly lower in oocytes from older compared to young mice (69.9 ± 0.9% vs. 80.9 ± 1.2%; 33.9 ± 0.3% vs. 56.4 ± 1.9%). The pregnancy rate in old mice (46.7 ± 6.7%; n = 15) was lower ($p < 0.05$) than in young mice (100 ± 0.0%; n = 15). The rate of stillbirth and fetal malformations was higher ($p < 0.05$) in the old group (17.2 ± 0.9%; n = 17) than in the young group (2.9 ± 0.8%; n = 6). In conclusion, DNA methylation levels in oocytes and embryos decreased during the aging process in mice. The decrease in the DNA methylation pattern was correlated with a reduction in the developmental potential of the oocytes.

Key Words: Aging, DNA methylation, fertility, oocyte, embryo

Table 1. Immunofluorescence results on 5-MeC of oocytes and embryos

		Average fluorescence intensity			
Group	MII oocyte	2-cell	4-cell	8-cell	Morula
		Old			
IVF	2.13 ± 0.04a	0.81 ± 0.04a	0.81 ± 0.03a	0.74 ± 0.02a	0.68 ± 0.03a
Natural mating	2.13 ± 0.04A	0.97 ± 0.03A	0.96 ± 0.03A	0.88 ± 0.03A	0.84 ± 0.02A
		Young			
IVF	2.38 ± 0.05 b	1.00 ± 0.04 b	0.96 ± 0.04 b	0.86 ± 0.02 b	0.80 ± 0.02 b
Natural mating	2.38 ± 0.05 B	1.11 ± 0.03B	1.06 ± 0.03B	1.01 ± 0.03B	0.93 ± 0.03B

In the same row, values with different small or capital superscripts letters means significant difference ($p < 0.05$).

0152

Generation and characterization of induced pluripotent stem cells (iPS) from adult canine fibroblasts

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Pluripotent stem cells such as embryonic stem (ES) and iPS cells can give rise to derivatives of all three germ layers and thus have great potential in regenerative medicine. Here we report the derivation of iPS cells from adult canine fibroblast using retroviral transduction of Oct3/4, Sox2, Klf4 and c-Myc (OSKM). Retroviruses containing OSKM were transduced into adult dog skin fibroblasts and replated onto γ – irradiated mouse embryonic fibroblasts. ES-like colonies were picked at day 21 post-infection and expanded in three different ES culture media containing either basic FGF (bFGF, 10 ng/ml), human LIF (hLIF, 103 units/ml) or both bFGF and hLIF, and supplemented with two chemical inhibitors (3 μM CHIR99021 and 20 μM PD98059). The isolated cells cultured in bFGF and hLIF had the strongest alkaline phosphatse activity and those were further analyzed by RT-PCR and immunocytochemistry (ICC), and found to express the pluripotency markers, POU5F1 (OCT4), SOX2, NANOG and LIN28 as well as ES cells-specific genes (PODXL, DPPA5, FGF5, REX1 and LAMP1). Subcutaneous injection of the canine iPS cells into SCID mice created teratomas, and expression of markers for all three germ layers in the tumor were confirmed by both RT-PCR and ICC. Karyotyping analysis using array comparative genomic hybridization (aCGH) and fluorescence in situ hybridization (FISH) showed that genomic aberrations were gained from CFA 4, 8, 13 and 16 after extended culture periods but not by induction of reprogramming. All four

chromosomes examined were found to have gained aneuploidy in all four lines at passage 17 (S1: 58.8%, S2: 61.8%, S3: 57.1%, S4: 56.4%), with a smaller percentage showing aneuploidy at earlier passages (P7, S1: 7.3%, S2: 13.5%, S3: 17.5%, S4: 15.9%). Gains of CFA4 by fusion of homologous chromosomes was the most commonly observed aberrations in all four lines. *in vitro* differentiation by embryoid body formation and directed differentiation resulted in cells representative of all three germ layers as confirmed by both ICC and RT-PCR. This work was funded by a grant to JP and NO from the Canine Health Foundation, grant #01272.

Key Words: Stem cells, canine, reprogramming, iPS, pluripotency

0153

In vivo heat stress affects development of preimplantational rabbit embryos

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The fertility of farm animals decreases during the summer season. Previous studies have been done to evaluate the effect of heat stress (HS) on oocyte and embryo development in *in vitro* models. Since rabbits are especially susceptible to HS, the establishment of an *in vivo* animal model of heat stress in rabbits would be advisable. At the present study, male and female rabbits at the HS group were subjected to continuous temperature cycles by simulating daily periods of high temperatures. The temperature was increased to a maximum of 31°C at 15 h for 3 h; this temperature corresponded to the average maximum temperature of the three previous summer periods. The control group was maintained at 17°C. Embryos were recovered at 72 h post-insemination from uterus and oviduct, classified according to their stage of development and morphologically evaluated for quality. Embryos were then cultured for 24 h at 38.5°C and 5% CO2 in air. Embryos were mainly recovered at compacted morula and morula stages and a few of them in earlier stages. Females of the HS group produced a lower number of morphologically normal embryos (HS: 8.4 ± 0.9 vs. Control: 6.1 ± 0.9) as well as a lower number of embryos at the compacted morula stage (HS: 50.6% vs. Control: 73.5%), meanwhile the number of morulae (M) and oocytes and degenerated embryos (DE) were higher compared to the control one (M: 22.4% in HS and 13.7% in Control; DE: 23.5% in HS group vs. 12.7% in Control). After 24 h of *in vitro* culture, embryos from HS and Control groups showed similar percentages of development to the blastocyst stage (90.4% in Control group and 86.8% in HS group). In conclusion, the *in vivo* model of heat stress developed for this study affected negatively the prolificacy of females in terms of number of morphologically normal embryos and produced a phenomenon of retardation in embryo development (compacted morula vs. morula stages), whereas the embryonic developmental competence was apparently not affected after *in vitro* culture and embryos developed to the blastocyst stage in similar percentages.

Key Words: Heat stress, embryo, *in vivo*, rabbit, fertility

02. Artificial insemination

0200

Differences in age at first calving as a result of various usage of sex sorted semen

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The use of sexed semen is mostly limited to heifers that have greater fertility compared to higher lactation cows. Due to lower conception rate (CR) of sex sorted semen, the age at first calving and subsequently generation interval would tend to increase after utilization of this kind of sperm. The objective of the current study was to evaluate the age at first calving (AFC) under different sexed semen strategies and various ranges of utilization of sexed semen in heifers. The study was conducted using a deterministic computer modeling program in Matlab R2010a. Three different strategies were simulated including the continuous usage of sexed semen (CS), the usage of the sexed semen at the first insemination (S1) and the first and the second inseminations (S2) followed by conventional semen. All strategies were evaluated reaching to a minimum cumulative pregnancy rate of 90%. Utilization of sexed sorted semen was changed from 1% to 100% (1% interval). The differences of age at first calving of various strategies from the AFC of conventional semen (as control strategy) were obtained. Conception rate of heifers was assumed to be 56% and 39% for conventional and sexed semen, respectively. Estrus detection rate was set at 80%. When the use of sex sorted semen in herd was < 10%, AFC of different strategies did not make great differences. By increasing the use of sex sorted semen, AFC of CS strategy increased consistently with increase in number of services. Utilization of sexed semen by 15 to near 60% led to similar AFC under CS and S2 strategies and lower AFC for S1 strategy. Greater utilization of sex sorted semen (more than 60%) increased differences in AFC of various strategies. Despite more utilization of sex sorted semen in large compared with small herds, its use is usually limited to 10–20% of heifers. Hence, the utilization of sex sorted semen at realistic rates does not considerably increase AFC. However, increasing the use of sexed semen by 60% or more would lead to increased AFC up to 20 days under different strategies.

Key Words: Sexed semen, age at first calving, heifer

0201

Reproductive performance in beef heifers supplemented with rumen-protected fat prior or after fixed-time AI

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The use of protected fat sources in dairy cows is a common strategy to increase the energy density of the diet to improve productivity and reproductive performance. The addition of Megalac-E to the diet was already tested in the period after fixed-time AI (FTAI) protocol to increase pregnancy rate by reducing embryonic losses in beef cattle. However, low pregnancy rates after FTAI were associated with reduced ovulation rates in estrus synchronization treatments in heifers. The objective of this study was to evaluate whether supplementation of 200 mg of rumen-protected fat (Megalac-E) in the diet 30 days prior to or after FTAI positively affects the size of the ovulatory follicle (OF), ovulation and pregnancy rate in beef heifers raised on pasture. One hundred and thirty-eight Brangus heifers, body condition score 2.85 ± 0.17 (1–5 scale), were randomly sorted into two groups. Group 1 (n = 70) was supplemented with 200 mg/heifer/day of Megalac-E for 30 days before the date of FTAI while Group 2 (n = 68) received the same supplement 30 days after FTAI. All animals were submitted to hormonal treatment (Day 0) initiated by the insertion of intravaginal device with 1 g of progesterone (DIB, Coopers Saúde Animal, Brazil) and an injection of 2 mg of estradiol benzoate (EB) i.m. (Gonadiol, Coopers Saúde Animal, Brazil). On Day 7, the devices were removed and injected 150 μg of cloprostenol i.m. (Veteglan, Laboratórios Calier, Brazil). On Day 8, heifers received 1 mg of EB i.m. All heifers were FTAI 52–54 h after DIB removal. Ovarian ultrasonographic examinations were done in a sample of 30 heifers per group 48 h after progesterone device removal to measure the diameter of OF and 24 h after AI to determine ovulation rate. Pregnancy diagnosis were done 30 days after the FTAI. The diameter of the OF was significantly greater ($p < 0.05$) in the Group 1 (11.2 ± 2.5 mm) compared to Group 2 (10.1 ± 2.1 mm). Ovulation (90% vs. 75%) and pregnancy rate (67.1 vs. 48.5%) were significantly higher ($p < 0.01$) in Group 1 comparing to Group 2. According to our data, the supplementation with 200 mg of Megalac-E in the period of 30 days prior to FTAI had a positive effect on OF size and improved ovulation and pregnancy rate.

Key Words: Megalac-E, fixed-time AI, beef heifer

0202

Incorporation of sexed semen into reproductive management of range cow-calf operations

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The objective was to evaluate pregnancy rates of suckled beef cows reared in extensive systems and assigned to artificial insemination (AI) with sexed or conventional semen. A total of 441 postpartum beef cows originated from the Oregon State University – Eastern Oregon Agricultural Research Station (Burns location, n = 242 cows, Union location n = 199 cows) received a 100-μg treatment of GnRH and a controlled internal drug releasing device containing 1.38 g of progesterone (CIDR) on day 0 of the study, prostaglandin F2α treatment (25 mg) and CIDR removal on day 7, and a second GnRH treatment (100 μg) and fixed-time AI 66 h after the prostaglandin F2α treatment. In addition, at the Union station, estrus behavior was evaluated between CIDR removal and the second GnRH injection, and cows were inseminated 12 h after the onset of estrus. At the time of AI, cows were stratified according to parity and assigned to be inseminated with: (i) conventional semen (total n = 223); and (ii) GenChoice 90™ sorted for male calves (Genex Cooperative, Inc., Shawano, WI, USA; total n = 218). Blood samples were collected at AI and 7 days later to determine concentrations of progesterone and assess cow response to the estrous synchronization protocol. Only cows that had progesterone concentrations below 1 ng/ml at AI, but concentrations above 1 ng/ml 7 days after AI were considered responsive. Transrectal ultrasonography was performed 90 day after AI at the Burns station, and 45 day after AI at the Union station, to determine semen source effects on pregnancy rates. No differences were detected ($p = 0.37$) in synchronization rate between cows assigned to receive sexed semen or conventional semen (81.7% vs. 85.0%, respectively). Across both locations (Burns and Union) and all cows (responsive or not to the estrous synchronization protocol), cows inseminated with sexed semen had reduced ($p < 0.01$) pregnancy rates compared to cows that received conventional semen (36.6% vs. 51.9%, respectively). Similarly, across locations and only within cows that effectively responded to the synchronization protocol, cows inseminated with sexed semen had reduced ($p < 0.01$) pregnancy rates compared to cows that received conventional semen (43.8% vs. 60.1%, respectively). However, within cows inseminated after estrus detection at the Union station, those receiving sexed semen had similar ($p = 0.44$) pregnancy rates compared to cows receiving conventional semen (75.0% vs. 63.6%, respectively). Conversely, cows at the Union station that were timed-inseminated had reduced pregnancy rates if received sexed semen compared to conventional semen (52.2% vs. 68.7%, respectively). In summary, cows timed-inseminated with sexed semen had reduced pregnancy rates compared to cows inseminated with conventional semen, whereas the same outcome was not observed in cows inseminated upon estrus detection.

Key Words: Artificial insemination, beef cows, sexed semen

0203

Effect of GnRH on conception rate of heifers and suckling beef cows not in estrus at fixed time AI

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The objective of this study was to evaluate the effect of GnRH at fixed time AI (TAI) in animals that did not exhibit estrous in response to the synchronization protocol as assessed by degree of tail-paint removal. Aberdeen Angus cows (n = 254) and heifers (n = 299) were assigned to three herds balanced by body condition and cyclicity as assessed by ultrasound. All animals received an intravaginal progesterone releasing and an injection of 2 mg of estradiol benzoate on day 0. On day 7 the devices were removed and animals received a luteolytic agent (0.150 mg cloprostenol), ECP (1 mg), and tail-paint was applied for estrus detection. All animals were bred TAI between 48 and 53 h after device removal (day 9) by a single technician using frozen semen. At the time of AI, degree of estrous activity was assessed based on tail-paint removal on a scale of 0 (tail-paint completely removed) to 5 (tail-paint intact). Animals with limited indication of estrous activity (score 4 and 5) were randomly assigned to receive 100 mg of GnRH or no further treatment (Control). Pregnancy diagnosis was performed by rectal palpation 35–45 days post TAI. The data were analyzed by Chi-square test. At TAI, 74% of cows (187/254) and 70% of heifers (209/299) were determined to have exhibited estrous activity (tail-paint score 0–3). The conception rates for cows and heifers detected in estrus were 61% (114/187) vs. 59% (123/209), respectively. Among heifers not in estrus, GnRH treatment resulted in greater ($p < 0.01$) conception rates (61%, 26/43) compared with untreated controls (29%, 14/47). In suckling cows, GnRH had no effect ($p > 0.05$) on conception rates (54%, 19/35 vs. 53%, 17/32 for GnRH vs. control, respectively). In conclusion, among females not detected in estrus, as assessed by tail-paint, GnRH administered at TAI enhanced conception rates in heifers but not in cows.

Key Words: GnRH, fertility, heat, timed AI, beef cattle

0204

Use of eCG or hCG on day 7 after FTAI to improve pregnancy rate in beef cows

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This study aimed to evaluate the development of corpus luteum (CL), dominant follicle (DF) and pregnancy rate in beef cows treated with hCG or eCG on Day 7 after fixed-time AI (FTAI). Two hundred and nineteen Brangus cows had their estrus synchronized for FTAI using an intravaginal insert with 1 g of progesterone (DIB, Coopers Saúde Animal, Brazil) for 8 days. On Day-9, cows received the inserts and 2 mg of estradiol benzoate (EB) (Gonadiol, Coopers Saúde Animal, Brazil), i.m. were injected. At the removal of the insert (Day-2), 150 μg cloprostenol (Veteglan, Calier Laboratórios, Brazil), i.m., were injected and 24 h later 1 mg EB, i.m. The FTAI were performed 52–56 h after removal of the insert on Day 0. At Day 7 after FTAI, cows were divided into three groups: hCG (n = 40) receiving 1.500 i.u., i.m. (Vetecor, Laboratórios Calier, Brazil), eCG (n = 41) injection of 400 i.u., i.m., (Novormon, Coopers Saúde Animal, Brazil), Control (n = 138) without hormonal treatment. Ovarian ultrasonographic examinations (Chison 8300, linear transducer, 5 MHz, China) were done in the subgroups hCG (n = 23), eCG (n = 25) and Control (n = 16). Cows were scanned to determine the diameter of the ovulatory follicle (OF), on Day 0 (estrus), and on Days 7 and 12, to establish area of CL, the presence of accessory CLs and DF size. The pregnancy diagnosis was performed 30 days after TAI. Statistical analyses were performed by chi-square test for pregnancy rate and by ANOVA for all ultrasonografic parameters. Pregnancy rates of the FTAI were 45% (18/40), 61% (25/41) and 41% (56/138) for hCG, eCG and Control group, respectively, and were not significantly different ($p = 0.07$). Our results indicated that hCG treatment stimulated the CL development and increased ovulation rate of DF between Day 7 and Day 12 while eCG injection increased the size of CL and of the DF in the same period.

Key Words: eCG, hCG, FTAI, beef cow

Table 1. Mean (±SEM) diameter of ovulatory follicle (OF), diameter of the dominant follicle (DF), area of CL, number of CL in Brangus cows treated with hCG or eCG on Day 7 and Control.

	hCG	eCG	Control
Diameter OF D0	9.77 ± 3.21	10.0 ± 2.78	11.73 ± 1.79
Area CL D7	1.90 ± 1.07	2.05 ± 0.48	2.18 ± 0.59
Area CL D12	3.97 ± 1.82[a]	3.05 ± 1.22[b]	2.65 ± 1.22[c]
No. CL D12	1.43 ± 0.67[a]	1.04 ± 0.2[b]	1 ± 0[b]
Diameter DF D7	5.66 ± 6.16	3.67 ± 3.63	3.32 ± 4.89
Diameter DF D12	3.20 ± 6.85[b]	8.60 ± 5.69[a]	3.74 ± 4.70[b]

[abc]Values with row differ ($p < 0.01$).

0205

Progesterone supplementation improves fertility in lactating dairy cows without corpus luteum at the initiation of the 5-day timed AI protocol

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Absence of a functional corpus luteum (CL) during growth of the ovulatory follicle impairs reproduction in cattle. Timed artificial insemination (AI) programs based on gonadotropin-releasing hormone (GnRH) and prostaglandin (PG) $F_{2\alpha}$ allow for strategic progesterone (P4) supplementation targeting this low fertility cohort. Supplementing P4 to lactating dairy cows with a single intravaginal insert results in small increases in circulating concentrations of P4 (0.8–1 ng/ml) and minor improvements in fertility of anovular cows, which typically remains below that of cows subjected to timed AI programs during diestrus. It is hypothesized that P4 supplementation with two intravaginal inserts during the timed AI protocol will restore fertility of cows without CL similar to that of cows in diestrus. Therefore, objectives were to determine the effects of supplemental P4 on fertility responses of lactating dairy cows lacking a CL at the initiation of the 5-day timed AI program (day-8 GnRH, day-3 and $-2PGF_{2\alpha}$, d0 GnRH + AI). Cows had their ovaries evaluated by ultrasonography on day-8 and those without CL were assigned randomly to receive either 0 (Control; n = 234) or two controlled internal drug-release (CIDR) inserts containing P4 from day-8 to day-3 (2CIDR; n = 218). Cows bearing CL on day-8 were kept as positive controls (Diestrus; n = 946). Cows had their ovaries scanned on day-3 to detect newly formed CL and again on d0 for assessment of ovulatory follicle diameter. Short estrous cycle was defined as cows detected in estrus from 5 to 17 day after AI. Pregnancy was evaluated by ultrasonography 34 and 62 day after AI. Pregnant cows on d34 diagnosed non-pregnant on d62 were considered to have lost their pregnancies. Data were analyzed using the LOGISTIC and GLIMMIX procedures of SAS. Treatment affected circulating concentrations of P4 during the growth of the ovulatory follicle and improved pregnancy per AI in cows without CL (Table 1). The proportion of cows bearing a newly formed CL at $PGF_{2\alpha}$ (day-3) was greater ($p < 0.01$) for Control and 2CIDR than for Diestrus (66.7%, 61.9%, and 52.0%, respectively). However, a greater ($p < 0.01$) proportion Diestrus cows had at least one CL at $PGF_{2\alpha}$ (day-3) compared with Control and 2CIDR cows (97.7%, 66.7%, and 61.9%, respectively). Nevertheless, circulating P4 on d2, 5, 8, 11, and 14 after AI did not differ ($p = 0.99$) among treatments. Current results clearly indicate that lack of CL at the initiation of the timed AI protocol reduces fertility of lactating dairy cows. Increasing P4 concentrations by adding 2 CIDR inserts during the development of the ovulatory follicle restored pregnancy per AI in dairy cows without CL at the initiation of the 5-day timed AI program similar to that of Diestrus cows.

Key Words: Progesterone, follicle, 5-day timed AI, dairy cow

Table 1. Effect of CL and supplemental P4 on fertility of dairy cows

	Treatment			
	Control	2CIDR	Diestrus	p
Ovulatory follicle, mm	18.0 ± 0.6	18.6 ± 0.6	18.9 ± 0.5	0.46
	P4, ng/ml			
day-8	0.2 ± 0.2[b]	0.2 ± 0.2[b]	2.5 ± 0.2[a]	< 0.01
day-7	0.3 ± 0.3[b]	3.0 ± 0.3[a]	2.7 ± 0.3[a]	< 0.01
day-5	0.3 ± 0.3[c]	2.5 ± 0.3[b]	3.5 ± 0.3[a]	< 0.05
day-3	1.1 ± 0.3[c]	2.5 ± 0.3[b]	4.1 ± 0.3[a]	< 0.01
day-0	0.13 ± 0.04[b]	0.14 ± 0.04[b]	0.28 ± 0.04[a]	0.01
Short cycle	11.1 (18/162)[a]	3.5 (4/116)[b]	5.7 (27/474)[b]	0.02
Pregnant d62	28.6 (67/234)[b]	43.7 (94/215)[a]	47.3 (445/941)[a]	< 0.01
Pregnancy loss	6.9 (5/72)	5.1 (5/99)	4.7 (22/467)	0.40

0206

A 50-day period of negative energy had no effect on pregnancy per artificial insemination in heifers

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It has been hypothesised that follicles that develop during a period of negative energy balance (NEB) may contain a developmentally incompetent oocyte with an impaired potential to establish a pregnancy. The objective of this study was to establish (i) the concurrent, and (ii) the carryover effects of a 50 day (d) period of dietary restriction on pregnancy per artificial insemination (P/AI) in heifers. A total of 156 reproductively normal beef heifers with an initial (mean ± SEM) live weight (LWT) and body condition score (BCS) of 581 ± 4.4 kg and 3.45 ± 0.05, respectively were used for the experiment. Heifers were randomly assigned to either: (i) control feed intake group (C: n = 68, 1.3 times estimated maintenance energy (M) requirements for 50 day; start = day 0) or (ii) restricted feed intake (R: n = 88, 0.65 M for 50 day). Following the 50-day dietary treatment period, all heifers were fed a 2 M diet until the end of the study. Live weight and BCS were recorded on day 0, 14, 21, 28, 35, 42, 50, 57 and 90. Plasma non-esterified fatty acids (NEFA), beta hydroxy butyrate (BHB), urea and glucose were measured on day 0, 14, 28, 42, 50, 57, and 90. Estrous was synchronised using two injections of PGF2α administered 11 day apart. Heifers were artificially inseminated (AI) on day 50–52 using frozen thawed semen from one high fertility bull. Pregnancy was determined by ultrasonography 30 day after AI. Following pregnancy diagnosis, pregnant heifers received PGF2α to induce luteolysis and allow return to estrous. All heifers were re-inseminated on day 93–95. Liveweight, BCS and metabolite data were analysed using the Mixed procedure of SAS for repeated measures ANOVA with terms for day, treatment and their interaction included in the model. Pregnancy per AI was analysed by Fisher's exact chi-square. During the first 50 day of differential feeding, heifers on the R diet lost more LWT (70.5 ± 2.8 vs. 5.8 ± 2.1 kg, respectively; ($p < 0.001$) and had greater BCS loss (0.45 ± 0.03 vs. 0.05 ± 0.03 units, respectively; ($p < 0.001$) than heifers on the C diet. A treatment × day interaction was observed for NEFA ($p < 0.001$), BHB ($p < 0.01$) and urea ($p < 0.05$) on day 14, 28, 42 and 50 respectively. Concentration of glucose remained unchanged in both treatment groups. Estrous response to the 2nd injection of PGF2α administered on day 48 was (C: 97%, R: 95%). The P/AI (C: 69%, R: 72%, $p > 0.05$) was similar following pregnancy diagnosis on day 80–82. Estrous response to the second injection of PGF2α administered on day 91–93 was (C: 91%, R: 90%). However, following ultrasound scanning on day 123–125 (C: 64%, R: 80%, $p = 0.03$), P/AI was lower in C heifers. There was no evidence that a dietary-induced 50 day period of NEB had any concurrent or carryover effects on P/AI in heifers despite an elevation in systemic concentrations of NEFA, BHB and Urea.

Key Words: Negative, energy, pregnancy, metabolites, cattle

0207

Inclusion of a GnRH analogue to improve a prostaglandin F2α-based protocol for timed artificial insemination (TAI) in sheep

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The objetive of this experiment was to improve the reproductive outcomes of Synchrovine® TAI protocol (two doses of PGF2α 7 day apart) including a GnRH analogue either at 24 or 36 h after second PGF2α dose. The experiment involved 205 multiparous and 89 nuliparous Corriedale ewes grazing natural pastures (600 kg DM, 8.5% CP), with 51.7 ± 8.1 kg and 3.2 ± 0.3 (means ± SD) live weight and body condition (score 1–5) respectively, during the breeding season (UTU ′La Carolina′, Flores Uruguay, 33°S–57°W). Ewes were allocated to three groups by parity, body condition and weight: (i) Synchrovine® (two doses of PGF2α 7 day apart, D-Cloprostenol 75 μg i.m., n = 101), (ii) Synchrovine® + GnRH-24 (Synchrovine® + GnRH 24 h after second PGF2α dose, busereline acetate 8.4 μg i.m., n = 98), and (iii) Synchrovine® + GnRH-36 (Synchrovine® + GnRH 36 h after second PGF2α dose, n = 95). Ewes were subjected to cervical TAI 46 ± 1.5 h after the second dose PGF2α with fresh extended semen from six rams (pooled and extended in UHT skim milk + 2% egg yolk + antibiotics; 160×10^6 sperms/ewe). Ovulation rate (ovulations/ovulated ewe); conception rate (pregnant/treated ewes), prolificacy (embryos/pregnant ewe), and fecundity (embryos/treated ewes) were evaluated by transrectal or transabdominal ultrasonography at 10 or 70 days after TAI, respectively (7.5 MHz linear-array or 3.5 MHz convex-array, respectively). Data were analyzed using the Genmod procedure and presented as LSM ± SEM. Differences were considered significant if $p < 0.05$ (Table 1). In conclusion, GnRH analogue at 24 or 36 h after second PGF2α dose reduced or did not improve reproductive outcomes of Synchrovine® protocol for TAI in sheep. Authors thanks to Laboratorio Uruguay S.A. for PGF2α (Sinchron D®) and Biogénesis Bagó for GnRH (Gonaxal®) donation, UTU ′La Carolina′ for facilities and animals, and Dra. Mariana Carriquiry for statistical help.

Key Words: Sheep, TAI, prostaglandin-F2α, GnRH, fecundity

Table 1. Reproductive response of GnRH associated to Synchrovine® protocol

	Ovulation rate	Conception rate	Prolificacy	Fecundity
Synchrovine® (n: 101)	1.20 ± 0.05[a]	46 ± 0.04[a]	1.09 ± 0.05[a]	48.8 ± 0.06[a]
Synchrovine® + GnRH-24 (n: 98)	1.11 ± 0.05[a]	8.7 ± 0.10[b]	1.00 ± 0.05[a]	8.6 ± 0.06[b]
Synchrovine® + GnRH-36 (n: 95)	1.23 ± 0.05[a]	34.5 ± 0.05[a]	1.08 ± 0.05[a]	37.2 ± 0.06[a]

[ab]Within a column, values without a common superscript differed ($p < 0.05$).

0208

Pregnancy rates in hair sheep ewes following vaginal insemination with fresh and short-term stored liquid semen

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Use of artificial insemination (AI) in sheep is not widespread in the U.S., partly due to the need for intrauterine insemination when using frozen-thawed ram semen. Success was reported using simple vaginal (shot-in-the-dark) AI with liquid semen in native breeds in Scandinavia, and we evaluated the feasibility of this approach for hair sheep now common on small farms in the U.S. All trials used semen extended in skim milk (11%; w/v) and egg yolk (5%; v/v) at a concentration of 350×10^6 sperm/ml and packaged in 0.5 ml straws. Estrus was initially synchronized by feeding melengestrol acetate (0.3 mg/head/day) or inserting a CIDR device for 11 day, and ewes were bred at a spontaneous estrus of the subsequent cycle 12 h after onset of estrus during a 5-day detection period using harnessed teaser rams. Ewes were inseminated by deep vaginal deposition of semen without speculum using a standard 0.5 ml AI gun. Pregnancy was determined 23 day after the last day of AI by transrectal ultrasound. The initial experiment was conducted in October with mixed breed yearling hair sheep ewes (n = 39). During the 5-day AI period 85% of ewes displayed estrus, and were inseminated with fresh semen (within 2 h of collection), and half of the ewes received a second fresh dose 12 h later. Overall pregnancy rate was 75%, and not different ($p > 0.1$) in single (67%) and twice-inseminated (86%) ewes, or between breeds (ranging from 60% in Barbados Blackbelly to 80% and 83% in St. Croix and Katahdin, respectively). The second experiment used mature, mixed breed ewes (n = 110) also in October. Ewes showing estrus (94%) were inseminated once with either fresh semen or semen stored at 5°C for 12 h collected from nine rams. Pregnancy rates were higher ($p < 0.05$) for fresh (59%) than 12 h-stored semen (39%), but were not different ($p > 0.1$) between breeds. Pregnancy rates for individual rams (with >12 inseminations) ranged from 31% to 68%. A third experiment with yearling ewes (n = 40) in December used pooled semen from four rams. Ewes showing estrus (85%) were inseminated with semen stored at 5°C for 12 h, and half of the ewes were inseminated with a second dose of semen (stored for 24 h) 12 h later. Pregnancy rates were 41% and 53% for once- and twice-inseminated ewes, respectively, and not different ($p > 0.1$). However, pregnancy rate was lower ($p < 0.05$) at the first day of the insemination period (10%) than the final three days (63%). In three trials conducted with larger groups (>100 ewes) during the transitional periods before (June) and after (March) seasonal breeding, estrus response to synchronization (<75%) and pregnancy rates (<15%) were generally poor, and masked potential differences related to length of semen storage. Results indicate vaginal AI should be conducted only during peak seasonal breeding, and that pregnancy rates will decline appreciably after 12 h of storage. Potential benefits from changes in extender composition and increased insemination dose should be evaluated.

Key Words: Hair sheep, vaginal AI, liquid semen, pregnancy, estrus synchronization

0209

Oral vitamin B1 supplementation prior to laparoscopic artificial insemination improves pregnancy and lambing rates in Pelibuey ewes

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The objective of this study was to evaluate the effect of vitamin B1 supplementation on pregnancy and lambing rates in sheep inseminated by laparoscopy. Ninety Pelibuey ewes, 2.5–4 years-old and in good body condition were used. Ewes received daily oral doses of 48 mg thiamine diphosphate mononitrate (in 20 ml) (supplemented group; n = 45) or 20 ml of placebo (control group; n = 45) for 14 days prior to insemination. Fluorogestone acetate-impregnated sponges maintained intravaginally for 14 days were used for estrus synchronization

and 350 IU eCG was administered intramuscularly on the day the sponges were removed. Laparoscopic artificial insemination was performed 56 h after sponge removal using refrigerated semen (50×10^6 sperm) from a Dorper ram. Pregnancy diagnosis was performed by ultrasonography ˜45 days after insemination. Differences between supplemented and control groups were determined by chi-square test. Pregnancy and lambing rates in the supplemented group (68% and 62%, respectively) were greater ($p < 0.05$) than in the control group (37% and 35%, respectively). The percentage of multiple births was not different between groups (10% and 6% in supplemented and control groups, respectively). These results indicate oral vitamin B1 supplementation for 14 days prior to laparoscopic artificial insemination improves pregnancy and lambing rates in Pelibuey ewes.

Key Words: Artificial insemination, sheep, pregnancy, calving, thiamine mononitrate

0210

Fixed-time artificial insemination in alpacas

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Artificial insemination is developing in alpacas, however, at present there are no effective timed AI protocols for this induced ovulating specie. This study was designed to synchronize the presence of ovulatory-sized follicles using exogenous progesterone. Twenty-two adult postpartum female alpacas with small (3–4 mm) ovarian follicles were synchronized with 5 mg i.m. progesterone (Steris). After 5 days of progesterone treatment, the alpacas were examined by ultrasonography for the presence of an ovulatory-sized follicle (8–12 mm), and were induced to ovulate with 750 IU hCG. All females were inseminated at 26-h following ovulation induction by uterine body depostion of a 1 ml insemination dose containing 8–10 million spermatozoa of either chilled (6 h storage at 4°C, n = 11) or frozen-thaw semen (n = 11) from two alpaca males. Ovulation was determined by acceptance or refusal of the male 7 days after insemination. Pregnancy was determined 30 days after artificial insemination by ultrasonography for the presence of an embryonic vesicle. All 22 females presented 8–12 mm follicle on day 5 of progestin treatment. Ovulation was induced in 86% of females. Among ovulating females, pregnancy rate was not different ($p > 0.05$) for chilled (63%, 5/8) vs. frozen-thawed semen (40%, 4/10). In conclusion, a 5-day progestin protocol combined with hCG successfully synchronized follicle size, induction of ovulation, and resulted in acceptable pregnancy rates among ovulating alpaca females using both chilled and frozen-thawed semen.

Key Words: Artificial insemination, alpacas, chilled semen, frozen-thawed semen

0211

Fertilization rate and number of embryos in sows reproductive tracts after intra-uterine insemination with cryopreserved boar semen supplemented with seminal plasma

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The objective of this study was to determine the effects of seminal plasma supplimentation in the thawing media on fertilization rate and the number of embryos following fixed time single intra-uterine insemination (IUI) with frozen-thawed (FT) boar semen. The seminal plasma was collected from the supernatant of semen extended (1:1 v/v) in ModenaTM and was therefore a mixture (˜1:1) of ModenaTM extender and seminal plasma. The supernatant was centrifuged twice and stored at −20°C until use. Twelve sows were treated with 750 IU human chorionic gonadotropin (hCG) at the onset of standing estrus to induce ovulation. The sows were divided into two groups: control (n = 7) and treatment (n = 5). The control sows were inseminated with 2×10^9 motile FT boar sperm diluted with 40 ml ModenaTM. Treated sows were inseminated with the same amount of FT boar sperm diluted with 40 ml autologous seminal plasma. Time of ovulation was determined every 4 h using transrectal realtime B-mode ultrasonography. All the sows were slaughtered at 61.0 ± 3.8 h after insemination. The sow's reproductive tracts were collected; embryos and non-fertilized oocytes were determined under a stereomicroscope. The number of corpora lutea (CL) were counted on both ovaries to estimate the number of ovulations. The recovery rate was defined as the number of embryos and oocytes recovered divided by the number of CL. Fertilization was assumed when at least two cells-stage embryos were found. The fertilization rate was determined as the number of embryos (two cells) divided by the sum of embryos and oocytes recovered. Continuous variables, i.e. the numbers of CL, embryos, oocytes, sperm motility, interval from weaning-to-estrus, onset of estrus-to-ovulation and hCG treatment-to-ovulation were analyzed using the General Linear Model procedure of SAS. Recovery rate and fertilization rate were compared using Fisher's exact test. The mean post-thaw motility was 42.1% and 40.8% for control and treated groups, respectively ($p = 0.257$). The interval from weaning-to-estrus, onset of estrus-to-ovulation and hCG treatment-to-ovulation, in control and treatment groups, were 4.7 ± 0.9 and 4.5 ± 0.5 days, 50.7 ± 5.0 and 54.6 ± 4.8 h, 43.5 ± 6.4 and 45.7 ± 4.8 h, respectively ($p > 0.05$). The ovulation rates were 22.3 ± 3.0 and 22.4 ± 8.8 ($p = 0.975$) and the number of embryo was 9.1 ± 3.8 and 9.6 ± 4.9 ($p = 0.941$) in the control and treatment groups, respectively. The recovery rate was 93.6% and 84.8% ($p = 0.02$) and the fertilization rates were 43.8% and 49.5% ($p = 0.39$) in the control and treatment groups, respectively. In conclusion, seminal plasma addition to thawing medium had no effect on post-thaw sperm motility, time of ovulation, fertilization rate, or number of embryos in sows.

Key Words: Pig, artificial insemination, hCG, frozen semen, seminal plasma

0212

Effect of semen extender on frozen-thawed sperm characteristics and distribution in the female genital tract after deep intrauterine insemination in pigs

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The objects of this study were to compare the effects of sperm thawing media on post-thaw sperm motility, membrane integrity, and distribution of spermatozoa in the genital tract after fixed-timed deep intrauterine insemination (DIUI) in sows. Semen from six boars were frozen in 0.5-ml straws, thawed at 38°C, and diluted to 1×10^8 spermatozoa/ml in either Modena solution (MS) or porcine fertilization medium (PFM; Research Institute for the Functional Peptides, Yamagata, Japan). Sperm quality, as assessed by motility using computer-assisted sperm analyzer and integrity of the plasma and acrosome membranes using flow cytometry, was evaluated at 0.5, 1.5, 3 and 6 h after thawing. Prior to the flow cytometry, sperm samples were stained with SYBR-14, propidium iodide (PI) and Alexa-Fluor 647 peanut agglutinin (PNA). Proportion of progressive spermatozoa and straight-line velocity in PFM were significantly higher than those in MS throughout 6 h after thawing ($p < 0.05$). The curvilinear velocity and amplitude of lateral head displacement in PFM were significantly lower than in MS until 3 h after thawing ($p < 0.05$). Percentage of spermatozoa with intact plasma and acrosome membranes (PI−/PNA−) in PFM was significantly lower than those in MS throughout 6 h after thawing ($p < 0.05$). The percentage of spermatozoa with damaged acrosome membranes (PNA+) in PFM was significantly higher than that in MS throughout 6 h after thawing ($p < 0.05$). Sows synchronized to estrus using $PGF_{2\alpha}$, eCG and hCG were inseminated once with frozen-thawed semen (5×10^8 spermatozoa in a 0.5-ml straw) diluted in 5 ml of MS or PFM and then infused with 5 ml of the same extender by DIUI at 34 h after hCG injection. At 4 h after DIUI, reproductive tracts were recovered from 16 sows. There was no significant difference in the number of spermatozoa in the utero-tubal junction and uterus between MS and PFM. However, the number of

polymorphonuclear leucocytes (PMNs) capable of phagocytosing sperm in the uterus of the PFM group was significantly reduced compared with the MS group ($p < 0.05$). When 14 sows were inseminated deep into the uterine horn once with 10×10^8 frozen-thawed spermatozoa in MS or PFM at 36 h after hCG, the pregnancy rate was not significantly different between the MS (42.9%) and PFM (71.3%) groups. Our results demonstrate that PFM enhanced the progressive motility of spermatozoa, while it increases sperm membrane damage compared with MS. We also indicate that PFM suppresses PMNs migration into the uterine lumen.

Key Words: Deep intrauterine insemination, frozen-thawed spermatozoa, pig, semen extender

0250

Hormonal treatment to improve embryo survival in beef cows after fixed time artificial insemination

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Successful maternal recognition of pregnancy (MRP) is directly involved with early survival of the bovine embryo. This study evaluated strategies to enhance ovarian function of cows submitted to fixed time artificial insemination (FTAI) and thereby optimize MRP. Twenty-eight cycling non-suckled Nelore (*Bos taurus indicus*) cows received one progesterone (1.9 g) intravaginal device and 2 mg estradiol benzoate. Seven days later received 250 μg of Dinoprost Thrometramine (PGF2alpha). The device was removed two days later and 0.5 mg of estradiol cipionate was given. FTAI (Day 0) occurred 48 h after device removal. Females were assigned to receive either no further treatment (Control, n = 14) or 200 μg of gonadorrelin (GnRH, i.m.) on D5 and 2500 IU of hCG (i.m.) on D12 (Treated, n = 14). Cows were submitted to ultra-sound examination of ovaries and plasma progesterone determination (P4) on D5, D12, D18 and D28. Categorical data were analyzed by Chi-square test and continuous variables were assessed by ANOVA in a 2×2 factorial design (pregnancy status on day 28 vs. treatment group). Conception rate was not different ($p > 0.05$) between control (36%) and treated cows (50%). All treated cows developed at least one accessory corpus luteum and had greater P4 on D12, D18 and D28 than control cows ($p < 0.05$) regardless of pregnancy status. Treated non-pregnant cows (n = 7) had greater ($p < 0.05$) dominant follicle diameter on D18 (13.7 ± 3.2 mm) than pregnant contempories (10.5 ± 2.2 mm, n = 7). A similar trend was observed among control non-pregnant cows (11.9 ± 3.0 mm, n = 5) and pregnant cows (9.7 ± 2.2 mm, n = 9). Hormonal treatment did not influence ($p > 0.05$) size of original CL on D12 or D18 as compared with control cows on D12 and D18. Treated cows that were detected as non-pregnant (D28, n = 7) had greater ($p < 0.05$) P4 on D18 (6.28 ± 3.74 ng/ml) than control cows regardless of their pregnancy status. Treated cows bearing a conceptus had greater ($p < 0.05$) P4 on D18 (8.63 ± 3.44 ng/ml) and D28 (9.09 ± 4.87 ng/ml) than control cows (5.02 ± 2.24 and 5.79 ± 3.30 ng/ml, respectively). In summary, hormonal treatment induced the formation of accessory corpus luteum and increased P4 around the time of MRP regardless the presence of a conceptus.

Financial support: EMBRAPA (03.09.02.004).

Key Words: Cattle, early pregnacy, GnRH, hCG, progesterone

0251

Reproductive efficiency of Holstein cows submmited to fixed time artificial insemination in semi-arid regions

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This study was conducted at the Experimental Station of São Bento do Una (ESSBU/IPA) Pernambuco, Brazil, (Latitude 08 31′35″ and Longitude 036 27′34.8″) with the objective to evaluate the efficiency of protocols for timed artificial insemination (TAI) in Holstein cows during summer in semiarid conditions (53.5% of maximum relative humidity and minimum of 39.1%, maximum ambient temperature 32.5°C and minimum 20.9°C). For the study 61 cyclic cows were used, up to 100 days in milk production with an average of 24.8 kg/milk/day and age ranging from 28 to 108 months. The cows were kept in semi-intensive system, receiving a diet composed of cactus pear (Opuntia ficus-indica Mill), sorghum silage [Sorghum bicolor (L) Moench] and protein concentrate with 18% of crude protein (CP) besides mineral supplement and water *ad libitum*. Females used were between 100 and 150 days postpartum and were submitted to gynecological examination by rectal palpation, being randomly distributed into two treatments T1 and T2. On day 0 (D0) all cows of treatment T1 (n = 28) received an intravaginal device containing 1.9 g of progesterone and 2 mg of estradiol benzoate (EB). On day 7 (D7) application of 0.265 mg of sodium cloprostenol and 300 IU eCG, on day 8 (D8) intravaginal devices were removed. Twenty-four hours after device removal (D9) 1 mg EB was injected and 54 h after device removal all cows were fixed-timed AI at day 10 (D10). Cows in treatment T2 (n = 33) were basically treated as described for the T1 except for the eCG dose which was 400 IU and 0.530 mg of sodium cloprostenol instead of 0.265 mg. All cows in both treatments (T1 and T2) were subjected to ultrasound for evaluation of follicular diameter at D9 and were TAI with semen from Holstein bulls of a reputable center. The data were subjected to analysis of variance and means were compared by *T*-test. Follicular diameter at D9 was 10.0 and 14.5 mm for T1 and T2, respectively, ($p < 0.05$). Pregnancy diagnosis was performed by ultrasonography 30 and 60 days after TAI. Conception rates were 29% (8/28) and 61% (20/33) for the T1 and T2, respectively at day 30% and 25% (7/28) and 58% (19/33) for T1 and T2, respectively at day 60 ($p = 0.01$). In conclusion, T2 resulted in greater mean follicle diameter on D9 than T1 and appeared to increased conception rates albeit the sample size was insufficient for a meaningful comparison of binomial data.

Key Words: Cattle, TAI, gonadotropin, eCG, Holstein

0252

Cows diagnosed with ovarian cysts derive no benefit from progesterone supplementation but conceive normally to a GnRH-based timed A.I. protocol

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Gonadotropin releasing hormone-based protocols for synchronization of ovulation (e.g. Ovsynch) followed by timed artificial insemination (timed A.I.) have resulted in acceptable rates of pregnancy in dairy cows diagnosed with ovarian cysts. The addition of progesterone to such protocols may further improve pregnancy rates. Our objective was to monitor treatment responses and pregnancy rates to an Ovsynch/timed-A.I. protocol with or without supplemental progesterone in cows diagnosed with ovarian cysts. Lactating dairy cows (n = 109; > 60 day postpartum) from nine herds diagnosed with one or more ovarian cyst (≥25 mm) received two treatments of 100 μg GnRH (Fertiline; Vetoquinol Canada) 9 day apart (Days 0 & 9), a single treatment of 500 μg cloprostenol (Estrumate; Schering Canada) on Day 7, and timed A.I. on Day 10, ˜20 h after the second GnRH. Fifty-three of 109 cows received an intravaginal progesterone (1.55 g) releasing device (PRID, Vetoquinol Canada) during the first 7 day of the protocol. Ovarian ultrasonography was performed on Days 0, 7, 10, 11 to monitor

treatment responses, and pregnancy was determined 32 day after timed AI. Ovarian structures were categorized into follicular cyst (FC; no evidence of luteal tissue), luteinized cyst (LC; cyst wall luteinized) and FC plus corpus luteum present on either ovary (FCL). The mean (± SD) cyst diameters (mm) for FC, LC, and FCL were 32.5 ± 5.6, 35.8 ± 6.1 and 32.4 ± ±6.0, respectively. The incidence of cysts on the right ovary, left ovary, and both ovaries were 56.9, 25.7 and 17.4%, respectively. More than one cyst was present in 26.6% of the cases. The cyst became larger in 25.6% of the cows after initiating the Ovsynch protocol, whereas a new cyst developed in 7.3% of the cases. Ovulatory responses to the first and second GnRH treatments were 62.3 and 83.4%, respectively. Pregnancy rates (%) were neither affected by progesterone treatment (no PRID: 44.6 vs. PRID: 37.7; $p = 0.46$) nor by cyst category (FC: 37.5; LC: 50.0; FCL: 40.5; $p = 0.59$). Cows diagnosed with ovarian cysts conceived normally to an Ovsynch/timed A.I. protocol but supplemental progesterone given during the first 7 day of the protocol did not improve pregnancy rates.

Key Words: Cystic ovaries, dairy cow, progesterone, Timed AI, pregnancy

0253

Genetic and environment factors as leverage effects on fertility at first service in Holstein cows together with milk production and insemination conditions

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Fertility at first service had continuously decreased in cattle during the two last decades. In order to evaluate and to quantify factors influencing fertility at first artificial insemination (AI) in Holstein cows, a study based on 4621 first AI's was conducted in 135 French dairy herds. For each AI, fertility was phenotyped by measuring progesterone concentration in milk on the day of AI and 21–23 days later, allowing to determine precisely the incidence rates of AI performed in luteal phase (LPAI, 4.5%), non-fertilization or early embryonic death (NF-EED, 36.9%), prolonged luteal phase or late embryonic death (PLP-LED, 31.9%). Both NF-EED ans PLP-LED presented high incidence rates. Pregnancy was evaluated once between day 45–75 using transrectal ultrasonography or palpation or PSPB (Pregnancy Specific Protein B) assay from blood and averaged 47.1%. Individual data for each 'cow-AI' event were recorded by breeders and AI technicians. Milk production (MP) data during the first 3 months of lactation and genetic milk and fertility breeding values (FBV) were extracted from the national database. Multivariate statistical analysis using mixed models for logistic regression (with herd as a random effect) showed that cows' FBV ($p \leq 0.001$), length of calving to first AI interval ($p \leq 0.02$), parity ($p \leq 0.01$), first 3 months MP ($p \leq 0.01$), first 3 months somatic cell count ($p \leq 0.01$), calving conditions ($p \leq 0.01$), type of estrus sign used by the breeder when asking for AI ($p \leq 0.01$), length of estrus detection to AI interval ($p \leq 0.02$) and feeding management ($p \leq 0.01$) strongly affected conception rate at 45 days. Table 1 shows that FBV and MP data impacted PLP-LED whereas AI modalities impacted LPAI and NF-EED rates. Combining these results, updated references are applied by AI technicians to give breeders practical recommendations for better breeding management from time of calving to AI, as well as mating plans including FBV. The results of this study should contribute to improve reproductive efficiency in Holstein cows through better management and increased use of fertility genetic information.

Key Words: Fertility, dairy cattle, insemination, feeding, genetics

Table 1. Factors affecting various types of pregnancy failures after 1st AI in Holstein cows

p Values	LPAI	NF-EED	PLP-LED
Calving conditions			c
Calving to AI interval	b	a	
Estrus sign used to inseminate	d		d
Estrus detection to AI interval		d	
Nb of cows silumtaneously inseminated	c		
Easiness of passing cervix	c		
Cows' restraining quality at AI		c	
Parity			d
Milk production*			c
Maximum fat/protein ratio*		c	
Somatic cell count*		c	b
Feeding management		a	b
Fertility breeding value			c

[a]$p < 0.1$, [b]$p < 0.05$, [c]$p < 0.01$, [d]$p < 0.001$.
*In the 1st three months.

0254

Reproductive outcome of prostaglandin based protocol for timed artificial insemination (TAI) in ewes: effects of focus feeding and GnRH treatment

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Two doses of PGF2α (PG) 7-days apart and TAI 48 h after the second dose (Sychcrovine® protocol) in ewes yields variable pregnancy rate to TAI. The aim of this experiment was to compare the effect of six days focus feeding after the first PG and a GnRH dose 36 h after the second PG on reproductive outcome to TAI. In a 2 × 2 factorial design (April, S 33°–W 57°), multiparous (261) and nulliparous (118) Corriedale ewes grazing natural pasture (600 kg DM, 8.5% CP) were homogeneously allotted by body condition (3.2 ± 0.3, score 1–5) and weight (51.3 ± 7.3) to four groups: control (n = 101, Sycnhrovine®, PG analog D-Cloprostenol 75 μg i.m.), GnRH (n = 95, busereline acetate 8.4 μg i.m. 36 h after second PG), SUP (n = 93; 410 g/ewe/day from first PG, extruded soy, 34% crude protein), and SUP + GnRH (n = 90). Fresh pooled semen from six healthy rams (extended in UHT skim milk with 2% egg yolk and antibiotics to 160×10^6 sperm/dose/ewe) were used for cervical TAI. Reproductive outcome (ovulation rate: ovulations/ovulated ewe; fertility rate: pregnant/treated ewes; prolificacy: 70 day embryos/pregnant ewe; and fecundity: 70 days embryos/treated ewes) were evaluated by ultrasonography (transrectal 10 day after TAI or transabdominal 70 days after TAI; Aloka® 500, Japan; 7.5 linear- or 3.5 MHz convex-array). Results were analysed using the GENMOD procedure of SAS, including in the model the fixed effect GnRH treatment, feeding group and their interaction. From the results (LSM ± SEM) we concluded that the treatments and their interaction did not improved the reproductive outcome. Fertility

Table 1. Effect of focus feeding (6 day), GnRH (36 h after second PGF2α) and their interaction on reproductive outcome of ewes under TAI (two PGF2α 7-day apart)

	Ovulation rate	Fertility (%)	Prolificacy	Fecundity (%)
Control (n = 101)	1.20 ± 0.05^{a}	46 ± 0.05^{ab}	1.09 ± 0.04^{a}	48.8 ± 0.06^{ab}
GnRH (n = 95)	1.23 ± 0.05^{a}	35 ± 0.05^{b}	1.08 ± 0.04^{a}	37.2 ± 0.06^{b}
Supplemented (n = 93)	1.22 ± 0.05^{a}	56 ± 0.05^{a}	1.06 ± 0.04^{a}	59.2 ± 0.06^{a}
Supplemented + GnRH-36 (n = 90)	1.10 ± 0.05^{a}	52 ± 0.05^{a}	1.03 ± 0.04^{a}	52.7 ± 0.06^{ab}

Different superscript within a column denote $p < 0.05$.

and fecundity were depressed by GnRH. We thanks to Laboratorio Uruguay for PG, Biogenesis Bago for GnRH, and Biogran for concentrate; UTU La Carolina for facilities and animals, and Dra. Carriquiry for statistical analysis.

Key Words: Sheep, estrus synchronization, nutrition

0255

Vaginal mucus and hyperemia affects fertility of artificial insemination in Assaf breed sheep

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Vaginal environment at the time of artificial insemination (AI) is associated with ovarian estrous status and may influence fertility. The aim of this work was to relate the amount of vaginal mucus and degree of vaginal hyperemia with fertility after cervical insemination. Adult Assaf sheep (n = 1001) were synchronized with intravaginal sponges (FGA-14 days; 20 mg Chronogest® MSD) and 500 IU of eCG (Folligon® MSD). Cervical AI was performed at 55 ± 1 h after sponge removal with a straight catheter. Semen was collected from adult males and diluted to 1600×10^6 spermatozoa/ml, refrigerated to 15°C and placed into 0.25 ml straws. Chilled semen (400×10^6 spermatozoa) was deposited into first cervical folds. The amount of cervical mucus observed at AI was classified into three categories: mucus 1 (no mucus), mucus 2 (external uterine orifice covered with mucus) and mucus 3 (1/3 of the vaginal cavity covered with mucus) and degree of hyperemia of vaginal mucosa as: pale, rose (intermediate between pale and red) and red (hyperemic). The distribution of mucus volume classifications were 38.4, 49.4 and 12.2% for mucus categories 1, 2 and 3, respectively. Ewes classified as a category two acheived greater ($p < 0.05$) conception rates (39.4%, n = 494) than ewes classified in categories 1 (32.9%, n = 385) or 3 (25.4%, n = 122). Ewes with vaginal mucosa classified as pale had lower ($p < 0.05$) conception rates (22.0%, n = 50) compare with ewes classified as rose (35.7%, n = 590) or red (36.8%, n = 361). In conclusion, the amount of vaginal mucus (mucus 2) and the degree of hyperemia of vaginal mucosa (no pale) at the time of AI was associated with conception rates in synchronized Assaf ewes.

Key Words: Artificial insemination, sheep, vaginal mucus, fertility

03. Conservation of genetic- and bio-diversity

0300

Monitoring salivary progesterone and estradiol in a captive female Amazonian manatee (*Trichechus inunguis*)

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Information about the reproductive endocrinology of the Amazonian manatee (*Trichechus inunguis*, Mammalia: Sirenia) is scarce; therefore, the aim of this study was to monitor salivary progesterone and estradiol patterns during the estrous cycle in this species. Salivary samples (daily) were collected during 12 weeks from an adult female animal housed at the Laboratory of Aquatic Mammals of the National Institute of Amazonian Research – LMA/INPA (Manaus, AM, Brazil). For sample collection, the pool was drained and the saliva was collected from mouth mucosa using a metal spoon. We selected 2 samples/week for progesterone assay, and 4–7 samples/week for estradiol assay. The salivary steroids were measured by enzyme immunoassay. The results were analyzed in an iterative process of exclusion of higher values than mean ± 2 standards deviations until determination of basal values. The luteal (high salivary progesterone) and nonluteal (basal salivary progesterone) phases were determined, and the cycle length was calculated. The mean basal value for salivary estradiol was 11.88 ± 7.02 (range 3.04–27.28 pg/ml). The means of salivary progesterone were 86.19 ± 43.54 pg/ml (range 33.51–152.78 pg/ml), and 11.97 ± 10.56 pg/ml (range 0.03–29.28 pg/ml) for luteal and nonluteal phases, respectively. We observed one and a half estrous cycles, and the cycle length was 44 days. Four salivary estradiol peaks (values > 29.3 pg/ml) were observed, in which the first two peaks occurred before the first luteal phase and the other two before the second luteal phase. These results suggest that Amazonian manatees apparently show similarities with the elephant reproductive physiology, with an anovulatory follicular wave before ovulation. In elephants, this anovulatory wave is important for luteinization of the multiple corpora lutea present during the estrous cycle, and sirenians, as elephants, also show multiple corpora lutea in ovaries. This is the first in-depth study about estrous cycle in Amazonian manatees; however, it is necessary a long-term study, analyzing a greater number of individuals to confirm these findings.

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Key Words: Estrous cycle, steroid, manatee, sirenian, *Trichechus inunguis*

0301

Effect of chilling duration on post-thaw characteristics of sperm from the north american bison (*Bison bison*)

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Development of functional reproductive technologies is important for genetic preservation and propagation of North American bison (*Bison bison*). In that regard, transport of chilled semen from the site of collection to the laboratory would facilitate cryopreservation. Therefore, the objective was to determine the maximum duration of pre-cyropreservation chilling which would not compromise post-thaw quality of bison sperm. Epididymal sperm from plains bison (n = 11) and ejaculated sperm from wood bison (n = 3) were collected, extended and held at 4°C for 24, 48, or 72 h prior to cryopreservation. There was no significant effect of duration of chilling on total and progressive motility (computer assisted sperm analysis) of epididymal sperm from plains bison at post-thaw. For ejaculated sperm from wood bison, total and progressive motility were sustained until 48 h, but declined significantly at 72 h. Representative plains bison sperm samples (n = 3) were evaluated for their *in vitro* fertilizing ability in a heterologous system using bovine oocytes. One of two bulls had significantly lower fertilization with sperm chilled for 72 compared to 24 or 48 h prior to freezing. In summary, motility of frozen-thawed epididymal sperm were sustained with pre-cryopreservation chilling for 72 or 48 h for plains and wood bison, respectively, whereas the effects of duration of sperm chilling on *in vitro* fertilizing ability differed between bulls. We concluded that extended semen or epididymal sperm from North American bison could be maintained at 4°C for at least 48 h before cryopreservation, without compromising post-thaw motility or *in vitro* fertilizing ability.

Key Words: Cryopreservation, epididymal sperm, sperm analysis, heterologous IVF, American bison

0302

Effects of reproductive seasonality on gamete quality in *Bison bison bison*

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The objective was to investigate the effects of reproductive seasonality on gamete quality and ovarian activity in North American bison (*Bison bison*) using plains bison (*Bison bison bison*) as a model. Epididymal sperm were collected during the breeding (July–September) and non-breeding (January–March) seasons (n = 61 per season). Three out of 13 parameters: post-thaw total motility (36.76 ± 14.18 vs. 31.24 ± 12.74%), linearity (0.36 ± 0.06 vs. 0.39 ± 0.04) and wobble (0.49 ± 0.04 vs. 0.51 ± 0.03; mean ± SD), were significantly higher for breeding season samples compared to those from non-breeding season ($p < 0.05$, unpaired *t*-test on arc-sine transformed data). A subset of samples (n = 4) from each season were used in heterologous IVF trials using domestic cattle oocytes, to determine fertilizing ability of bison sperm. Cleavage rate and morulae and blastocyst production (as a percent of cleaved embryos) were higher for breeding vs. non-breeding season samples (81.88 ± 6.8 vs. 49.94 ± 6.77; 41.89 ± 13.40 vs. 27.08 ± 23.21; and 30.49 ± 17.87 vs. 13.72 ± 18.98, respectively). Plains bison ovaries collected during the breeding (n = 97 pairs) and non-breeding (n = 100 pairs) seasons were classified as luteal or follicular (based on structures present). Oocytes recovered from these ovaries were classified into five grades based on morphological criteria. There was no significant difference between seasons in the number of luteal ovaries or in the grades of oocytes recovered. Oocytes were matured (TCM 199, 10% fetal calf serum sodium pyruvate, gentamicin, FSH, LH and estradiol), fertilized (FERT-TALP, BSA, penicillin, streptomycin, sodium pyruvate, PHE, heparin) with frozen – thawed bison sperm from breeding season (three bulls), and cultured (SOF, BSA, essential and non-essential amino acids), *in vitro*. Cleavage rate was higher for oocytes collected during breeding vs. non-breeding season (83.72 ± 6.42 vs. 73.98 ± 6.43; $p = 0.004$, Chi-square test), with no significant difference in subsequent development of these cleaved embryos to blastocysts. In summary, epididymal sperm from non-breeding season had decreased total motility and *in vitro* embryo production. Oocytes collected during non-breeding vs. breeding season had reduced ability to be matured, fertilized and/or undergo cleavage *in vitro*. Therefore, we inferred that season influenced quality of gametes in plains bison.

Key Words: Bison, reproductive technologies, sperm, oocyte, reproductive seasonality

0303

Assessment of sperm morphology of tambaqui (*Colossoma macropomum*)

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The tambaqui (*Colossoma macropomum*), is a native fish from Brazil which has great potential for fish farming. It has migratory reproductive patterns and asynchrony between the sexes. Biotechnological conservation of gametes is a good strategy for optimizing the reproductive processes of the species. However, there is currently no standardized protocol for chilling and freezing of semen of this species in captivity. Moreover, it is not known whether such a protocol would result in good fertility. In fish, almost all indicators of semen quality have not yet established minimum standards, making difficult the evaluation of semen quality and selection of breeding stock. Analysis of sperm defects is considered one of the most important criteria for evaluation of sperm quality, they may change fertility rates both in nature, such as chilled or frozen samples. The purpose of the present study was to evaluate the morphological changes of fresh and chilled semen, by light microscopy in stained samples. For this, semen samples were collected from tambaquis aged between four and six years, from a commercial herd located in the Brazilian Amazon, and chilled with dimethyl sulfoxide (DMSO) and dimethylformamide (DMF). The samples were diluted in saline buffered formalin at a ratio of 1:1000, stained with Rose Bengal 3% and evaluated in optical immersion. The classification of defects was discussed and standardized. The average value of abnormalities observed was about 70% in both samples diluted in DMSO and in DMF. The most common defects were the tail (folded and wrapped around the distal end). The next stage of the research will be conducted to assess the same samples using phase contrast microscopy, which reduces the changes under both the preparation of the smear and the use of dyes.

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Key Words: Fish semen, tambaqui, native fish

0350

Presence of MT1 melatonin receptor gene polymorphism in Araucana Creole Sheep

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Melatonin is a hormone that regulates circadian rhythms and many of the reproductive aspects of mammals and is secreted by the pineal gland during the hours of absence of light. This hormone has high affinity receptors coupled to G-like proteins, termed MT1. Some studies show a polymorphism of the sequence coding for these receptors is involved in the control of seasonal reproduction in sheep. Araucana creole sheep is a local breed raised by small holder farmers in southern Chile, which have recorded a short reproductive anestrous and high prolificacy. The purpose of this study was to determine the presence of MT1 receptor polymorphism in Araucana creole ewes and rams. To carry out this work, we used the PCR-RFLP, for which samples were obtained from genomic DNA of 127 Araucana sheep from six flocks, which were digested with the restriction endonuclease Mnl1. It was possible to identify the presence of MT1 receptor polymorphism in Araucana creole sheep. The genotypes were found in a genotype frequency of 66% for genotype +/+, 30% for genotype +/− and 4% for genotype −/ −. These results lead us to conclude that this polymorphism is highly distributed in this flocks and could be associated with short reproductive anestrous.

Key Words: Sheep reproduction, molecular markers, seasonal reproduction

04. Cryobiology

0400

Sequestration of BSP-A1/-A2 minimizes cryoinjury to bull spermatozoa

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The bovine seminal plasma protein (BSP-A1/-A2) present in seminal fluid binds to choline phospholipids of spermatozoa membrane

causing efflux of cholesterol. Continuous efflux of cholesterol leads to destabilization of sperm membrane rendering it susceptible to cryoinjury. This is evidenced as increased percentage of acrosome reacted spermatozoa during freeze-thaw process. Therefore, a study was carried out to assess the effect of sequestration of BSP-A1/-A2 protein on minimization of cryoinjury to crossbred bull spermatozoa. The efficacy of egg yolk used in extender as compared to anti-BSP-A1/-A2 to minimize cryoinjury was also evaluated. Protein was isolated using Heparin-Sepharose affinity and Ion-Exchange chromatography and isolated protein was characterized to raise anti-sera in rabbits. Raised antibodies was quantitated and coated in tubes used for collection of ejaculates. Semen ejaculates thus collected were cryopreserved using Egg Yolk Tris Glycerol (EYTG) extender. Chlortetracycline (CTC) assay was employed to classify spermatozoa in non capacitated or acrosome intact (pattern F), capacitated (pattern B) and acrosome reacted (pattern AR) population as indicators of cryoinjury at pre freeze and post thaw stages. The pre freeze and post thaw cholesterol content (μg/100 million spermatozoa, n = 6) was also estimated which was significantly ($p < 0.05$) higher in antibodies treated group (group III) as compared to group I (Control, routine collection and cryopreservation) and group II (direct collection of ejaculates in EYTG). At pre freeze stage, the CTC assay revealed significantly ($p < 0.01$) higher non capacitated spermatozoa in group III than group II and I (28.83 ± 1.31, 29.50 ± 1.36 and 59.67 ± 1.31% in group I, II and III, respectively, n = 6). At the same time, mean values for pre freeze spermatozoa showing pattern B (46.83 ± 1.37, 51.83 ± 1.57 and 29.33 ± 1.32% in group I, II and III, respectively, n = 6) and AR (24.33 ± 1.36, 18.67 ± 1.90 and 11.0 ± 1.74% in group I, II and III, respectively, n = 6) were significantly ($p < 0.01$) lower in antibodies treated group than other two groups. Similar pattern of distribution in three different groups of spermatozoa was observed at post thaw stage. The post thaw mean values for spermatozoa showing pattern F (14.83 ± 1.29, 16.83 ± 1.30 and 44.50 ± 1.36% in group I, II and III, respectively, n = 6) was significantly ($p < 0.01$) higher whereas pattern B (52.00 ± 1.42, 55.17 ± 1.59 and 41.50 ± 2.1% in group I, II and III, respectively, n = 6) and AR (33.17 ± 1.84, 28.00 ± 1.72 and 24.00 ± 1.74% in group I, II and III, respectively, n = 6) was significantly ($p < 0.05$) lower in group III compared to other two groups. It was evident that per cent increase in acrosome reacted spermatozoa was minimum in antibodies treated group. This study revealed sequestration of BSP-A1/-A2 protein from semen samples significantly minimizes cryoinjury to bull spermatozoa during cryopreservation.

Key Words: BSP-A1/-A2, sequestration, bovine, semen, cryoinjury

0401

Evaluation of lipid composition and localisation with spectrophotometry in single bovine embryos

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Bovine embryos produced *in vitro* are more sensitive to cryopreservation. This is associated with excessive triglyceride and less phospholipid content. Before embryo cryopreservation and transfer, it appears to be very important to have criteria for evaluating the lipid content and composition of individual embryos. The aim of the present study was to localize and measure the lipid content and composition of single bovine embryos at the blastocyst stage by confocal spectral imaging after embryo staining with fluorescent Nile Red dye and to test the reliability of the applied method. Three groups of embryos were produced, (i) *in vivo*, (ii) *in vitro* in basic medium (modified synthetic oviduct fluid, mSOF, supplemented with 5% foetal calf serum), and (iii) *in vitro* on a bovine oviduct epithelial cell (BOEC) monolayer with basic medium. Embryos produced *in vivo* were recovered from four superovulated beef heifers at Day 7.5 post insemination. All blastocysts were fixed, stained with the Nile Red dye and then individually placed between glass coverslips in order to maintain embryo volume before submission to laser excitation. Thousands of complete fluorescence spectra of Nile Red were recorded by scanning, step-by-step, each embryo in its equatorial optical section. The polarity of the lipid environment was visualized as a parametric map based on spectral changes in fluorescence. Spectral data were compared according to embryo culture environment with a Kruskal–Wallis test and a principal component analysis after Arcsin transformation. Forty embryos were analysed, eight produced *in vitro* in basic medium, 11 on BOEC, and 21 *in vivo* (10, 5, 3, 3 from each donor respectively). Homogeneous lipid profiles were established between embryos belonging to the same treatment ($p < 0.05$). According to individual factor maps, embryos produced in basic medium or on BOEC were different. A more polar lipid environment (e.g. phospholipids) was found in embryos produced on BOEC vs. a more apolar lipid environment (e.g. triglycerides) in embryos produced in basic medium. Variability between donors was also observed, one donor produced embryos with a polar lipid environment, one with apolar lipid environment and two with an intermediate lipid environment.

The confocal spectral imaging technique associated with environment-sensitive fluorochrome staining has enabled reliable analysis of the lipid environment of individual bovine embryos, and highlighted the variability between groups of embryos produced, as well as between donors.

Key Words: Lipid profile, individual embryo, confocal spectra imaging, cryopreservation, bovine

0402

Cryopreservation method affects the extent of apoptosis in trophectoderm and the speed of re-expansion in bovine *in vitro* produced blastocysts

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The objective of this study was to investigate re-expansion dynamics, extent and localization of membrane damage, and DNA fragmentation in bovine re-expanded blastocysts during culture after cryopreservation either by slow-freezing or vitrification. Bovine expanded blastocysts were produced by *in vitro* fertilization and culture of slaughterhouse-derived *in vitro* matured oocytes in CR1aa medium supplemented with 5% calf serum (CS) according to Imai et al. (*J Reprod Dev* 2006;52:19–29). Expanded blastocysts were frozen in D-PBS supplemented with 1.5 M ethylene glycol (EG), 0.1 M sucrose (Suc), 20% CS, and 4 mg/ml bovine serum albumin, or vitrified in D-PBS supplemented with 2.2 M EG, 2.7 M dimethyl sulfoxide, 0.5 M Suc, and 20% CS using Cryotop. Warming of vitrified embryos was performed in D-PBS supplemented with 20% CS at 38.5°C. After thawing/warming blastocysts were cultured *in vitro* for 24 h in TCM 199 medium supplemented with 20% fetal bovine serum. Rates of re-expanded embryos were recorded in 4 h intervals. Re-expanded blastocysts were either stained with Hoechst 33 342 and propidium iodide to assay membrane damage of blastomeres or subjected to TUNEL assay combined with differential inner cell mass (ICM) and trophectoderm (TE) staining to study the rate and location of apoptotic cells. Five replications were performed; data were analyzed by ANOVA and Fisher's PLSD test. Frozen embryos showed a significantly lower re-expansion rate during 24 h of post-thawing culture compared to vitrified embryos (49.6%; n = 201 and 86.5%; n = 136, respectively) ($p < 0.05$). Vitrified embryos reached the maximum level of re-expansion rate by 16 h of culture, whereas frozen embryos showed a gradual increase in re-expansion rate until 24 h of culture. There was no significant difference in the rates of membrane damaged cells between re-expanded frozen (n = 27) and vitrified (n = 41) embryos (7.2% and 7.6%, respectively). However, significantly higher ($p < 0.05$) rates of TE cells and total blastomeres showed DNA-fragmentation in frozen embryos (n = 33) compared to vitrified embryos (n = 31) (16.4% and 18.5% vs. 10.1% and 12.2%, respectively). The rates of DNA fragmentation in TE and total cells in non-cryopreserved control embryos (n = 28) (6.3% and 7.9%, respectively) were significantly lower than those in both cryopreserved groups. There was no significant difference in extent of DNA fragmentation in ICM among the groups. These results indicate that slow-freezing is more detrimental to embryo viability, especially to that of TE cells compared with vitrification. Reduced viability of TE cells in frozen-thawed blastocysts is associated with an increased grade of apoptosis and delayed re-expansion.

Key Words: Cryopreservation, apoptosis, IVF, blastocysts, bovine

0403

Bovine embryo culture in the presence of antioxidants: implications for blastocysts development, cryotolerance and levels of intracellular reactive oxygen species (ROS)

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The production of ROS is a normal process that occurs in cellular mitochondrial respiratory chain. However, the increase in intracellular ROS due to the high oxygen tension during *in vitro* culture (IVC) induce oxidative stress, leading to embryonic developmental failure. Addition of antioxidants during IVC appears to increase the resistance of bovine embryos to the oxidative stress and consequently improve cryotolerance. Thus, the aim of this study was to evaluate the effects of intracellular (cysteine and β-mercaptoethanol) and extracellular antioxidants (catalase) during IVC on the embryo development and cryoresistance, as well as the amounts of intracellular ROS produced during embryo culture. *Cumulus* oocyte complexes (n = 1378) were matured and fertilized *in vitro* for 24 h in a humidified incubator at 38.5°C in 5% CO_2 in air. Presumptive zygotes were IVC in Synthetic Oviduct Fluid medium (SOFaa), at 38.5°C in 5% CO_2 in air, with 0.6 mM cysteine (CIST), 100 μM β-mercaptoethanol (βME), 100 IU catalase (CAT) or without antioxidants (Contr) up to 3 days of culture. From day 3 to the end (day 7), all embryos were cultured in SOFaa medium. The cleavage rates and the embryonic development were evaluated, respectively, at 72 and 168 h pos-insemination, when the blastocysts were vitrified (n = 154) (Ingámed®; Maringá-PR, Brasil) or stained (n = 250) with the fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Invitrogen, Oakville, Canada), at 5 μM. The embryos vitrified were thawed and cultured for 24 h to evaluate the re-expansion rates. The stained embryos were imaged in an inverted microscope and analysed by Q-Capture Pro image software to measure the levels of intracellular ROS. The signal intensity values of embryos were subtracted by the average of backgrounds in the images. The cleavage, embryo development and the levels of ROS were analyzed by ANOVA followed by Tukey's test ($p \leq 0.05$), and re-expansion rates by Chi-square test. In terms of percentage of cleavage, no differences were found between the treatments ($p = 0.4174$) and the results were 84.5 ± 2.4% (Contr), 82.5 ± 2.4% (CIST), 79.4 ± 2.4% (βME) and 79.4 ± 2.4% (CAT). The blastocyst frequencies were similar ($p = 0.7529$) and the results were 47.9 ± 2.2% (Contr), 48.5 ± 2.2% (CIST), 43.7 ± 2.2% (βME) and 44.4 ± 2.2% (CAT). The re-expansion rates were not affected by the treatments ($p \geq 0.05$), 76.1% (Contr), 79.5% (CIST), 70.2% (βME) and 68.2% (CAT). The fluorescent intensity were similar ($p \geq 0.05$), and the results were 1.0 ± 0.06 (Contr), 0.9 ± 0.05 (CIST), 0.8 ± 0.04 (βME) and 0.8 ± 0.04 (CAT). H_2DCFDA staining showed a consistent but non-significant ($p > 0.05$) difference in intracellular ROS content between embryos cultured in the presence of antioxidant and control. In conclusion, the supplementation with antioxidants during IVC of bovine embryos did not improve any of the parameters measured.

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Key Words: Antioxidants, intracellular ROS, *in vitro* culture, cryotolerance, bovine embryo

0404

Effects of the use of antioxidants trolox, catalase and glutathione in the extender glycine-yolk-milk with 5% egg yolk on sperm kinetics, membrane integrity and lipoperoxidation in cryopreserved ram semen

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Oxidative damage to sperm resulting from reactive oxygen species (ROS) promotes a biochemical imbalance, which results in decreased sperm motility and sperm membrane injury during the freeze-thawing process. The objective was to determine the effect of antioxidants in an extender with low proportion of egg yolk on the kinetics and functional integrity of sperm membranes and lipid peroxidation in cryopreserved ram semen. Five ejaculates were used from five Dorper rams obtained by artificial vagina. After macro and microscopic evaluation was performed, semen was kept at 32°C, the ejaculate of each ram was diluted to achieve concentrations up to 400×10^6 sperm/ml and divided among the following treatments: glycine-yolk-milk with 5% egg yolk – control (GYM), GYM + trolox, 100 μM/10^8 sperm (GYM-T), GYM + catalase, 12.5 μg/ml (GYM-C) and GYM + reduced Glutathione, 5 mM/100×10^6 sperm (GYM-G). The semen was packaged in 0.25 ml straws, and then samples were cooled (−0.5°C/min) and frozen (−20°C/min) in automated control system. After thawing (40°C/20 s), sperm kinetics were determined using Computer Assisted Sperm Analysis (CASA), and overall integrity of sperm membranes was assessed by the combination of fluorochromes propidium iodide and *pisum sativum* agglutinin conjugated to fluorescein isothiocyanate in flow cytometry. The quantification of lipid peroxidation was determined by UV-VIS, double beam spectrophotometer method of thiobarbituric acid-reactive substances. Data was analyzed by ANOVA followed by Tukey′ test ($p < 0.05$). Neither total nor progressive sperm motility differed between treatments (GYM: 52.5/38.8%; GYM-T: 56.0/42.6% GYM-C: 54.2/39.9% and GYM-G: 50.4/35.2%) ($p > 0.05$). The percentage of sperm with intact plasma membrane and unreacted acrosome in GYM-C (35.9%) was similar to GYM-T (33.3%) ($p > 0.05$) and higher than GYM (30.1%) and GYM-G (26.9%) ($p < 0.05$), while results of GYM-T and GYM did not differ ($p > 0.05$). The levels of spontaneous lipid peroxidation in treatments GYM (3.9 nmol/10^8 sperm), GYM-T (3.7 nmol/10^8 sperm), GYM-C (3.6 nmol/10^8 sperm) and GYM-G (3.5 nmol/10^8 sperm) did not differ ($p > 0.05$). In the lipid peroxidation catalyzed by $FeSO_4$ and sodium ascorbate in the reaction medium, we observed an increased generation of TBARS in treatments GYM (4.9 nmol/10^8 sperm), GYM-C (4.9 nmol/10^8 sperm) and GYM-G (4.5 nmol/10^8 sperm), except in GYM-T (3.5 nmol/10^8 sperm) ($p < 0.05$), demonstrating the ability of trolox to inhibit the process of lipid peroxidation by scavenging peroxyl radicals, interrupting the chain reaction of lipid peroxidation. The antioxidants trolox and catalase, when added to the extender Glycine-yolk-milk with 5% egg yolk, improved the integrity of sperm membranes. Trolox appers to be able to control lipid peroxidation of cryopreserved ram spermatozoa.

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Key Words: Semen, ram, cryopreservation, antioxidants

0405

Gelatin in the cooling medium of ram semen

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It has been reported that the gelatinization of the diluents might standardize the medium, prevent sedimentation of spermatozoa during semen storage, protects the sperm from cold shock, and decreases the energy expenditure of the sperm when compared to liquid diluent.

Other positive results that have been attributed to the medium gelatinization is the limits the reflux of the semen to the vagina, favoring cervical and transcervical insemination. The objective of this study was to evaluate the quality of cooled ram semen diluted in medium with gelatin. Four ejaculates from six Santa Inês rams were collected and diluted in a Glycine–Yolk –Milk-based medium (GYM), with and without addition of 1.5% gelatin type B. The semen was stored in 0.25 ml straws containing 100×10^6 spermatozoa subjected to cooling for 48 and 72 h at 5°C, until the assessment *in vitro* and *in vivo* by artificial insemination. One hundred and eighty two Santa Inês ewes were randomly distributed into four experimental groups ($n \asymp 46$ females/group), in such a way that each donor ram ($n = 6$) inseminated approximately a similar number of females in each group ($n \asymp 15$ females/male/group) with their cooled semen depending on the type of diluent used (Control – GYM without gelatin; and Treatment – GYM × 1.5% gelatin) or period of cooling (48 and 72 h). The ewes were subjected to a protocol of estrus synchronization and submitted to fixed–time artificial insemination (FTAI) using the transcervical technique to determine the pregnancy rate. The normal data were subjected to ANOVA by the F test and the non–normal data to the Wilcoxon test. The pregnancy rate was analyzed by the chi-square test. In semen samples refrigerated for 48 h, the gelatin-treated group showed better ($p < 0.05$) integrity of plasma and acrosomal membranes measured by propidium iodide and carboxyfluorescein diacetate: 77.3 vs. 72.4% and 85.6 vs. 81.9%, respectively. However, in semen samples cooled for 48 h, the gelatin–treated group showed lower values ($p < 0.05$) of some sperm kinetic parameters compared to control, respectively: progressive motility – PM: 46.2 vs. 58.0%, straight–line velocity – VSL: 172.7 vs. 197.8 μm/s; average path velocity – VAP: 229.3 vs. 249.5 μm/s; straightness – STR: 75.4 vs. 79.3%: and flagellar beat–cross frequency – BCF: 51.1 vs. 53.7 Hz. In semen cooled for 72 h, the gelatin–treated group showed lower values ($p < 0.05$) of some sperm kinetic parameters compared to control: total motility – TM: 92.9 vs. 96.6%; PM: 41.9 vs. 49.6%; and BCF: 52.7 vs. 55.4 Hz. The pregnancy rates of ewes inseminated with semen treated with gelatin and control showed no differences ($p > 0.05$) in semen cooled for 48 h (26.1 vs. 11.1%) and 72 h (4.4 vs. 13.1%), respectively. The fertility results were not affected by the apparent disadvantages caused by gelatin to the sperm kinetics. The addition of gelatin improves some aspects of the sperm integrity however does not improve the FTAI fertility of ram semen cooled for 48 h. The addition of gelatin did not bring benefits to the ram semen cooled for 72 h.

Key Words: Diluent, gelatinization, sperm integrity, sperm kinetic, artificial insemination

0406

Custom microarray analysis of gene expression of ovine oocytes subjected to vitrification

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Even if structural and morphological damages induced by cryopreservation in gametes and embryos have been extensively investigated, the information available on the molecular events is still limited. Aim of this work was to evaluate the effects of vitrification procedures on the transcriptome (mRNA levels) of ovine oocytes using a customized ovine cDNA microarray. The analysis was performed on vitrified-warmed *in vitro* matured oocytes (VTR) and on control oocytes (CTR) subjected only to *in vitro* maturation. Abbattoir–derived oocytes were matured *in vitro* in standard conditions. IVM oocytes were randomly divided in two classes and (i) subjected to vitrification procedures, or (ii) collected for gene expression analysis (added to 2 μl diethylpyrocarbonate (DEPC) treated water, snap frozen in liquid nitrogen and stored at −80°C). Vitrification was performed with the minimum essential volume method using cryotop as device (Kuwayama et al., 2005). Briefly, oocytes were cultured in holding medium (HM; 20 mM Hepes-buffered TCM-199 with 20% FCS) with growing concentration of cryoprotectants [10% ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO) for 30 s and successively 20% EG and 20% DMSO for 20 s], loaded on cryotop and immediately plunged into liquid nitrogen for storage. Warming was performed plunging the cryotops directly into HM supplemented with 1.25 M sucrose and then in solutions with decreasing sucrose concentration (0.62, 0.31 M) for 30 s. Oocytes were washed in HM and collected for gene expression analysis after 2 h culture. We generated a medium density microarray based on Illumina DASL technology, comprising 1536 independent assays addressing 1039 transcripts. Sequences were selected in the OAGI (Ovis Aries Gene Index) database of the Dana-Farber Cancer Institute Gene Index Gene Ontology (GO) section (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=sheep). The selected transcripts are relevant to several cell functions such as transcription, cell cycle regulation, signal transduction and immune response. VTR (nine groups of 20 oocytes) and IVM (three groups of 20 oocytes) oocytes were subjected to mRNA isolation with Dynal microbeads kit (Oslo, Norway) and subsequently hybridized to the customized microarray. Comparison of mRNA content between VTR and CTR oocytes revealed 29 differentially expressed genes, mainly down-regulated (25 transcripts) after vitrification ($p < 0.01$). Microarray analysis results were validated by quantitative reverse transcription Real Time PCR of six selected transcripts. This study highlights alterations in the transcriptome of ovine oocytes as a consequence of vitrification procedures. The identification of the molecular mechanisms involved in the oocyte reaction to cryopreservation is an important step for the improvement of the experimental procedures.

Key Words: Oocyte, cryopreservation, ovine, gene expression, microarray

0407

Use of collagenase type-1 to Improve the seminal characteristics of dromedary camel (*Camelus dromedarius*) semen

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Camel semen evaluation is difficult due to the presence of a thick gel. The objective of this study was to evaluate the effect of addition of collagenase type-1 enzyme on the physical and functional properties of camel semen. A total of forty semen ejaculates were collected during the breeding season from eight different stud camels using artificial vagina. All ejaculates were split into three equal aliquots. One aliquot was kept as control (A1) and two of these were diluted with Tris buffer media in 1:1 with (A3) or without (A2) addition of 0.1% collagenase type-1 enzyme (for 20 min) and evaluated for macroscopic and microscopic semen characteristics. All aliquots were pipetted to observe the macroscopic examination (Consistency and rheological properties). Aliquot (A3) did not form thread when pipetted and showed thin watery consistency while the other two aliquots (A1 and A2) did evidence thick viscid, thick and thin watery consistency in different proportions. Only aliquot (A3) showed initial individual sperm motility and functional activity (HOST) curled tailed spermatozoa with overall average over 70%. There were significant differences ($p < 0.01$) between all the aliquots for sperm motility and sperm with functional membrane where as non-significant differences ($p > 0.01$) were observed between all aliquots for live spermatozoa and sperm abnormalities percentage. An overall mean of sperm concentration in the camel semen treated with collagenase enzyme was 331.75 ± 13.71 million/ml. The results showed that treating semen with 0.1% collagenase in Tris buffer media improves semen macroscopic and microscopic seminal characteristics and also facilitates the separation of spermatozoa from thick gel of the seminal plasma in dromedary camel semen.

Key Words: Camel, collagenase type-1, semen, tris-buffer, motility

0408

Evaluation of two cryopreservation systems for asinine sperm

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Nevertheless some asinine breeds are considered endangered. The use of frozen semen is a powerful tool to help not only the conservation of species, but also the expansion of herds. However the extremely low fertility impede the widespread use of this technology. The present study aimed to comparer the styrofoam box (G1) and a programmable machine (G2) for freezing donkey semen. It was used 16 sperm samples from eight donkeys (each of them provided two samples). Samples were diluted (1:1) in a milk base extender and centrifuged at $600 \times g$ for 10 min. Pellets were resuspended in Botucrio extender (Botupharma, Brazil), in to a final concentration of 200 million sperm cells/ml and loaded into 0.5 ml straws. All straws were labeled with the name of the corresponding stallion, book registration number, and freezing protocol. The samples from the machine group were cooled at a cooling rate of 0.5°C/min from room temperature to 5°C. For G1 the loaded straws were kept at 5°C for 20 min in a commercial refrigerator (Minitub, Germany). Then straws were frozen in accordance with each group protocol: for G1 straws were placed on a metal frame and inserted into the styrofoam box containing liquid nitrogen and frozen in nitrogen vapor (6 cm height from liquid nitrogen) during 20 min, providing a freezing rate of 10°C/min from 5 to −60°C and a freezing rate of 8°C/min until – 120°C; for the machine group (G2) samples were placed inside the machine and frozen at a freezing rate of 15°C/min between 5 and −10°C, and then 40°C/min untill −140°C. After freezing, samples were plunged in liquid nitrogen. Data were analysed by ANOVA. The computer-assisted sperm analysis assessed the following parameters: total motility (TM), progressive motility (PM), angular path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), percentage of rapid cells (RAP). Plasma membrane integrity (PMI) was assessed using fluorescent probes. There was no statistical difference ($p > 0.05$) for average values of the following parameters for G1 and G2, respectively: TM (64.3% and 65.3%), VAP (83.9% and 84.4%), VSL (158.6 and 161.9) and RAP (52.1% and 52.1%), PMI (42.9% and 43.1%). However, PM (39.9% and 44.5%) and RAP (48.3% and 50.9%) values were higher ($p < 0.05$) in G2 compared with G1. This is the first study assessing different methods and freezing rate for asinine sperm. We have noticed that despite the fact that several parameters displayed similar results, the machine improved ($p < 0.05$) PM and RAP, suggesting that for donkey sêmen a quicker freezing rate is beneficial. In conclusion we can affirm that automatic machines shall contribute to the improvement of asinine sperm cryopreservation.

Key Words: Semen, frozen, donkey, freezing rates, asinine

0409

Spatial temporal relationship of mitochondria and lipid droplets of swine embryos may reflect successful embryo cryopreservation for gene bank conservation

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Pig pre-implantation embryonic development differs from other species. Domestic ungulates have an extended pre-implantation developmental period and the formation of a non-invasive epitheliochorial placentation in contrast to the invasive hemo-choreal placenta characteristic of man and mouse. The increasing focus on the pig as a biomedical model raises the need for studies investigating morphological and molecular mechanisms during the pre-implantation embryo development in this species. The objective of this study was to characterize the ultrastructure of lipid droplets and mitochondria in Piau (fatty pig breed nearing extinction) embryos with the intention of correlating these observations with success following cryopreservation. Twelve grade 1 blastocysts from 12 different Piau gilts mated with a Piau boar were recovered 6 days after the first mating. Embryos were prepared for transmission electron microscopy. Briefly, they were fixed in 0.1 M Na-phosphate buffer (pH 7.3) containing 3% glutaraldehyde at room temperature for 1 h and then stored at 4°C in Na-phosphate buffer for later routine transmission electron microscopy. Ultrathin sections were examined on a Zeiss EM 109 and CM 100 Philips transmission electron microscope. Seven to 10 random micrographs were taken from the trophoblast and inner cell mass of each embryo. All embryos were examined at magnifications ranging from 600× to 25 000×. All micrographs showed the presence of small rounded mitochondria and low numbers of lipids droplets in embryo cell cytoplasm. Mitochondria had an electron translucent, filamentous appearance and possessed many lamellar cristae. Moreover, mitochondria and myelinic figures were observed around the low number of lipids droplets. The smooth endoplasmic reticulum formed a spatial network next to mitochondria and lipid droplets. Myelinic figures contained lipid droplets and homogeneous material similar to organelle membranes. We hypothesized that pig oocytes and embryos may use intracellular lipids as an energy source and that most of the ATP produced during embryo development comes from oxidative phosphorylation. As it would appear that the number of lipid droplets in embryo cells in this breed is less than that reported for other swine breeds, we speculate that there may be a high rate of embryo survival after embryo cryopreservation. Research should now focus on the cryopreservation of embryos from the Piau breed which would have immense benefit in establishing an embryo gene bank for this critically endangered swine breed. This is the first report of the ultrastructure of embryos from this fat type native pig breed; this knowledge will be important for the development of embryo cryopreservation studies aimed at this species.

Key Words: Cryopreservation, endagered breed, fatty pig, swine embryo morphology, ultrastructural

0410

The effect of homologous prostatic fluid on the parameters of dog semen extended and frozen in Tris-egg yolk and INRA Freeze: preliminary results

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Recent studies showed that the cryoprotector component of egg yolk for the freezing of canine semen is represented by the low density lipoproteins (LDL), while the others components of egg yolk have a number of detrimental effects on the frozen-thawed canine semen. The aim of this study was to compare the effect of a LDL commercial extender used for stallion semen – INRA Freeze® (IMV Technologies, L'Aigle, France) with a standard extender for dog semen containing Tris, fructose, glycerol and 20% egg yolk (TFG-EY). Since there are some conflicting reports concerning the effect of prostatic fluid on sperm function, we also investigated if the effect of post-thaw dilution with autologous prostatic fluid on viability, motility and acrosome status of frozen-thawed canine spermatozoa is correlated with the composition of the extender. Semen was collected from six Beagle dogs and each sperm rich fraction was divided in two parts: one was diluted with INRA Freeze® and the other with TFG-EY and frozen with the same protocol. The third fraction of each ejaculate was collected separately, centrifuged at $1118 \times g$ for 10 min and the supernatant was frozen at −18°C until use. For each extender, two straws were thawed: one straw was diluted 1:2 with autologous prostatic fluid, while the other was not diluted at all. Motility (CASA, IVOS; Hamilton Thorne, USA), viability and acrosome status (Guava EasyCyte® flow cytometer; IMV Technologies, L'Aigle, France), morphology (Diff-Quick stain) were assessed at 5 min, 1 h and 2 h post-thaw (T0, T1, T2). Statistical analyses were performed with IBM SPSS software (ver. 19 for Windows; IBM, New York, NY, USA). The results are presented as mean values and a p value <0.05 was considered statistically significant. Viability, total and progressive motility and the percentage

of intact acrosomes were higher ($p < 0.05$) for the TFG-EY group with or without the addition of the prostatic fluid. The results for motility in the INRA Freeze group were dramatically low compared to the TFG-EY group. There were no significant differences regarding the morphology of fresh and frozen semen for any of the four groups. Addition of prostatic fluid for the TFG-EY group significantly reduced the total and progressive motility at T0, T1 and T2 ($p < 0.05$), but showed no influence for the INRA Freeze group. This data show that the effect of prostatic fluid regarding motility differs between the two extenders. This may illustrate either an inhibitory effect of some components of the INRA Freeze® on the noxious effects of the prostatic fluid or conversely an enhancing effect of the TFG-EY on those. Alternatively, it could be that low values obtained with INRA Freeze® did not permit highlighting the effects of prostatic fluid.

Key Words: Dog semen, cryopreservation, prostatic fluid

Table 1. Mean values for viability, progressive motility and acrosome status for the TFG-EY and INRA groups

Post-thaw	T0	T1	T2
TFG-EY			
Viability (%) (V)	81.75	70.70	67.85
Progressive motility (%) (PM)	75.87	50.05	27.75
Acrosome intact (%) (AI)	84.98	78.14	77.46
TFG-EY + prostatic fluid (PF)			
V (%)	77.31	74.18	72.50
PM (%)	52.78	18.58	16.80
AI (%)	83.31	82.56	69.81
INRA			
V (%)	48.53	40.62	40.06
PM (%)	15.97	6.33	3.10
INRA + PF			
AI (%)	77.16	61.35	43.31
V (%)	45.25	33.41	32.12
PM (%)	14.03	5.22	2.72
AI (%)	88.09	74.32	88.29

0411

Use of freezing machine (TK3000) on cryopreservation of dog epididymal sperm

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Sperm freezing can cause irreversible damage to sperm membranes and this can interfere with cell viability and fertility. The aim of this study was to evaluate the effect of compact automatic freezers (TK 3000 SE compacta, TK Equipamentos para Reprodução, Uberaba, MG, Brazil) and traditional method to freeze the semen on epididymal sperm viability. Epididymides were obtained from 13 adult dogs by elective orchiectomy. After surgery, the testis/epididymis were kept at 5°C for 24 h in saline solution. The tail of epididymis was dissected and squeezed toward the vas deferens with a clamp into a Petri dish containing Ringer's solution. Immediately after collection, sperm from all dogs was pooled and evaluated for sperm motility and concentration. The pooled sperm suspension were divided into 12 samples and centrifuged at $800 \times g$ for 10 min. Supernatant was removed and the pellet was resuspended in one step, with three extenders (Bovimix®, BotuCrio® and one with Tris/yolk/glycerol). The extended sperm was loaded into 0.5 ml French straws containing 80×10^6 sperm/straw, at room temperature. Pairs of samples from each extender were frozen by either the traditional or automated method. In the traditional method the straws were equilibrated at 5°C for 1 h and frozen in nitrogen vapor for 20 min and stored at −196°C. In the automated method, straws were placed in a compact freezing apparatus (TK 3000 SE Compacta) and frozen using a curve for stallions (curve Eq P2S1). After were stored in cryogenic container for a month, straws were thawed at 56°C for 10 s. Thawed sperm samples were evaluated for motility by CASA (Computer Assisted Sperm Analyzer; Hamilton-Thorne IVOS, Beverly, MA, USA). Statistical analysis was performed by the program Sigma Stat for Windows, version 11.0, 2008. Values were considered significant at $p \leq 0.05$. The fresh sperm samples showed mean of 80% motility and 85% of membrane integrity. After thawing, the results, mean ± SD obtained in samples frozen in traditional method vs. compact freezing apparatus were: motility 57.2 ± 10.1% vs. 60.2 ± 8.2% and progressive motility 27.5 ± 8.6% vs. 32.0 ± 10.6%. The sperm velocity parameters are described in Table 1. There were no statistic diference between the two freezing methods ($p \geq 0.05$), we conclude that the automatic machine TK3000 SE Compact can be used to freeze dog epididymal sperm.

Key Words: Sperm freezing, canine, epididymal, automatic freezers, CASA

Table 1. Mean ± standard deviation of velocity parameters sperm canine epididymides, evaluated with CASA system, after frozen in tradition or automated method and thawed. Londrina-PR, Brazil, 2011

Methods/ parameters	VAP	VSL	VCL	ALH	BCF	STR	LIN
Traditional	79.2 ± 6.3	66.0 ± 4.7	113.8 ± 10.8	5.9 ± 0.4	14.2 ± 2.5	83.5 ± 1.6	60.7 ± 3.0
TK 3000	83.9 ± 8.2	69.7 ± 7.7	121.6 ± 10.0	6.1 ± 0.6	13.7 ± 2.2	82.5 ± 2.1	59.8 ± 5.6

0450

Osmotic reaction of Jersey dairy cattle embryos exposed to various cryoprotective additives

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Retrospective analysis of data from commercial embryo transfer companies previously revealed a reduced pregnancy rate following transfer of frozen-thawed embryos from Jersey compared with other breeds of dairy cattle (Steel and Hasler, 2004, Reprod. Fertil. Dev. 16:182; Kieler, 2008, Proc. AETA Ann. Conf., p. 49). Although increased lipid content of Jersey embryos has been implicated as a cause (Pegoraro et al., *Reprod Fertil Dev* 2004;16:268), the exact biological reason for the reduced cryotolerance of Jersey embryos has not yet been elucidated. The objective of this study was to study the permeability of Jersey embryos to various cryoprotective additives (CPAs). We hypothesized that inadequate dehydration of the high lipid content Jersey embryos prior to freezing may contribute to their lower post-thaw survival. Thirty *in vivo* derived Jersey compact morula to blastocyst stage quality grade 1 and 2 embryos were exposed for 10 min at room temperature to a 2.0 M concentration of one of four different CPAs: ethylene glycol (EG; n = 8), glycerol (GLYC; n = 8), propylene glycol (PG; n = 7), or dimethylsulfoxide (DMSO; n = 7). Photomicrographs obtained at Time 0 (in embryo holding medium; ViGro, Bioniche) and at 1-min intervals thereafter (in CPA) were subjected to computerized image analysis to monitor volume changes (total of 11 images per embryo). Data were analyzed by JMP, and means are expressed as mean ± SD. Average embryo volumes, expressed as a percentage of Time 0 volume, were 93.8 ± 7.3 (EG), 75.6 ± 7.6 (PG), 75.2 ± 11.6 (GLYC), and 60.6 ± 6.9 (DMSO). Minimum relative embryo volumes during the 10-min pre-freeze equilibration period were 93.6 ± 7.2 (EG), 74.7 ± 6.8 (PG), 75.0 ± 11.4 (GLYC), and 59.8 ± 5.9 (DMSO). Results revealed that EG permeated Jersey embryos more easily ($p < 0.05$) than did DMSO, with GLYC and PG being intermediate. Each of the four CPAs investigated in this study permeated Jersey dairy cattle embryos, and results suggest that inadequate embryo dehydration prior to slow freezing does not contribute substantially to reduced post-thaw viability of cryopreserved Jersey dairy cattle embryos. To the best of our knowledge, these data are the first to be published concerning osmotic reaction of Jersey dairy cattle embryos to various CPAs. (Donation of Jersey cattle by Eric Lyon and partial funding from the AJCC Research Foundation are gratefully acknowledged.)

Key Words: Cryopreservation, embryo, Jersey, cattle, osmosis

0451

Aloe vera sp. is an acceptable alternative to egg yolk for preserving goat semen at 4°C

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Diluents containing egg yolk are the most practical for preserving semen in low temperatures. However, due to the recent requirements for disease control and security with biological products, it has been suggested that animal products be eliminated from diluents used for semen conservation. Though a few studies have been performed on the effect of *Aloe vera* sp. in ram semen, to date *in vitro* evaluation of goat semen after cooling with use of *Aloe vera* sp. has not been studied. Therefore, this study assessed the effect of 5 or 10% (wt/vol) *Aloe vera* sp. (T4 and T5) or 5% or 10% (vol/vol) egg yolk (T2 and T3) in a coconut water powder extender (ACP-101®) for preservation of goat semen at 4°C. The control group (T1) received only ACP-101®. For this, a pool of ejaculates from four male goats (one ejaculate per goat) was used. Kinetic parameters and sperm viability were assessed using a computer-assisted sperm analyzer (CASA; SCA®, Microptics SL, Spain) and an Eosin-nigrosine test, respectively, at 0 h (fresh and diluted semen before cooling) and at 6, 12, 24 and 48 h after cooling. The best results in terms of total motility was observed for 5% egg yolk at 0 and 6 h ($p < 0.05$). Nevertheless, from 24 to 48 h of evaluation, 5% *Aloe vera* sp. revealed the best average values for total motility (70.3 ± 4.4% and 71.6 ± 4.1%, respectively) when compared to the other treatments ($p < 0.05$). In relation to progressive motility, during the first 24 h, treatments with *Aloe vera* sp. were similar to control group but presented significant higher averages values when compared to treatments with egg yolk ($p < 0.05$). Regarding kinetic parameters (curvilinear velocity, VCL; linear velocity, VSL; mean velocity, VAP, and linear coefficient, LIN), it was observed that treatments with 5% *Aloe vera* sp. and 5% egg yolk and the control group had the best values when compared to the other treatments for the first 24 h of storage ($p < 0.05$). However, from this time to the end of the study, only treatment with 5% *Aloe vera* sp. maintained their values ($p < 0.05$). Regarding sperm viability, at 24 and 48 h, significantly higher percentages values were observed in 5% (63.8 ± 6.2% and 60 ± 5.7%) and 10% *Aloe vera* sp. (54.58 ± 6.85 and 55 ± 4.59) when compared to 5% (37.3 ± 8.6% and 21.6 ± 7.8%) or 10% egg yolk (37.0 ± 8.1% and 22.5 ± 8.8%) ($p < 0.05$). To our knowledge, this is the first report regarding the use of *Aloe vera* sp. as a substitution for egg yolk in water coconut powder extender (ACP-101®) for preserving chilled goat semen. According to our results, we suggest that the optimal concentration of *Aloe vera* sp. is 5% in the ACP-101® extender. In addition, we conclude that *Aloe vera* sp. can be used as a substitute for egg yolk in ACP-101® extender for preserving goat semen for 48 h at 4°C.

Key Words: *Aloe vera* sp., cooling, semen, CASA, goat

0452

Vitrification of two-cell mouse embryos has no effect on gene expression and histone marks of *H19*, *Igf2* and *Mest* genes in blastocyst stage

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Vitrification of the embryos is a routine procedure in many IVF labs. However the impact of embryo vitrification on epigenetic changes has not been evaluated. Allele–specific DNA methylation and chromatin composition are two epigenetic modification systems that control gene expression. *H19*, *Igf2* and *Mest* are imprinting genes that are sensitive to any changes in culture conditions. The effect of vitrification on gene expression and some modifications of H3 histone in *H19*, *Igf2* and *Mest* genes in blastocysts resulted from non-vitrified and vitrified 2-cell embryos was investigated in the present study. Non-vitrified (group1) and Vitrified (group2), 2-cell embryos obtained from superovulated NMRI mice were cultured in KSOM medium into blastocyst stage. Vitrification was carried out in two steps, 1– equilibration (7.5% ethylene glycol, 7.5% dimethyl sulphoxide in Hams F10 supplemented with 20% HSA for 7 min). 2-vitrification (15% EG, 15% DMSO, 0.5 M sucrose in 20% Hams F10 for 60 s). *H19*, *Igf2* and *Mest* expressions compared between the two experimental group as well as to those of *in vivo*-derived blastocyst (Control). Also some modifications of H3 histone (H3K9me2, H3k4me3 and H3K9ac) in regulatory regions of the genes were compared with control group. Real-Time PCR (35 embryos/group) and ChIP assays (75 embryos/group) were carried out for gene expression and histone modifications respectively. Thirty-five embryos were used for gene expression analysis and 75 embryos were used for assessing histone modification. Data analysis was performed using Kruskal–Wallis test. The results of this study showed that the expression level of observed imprinting genes were increased significantly in all experimental groups in comparison to control group ($p \leq 0.05$). H3K9me2 ($p \leq 0.05$) decreased while H3K9ac ($p \leq 0.05$) increased in the experimental group compared with the control group. There was no significant difference in H3k4me3 mark between experimental and control groups. Also no significant difference was seen between groups 1 and 2 in expression level of the genes and the histone marks. Our results demonstrate that vitrification and culture condition lead to changes in expression level and modification of histone in *H19*, *Igf2* and *Mest* genes but these changes were not different between vitrified and non vitrified groups. It seems that vitrification has no additional effect on these histone marks and genes and can be considered as a safe method in ART and clinical outcomes.

Key Words: Vitrification, histone modification, *H19*, *Igf2*, *Mest*

05. Cryopreservation of oocytes & embryos:

0500

Pregnancy rates following transfer of bovine embryos crypreserved in ethylene glycol for direct transfer or vitrified using Cryotops

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Several ultrarapid vitrification methods, such as Cryotop (Kitazato BioPharma, Japan) have been developed to increase survivability of embryos. The objective of this study was to evaluate the practicability of vitrification with cryotops for bovine embryos under on-farm conditions, and to compare pregnancy rates with those frozen in ethylene glycol for Direct Transfer. Embryos ranging from compacted morula to blastocyst stages were recovered from superovulated Holstein (Hol) and Japanese Black (JB) donors 7 days after estrus. Embryos were cryopreserved by vitrification or slow, controlled freezing in ethylene glycol. Vitrification was performed using the Cryotop device which is constructed with a fine, thin sheet attached to a plastic handle. Embryos were exposed to an equilibration solution containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in holding medium (HM; 20% calf serum in TCM-199) for 3–4 min and were then equilibrated for 1 min in vitrification solution containing 15% EG, 15% DMSO, and 0.5 M sucrose in HM. Embryos were loaded onto a cryotop and vitrified by plunging in liquid nitrogen. Vitrified embryos were warmed in HM containing 1.0 M sucrose for 1 min, transferred into HM containing 0.5 M sucrose for 3 min, and then transferred into HM for 10 min. The warming and dilution were done on at 38°C. Each embryo was loaded into a 0.25-ml straw with PBS containing 20% fetal calf serum and transferred nonsurgically to synchronous recipients. Slow, controlled freezing was performed with EG. Embryos were equilibrated in 1.5 M EG and 0.1 M sucrose in HM for 15–20 min. Each embryo was loaded into a 0.25-ml straw with freezing medium. The straws were placed directly into a precooled chamber of a programmable freezer at −7°C. After 2 min, the straws were seeded, maintained at −7°C for another 13 min, and then cooled to −30°C at 0.3°C/min before being plunged into liquid nitrogen. Straws were

thawed by holding in air for 8 s, followed by plunging into a 30°C water bath. The frozen-thawed embryos were transferred to synchronous recipients by Direct Transfer. Pregnancy was diagnosed by ultrasonography 30 days after estrus. The pregnancy rates were compared by chi-square test. The pregnancy rates did not differ between vitrification (63.2%; 84/133) and Direct Transfer (55.5%; 949/1710). There was also no significant differences in pregnancy rate between donor breeds (Direct Transfer, Hol: 50.4%, 59/117 and JB: 55.9%, 890/1593; vitrification, Hol: 59.0%, 36/61 and JB: 66.7%, 48/72). However, pregnancy rate after cryoprservation was affected by class of recipient (Direct Transfer, heifers: 59.8%, 735/1230 and cows: 44.6%, 214/480, $p < 0.01$; vitrification, heifers: 73.7%, 42/57 and cows: 55.3%, 42/76; $p < 0.05$). Results demonstrate that Cryotop vitrification can be used under on-farm conditions, and that pregnancy rates are likely to be at least as high as with Direct Transfer in ethylene glycol.

Key Words: Vitrification, Cryotop, embryo, pregnancy rates, direct transfer

0501

Supplementation of maturation medium with L-carnitine improves developmental competence of *in vitro* matured bovine oocytes after vitrification

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The efficiency of embryo development after vitrification of bovine oocytes is still unsatisfying. A large amount of cytoplasmic lipids contribute to the low cryotolerance of oocytes. L-carnitine (LC), an element related to lipid metabolism, is a substance with the potential to reduce the amount of lipids in the ooplasm during *in vitro* maturation (IVM). This study examined the effect of LC during IVM on maturation, cryotolerance and developmental competence of oocytes. COCs were subjected to IVM in the absence (control; n = 790) or presence of 0.6 mg/ml LC (LC group; n = 764). After IVM some COCs were vitrified by Cryotop (Sripunya et al., *J Reprod Dev* 2010;56:176–81). *In vitro* fertilization and embryo culture were performed according to Imai et al. (*J Reprod Dev* 2006;52:19–29). Live oocytes were evaluated post IVF on the basis of the integrity of zona pellucida and oolemma. Cleavage and blastocyst rates were recorded on Days 2, and 8, respectively. Also, ATP content in IVM oocytes was measured by an ATP assay kit (Sigma), and intracellular lipid droplets were observed by Nile red staining and confocal microscopy, and then were evaluated using NIH Image J (v.1.40) software. Data were analyzed by GLM-procedure. LC did not improve the maturation rate of oocytes compared with control group. Vitrification similarly reduced ($p < 0.05$) the rates of live oocytes both in control (80.6 ± 12.5%) and LC groups (82.7 ± 4.6%) compared to non vitrification groups. After IVF, cleavage rates of the surviving oocytes in vitrified control and LC groups (56.5 ± 9.6% and 62.8 ± 12.6%) were lower ($p < 0.05$) than those in non-vitrified control and LC groups (83.9 ± 10.4% and 84.3 ± 3.3%). After vitrification, blastocyst formation rate in the LC group was higher ($p < 0.05$) compared with the control group (54.4 ± 12.6% and 34.9 ± 10.9%), but did not differ from those in non-vitrified control and LC groups (52.1 ± 10.3% and 52.8 ± 7.3%). ATP levels in non-vitrified and vitrified oocytes tended to be higher in the LC groups compared with control (2.33 ± 0.2 vs. 2.14 ± 0.3 pmol; $p = 0.082$ and 2.24 ± 0.3 vs. 2.11 ± 0.1 pmol; $p = 0.091$, respectively). The density of lipid droplets was not different between control and LC groups. However, LC treatment affected the location of lipid droplets in oocytes; in LC group lipid droplets were located throughout the cytoplasm whereas in control lipid droplets were mainly localized in the peripheral area. Conclusion, LC supplementation during IVM improved developmental ability of oocytes after vitrification but not of non-vitrified ones. Improved oocyte cryotolerance by LC treatment might be related to dislocation of lipid droplets from plasma membrane. Further research will be necessary to clarify the exact cellular processes of LC treatment.

Key Words: Vitrification, oocytes, L-carnitine, embryo development, bovine

0502

Comparison of vitrification and slow controlled freezing protocols for the cryopreservation of bovine blastocysts

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In vitro-produced bovine embryos are more sensitive to cryoinjury than those produced *in vivo*. This study compared the effectiveness of a slow, controlled freezing and a vitrification protocol for the cryopreservation of *in vitro*-derived bovine embryos. Ovaries of Nelore cows were obtained from a slaughter house. Cumulus oocyte complexes (COCs) were collected from follicles ≥ 4 mm in diameter. COCs were matured in TCM-199 and fertilized with frozen thawed Nelore bull semen (IVF = Day 0). On Day 1, presumptive zygotes were cultured with SOFaa supplemented with fetal bovine serum (FBS) until Day 7. Slow, controlled freezing of blastocyst stage embryos was performed with 1.5 M ethylene glycol (EG) in Dulbecco's PBS (PBS). Embryos (n = 205) were loaded into 0.25 ml straws with the freezing medium. The straws were seeded at −6°C, maintained for 5 min, and then cooled at 1.2°C/min to −31°C and plunged into liquid nitrogen. Vitrification was performed using a tiny hook (WTA®; Cravinhos, SP, Brazil) as a carrier device. Embryos (n = 186) were equilibrated in 500 µl of solution 1 (Achilles Genetics Vitrification Kit; Achilles Genetics®; Garça, SP, Brazil) at room temperature for 2 min. Then, embryos were transferred into a 7 µl drop of Solution 2 (Achilles Genetics Vitrification kit) at room temperature for 30 s, placed on a vitrification hook and immersed into liquid nitrogen. Slow frozen embryos were thawed by holding the straws at 24°C in air for 7 s and then placed in a waterbath at 25°C for 20 s. Vitrified embryos were warmed by holding the vitrification hook in air at 24°C for 7 s. Then freezing or vitrification media were removed from embryos by washing them in a mixture of 0.5 M trehalose and 0.5 M sucrose, 0.1 mM β-mercaptoethanol for 3 min followed by 0.25 M trehalose and 0.25 M sucrose in PBS for 3 min. Some of embryos (slow freezing n = 119 and vitrification n = 21, respectively) were subsequently cultured in SOFaa supplemented with 5% FBS for 48 h to verify viability of embryos by re-expansion of blastocysts. The remainder (slow freezing n = 86 and vitrification n = 21) were transferred to synchronous recipients. Chi-square test and Z-test were used for statistical analysis. The re-expansion rate of the vitrified embryos in culture 48 h after warming was significantly higher ($p < 0.001$) than slow frozen embryos (88.5% vs. 36.1%, respectively). Conception rates did not differ between vitrification (46.5%; 40/86) and slow freezing (38.1%; 8/21). Effectiveness as calculated by percent re-expansion in culture time conception rate was higher ($p < 0.01$) following vitrification than following slow freezing (33.4% vs.16.5%, respectively). Results indicate that the vitrification method used in this study was more effective for the cryopreservation of *in vitro*-produced bovine blastocysts than the slow, controlled freezing protocol used in this study in terms of maintaining embryo viability and achieving conception following embryo transfer. Financial support from CNPq and FAPESP.

Key Words: Cattle, vitrification, cryopreservation

0503

In-straw cryoprotectant dilution of IVP bovine embryos vitrified using a solid-surface system

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Although vitrification has become the preferred method of cryopreservation of *in vitro*-produced (IVP) bovine embryos, simple methods for dilution in the straw are needed to facilitate embryo transfer in the field. An experiment was designed to determine the post-thaw survival of IVP embryos that were vitrified using a solid-surface vitrification system and cryoprotectant dilution in the straw after warming. Bovine, Grade 1 IVP blastocysts (n = 295) derived from slaughterhouse oocytes were

randomly allocated to four groups (two vitrification solutions and two diluting solutions) and a control group (not vitrified but maintained in culture until hatching). Embryos in the EG group were exposed to a 15% ethylene glycol (EG) + 0.25 M trehalose (TR) solution for 1 min and a 30% EG + 1 M TR solution for 30 s prior to vitrification. Embryos in the EG + PROH group were exposed to a 10% EG + 10% propylene glycol (PROH) solution for 1 min and then to a 20% EG + 20% PROH + 0.5 M TR solution for 30 s prior to vitrification. In all cases cryoprotectants were diluted in Hepes-buffered TCM 199. All embryos were vitrified by the CVM system (Cryologic, Australia). Briefly, a 0.6 µl droplet of the vitrification solution containing an embryo was placed in a hook attached to a straw plug (called fiberplug), exposed to a solid metal surface cooled at −196°C and then inserted into a 0.5 ml plastic straw that contained 450 µl of either of the two diluting solutions (Vigro Holding, Bioniche, Canada; or a 0.25 M sucrose solution) under liquid nitrogen. The volume was calculated to provide for space in the straw for the fiberplug and a 5 mm air space, to avoid direct contact between the diluting solution and the vitrified embryos. Straws containing vitrified embryos and diluting solutions were thawed in a 30°C water bath for 30 s and the solutions were mixed by gently agitating the straw. Three minutes after mixing, embryos were placed in SOFaa culture medium under mineral oil and a controlled atmosphere (5% CO2, 5% O2 and 90% N2) for 72 h to determine re-expansion and hatching. Data were transformed by square root and analyzed by ANOVA. All the vitrified embryos were recovered from the straws. Hatching (51/65, 78%) rates were highest ($p < 0.05$) in the control group. Among the vitrified groups, EG resulted in higher ($p < 0.05$) re-expansion and hatching rates (87/115, 75% and 67/115, 58%, respectively) than EG + PROH (71/115, 61% and 42/115, 36%, respectively). Furthermore, re-expansion and hatching rates in embryos vitrified with EG did not differ between embryos exposed to 0.25 M sucrose (45/60, 75% and 34/60, 57%) and those exposed to holding medium (42/55, 76% and 33/55, 60%) after warming. However, 0.25 M sucrose resulted in higher re-expansion and hatching rates (41/60, 68% and 28/60, 47%) than holding medium (30/55, 55% and 14/55, 25%) when embryos were vitrified in EG + -PROH. In conclusion, in-straw dilution with sucrose or holding solutions may be a practical alternative for direct transfer of vitrified IVP embryos using the CVM system and an EG vitrification solution.
Key Words: Bovine, embryo, IVP, Vitrification, Direct Transfer

0504

Effect of trehalose supplementation in vitrification medium on developmental potential of vitrified ovine oocytes

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Oocyte vitrification solutions have high concentrations of cryoprotectants that lead to known biological and physico-chemical toxic effects. One approach to minimize freezing injuries could be the inclusion of non toxic cryoprotectants, such as sugars. For example, trehalose has proven to be particularly effective to stabilize frozen and dried cells. Both *in vivo* and *in vitro* it protects biostructures, such as proteins, membranes and nucleic acids, from damage because of dehydration, heat or cold. Vitrification solutions containing trehalose have been used to vitrify oocytes from different species, but it not always proved to preserve oocyte survival and developmental potential better than sucrose or other sugars. The current study aimed to verify if trehalose supplementation in the vitrification medium enhances *in vitro* developmental potential of vitrified ovine oocytes. After *in vitro* maturation, oocytes were dehydrated by 3 min exposure to equilibration solution containing 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol (EG) in Dulbecco Phosphate Buffered Saline without Ca^{++} and Mg^{++} containing 20% FCS ($PBS^{CaMgfree}$/FCS). The oocytes were then transferred to the vitrification solution containing 16.5% DMSO, 16.5% EG and 0.5 M trehalose in $PBS^{CaMgfree}$/FCS before being loaded on cryotop devices and directly plunged into liquid nitrogen. For warming, the content of each cryotop was transferred from liquid nitrogen into 200 µl drops of 1.25 M trehalose in $PBS^{CaMgfree}$/FCS for 1 min and then oocytes were transferred stepwise into 200 µl drops of decreasing trehalose solutions (0.5, 0.25 and 0.125 M). In the control group, oocytes were vitrified and warmed following the same procedures but using sucrose instead of trehalose at the same concentrations. After warming oocytes of both groups were fertilized and cultured *in vitro*. Embryos were observed daily starting from the sixth until the eighth day of culture and the newly formed blastocysts were recorded. Data were analyzed using Chi square test and differences were considered to be significant when $p \leq 0.05$. The rate of cleaved oocytes was higher ($p < 0.05$) in vitrification solutions supplemented with trehalose compared to sucrose, i.e. 63% (117/185) vs. 53% (96/182). No difference was observed in blastocysts output between trehalose and sucrose groups. However, trehalose accelerated early embryo development as the number of blastocysts produced by the seventh day of culture post fertilization was 72% (13/18) in trehalose and 20% (2/10) in sucrose groups ($p < 0.01$). Our data showed that trehalose supplementation in vitrification solutions improves *in vitro* development potential of vitrified ovine oocytes, in terms of cleavage rate and blastocyst rate at seventh day of development (Supported by RAS-Special Project Biodiversity).
Key Words: Oocyte, vitrification, trehalose, cryotop, sheep

0505

Catalase prevents lipid peroxidation and maintains survival of caprine preantral follicles cryopreserved in a 1,2-propanediol-freezing medium

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Ovarian tissue cryopreservation consists in a valuable tool to preserve female fertility, as reported by the live births obtained after transplantation of frozen-thawed ovarian tissue. However, cold or freezing stress results in cell membrane damage through lipid peroxidation. The aim this research was to examine the effect of cryopreservation medium (1.0 M PROH) supplemented with trolox® (0.1, 0.5, or 1.0 mM) or catalase (5, 10, or 20 IU/ml) on follicular morphology, viability and lipid peroxidation. Goat ovaries obtained from abattoirs were fragmented and frozen in absence or presence of antioxidants (0.1, 0.5, or 1.0 mM trolox® or 5, 10, or 20 IU/ml catalase). Fragments of ovarian tissue fresh and frozen/thawed were then submitted to analysis of follicular morphology, viability and lipid peroxidation. Data were submitted to ANOVA and Student *t*-test. Values were considered statistically significant when $p < 0.05$. Cryopreservation without the addition of antioxidants (trolox® or catalase) decreased percentages of normal follicles (56.6%) compared to the fresh tissue (87.7%) ($p < 0.05$). However, supplementation of the cryopreservation medium with catalase (10 or 20 IU/ml) or trolox® (0.1 mM) resulted in follicular morphology and viability similar to that in the fresh tissue ($p > 0.05$). The lipid peroxidation was reduced only when 20 IU/ml catalase was added to the cryopreservation medium (22.69 nmol malonaldehyde/mg protein) compared to ovarian tissue cryopreserved in the absence of antioxidants or in the presence of 10 IU/ml catalase or 0.1 mM trolox® (40.33; 38.40 and 37.18 nmol malonaldehyde/mg protein, respectively) ($p > 0.05$). In conclusion, supplementation with 20 IU/ml catalase was effective for the preservation follicular morphology and viability and reduce the level of cellular lipid peroxidation.
Key Words: Slow freezing, ovarian tissue, goat, lipid peroxidation

0506

Apoptotic changes in equine embryos vitrified by two different methods

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To date, pregnancies have been obtained after transfer of vitrified equine embryos which were < 300 µm in diameter (Eldrige-Panuska et al. 2005). Effective cryopreservation of large blastocysts (> 300 µm) in straws has been difficult because of volume of blastocoele fluid or presence of equine embryonic capsule (Choi et al. 2011). Therefore, cryopreservation of equine embryos is still not a routine procedure and

there is a need to develop appropriate vitrification technique. Previous investigations indicate that the lowered viability of embryos after freezing is a result of increased apoptotic changes. The aim of this study was to determine the extent of apoptotic changes in equine embryos at the blastocyst stage before and after freezing. Embryos were vitrified using two different devices: traditional 0.5 ml straws and ultra-fast modification of cryotop, Rapid-i (Vitrolife, Goteborg, Sweden). Type of carrier plays a vital role in post-warming survival. The cryotop is outstanding as a carrier for vitrifaction and had never been used for equine blastocysts cryopreservation. Twenty-four embryos were recovered after 69 uterine flushes from Polish pony mares on day 6, 7 or 8 after ovulation. Out of these 10 were evaluated directly after flushing (control group) and the rest were vitrified and thawed by EquiPro VitKit (Minitube, Tiefenbach, Germany). Embryos were loaded onto the top of the film strip in Rapid-i devices or into 0.5 ml straws in commercial Vitrification Solution (EquiPro VitKit), then quickly immersed into liquid nitrogen. Embryos vitrified in straws were warmed by placing into water bath at 20°C for 10 s and moved into Dilution Medium (EquiPro VitKit) in room temperature. Embryos vitrified by Rapid-i were moved directly into Dilution Medium. After thawing embryos were evaluated using TUNEL method to detect nuclei with DNA damage, which is characteristic of the apoptotic process. For every blastocyst Dead Cell Index (DCI = total number of apoptotic nuclei/total number of nuclei x100) was calculated. The average sizes of embryos were: for control group (n = 10) 458.3 ± 130.9 μm (298–690 μm), for vitrified in straws (n = 7) 430.7 ± 126.4 μm (239–591 μm), and for vitrified in Rapid-i (n = 7) 433.1 ± 112 μm (290–587 μm). The overall DNA fragmentation was 30% (3/10) for fresh embryos, 71% (5/7) for embryos vitrified in straws, and 28.6% (2/7) for those in Rapid-i. Data were analyzed using one-way ANOVA and Tukey's Test. DNA damage significantly increased (DCI = 5.8%, $p < 0.01$) in embryos cryopreserved in straws compared to fresh embryos (DCI = 0.6%). Cryopreservation in Rapid-i carrier did not cause DNA damage. There were no statistically significant differences between extent of apoptotic changes in embryos vitrified in Rapid-i (DCI = 0.7%) and fresh embryos (DCI = 0.6%). Rapid-i vitrification protocol allows for freezing horse embryos up to 600 μm in diameter without reducing their quality and developmental competence. This will enable obtaining pregnancies after embryo transfer.

Key Words: Embryo, equine, vitrification, apoptosis

0507

In vitro evaluation of equine embryo viability after 6 or 24 h of cooling in different media

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The satisfactory pregnancy rates are obtained after transfer of shipped equine embryos maintained around 5°C in commercial zwitterion-based holding media. Cooling horse embryos in flushing media could be an easier and cheaper option. The aim of this study was to compare the number of embryo dead cells after 6 and/or 24 h of cooled preservation in three different media. Day 8 embryos were collected by uterine flushing with Ringer Lactate at 37°C in an Ez-Way Filter (PETS, Canton, TX, USA) from 17 Standardbred mares artificially inseminated with fresh or frozen semen. Embryo quality, stage and diameter were evaluated before embryos were randomly allocated to one of these treatments: Emcare Holding Solution (ICPbio Reproduction, USA), 24 h (EHS24; n = 6), using the standard protocol (control); Emcare Flushing Solution (ICPbio Reproduction, USA), 6 h (EFS6; n = 6) and 24 h (EFS24; n = 4); Ringer Lactate, 6 h (RL6; n = 7). In EFS's and RL6 groups, the recovered embryos were kept in the filter and rinsed with 1 l of EFS or RL, which replaced the medium flushed from the uterus. Fluid filled filters were then placed in the Equitainer® and after cooling for the due time, the embryos were incubated with the cells stain DAPI and the dead cells were counted using epifluorescence UV-illumination on a Leica DM LB microscope. Embryo's total cell number was estimated using the correlation: $n = 0.0106d_2 + 2.0542d - 375.28$ (n = cells number, d = embryo diameter in μm) (Moussa et al., 2004;160). The effects of treatment and diameter group (< 300, 300–1000, > 1000 μ) on the percentages of dead cells/estimated total embryo cells were evaluated by ANOVA (GLM). All the recovered embryos were excellent or good quality blastocysts. Data on embryo diameters, total cells, dead cells and dead/estimated total cells ratios (Mean ± SD) are described in Table 1. No differences in proportion of dead cells were observed between diameter and treatment. Holding for 24 h or in RL for 6 h, 8 days old, good quality equine embryos in EFS didn't increase the proportion of dead cells compared to cooling in EHS for 24 h. If *in vivo* studies will confirm the absence of major embryo damage, these protocols will simplify equine embryo cooling and shipping, before transfer.

Key Words: Horse, embryo, cooling, DAPI, cooling media

Table 1. Diameter, total cells number, dead cells number and percentage of 8 days old equine embryos cooled in different media for different times (mean ± SD). Differences not significative

Treatment group	Diameter (μm)	Total cells (n)	Dead cells (n)	Dead cells (%)
EHS24	608.9 ± 435.4	6412.9 ± 8029.0	56.8 ± 108.3	0.58 ± 0.46
EFS6	842.8 ± 614.4	12 219.6 ± 14 366.8	2.2 ± 1.2	0.11 ± 0.17
EFS24	877.2 ± 433.3	11 075.4 ± 7063.8	53.8 ± 54.9	0.41 ± 0.34
RL6	1002.5 ± 507.6	13 297.9 ± 7290.5	23.3 ± 34.3	0.26 ± 0.35

0508

Vitrification of GV and IVM horse oocytes with two different equilibration methods

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Aim of the work was to compare two different equilibration methods in vitrification of GV (germinal vesicle) and IVM (*in vitro* matured) horse oocytes by assessing their viability after thawing. Oocytes were recovered from slaughterhouse ovaries and divided, on the basis of the morphology of cumulus cells, in cumulus-expanded (CE) and cumulus-compacted (CC) oocytes. Groups of CE and CC oocytes were vitrified immediately after recovery at the GV stage and after *in vitro* maturation at the MII stage according to two different equilibration methods as follow: E1) equilibration in 20 mM Hepes-buffered TCM-199 supplemented with 20% FCS (holding medium, HM) with 10% ethylene glycol (EG) + 10% dimethyl sulfoxide (DMSO) followed by incubation in HM with 20% EG + 20% DMSO + 0.25 M sucrose for 20 s; E2) equilibration for 15 min in increasing concentrations of EG and DMSO to a final concentration of 7.5% (v/v) EG plus 7.5% DMSO (v/v) in HM (three steps), incubation in HM containing 15% (v/v) EG, 15% (v/v) DMSO and 0.5 M sucrose for 1 min. In both systems oocytes were placed on the Cryotop strip and immediately submerged in liquid nitrogen. Thawing was performed at 38.5°C by washing the oocytes in HM with decreasing sucrose concentrations (1.25, 0.62, 0.31 M). Oocytes vitrified at GV stage were *in vitro* matured in TCM 199 + 10% FCS + FSH/LH (0.1 IU/ml) + cysteamine, fixed and stained with glycerol-Hoechst 33 342 to assess nuclear maturation. Oocytes vitrified at the MII stage were cultured for 2 h to evaluate their morphological survival on the basis of the presence of an intact zona pellucida and membrane. Non-vitrified oocytes undergoing the same maturation protocol were used as control. Results (see table) indicate that *in vitro* maturation rate of CE oocytes vitrified at GV stage was significantly higher with E2 method (61.3% vs. 37.1%), while no difference existed for CC oocytes. After cryopreservation in MII, a higher percentage of CC oocytes survived after 2 h of culture with E2 (83.3%) method compared to E1 (61.4%). In conclusion, our results show that the multi-step equilibration method for vitrification of horse oocytes allows better survival rates after thawing for CE oocytes when vitrified at GV stage and for CC oocytes when vitrified at MII stage.

This study was supported by RAS, L.R. 7/2007.

Key Words: Oocyte, horse, vitrification

Table 1. Maturation and survival rates of oocytes vitrified with the two methods

Vitrification system	E1	E2
	Maturation stage	
GV	%MII after IVM – n	$61.3^{b\#}$ – 38/62
	CE 37.1^{a} – 25/62	38.6^{a*} – 22/57
	CC 41.5^{a} – 22/53	
MII	%MII viable after thawing/culture – n	74.2^{a} – 23/31
	CE 69.4^{a} – 25/36	83.3^{b} – 45/54
	CC 61.4^{a} – 27/44	
CTR	%MII after IVM – n	
	CE 56.3 – 67/119	
	CC 53.3 – 98/184	

$^{ab\#}$*Values with different letter/symbol within a raw/column differ significantly ($p < 0.05$; chi-squared test).

0509

Hollow fiber vitrification (HFV) method enables cryopreservation of cryosensitive porcine embryos

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Current embryo vitrification methods with the minimum volume cooling (MVC) concept allow for high post-thaw survival of mammalian ova/embryos in various species. The present study aimed to develop a novel vitrification method that enables efficient cryopreservation of cryosensitive embryos such as porcine embryos. We developed a novel vitrification method based on use of a hollow fiber device, which can easily hold as many as 40 mouse or 20 porcine embryos in <0.1 µl of solution (HFV method). Survival rates of up to 100% were obtained for mouse embryos vitrified in the presence of 15% dimethyl sulfoxide, 15% ethylene glycol and 0.5 M sucrose using the HFV method, regardless of the developmental stage of the embryos tested (1-, 2-cell, morula or blastocyst; n = 50/group). The HFV method was also proven to be effective for vitrifying porcine *in vitro*- and *in vivo*-derived embryos that are known to be highly cryosensitive. Statistical analyses were performed using SPSS 16.0 software. Differences in proportional data between the two groups were analyzed using the chi-squared test. The blastocyst formation rate (48/65, 73.8%) of parthenogenetic morulae derived from *in vitro* maturation after vitrification was comparable to that of non-vitrified embryos (59/65, 90.8%). Post vitrification survival of morulae derived from *in vitro* maturation/fertilization morulae was also comparable to that of non-vitrified embryos (76/97, 78.4% vs. 84/96, 87.5%). Transfer of 72 *in vivo*-derived embryos vitrified at the morula/early blastocyst stages to three recipients gave rise to 29 (40.3%) piglets. These data demonstrate that the HFV method enables simultaneous vitrification of multiple embryos while still adhering to the MVC concept and this new method is very effective for the cryopreservation of murine and porcine embryos.

Key Words: Cryopreservation, Hollow fiber, Mouse embryos, Pig embryos, Vitrification

0510

Short-term storage of collared peccaries (*Tayassu tajacu*) ovarian tissue using phosphate buffered saline (PBS) solution or powdered coconut water (ACP®) based medium

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The Tayassu tajacu, also known as collared peccary, is an exclusive species from the Americas constantly threatened from poaching and deforestation of their habitats. The recovery of oocytes destined to *in vitro* culture or to germplasm banking has been considered a very important tool for multiplication of wild animals. Therefore, the development of protocols for oocytes storage during transportation are of great importance since that most of ovary donors are far from specialized laboratories. The objective of this study was to establish a protocol for the short-term preservation of collared peccary's preantral follicles (PAFs) using phosphate buffered saline (PBS) or powdered coconut water (ACP®) based medium. Pairs of ovaries were collected from eight females and each pair was divided into nine fragments. One fragment was immediately destined for classical histology and transmission electron microscopy (TEM), constituting control group; the other fragments were placed in tubes with PBS or ACP® at room temperature (27°C) and packed in 5 l Styrofoam boxes containing biological ice, that provides an initial temperature of 3°C. The boxes were sealed and opened at 4, 12, 24 and 36 h after the start of the experiment; the temperature of the boxes and the pH of the medium were monitored. After opening the boxes, the ovarian fragments were submitted to the histology analysis and PAFs were classified as primordial, primary or secondary, and also as normal or degenerated. Some PAFs were mechanically isolated and evaluated for viability by using a Trypan blue die test. Finally, the treatments that did not differ from the control group were evaluated through TEM. Data (mean ± SE) were analyzed by Two Way Repeated Measures ANOVA ($p < 0.05$). After 4 h storage in ACP® medium (87.8%), follicular integrity was similar to control group (94.4%). However, ultrastructural analyses revealed the first signals of PAFs degeneration, as swollen mitochondria in the chilled PAFs. Furthermore, ACP® (66.7%) was more efficient than PBS (49.4%) for preserving the morphological integrity of PAFs after 36 h storage ($p < 0.05$), but no differences were verified among treatments for any evaluation time with regards to follicular viability ($p > 0.05$). During the experiment, an increase in the temperature into the boxes was verified after 24 h storage ($p < 0.05$), but this temperature was always lower than 8.4°C. The pH of the media remained constant closer to 7.0. In conclusion, we recommend the use of the ACP® as an alternative medium for the short term preservation of Tayassu tajacu preantral follicles.

Key Words: Preantral follicle, collared peccary, conservation, oocyte, powdered coconut water

0511

Ultrastructure of fresh and frozen ovarian tissue in doe rabbit: effect of different combinations of cryoprotective agent and sugar

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Among different cryoprotective agents, dimethyl sulfoxyde (DMSO) and 1,2-propanediol (PROH) are widely used to preserve the ovarian tissue, in association with disaccharides (sucrose or trehalose) to improve the resistance of cells during the cryopreservation process. The objective of this study was to investigate the effect of each combination of one permeating CPA and one non-permeating CPA on the cells ultrastructure of frozen/thawed ovarian cortices in the doe rabbit.

Ovarian cortices were slowly frozen (0.3°C/min, 1 ml CBS straws) in TCM199 supplemented with 1.5 M DMSO or PROH, 0.3 M trehalose or sucrose and 10% fetal calf serum, after a three steps (5 min each) equilibration phase. After thawing, ovarian tissue were treated for transmission electron microscopy. TEM showed that most follicles from the fresh tissue (control) had normal ultrastructure. After cryopreservation, the oocyte ultrastructure appeared to be similar to the control, regardless of the freezing medium that was used. The cellular organelles showed few signs of damage. The mitochondria were well distributed, but a few membranes were ruptured. The Golgi apparatus and the endoplasmic reticulum did not show any signs of damage. Vesicles and vacuoles were rarely observed. The chromatin of the oocyte was diffused and well preserved. Dark follicular cells or follicular cells without any content were most frequently observed after cryopreservation, and follicular cells seemed to be more affected than oocytes by cryopreservation injuries. This observation is in agreement with results obtained by Siebzehnrübl (2000) in human. On the contrary, the follicular cells seemed to maintain good cohesion with oocytes after cryopreservation, as interdigital structures were present between oocyte and follicular cells without any differences when compared to the control group. This observation is different from that of Navarro-Costa (2005), who observed a partial destruction of the transzonal processes containing filamentous actin after granulosa-oocyte complexes were cryopreserved with DMSO or glycerol in the mouse. For all of the cryopreserved groups, some follicles showed partial or total disruption of the nuclear membrane, with alteration of the inside of the nucleus. Nevertheless, the most important damage observed after cryopreservation was the disorganization of the ovarian stroma. Indeed, fibroblasts appeared to display a lack of cytoplasm or important vacuolization; this is consistent with recent observation made by Gosden et al. (2010) in rabbit. In general, the damages were less frequently observed after cryopreservation using PROH and trehalose, and no disjunction was observed between oocytes and follicular cells, except in one follicle after cryopreservation using DMSO and sucrose. Our results also support the hypothesis that supporting tissues (stroma) are more widely damaged than follicles by the slow freezing process. However, fibroblasts can easily be reproduced by cell division, which suggests that damages to the stroma can be repaired.

Key Words: Slow-freezing, ovarian tissue, ultrastructure, rabbit

0550

Effect of hyperbaric oxygen therapy on the follicular reserve of canine ovarian tissues after xenotransplantation

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The cryopreservation of ovarian tissues is a potentially significant technology for the preservation of the genetic resources of working dogs as well as other laboratory and domestic animals. However, it has been reported that a large proportion of follicles are lost during the initial ischemia which occurs after transplantation of mouse, dog, sheep and human ovaries. Most follicles that survive cryopreservation undergo ischemic loss during neovascularisation. Several attempts have been made to prevent or at least decrease the follicular loss of cryopreserved ovarian tissues after transplantation. However, an effective solution has not been found to date. Recently, effects of hyperbaric oxygen therapy (HBO) on transplantation have been reported in several organs and tissues such as the liver, bone, thyroid and pancreatic islet cells. Thus, we examined the effect of HBO on follicular survival in the canine ovarian tissues after xenotransplantation. Ovarian slices ($1 \times 1 \times 1$ mm) from a bitch were transplanted under the kidney capsule in ovariectomized SCID mice. Hyperbaric oxygen with 100% oxygen was initiated for 30 min at 2.5 atmospheres absolute immediately after transplantation, and this treatment was repeated at 24-h intervals for 2 days. The number of follicles was dramatically reduced at 2 weeks post transplantation. The average numbers of primordial, primary, secondary and antral follicles in ovarian tissues of mice decreased from 1.2, 3.9, 1.3 and 0.6 to 0.8, 1.7, 0.4 and 0/mm^2 at 2 weeks after transplantation, respectively. However, higher numbers of secondary and antral follicles were retained when recipient mice were treated with hyperbaric oxygen. The mean numbers of primordial, primary, secondary and antral follicles per square millimeter in ovarian sections were 0.5, 1.1, 1.6 and 0.3 in the HBO group. The numbers of secondary follicles in ovarian tissues of mice with HBO were significantly higher than those without HBO. These results indicate HBO can be effectively used for the enhancement of follicular reserve in transplanted ovarian tissues.

Key Words: Dog, ovary, transplantation, cryopreservation

0551

Optimization of a warming and dilution protocol for modified open pulled straw vitrification of murine blastocyst stage embryos

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Vitrification for embryo cryopreservation has acquired great relevance worldwide because of the easiness of the procedure, the low cost of the equipment, and the high levels of embryo viability after cryopreservation. Nevertheless, a great variability has been reported among protocols, especially regarding the steps of warming and dilution. In order to contribute to the design of a standard warming and dilution protocol for modified open pulled straw (mOPS) vitrification, a series of experiments was conducted. In every experiment, NIH strain murine blastocyst stage embryos with excellent quality were used, and vitrified using a solution of 25% of ethylene glycol and 25% of glycerol by three-step equilibration procedure. After vitrification, morphological viability of embryos was evaluated under stereoscopic microscopy, to establish the percentage of intact embryos. Variables were analyzed using the Kruskal–Wallis test. Differences were considered significant at a probability value of $p < 0.05$. In experiment 1, 80 embryos were used to test the effect of warming rate on post vitrification embryo viability. The four warming rates used were: very fast (4194°C/min) in two steps (−196 to 25°C in 10 s and 25 to 37°C in 10 s); fast (3960°C/min) in two steps (−196 to −100°C in 10 s and −100 to 25°C in 10 s); medium (1398°C/min) in one step (−196 to 37°C in 10 s) and slow (1326°C/min) in one step (−196 to 25°C). The highest morphological viability (84.2%; $p < 0.05$) was obtained using the very fast warming rate. In experiment 2, 320 embryos were used to test the effect of number of steps (1, 2, 3 and 4 steps) and time (5, 10, 15 and 20 min) of dilution on embryo viability. The highest viability rate (89.5%; $p < 0.05$) was obtained using the protocol three steps and 15 min. In experiment 3, 60 embryos were used to test the effect of sucrose concentration (1, 0.5 and 0.25 M) during the dilution on the embryo morphological viability. The concentration at 0.5 M solution gave the highest viability (89.5%; $p < 0.05$). Therefore, the optimum vitrification protocol for murine blastocysts was the one that used a very fast warming rate (4194°C/min) using three steps and 15 min dilution with 0.5 M sucrose; these results give very important details to the optimization of a vitrification protocol in murine embryos.

Key Words: Vitrification, embryo, murine

0552

Improvement of new vitrification method for mouse preimplantation embryos

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This study was carried out to evaluate a new vitrification method for mouse embryos to improve the survival potential of post-thaw embryos and overcome the contamination of frozen embryos in LN2 storage. Most vitrification methods expose the embryos directly to LN2 whether using droplet vitrification or manipulated straws. When embryos are exposed to LN2, they can be contaminated following this step if the embryos were exposed to the same LN2. Therefore,

techniques that limit direct exposure of embryos to LN2 are necessary. The new vitrification method described here was developed to freeze embryos in 0.25-ml standard straws. To do this experiment, 8-cell and blastocyst-stage embryos were obtained by natural mating of ICR females with C57BL6 males. Collected embryos were washed three times with holding medium (HM: D-PBS with 0.3% BSA) and exposed to vitrification solution 1 (VS1: HM plus 10% ethylene glycol) for 5 min and then loaded in 0.25-ml straws using a fine glass pipette to transfer embryos in vitrification solution 2 (VS2: HM plus 35% ethylene glycol + 5% PVP + 0.5 M sucrose). Straws were sealed with heated forceps and immersed in LN2 by exposing the embryo in VS2 portion of the straws firstly within 1 min and then slowly immersing last portion into LN2 to prevent the straw from collapsing. The frozen straws were exposed to room temperature for 10 s and immersed in a 37 Â°C water bath for 30 s to thaw. After diluting the embryo in VS2 solution with VS1 solution by shaking the straws after thawing, the sealed upper portion of straws were cut and thawed semen was poured into 30-ml Petri dish to dilute in dilution solution 1 (DS1: HM plus 0.5 M sucrose) for 2 min. After this short incubation, embryos were transferred to dilution solution 2 (DS2: HM plus 0.25 M sucrose) for another 2 min and then wash three times with HM. Eight-cell stage and blastocyst-stage embryos were recovered and cultured in M-16 medium with 10% FBS for 48 or 24 h, respectively to evaluate post-thaw survival. Total cell numbers were counted after the incubation periods. The survival rates of post-thaw blastocysts and 8-cell embryos were 100% (52/52) and 93.1% (108/116), respectively. There was no significant difference between each cell stage. Total cell numbers for non-frozen and frozen blastocysts were significantly higher than that in frozen 8-cell embryos (99.7 ± 12.4 and 94.8 ± 15.1 vs. 74.7 ± 14.6), but apoptotic cell numbers in frozen blastocysts were significantly higher than those in non-frozen blastocysts and frozen 8-cell embryos (5.4 ± 4.4 vs. 0.0 ± 0.0 and 1.9 ± 3.1) ($p < 0.05$). This new vitrification method can result in high post-thaw survival rates for 8-cell and blastocyst-stage embryos and overcome the contamination problems when using LN2. This method may be applied with human and bovine pre-implantation stage embryos but further research is needed to verify this. This study was supported by Next generation of BioGreen21 (PJ007990012011).

Key Words: Vitrification, mouse, pre-implantation embryo, 0.25 ml straw

0553

The exposure of bovine immature oocytes to 'Nitrocooler' negative pressure enhances cryotolerance of *in vitro* produced blastocysts

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Cryopreservation allows tissues and embryos to retain their viability for long term. Amongst cryopreservation methods, vitrification is the most suitable for *in vitro* produced embryos. Recently, many groups showed that submission of gametes and embryos to controlled stress activates cellular defense pathways and improves cryotolerance. The aim of this study was to assess IVF efficiency of (GV) oocytes submitted to three different intensities of negative pressure, as well as further blastocyst cryotolerance. Negative pressure has been produced through the custom device 'Nitrocooler' (NC) (Mezzalira et al., *Reprod Fert Dev* 2010;22:1, 210) for 5 min, and measured in Millibar (mbar) through a manometer adapted to the vacuum pump input. Bovine oocytes recovered from abattoir ovaries were randomly allocated into one of the following experimental groups: control (non-stressed), NC 220 mbar, NC520 mbar, NC820 mbar. After conventional *in vitro* maturation, fertilization and culture, cleavage and blastocyst rates were assessed as embryo viability criteria. Expanded blastocysts were vitrified/re-warmed in glass micropipettes, according to Vieira different intensities of negative pressure 2007 (Anim Reprod Sci, 99:377). Cryotolerance was assessed based on fresh and vitrified blastocysts hatching rates (day 10). All data were analyzed by the Chi-square test with 5% of significance level. Cleavage rate of NC 220 group (75.4%) was significantly lower than NC 820 (80.1%, $p < 0.05$). Average blastocyst rates did not differ among all the experimental groups. Non-stressed blastocysts showed statistical difference ($p < 0.05$) between fresh and vitrified groups, whereas NC stressed blastocysts did not show difference ($p > 0.05$) between fresh (average 85.4%) and vitrified (average 75.3%) groups. Moreover, oocyte exposure to 500 mbar of negative pressure improved blastocyst cryotolerance in comparison to its not stressed vitrified counterpart (hatching rates respectively 80.0% and 50.0%; $p < 0.05$). Results show that application of short-term negative pressure to immature oocytes does not affect embryo production. Immature oocytes exposed to 'Nitrocooler' device have enhanced further blastocyst cryotolerance.

Table 1. *In vitro* development of bovine IVF embryos after exposure of GV oocytes to different intensities of negative pressure and further blastocysts cryotolerance

Group	N Cultured	% Cleavage	% Blastocyst	Blastocyst vitrification	N (%) Hatching
Control	772	77.5[ab]	31.0	-	21/26 (82.1)[a]
				+	15/30 (50.0)[b]
NC 220	763	75.4[b]	29.2	-	23/28 (82.2)[a]
				+	20/30 (66.7)[ab]
NC 520	754	78.6[ab]	30.4	-	24/25 (96.0)[a]
				+	20/25 (80.0)[a]
NC 820	770	80.1[a]	31.6	-	23/29 (79.3)[ab]
				+	20/30 (66.7)[ab]

[ab]In the same column indicate a difference ($p < 0.05$).

0554

Cryopreservation of doe rabbit ovarian tissue: toxicity of freezing solutions and effect of post-seeding freezing rate on the morphology and on the DNA fragmentation of preantral follicles

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The objective of this study was to evaluate the toxicity of two freezing solutions [1.5 M dimethyl sulfoxide (DMSO) or 1.5 M 1,2-propanediol (PROH) supplemented with 0.2 M trehalose] and to compare the effects of two post-seeding freezing rates (2 vs. 0.3°C/min) on the morphology of preantral follicles and on the DNA fragmentation (TUNEL) of oocytes after cryopreservation of the doe rabbit ovarian tissue. A one way analysis of variance for equally size groups was used to compare the proportion of different types of follicles (normal intact, defective nucleus, defective cytoplasm and both defects) or labelled follicles before and after cryopreservation. Differences were considered significant when $p < 0.05$. Equilibration into freezing solutions ($n = 15$) before freezing did not induce morphological damages of the follicles (71.4 ± 7.2% and 80.6 ± 5.9% of intact follicles in DMSO and PROH, respectively; $n = 15$ per group) compared to control (81.0 ± 9.4%, $n = 15$). Each protocol induced a significant decreasing of morphologically normal follicles after freezing compared to controls. Proportions of intact follicles were 80.2 ± 4.6% (control), 37.3 ± 7.1% (PROH) and 25.3 ± 4.2% (DMSO) after freezing at 2°C/min ($n = 15$ per group), and 77.7 ± 3.9% (control), 65.0 ± 3.3% (PROH) and 51.1 ± 1.8% (DMSO) after freezing at 0.3°C/min ($n = 15$ per group). A significant difference between PROH and DMSO was observed for the latter freezing rate. Proportions of follicles with non-fragmented DNA (unlabeled) were 78.0 ± 6.9% (control), 32.4 ± 4.2% (PROH) and 35.2 ± 8.8% (DMSO) using a freezing rate of 2°C/min ($n = 15$ per group), and 65.2 ± 12.5% (control), 40.8 ± 17.5% (PROH) and 57.2 ± 14.1% (DMSO) using a freezing rate of 0.3°C/min ($n = 15$ per group). No statistical difference was observed before and after cryopreservation using a freezing rate of 0.3°C/min. Our results confirm that the use of a very slow freezing rate is more suitable for the cryopreservation of doe rabbit ovarian tissue. This may be related to the limitation of the evolution of the thermal gradient from the surface to the interior of the tissue.

Key Words: Slow-freezing, rabbit, ovarian tissue

06. Cryopreservation of semen:

0600

Evaluation of epididymal bovine sperm after postmortem short-term storage at 5°C

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Now-a-days, more attention is given to recovery and preservation of epididymal sperm function that can be collected after death of endangered or high genetic value species. However, postmortem specimens have a limited time before decomposition affects functionality. The aim of this work was to determine if epididymal sperm could be successfully recovered with acceptable viability after 12 and 36 h postmortem storage. Although it was expected that sperm would present a better quality at 12 h postmortem, another aim was to know if this 'window of opportunity' to collect and preserve epididymal sperm could be expanded. The complexes epididymis-testicles of bovine males were collected as pairs (n = 10) at an abattoir in Bom Jesus, Piaui, Brazil. Each pair was individually transported to laboratory packaged in Ziploc® bags, placed on ice to cool to 5°C in a Styrofoam container. At the laboratory the complexes epididymis-testicles were maintained for 12 or 36 h in a refrigerator at 5°C. Testicles were allowed to warm to room temperature (25°C) from cool storage (4°C) for 30 min and were dissected away from the tunica vaginalis and other extraneous tissues. The epididymis was then dissected away from the testis, sectioned and the cauda epididymis was placed in a 60 mm petri dish. Cauda epididymides were minced and rinsed with 3 ml warmed saline solution and allowed to incubate for 5 min. After this, epdidymal sperm were placed in a plastic tube and centrifuged ($700 \times g$ for 6min). The epididymal sperm pellet was resuspended in saline warmed solution and submitted to evaluation: motility (%), vigor (0–5), morphology and viability (%). Results were expressed as a mean ± standard deviation and data analysis was performed using ANOVA in Statview with a Tukey test ($p < 0.05$). Total mean sperm number recovered from each cauda epididymis was 3.9 and 4.1×10^8 at the 12 and 36 h intervals postmortem, respectively. Mean sperm motility was higher at 12 h (60%) than after 36 h (18.8%) postmortem. A significant reduction of vigor was observed when comparing the 12 and 36 h intervals (2.9 vs. 1.2). There was no difference between intervals (12 and 36 h) regarding viability (55.1% vs. 52.6%) and morphological defects (62.5% vs. 54.3%). Regarding morphological abnormalities, the most major defects observed were proximal droplet but the distal droplets were also observed. In conclusion, maintaining the epididymis at 5°C for 12 and 36 h allowed a successful sperm recovery. However, only the 12 h postmortem interval allowed collecting semen presenting acceptable values of motility and vigor.

Key Words: Epididymis, sperm, bovine

0601

Mass spectrometric plasma lipid fingerprinting kynetics after birth in calves as a tool to track metabolic changes in the extra uterine life adaptation

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Adaptation to the extra uterine life necessitates major physiological adjustments. It has been observed that calves produced by the biotechnologies such as *in vitro* fertilization, nuclear transfer and transgenesis present high morbidity in the first week after birth. The objective of this work is to report the lipid species detected by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) fingerprinting and the changes observed in the first eight days after birth in plasma samples from calves produced by natural fertilization. Plasma samples were collected from eight Nellore calves from day 1 to day 8 after birth, at eight different times, in a total of 64 samples. Plasma samples (500 μl) had the lipids extracted by the Bligh & Dyer method. The volume of 1 μl of the lipid extract was spotted onto the MALDI target. Mass spectra were acquired on a Bruker Autoflex mass spectrometer in the positive ion mode in the mass range of m/z 700–900. The conditions of MALDI-MS ionization (positive ion mode and the use of DHB matrix) favor the detection of protonated and sodium adducts of sphingomyelins (SM) and phosphatidylcholines (PC) as the most intense ions, which are signaling and structural lipids, respectively, present in the cellular membranes. Species of SM were mostly with 16 and 24 carbons and with no or one unsaturation (such as SM 16:0 and SM 24:1). Species of PC with 32–40 carbons and zero to six unsaturations have been observed, such as PC 32:1 (of m/z 754), PC 34:2 (of m/z 758) and PC38:6 (of m/z 806). It was possible to observe changes in the ion intensities of diverse lipid species during the first eight days after birth, as an example, relative ion intensities of SM 22:0 (m/z 787), which have signaling transduction functions, have consistently increased after birth, while the lipid membrane PC 40:5 has consistently decreased. We hypothesize that the changes observed are related to the plasmatic cellularity changes. We conclude that the MALDI-MS lipid fingerprinting can bring novel physiological information relevant to the application of bovine biotechnologies and to the understanding of the physiological lipid changes plasma after birth.

Key Words: MALDI, Nelore, neonatal, lipids

0602

Studies on assessment of optimal osmotic pressure of citrate egg yolk extender for cryopreservation of buffalo bull semen

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The study was designed to determine the optimum osmolarity (mOsm/l) of citrate egg yolk extender (CEYE) for cryopreservation of buffalo bull semen. For this purpose, semen from Nili-Ravi buffalo bulls (n = 4) kept at the Semen Production Unit, Qadirabad, Sahiwal, Pakistan was collected at weekly intervals for 5 weeks with artificial vagina (42°C). Five solutions of tri-sodium citrate dihydrate and D (−) Fructose with increasing osmolarities (255, 265, 275, 285 and 295 mOsm/l) were used to prepare a CEYE, having glycerol as a cryoprotectant, in bi-distilled water; pH was maintained between 6.9 and 7.3. Semen was frozen in 0.5 ml straws at −196°C at a concentration of 20 million spermatozoa per straw. Thawing of semen was carried out in a water bath at 37°C for 30 s and the semen was immediately (within 1–5 min) evaluated for sperm motility, viability, acrosomal integrity, plasma membrane integrity (PMI), sperm DNA integrity and lipid peroxidation through phase contrast microscope, supravital staining, normal apical ridge, hypo-osmotic swelling test (HOS), acridine orange (AO) staining and thio-barbituric acid assay, respectively. The data were evaluated by using one-way analysis of variance (ANOVA) with $p \leq 0.05$ being the significant level. Sperm motility was affected ($p \leq 0.05$) by osmolarity, however, effects on all other parameters were non-significant. The osmolarity of semen extender in the range of 285–295 mOsm/l provided the highest ($p \leq 0.05$) sperm motility. Although the optimal osmolarity of the semen extender for cryopreservation of buffalo bull semen may not have been achieved, the results of this study suggest that it might be near 295 mOsm/l.

Key Words: Cryopreservation, buffalo bulls, osmotic pressure

Table 1. Post-thaw sperm characteristics under various osmotic conditions

Osmotic pressure mOsm/l	Motility, %	Viability, %	Acrosomal integrity, %	Plasma membrane integrity, %	DNA integrity, %	Lipid peroxidation, nm
255	37.0 ± 3.6[b]	65.1 ± 2.5	58.7 ± 2.9	58.1 ± 2.9	97.6 ± 0.4	42.8 ± 8.2
265	36.0 ± 3.3[b]	66.4 ± 3.8	59.7 ± 2.3	54.8 ± 5.0	97.8 ± 0.2	27.0 ± 5.0
275	38.6 ± 3.7[b]	62.6 ± 4.4	59.8 ± 3.7	55.1 ± 3.6	98.0 ± 0.2	46.2 ± 9.0
285	45.6 ± 3.8[ab]	61.2 ± 4.3	67.6 ± 2.9	49.7 ± 3.9	98.0 ± 0.1	53.0 ± 12.7
295	50.3 ± 4.1[a]	60.3 ± 3.7	63.9 ± 3.1	54.1 ± 3.3	98.3 ± 0.1	41.3 ± 10.6

Data is mean ± SE. Significance at $p < 0.05$.

0603

Capacitation status and fertilization ability of thawed ram sperm obtained with artificial vagina and electroejaculation

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Freezing temperatures alter the function of spermatozoa (SPZ), causing premature sperm capacitation and decreasing fertilization ability. Semen for artificial insemination can be collected with AV or EE, however the components of the ejaculated semen obtained with both methods are very different. It has been reported that the seminal plasma (SP) can prevent premature sperm capacitation at thawing, however little is known about the function and quality of SP obtained from EE nor the effect of this technique on the spermatozoa itself. Therefore, the objective of this study was to evaluate if the collection method affects the capacitation status and the fertilization ability of thawed ram semen through the modification of seminal plasma or sperm. Ejaculates were collected from five adult rams during 12 weeks, twice a week by AV and EE, alternatively. During weeks 0–6, the AV ejaculates were pooled within 30 min of collection, as were EE ejaculates, centrifuged and the supernatant recovered to obtain SP that was frozen. During weeks 7–12, semen samples were pooled by method of collection and washed to obtain SPZ. For washing (twice), each pool was diluted 1:9 (v:v) in washing solution at 37°C, centrifuged at 700 × *g* for 15 min and supernatant was discarded. Treatments were: (i) SPZ from AV + SP from AV; (ii) SPZ from AV + SP from EE; (iii) SPZ from AV + washing solution; (iv) SPZ from EE + SP from EE; (v) SPZ from EE + SP from AV and (vi) SPZ from EE + washing solution. SP was added at 57% of volume of each pool. Semen was loaded into 0.25 ml straws (50 millions SPZ/straw) and frozen. Straws were thawed (37°C) and 20 µl were used to assess capacitation status with chlortetracycline (CTC) assay. To evaluate fertilization ability using a heterologous *in vitro* fertilization assay, bovine oocytes were co-incubated with 1 million motile SPZ. Percentages of SPZ of each CTC-pattern and fertilized oocytes were compared by ANOVA using the GLM procedure of SAS (2000). The collection method was included as a fixed effect in the model. Collection week was used as a block. There was no general effect of treatments on SPZ in F-CTC (uncapacitated, acrosome-intact cells) and SPZ in B-CTC (capacitated, acrosome-intact cells) patterns. However, SPZ collected by EE resulted in lower number of SPZ in AR-CTC (acrosome-reacted cells) pattern (29 ± 1.2 and 36 ± 1.2 for EE and AV respectively; $p = 0.002$) and greater fertilization rates (41 ± 5 and 29 ± 5; for EE and AV respectively; $p \leq 0.0001$). In conclusion, ram semen collected by EE has decreased acrosome reaction and increased fertilization capacity after thawing than AV obtained semen, independent of the SP. This work was supported by National Institute of Agricultural Technology (INTA).

Key Words: Sperm, seminal plasma, electroejaculation, artificial vagina, ram

0604

Influence of cholesterol-loaded cyclodextrin pretreatment on osmotic tolerance of ram spermatozoa

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Cryopreservation induces irreversible damage to mammalian sperm plasma membranes and osmotic stress is one of the factors responsible for this damage. The objective of the present study was to investigate the effect of cholesterol-loaded cyclodextrin (CLC) pretreatment on the plasma membrane integrity of ram spermatozoa exposed to different osmotic challenges. Semen was collected through electroejaculator from three mature rams. The ejaculates (n = 9), with a progressive motility higher than 60% and abnormal sperm rate lower than 20%, were utilized in all replicates. Ejaculates were extended with tris-citric acid-glucose (TCG) solution to make a final concentration of 400×10^6/ml and then divided into two aliquots. The first aliquot was treated with CLC (at a dose level of 3 mg/120×10^6 spermatozoa) while, second aliquot remained untreated. The sperm samples from both aliquots were then incubated at 35°C for 15 min in one of the five fructose solutions, adjusted to 20, 80, 290, 500 or 1500 mOsm/kg. The hyperosmotic incubation samples (500 and 1500 mOsm/kg), were centrifuged to remove the supernatant and the sperm pellet was re-suspended in 80 mOsm/kg fructose solution for further incubation of 15 min. After osmotic challenges, the percent live sperms and the sperm plasma membrane integrity were estimated in both groups by a modified hypoosmotic swelling test (HOST) associated with a supra-vital eosin staining (HE-test). CLC pretreatment significantly increased the percentage of live, intact and, live-intact sperm cells ($p < 0.01$) following exposure to different osmotic challenges. In conclusion CLC pretreatment enhanced the osmotic tolerance of ram spermatozoa even in wide range of anisosmotic challenges.

Key Words: Cholesterol-loaded cyclodextrin, ram, spermatozoa, osmotic tolerance

0605

Efficacy of low density lipoproteins in replacement of whole egg yolk in a commercial extender 'Botu Bov®' for the preservation of motility and membrane integrity of frozen-thawed ovine sperm

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The aim of the study was to test the efficacy of different concentrations of low density lipoproteins (LDL) for cryopreserving ovine sperm. LDL was used in a commercial extender 'Botu Bov®' which is frequently used to freeze bovine, caprine and ovine sperm. LDL was extracted from hen egg yolk according to the technique described by Moussa et al. (2002) and modified by Neves et al. (2007). Four pools of ejaculates collected by artificial vagina from four Santa Inês rams were subjected to the freezing/thawing process. Extra gonadal sperm reserves were firstly exhausted and only ejaculates showing ≥80% motility and ≤30% abnormal sperm were used. The egg yolk of the extender (control) was replaced by 2, 8 or 16% (g/v) of LDL. Immediately after collection, seminal pools were divided into four aliquots and diluted in each preparation to reach 100×10^6 sperm/ml. The diluted semen, maintained at room temperature (˜20°C), was packaged in 0.25 ml straws. Straws were cooled to 5°C in 1 h, using a computerized device TK4000® (cooling rate −0.25°C/min), and were maintained at 5°C for 2 h. Thereafter, samples were frozen in 4 cm above the liquid nitrogen for 20 min. After a week of storage in liquid nitrogen, samples were thawed over 20 s in 46°C water bath. Motility was assessed by a computer assisted sperm analyzer

(SCA® v.4) and functional and structural integrity of sperm membranes was evaluated by the hypoosmotic swelling test (HOST) and fluorescent probes (CFDA/PI) respectively. Statistical Package for Social Sciences (SPSS® 13.0; Chicago, IL, USA) software was used for statistical analysis. All data were subjected to one-way ANOVA followed by LSD test to determine differences among experimental groups ($p < 0.05$). Post-thaw sperm motilities (mean ± SD) were 65.9 ± 25.3%, 47.7 ± 7.4%, 45.8 ± 16.9% and 78.5 ± 20.1%; percentage of swollen sperm were 7.0 ± 8.2%, 10.3 ± 8.8%, 11.5 ± 10.6% and 5.3 ± 4.6% and structural integrity of membranes was maintained at 20.8 ± 11.2%, 14.8 ± 5.1%, 19.5 ± 9.7% and 31.8 ± 7.1% in the control extender or with 2, 8 or 16% LDL respectively. There were no statistical differences between treatments for post-thaw sperm motility and tests for membrane integrity. Preliminary results indicate that LDL can replace whole egg yolk in the 'Botu Bov®' extender. Additional tests are required to determine the appropriate concentration of LDL for ovine semen cryopreservation. Acknowledgements:
Thanks are due to FAPEMIG and CNPq for their financial support.
Key Words: Ram, Santa Inês breed, low density lipoproteins, cryopreservation, spermatozoa

0606

The cryoprotective effects of soybean lecithin-based extender on goat sperm quality

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The aim of this study was to evaluate the effect of soybean lecithin (SL) in different concentrations at extender for sperm goat cryopreservation. Four sexually mature male Saanen goats with fertility history were used. Ejaculates were obtained by artificial vagina. Semen samples were pooled and diluted at skim milk-based extender [control group (CG)] and Tris extender supplemented with SL in different concentrations, according to experimental groups: G1 = 0.04% SL; G2 = 0.08% SL; G3 = 0.16% for a final concentration of 240×10^6 spermatozoa/ml. Semen samples were packed in straws (0.25 ml), frozen in automated system and stored in liquid nitrogen (−196°C). After thawing (37°C/30 s), samples were evaluated for sperm quality parameters such as sperm motility, membrane integrity (propidium iodide and 6-carboxy-fluorescein diacetate – PI/CFDA), acrosome integrity (Fluorescein Isothiocyanate conjugated peanut agglutinin – FITC-PNA) and mitochondrial activity (lipophilic cationic JC-1®) by epifluorescence microscopy. Data were analyzed using one-way ANOVA after arcsin transformation of percent values and Tukey-Kramer multiple comparison test, at 5% of significance. Results are summarized in Table 1. No significant difference was observed among experimental groups and control group for all parameters ($p > 0.05$). It is noteworthy that, whereas control group presented a significant lower mitochondrial potential compared to fresh semen ($p < 0.05$), the same did not occur with extender supplemented with soybean lecithin that did not differ from fresh sperm ($p > 0.05$). Extender containing soybean lecithin at different concentrations preserved sperm quality parameters in a manner similar to conventional skim milk-based extender. Thus, it concludes that extender containing soybean lecithin as the lipid/lipoprotein source can be an alternative to animal products replacement in extender preparation for freezing semen goat.
Key Words: Semen, cryopreservation, mitochondrial activity, chemical defined medium, skim milk

Table 1. Percentage (mean ± SD) of quality sperm parameters

Parameters (%)	Fresh sperm	CG	G1	G2	G3
Motility	75.0 ± 4.5^a	38.3 ± 4.1^b	38.3 ± 7.5^b	36.7 ± 5.2^b	38.3 ± 4.1^b
Acrossome integrity	70.3 ± 15.0	56.7 ± 14.2	62.2 ± 14.1	63.0 ± 17.4	61.3 ± 15.7
Membrane integrity	65.7 ± 2.9^a	45.2 ± 14.7^{ab}	39.7 ± 16.7^b	43.6 ± 14.8^{ab}	38.1 ± 19.3^b
Mitochondrial activity	64.5 ± 6.0^a	41.0 ± 20.3^b	51.7 ± 10.6^{ab}	58.0 ± 9.7^{ab}	57.4 ± 11.6^{ab}

Different letters in the same row denote $p < 0.05$.

0607

Storage of porcine semen at 4°C without antibiotics preserves semen motility and results in similar pregnancy rates, embryo development and litter size

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Antibiotics are commonly used in porcine semen extenders to prevent growth of bacteria that can contaminate the ejaculate during semen collection and handling. High concentrations of bacteria in semen doses may reduce shelf life and subsequent fertility after insemination. The animal production sector is under strong pressure to reduce the use of antibiotics in the animal industry. The objective of this study was to develop a semen extender that allows for storage of semen at 4°C to prevent bacterial growth without addition of antibiotics, while maintaining semen quality. In experiment 1, split ejaculates of nine boars from a commercial AI stud were used to compare motility, viability and bacterial growth of semen diluted with an antibiotic free extender (X) stored at 4°C with semen diluted with a commercial extender (Y), which contained antibiotics and was stored at 17°C. Extender X was based on a zwitterionic buffer with milk proteins and extender Y was a commercial BTS with antibiotics. Statistical analyses were done by analyses of variance. Motility at D0 was on average 77% for X and 79% for Y ($p > 0.1$). Daily motility was slightly lower on D1 for X than for Y (73% vs. 78%; $p < 0.05$), but was similar between the two extenders on the following days, 72% vs. 74% on D3 and 71% vs. 72% on D7, respectively for X vs. Y ($p > 0.1$). The percentage of viable spermatozoids was 89% vs. 90% on D1, 86% vs. 80% on D3 and 79% vs. 79% on D9, respectively for X vs. Y ($p > 0.1$). The bacteria count (mean ± SD) was 2622 ± 1592 vs. 2830 ± 1710 CFU/ml on D0 and 1522 ± 1129 vs. 0 ± 0 CFU/ml on D6, respectively for X vs.Y ($p > 0.1$). In experiment 2, 5 commercial boars were used to evaluate pregnancy rate, embryo development and litter size (D27 of gestation) of gilts (n = 21) inseminated with semen diluted in X and stored for 24–72 h at 4°C. Ovulation rate was 16 ± 2, no of fetuses was 12.9 ± 2.3 and average fetal weight was 0.818 ± 0.03 g. As a reference, three contemporary gilts that were inseminated with semen diluted in extender Y and stored for 24–72 h at 17°C, were also slaughtered at 27 days of gestation. Reproductive parameters were similar for X and Y gilts. The ovulation rate for Y was 15.3 ± 0.4, no of fetuses was 14 ± 1.3 and average fetal weight was 0.689 ± 0.03 g. In conclusion, this study shows that the new extender X can be used to store porcine semen at 4°C while maintaining sperm quality and subsequent fertility. Bacterial growth is prevented during storage at 4°C as the total bacterial count after 6 days of storage did not differ from the bacterial count after storage at 17°C in extender Y with antibiotics.
Key Words: Semen extender, swine, antibiotic-free, fertility, storage 4°C

0608

Methylformamide and glycerol as successful combination for canine semen cryopreservation

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It has already been demonstrated that methylformamide (MF) can be used as a cryoprotectant for canine semen, although it was shown to be inferior to glycerol (GL) (Futino et al., *Reprod Domest Anim* 2010;45:214–20). A diluter containing both 4% MF and 1% GL was recently used with good results for cryopreserving equine semen. This equine diluter, BotuCrio™, was therefore tested in the present study to evaluate its cryoprotective capacities for canine semen. Twelve ejaculates from five dogs between 2 and 7 years of four different breeds were collected and frozen as split ejaculates. Half of each ejaculate was frozen according to the Swedish protocol as described by Rota 2010 (*Reprod Domest Anim* 1998;33:355–361) using a final GL

concentration of 4–5% as sole cryoprotectant. The other half was frozen using BotuCrio™ (4%MF and 1% GL) with the cushionned protocol, as described for equine semen (Hoogewijs et al. *Equine Vet J* 2011;43:35–41). Fresh and frozen/thawed semen was analyzed for total motility (TM), progressive motility (PM) and percentage rapid (Rap) spermatozoa using CASA (Ceros 12.3, Hamilton Thorne), membrane integrity (MI; Sybr-14/PI assay), acrosome integrity (AI; FITC-PSA assay) and morphology (Morph; eosin/nigrosin assay). The differences between fresh and post-thaw semen quality (quality loss due to cryopreservation) were used as statistical endpoints (normally distributed, SPSS 19). The average decrease in TM, PM, Rap and MI for the Swedish protocol was higher ($p < 0.01$) compared to the counterparts frozen in BotuCrio™. No significant decrease in AI and Morph was noticed between groups. Based on these preliminary *in vitro* results, an extender combining MF and GL as cryoprotective compounds, can be used successfully for cryopreserving canine semen and may be an interesting alternative for dogs with low cryosurvival using classic extenders with GL as cryoprotectant.

Key Words: Canine semen, cryopreservation, methylformamide, glycerol, cryoprotectant

Table 1. Average decrease (±SD) in sperm quality parameters (TM, PM, Rap, MI, AI and Morph) for 12 canine split ejaculates frozen using 2 different protocols (Swedish vs. equine protocol)

	TM (%)	PM (%)	Rap (%)	MI (%)	AI (%)	Morph (%)
Swedish protocol	49.2 ± 9.4[a]	41.0 ± 7.4[a]	44.5 ± 8.0[a]	46.0 ± 8.9[a]	9.9 ± 2.0	10.1 ± 1.0
Equine protocol	40.0 ± 8.2[b]	34.7 ± 7.7[b]	37.2 ± 7.6[b]	37.0 ± 7.6[b]	8.1 ± 1.2	9.9 ± 1.1

[ab]Values with different superscripts within a column are statistically different ($p < 0.01$).

0609

The effect of suprazero and subzero freezing rates on lipid peroxidation in post-thaw rhesus macaque spermatozoa

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Cryopreservation damage to sperm has not been well studied in a valuable non-human primate model. Reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, are significant stress factors that are generated during semen cooling and cryopreservation and may be the main cause of sublethal damage. ROS have been shown to disrupt the sperm plasma membrane, induce lipid peroxidation, alter proteins and DNA, and decrease sperm motility and fertility. The main junctures during cryopreservation that cause lethal and sublethal cellular damages are lipid phase transitions in suprazero temperatures and ice crystal formation in subzero temperatures. The objective of this study was to determine whether ROS production varies during cryopreservation. In the rhesus model it has not been determined whether suprazero cooling or subzero freezing rates cause a significant amount of ROS damage to sperm. Semen samples were collected from three electroejaculated male rhesus macaques, washed, and resuspended in a TEST–yolk cryopreservation buffer to 100×10^6 sperm/ml. Sperm were frozen in 0.5 ml straws at four different combinations of suprazero and subzero rates. For this study, three different suprazero rates were used between 22°C and 0°C: 0.2°C/min (slow), 40°C/min (med), and 96°C/min (fast). These suprazero rates were used in combination with two different subzero rates for temperatures 0°C to −110°C: 46°C/min (Med) and 100°C/min (Fast). Once straws reached −110°C, they were plunged into liquid nitrogen. The different freezing groups were as follows: Slow-Fast, Slow-Med, Med-Med, and Fast-Fast. Samples were thawed for 30 s in a 37°C water bath and then evaluated. To determine ROS production of each rate, the fluorescent probe $BODIPY^{581/591}C_{-11}$ was used to detect lipid peroxidation, a result of ROS generation, using flow cytometry. Motility was also evaluated using a computer assisted sperm analyzer. Cells frozen using rates Slow-Fast, Slow-Med, Med-Med, and Fast-Fast exhibited 31%, 33%, 41%, and 78% lipid peroxidation, respectively. Total motility for Slow-Fast, Slow-Med, Med-Med, and Fast-Fast was 18%, 20%, 10%, and 9%, respectively. Slow-Fast and Fast-Fast were compared using the *T*-test to assess lipid peroxidation, which showed a tendency of increased lipid peroxidation ($p < 0.1$) as a result of suprazero cooling rate. There was no difference ($p > 0.1$) in total motility (18% and 9%) between Slow-Fast and Fast-Fast treatments. When comparing treatments with the same suprazero rates but different subzero rates, we found treatments of Slow-Fast and Slow-Med to have similar percentages of peroxidation (31% and 33%) and were not different, indicating subzero rates were not responsible for lipid peroxidation. In addition there was no difference ($p > 0.1$) in total motility (18% and 20%) for Slow-Fast and Slow-Med treatments. These data highlight the role of the suprazero cooling rate in generation of ROS and lipid peroxidation.

Key Words: Cryopreservation, oxidative stress, lipid peroxidation, spermatozoa, non-human primate

0650

Evaluation of *in vitro* fertilizing capacity of cryopreserved bull epididymal semen

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In the present study, the fertilizing potential of semen recovered from slaughtered bulls' epididymis has been evaluated after cryopreservation. For such purpose, testicles of seven Holstein Friesian bulls aged between 2 and 5 years were randomly recovered at the slaughterhouse. The epididymal tail was dissected, and sperm were recovered and evaluated for volume, sperm concentration, and membrane-acrosomal integrity assessment by FITC-PNA/PI fluorescence using a flow cytometry (FCM). For each sample, 10 000 sperm were analyzed at the rate of 500–1000 cells/s using flow cytometer equipped with CellQuest® software. Semen was frozen in 0.25 ml French straws and sperm assessments were performed after thawing. Sperm fertility potential was tested by *in vitro* fertilization. Bovine ovaries ($n = 210$) were collected at the local slaughterhouse and transported in phosphate-buffered saline (30–37°C). Cumulus–oocyte complexes ($n = 1134$) were aspirated from follicles and classified under a stereomicroscope on the basis of their morphological appearance. Good oocytes ($n = 1072$) were matured for 24 h, and fertilized *in vitro*. After 48 h, non-cleaved oocytes were fixed for polyspermy evaluation. On day 7, 9 and 10, embryos were classified as developed (blastocysts and hatched blastocysts). For each bull, three trials of *in vitro* fertilization were performed. Before freezing, on average, the sperm volume of each bull was 8.1 ± 1.5 ml with a sperm concentration of $21.6 \pm 7.3 \times 10^7$ sperm/ml. Sperm viability averaged 86.5 ± 4.2%, decreasing to 64.5 ± 5.0% ($p \leq 0.05$) after cryopreservation, as evaluated by microscopy. By FCM it was found that before freezing, the average percentage of sperm with membrane damaged and intact acrosome was 3.3 ± 0.8% increasing to 4.0 ± 1.2% after thawing ($p \leq 0.05$). In relation to the sperm with damaged membrane and acrosome, the incidence was 3.6 ± 2.2% and 4.5 ± 0.9% ($p \geq 0.05$), respectively in fresh and after thawing. The average percentage of sperm with intact plasma membrane and acrosome before and after thawing was 90.7 ± 2.9% and 90.8 ± 1.9% ($p \geq 0.05$) respectively. Sperm with intact membrane and damaged acrosome averaged 2.4 ± 2.8% and 0.7 ± 0.2% ($p \leq 0.05$) respectively. The fertilization rate, using frozen/thawed epididymal semen averaged 64.1 ± 3.9% fertilization with no significant differences between bulls ($p \geq 0.05$). For the bull considered as control, the fertilization rate was 72.2 ± 10.1%, with no significant differences ($p \geq 0.05$) between epididymal and control semen. In conclusion, the present study demonstrated that acceptable survival rates and fertilizing ability can be obtained from cryopreserved epididymal sperm of bulls. FCM can be used to evaluate membrane and acrosome integrity in frozen-thawed epididymal spermatozoa.

Key Words: Semen, cryopreservation, fertilization, epididymal, *in vitro*

0651

Using of low density lipoproteins and glutamine to improve frozen buffalo bull semen and fertility

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The aim of present study was to demonstrate the cryoprotective role of Low Density Lipoproteins (LDL) and glutamine for freezing and fertility of buffalo bull semen and its effects on the percentage of progressive motility, intact acrosome and membrane integrities of sperm following freezing. Semen from three Egyptian buffalo bulls was collected once weekly and ejaculates with more 75% progressive motility and more 85% normal sperm morphology prior to cryopreservation were pooled in order to have sufficient semen for a replicate and to eliminate the bulls effect. Three experiments were carried out in this study. In Exp.1, whole egg yolk 20% in Tris extender was replaced by different concentrations of 4%, 6%, 8%, 10%, 12% and 15% LDL. In Exp.2, glutamine was added to the Tris egg yolk extender at 20, 40, 60, 80, and 100 mM. In Exp.3, in taking into account the results of Exp. 1 and Exp. 2, glutamine was added to the 12% LDL (optimal level in Exp.1), at the following low concentrations 10, 20, 30, 40, 50 and 60 mM.
In Exp.1, the percentage of post-thaw progressive motility was higher ($p < 0.05$) in 12% LDL extender than control extender containing 20% EY (63.3% vs. 35%, respectively) and give higher conception rate [72.7% n = 11% vs. 50% n = 20 for control (20% egg yolk)]. In Exp.2, the post-thaw plasma membrane integrity was significantly higher ($p < 0.05$) in 20, 40 and 60 mM glutamine (71.33, 73.33 and 70.00% respectively) compared with control and other extenders, and give higher conception rate (72.2% n = 11, 64.3% n = 14 and 60% n = 10, respectively). In Exp.3, the highest percentage of post-thaw progressive motility was recorded in extender containing 20 mM glutamine + 12% LDL (68.33%) and gave the highest ($p < 0.05$) number of acrosome integrity in post-thaw buffalo spermatozoa (83%) in comparison with other media. The addition of 10, 20 and 30 mM of glutamine to the 12% LDL extender lead to an improvement in quality of buffalo frozen semen and offer higher conception rates (72.2% n = 18, 73.3% n = 15 and 66.6% n = 12, respectively). In conclusion, the present results helped in developing a suitable extender for freezing buffalo sperm (Tris supplemented with 12% LDL and 10–30 mM glutamine) without egg yolk and with cryopreservation qualities that are at least as much better as those of a widely used reference extender.

Key Words: Buffalo bull semen, cryopreservation, LDL extender, glutamine, fertility

0652

Evaluation of frozen-thawed Girolando bull semen diluted with commercial extender and rediluted or not with powder coconut water based solution (PCW-111®): *in vitro* and *in vivo* results

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The present study was conducted to evaluate the effect of redilution of frozen-thawed bovine semen in citrate-yolk-glycerol (commercial extender – CE) in powder coconut water based solution (PCW-111®) upon *in vitro* semen quality (Study 1) and *in vivo* fertility (Study 2).
Study 1.
Seventy doses of frozen semen in 0.5 ml straws from seven Girolando bulls were used. Thirty-five straws were thawed (37°C/30 s) and subjected to the thermoresistence test (TRT) at 38°C for 2 h (CE). The remaining 35 doses were subjected to TRT after thawing and redilution in PCW-111® (CE + PCW-111®). Using the Sperm Class Analyzer TM software (SCA TM 2005, Microptics, S.L. version 3.2.0, Barcelona Spain) kinetic parameters of sperm were evaluated 5, 30, 60, 90 and 120 min after TRT. Sperm viability was assessed by eosin-nigrosin staining at five and 120 min. Sperm parameters were compared between treatments by *t*-test. The variables obtained between treatments at different incubation times of TRT were evaluated using analysis of variance and Ducan's test (SAS, 2003).
Study 2.
Fertility was assessed after artificial insemination (AI) of 50 Girolando cows with the use of semen from the bull that presented the best results in study 1. Semen samples from this very bull were frozen with a commercial extender. After thawing, semen was used to inseminate cows without redilution (CE) or subjected to redilution in a powder coconut water-based solution (CE + PCW-111®). Thus, 25 cows were inseminated with control doses and other 25 with rediluted semen, from the same bull. As evaluated by ultrasound, cow fertility was asssessed on days 40 and 64 after AI and compared by Chi-square test ($p < 0.05$). In study 1, motility and percentage of live sperm during TRT did not differ between treatments ($p > 0.05$). Redilution of semen in PCW-111® had a positive effect on the percentage of fast sperm and all velocity parameters (VCL, VSL, VAP, LIN, STR, WOB) were influenced by redilution after 90 and 120 min during termoresistance test ($p < 0.05$). On day 40 post AI, fertility of cows was 36% for CE and 52% for CE + PCW-111®. On day 64, fertility was estimated as 24% for CE and 44% for CE + PCW-111® treatments (Table 1). In conclusion, the redilution of frozen-thawed bovine semen in PCW-111® has benefited *in vitro* sperm quality and *in vivo* fertility.

Key Words: Semen, artificial insemination, extender, PCW-111®

Table 1. Fertility of Girolando cows inseminated with frozen-thawed semen diluted with commercial extender (CE) only, or rediluted in powder coconut water based solution (CE + PCW-111®)

Treatments	Fertility rates (%)	
	40 days	64 days
CE	36[a]	24[a]
CE + PCW-111®	52[b]	44[b]

[ab]Different letters indicate significant differences between treatments (Chi-square test, $p < 0.05$).

0653

Effects of storage and whole or clarified egg yolk in a commercial semen extender on cooled preservation of Pelibuey hair ram (*Ovis aries*) semen

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Chicken egg yolk is a common component of extenders for cooling and cryopreservation of semen in most species. However, complete egg yolk may contain particles that could be detrimental to sperm. The aim of this study was to evaluate the effects of two egg yolk types (clarified egg yolk, CEY, prepared by centrifugation at $10\,000 \times g$ for 20 min and whole egg yolk, WEY) in a Triladyl® extender on cooled ram semen. Ejaculates were pooled, divided into two aliquots and extended in Triladyl® diluent + 20% WEY or CEY. The dilution was performed at room temperature to a final concentration of 400×10^6 spz./ml. Sperm motility was analyzed with CASA (ISAS, Proiser; Valencia, Spain), membrane integrity (MI) with Sybr-14/PI staining, normal acrosome ridges (NAR) with FITC-PSA and plasma membrane integrity in the tail (PMIT) by HOS test. Samples were analyzed immediately after dilution and 24, 48 and 72 h of cooled storage at 5°C. The effects of storage time and extender were analyzed by linear mixed-effects models. Progressive motility (PM) and PMIT decreased ($p < 0.05$) with storage time for WEY and CEY extenders. However, total motility (TM) did not vary between WEY and CEY extenders after semen storage at 48 h. MI did not show significant

differences among storage time and the two extenders. At 72 h, WEY yielded the highest percentage ($p < 0.05$) of damage acrosome (89 ± 2.3%) compared with CEY (78 ± 2.8%). Results showed that clarified egg yolk can potentially be used to replace WEY for preserving pelibuey ram semen over 72 h of storage at 5°C. Supported by CONACYT – Basic Science 164592. AE Domínguez-Rebolledo is supported by the Professor Improvement Program (PROMEP).
Key Words: Egg yolk, storage, semen, extender, hair ram

0654

Effect of combined addition of BSA and glutathione to semen extender on quality and fertility of boar semen stored at 17°C

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There are some long-term extenders for stored boar semen at 15–18°C, however they cannot maintain appropriate sperm fertility over day 5 of storage. It is known that some antioxidants maintain sperm quality during preservation by diminishing the detrimental effect of reactive oxygen species and lipid peroxidation on sperm membrane. The aim of the present study was to evaluate the protective effect of the combined addition BSA (Sigma) and reduced glutathione (GSH) to extender on sperm quality and fertility of liquid boar semen stored at 17°C. In experiment 1 the pooled sperm-rich fraction from three Large White boars were split and extended to 3×10^9 sperm in 100 ml with GTCS [distilled water – 1000 ml, glucose – 40 g, EDTA – 2.6 g, citrate Na – 3.8 g, bicarbonat – 0.5 g, ammonium sulfate $(NH4)_2SO4$ – 1.8 g, antibiotic combination (poligen) – 0.3 g] diluent (control) or GTCS diluent (treatment group) with addition of BSA (0.2 g) and GSH (0.02 g)]. Diluted boar semen stored at 17°C for 10 days and samples were evaluated for progressive sperm motility (CASA), acrosome morphology (Watson technique) and viability (eosin-nigrosin). In experiment 2, the effect of BSA and GSH on the results of insemination of sows was studied. Sows were inseminated twice in spontaneous oestrous (0 and 24 h after detection of standing oestrous) after 6 days sperm storage at 17°C. Statistical analysis was performed using SAS system for Windows. The progressive sperm motility after 0, 2, 4, 6, 8 and 10 day storage in treatment group was 82%, 80%, 80%, 75%, 70% and 64%, respectively, vs. 80%, 78%, 75%, 65%, 40% and 31% in control ($p < 0.01$). On day 8 and 10 the higher viability ($p < 0.05$) was found in treatment group (61% and 55%) vs. in control (50% and 43%). On day 0, 2, 4, 6, 8 and 10 acrosome integrity in treated semen was 79%, 76%, 74%, 71%, 68% and 62%, vs. 81%, 78%, 74%, 72%, 65% and 60% in control, respectively. Farrowing rates in treatment group after 6 day storage of semen at 17°C was 72 (18/25) vs. 54.4% (12/22) in control ($p < 0.05$). Sows did not show a significant difference ($p > 0.05$) in litter size among treatment group (11.0 ± 0.78) and control (10.4 ± 1.12). These studies demonstrated that combined use of BSA and GSH in GTCS diluent has a beneficial effect on quality and fertility of chilled boar semen stored for 6 days at 17°C.
Key Words: Boar sperm, antioxidants, quality, insemination, fertility

0655

Critical time-points in canine semen cryopreservation

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Cryopreservation of canine semen, fundamental for male genetic preservation, may be also an important biotechnique for wild canids with the domestic dog as a biologic model. However, research is still required in order to achieve the efficiency observed in other species. The aim of this experiment was to perform semen analysis during each step of the cryopreservation process, seeking to identify the critical time–point for the maintenance of sperm viability. Eleven semen samples collected from six dogs were cryopreserved in a two-step protocol with Tris–fructose–citric acid–egg yolk extender with 5% glycerol. Aliquots of fresh semen (F), chilled (C – after 1 h at 5°C), at glycerolization (G – 1 h after cryoprotectant supplementation) and post–thaw (T – 30 s at 37°C) were evaluated for: sperm motility (%), forward progressive velocity (arbitrary scale from 0 to 5), specific patterns of sperm motility (computerized motility analysis – CASA), oxidative stress through the thiobarbituric acid reactive substances assay (TBARS), mitochondrial activity through the oxidation of 3,3′diaminobenzidine (DAB, %), flow cytometry analysis using mitochondrial membrane potential – JC1 dye (%), plasma and acrosomal membrane integrity – FITC/PI dyes (%) and DNA fragmentation – SCSA (Sperm Chromatin Structure Assay, %). Data were analyzed by ANOVA and LSD at $p \leq 0.05$. Sperm processing promoted a decrease in motility and forward progressive velocity after cooling (F: 88.1 ± 1.9% and 3.7 ± 0.1%; C: 66.3 ± 6.3% and 2.9 ± 0.2%, respectively). Furthermore, post-thaw samples showed lower motility (T: 45 ± 5.7%) when compared to the previous evaluations. Similarly, CASA showed a decrease in total sperm motility at glycerolization, with pronounced reduction after thawing (G: 61.3 ± 5.9%; T: 28.18 ± 2.9%). Post-thaw samples presented the higher percentage of slow (T: 18.63 ± 3.63%) and static sperm (T: 18.63 ± 3.63%). Glycerolization and thawing are deleterious processes to mitochondrial activity (F: 72.9 ± 3.13%; G: 61.4 ± 5.4% and T: 39.3 ± 5.0%), corroborated by the significant decrease in mitochondrial membrane potential detected by JC1 probe (F: 52.6 ± 2.4%; G: 39.1 ± 3.8% and T: 14.1 ± 1.5%). There was a higher percentage of plasma and acrosome membrane damaged after thawing (F: 19.2 ± 3.8%; T: 31.6 ± 4.0%). Nevertheless, cooling promoted higher percentage of acrosome damage in relation to fresh semen (F: 17.04 ± 2.7%; C: 34.4 ± 7.6%). No DNA fragmentation was observed in the different time-points. Oxidative stress was higher in frozen–thawed samples (F: 279.3 ± 77.4 ng/ml; T: 4771.1 ± 539.4 ng/ml), which may explain the sperm changes observed after thawing. In conclusion, despite the decrease on sperm quality after cooling and glycerolization, the deleterious effect of freezing was significantly more pronounced. A hypothesis to explain such effect is that, independently of intracellular ice formation during semen freezing, the intense production of reactive oxygen species can be considered the main cause of these injurious factors.
Key Words: Canine, semen, cryopreservation

0656

Evaluation of the use of low-density lipoproteins (LDL) extenders in the cryopreservation of electroejaculated semen from brown bear (*Ursus arctos*)

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The use of alternatives to egg yolk in cryopreservation extenders may avoid problems such as the heterogeneity among extender batches. The objective of this study was to design a well-defined extender, with the aim of establishing a specific freezing protocol for brown bear semen. The extraction of LDL was performed in our lab according to the protocol by Moussa et al. 2002 (*Theriogenology* 2002;57:1695–1706). Egg yolk was obtained from hen eggs in aseptic conditions, and homogenized with the same volume of a 1.7 M NaCl solution. Then, we carried out a slow precipitation with ammonium sulfate (40%) for 1 h and, the supernatant obtained after centrifugation at 10 000 × *g* at 4°C for 45 min, was dialyzed for at least 6 h to remove the ammonium sulfate. The solution was again centrifuged and the floating residue, rich in LDL was collected. This fraction was frozen at −20°C and lyophilized, obtaining the final fraction rich in LDL. In this study, four brown bears were electroejaculated under general anaesthesia. Semen was extended with TTF-Base (TES – Tris-fructose 300 mOsm/kg, ph 7.1, with 6% glycerol, 2% EDTA and 1% Equex-Paste), supplemented with: EY20 (20% egg yolk), LDL5 (5% LDL), LDL10 (10% LDL) and LDL15 (15% LDL). Samples were frozen in a programmable biofreezer (−20°C/min) and were analyzed immediately after thawing

(65°C/6 s) by CASA and flow cytometry (PI/PNA–FITC and SYBR–14/PI). Results are represented as mean ± SEM. Values of $p < 0.05$ were considered statistically significant. Total motility (%) showed higher values for EY20, LDL5 and LDL10 ($p < 0.05$). Progressive motility (%), curvilinear velocity (μm/s) and straightness velocity (μm/s) were higher for EY20, but linearity index (%) was higher both for EY20 and LDL15. Contrasting with these results, flow cytometry showed that LDL might provide better plasma membrane protection than egg yolk. Viable spermatozoa (SYBR-14 ± PI−) were higher for both LDL10 and LDL15 (EY20: 39.2 ± 6.3; LDL5: 33.1 ± 4.3, LDL10: 48.4 ± 3.3 and LDL 15: 59.4 ± 4.7) whereas the proportion of damaged acrosomes (PNA− FITC+) showed no differences. The differences among motility and flow cytometry results may be caused by the presence of coarse particles in the LDL extenders, which might have increased the proportion of events identified as non-motile spermatozoa by the CASA. The use of LDL at both 10% and 15% might be a substitute of egg yolk, but it would be necessary to improve the purification process of LDL in order to prevent the presence of large particles in the extender. Supported partly by CICYT (CGL2010–19213/BOS), Cantur SA and Ramón y Cajal program (RYC–2008–02560, MICINN, Spain).

Key Words: LDL, brown bear, cryopreservation, sperm

0657

Conservation of genetic potentials of Eastern sarus crane (*Grus antigone sharpii*): effects of season and preservation techniques on semen quality

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Eastern sarus crane is classified as vulnerable on the IUCN Red List. While only a few thousands of Eastern sarus cranes have been estimated worldwide, they have become extinct from natural habitats of several countries including Thailand. This study was conducted to examine the influence of season on semen traits and also to investigate the effects cold storage and cryopreservation on semen quality of Eastern sarus crane. During April 2010 to September 2011, semen from 6 captive cranes was manually collected every 2–3 weeks at Nakhon Ratchasima zoo, Thailand (14°59′N, 102°12′E). The semen characteristics were examined. In experiment 1, the semen was diluted with crane semen extender and cooled in a refrigerator to 4°C. The semen was then examined for motility and viability at 24, 48 and 72 h after cold storage. In experiment 2, the semen was cryopreserved using different cryoprotectant (dimethyl sulphoxide, DMSO) equilibration techniques (1-step vs. 2-step). The frozen semen was then thawed by two techniques (37°C vs. in-air thawing) prior to examine the sperm motility and viability. The sperm concentration ranged from 0.03 ± 0.08 to 8.7 ± 10.8 ($\times 10^6$ per ejaculation) with a large variation of semen volume (1.8 ± 21–109.5 ± 68.4 μl). The increase in sperm quantity and quality (viability and motility) from June to August (rainy season) compared with April (summer) appeared to coincide with an increase of rainfall, indicating the effects of season on semen production. Cold storage significantly decreased the sperm quality over the times of cold storage. The viability and motility of semen exposed to 48 h of cold storage were significantly lower than fresh semen (58.5 ± 9.5% and 50.0 ± 0% vs. 74.0 ± 12.5% and 66.1 ± 10.2%, respectively). DMSO equilibrating techniques (1- vs. 2-step) did not significantly differ in terms of sperm motility and viability, when the semen was thawed by a particular technique. However, the 2-step DMSO equilibration and in-air thawing significantly increased the percentage of sperm motility (41.5 ± 11.6%) compared with 37°C thawing (33.0 ± 8.7%), but the viability was not affected. It is concluded that semen characteristics of Eastern Sarus crane is influenced by seasonality. We have described for the first time that cold storage is an efficient preservation technique for maintaining the semen quality of Eastern Sarus crane for at least 48 h, while cryopreservation is more detrimental to sperm quality. Fertility test of cooled/frozen-thawed semen by means of artificial insemination remained to be performed.

Key Words: Eastern sarus crane, season, semen, cryopreservation

0658

Effects of alternative cryoprotectants, diluents, straw size and cholesterol addition on cryopreserved rooster sperm

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Rooster sperm do not cryopreserve well. This is due in part to osmotic changes that sperm undergo during addition and removal of cryoprotectant (CPA), as well as membrane damage during the membrane phase transition from fluid at 37°C to gel at low temperatures. When a CPA is removed from sperm, it induces a transient osmotic gradient across the plasma membrane as CPA permeability is lower than that of water. An alternative CPA, methylacetamide (MA), with a lower molecular weight than glycerol (GLY) permeates the plasma membrane more quickly than GLY, potentially decreasing osmotic damage. To decrease membrane damage induced by the membrane phase transition, cholesterol can be added to plasma membranes, increasing membrane fluidity at lower temperatures. Rooster sperm were collected from several birds, pooled and diluted to 1 billion cells/ml in either a trehalose-based diluent (TD) or glutamate-based diluent (GD). In experiment 1, sperm were diluted 1:1 at 5°C with either 18% GLY or 18% MA resulting in a 9% CPA final concentration. Sperm were packaged in 0.25 or 0.5 ml straws and frozen in LN vapor. Motility analyses were conducted using CASA for sperm thawed in a 5°C water bath and diluted 1:10 in GD or TD containing 10% BSA. Data for all experiments were analyzed by ANOVA and means separated using Student–Newman–Keuls multiple comparison test. Higher motility rates were seen for treatments packaged in 0.5 ml straws ($p < 0.05$). In addition, for sperm frozen in 0.5 ml straws, cells frozen in TD exhibited higher motility (>60%) than sperm frozen in GD (46–53%). In experiment 2, cholesterol was added to sperm using cholesterol-loaded cyclodextrins (CLCs) at 0.5, 1, 2 and 3 mg/ml after initial sperm dilution and incubating at 5°C for 30 min. Sperm were then diluted 1:1 with either 18% GLY or 18% MA in either GD or TD, packaged in 0.25 ml straws and frozen. Addition of CLCs did not improve sperm post-thaw motility rates ($p > 0.05$). To determine if MA exposure is detrimental to sperm, in experiment 3, sperm were exposed to 9% MA at 5°C in TD for 0, 2.5, 5 and 10 min prior to freezing. MA exposure time prior to cryopreservation did not affect post-thaw sperm motility rates ($p > 0.05$). In experiment 4, sperm were left with 9% MA for 0, 5, 10, 15, 20, 30, 45 and 60 min after thawing, before analysis. Exposure to MA for up to 1 h post-thaw did not affect sperm motility rate ($p > 0.05$). In conclusion, altering sperm membrane composition by adding CLCs did not improve post-thaw motility of rooster sperm. Sperm frozen in TD with 9% MA or 9% GLY in 0.5 ml straws protected the cells from cryodamage most effectively, and exposure to MA prior to or after freezing did not affect sperm motility. Future experiments will determine the fertilizing capacity of sperm frozen using MA as the cryoprotectant.

Key Words: Poultry, sperm, cryoprotectants, cryopreservation

0659

The effect of omega-3 fatty acids addition in sperm extenders on the quality of frozen bovine semen

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The role of omega-3 polyunsaturated fatty acids (n-3 PUFAs) on sperm resistance to cooling and storage is controversial. Although previous studies demonstrated that the addition of omega-3 fatty acids to the diet of animals (e.g. pig and horse) can influence sperm membrane fluidity and integrity as well as male fertilizing capacity,

there is not enough data about their effects on semen quality *in vitro*, especially on bovine semen. So, the present experiment was designed to determine the protective effects of various levels of purified n-3 PUFAs on bovine semen quality *in vitro* in response to cryopreservation. Polyethylene glycol (PEG), as a more suitable solvent has to be added to introduce n-3 PUFAs to semen extenders. Media were finally sonicated. In treatment A, PEG was added alone to the samples and in treatments B, C and D three different concentrations of n-3 PUFAs (1%, 2.5% and 5%) in combination with PEG were added to the semen extender (Tris-citrate buffer medium containing egg yolk and glycerol). Five proven bulls were randomly selected; ejaculates were collected by artificial vagina and semen characteristics were recorded. Only ejaculates with normal characteristics (volume > 5 ml; concentration > 1.2 billion/ml; motility > 75%; normal morphology > 85% and viability > 85%) were chosen and diluted semen (40 millions/ml) was loaded into 0.5-ml straws, sealed and frozen. Motility, viability and morphology were investigated in frozen-thawed sperm after 1 month. Motility and other dynamic parameters of sperm were analyzed by computer aided sperm analysis (CASA). The results were evaluated by repeated measure ANOVA using SPSS and $p < 0.05$ was considered significant. Motility were 37.9%, 12.8%, 13.2%, 13.8% and 10.4%; viability were 54.8%, 25.7%, 27.6%, 26% and 19.2%; and normal morphology were 88%, 72.7%, 80.6%, 75.4% and 83.6% for control, treatments A, B, C and D, respectively. Our results showed that PEG has some detrimental effects on sperm motility and viability, whereas the addition of n-3 PUFAs to semen extenders could not attenuate the detrimental effects of PEG and did not significantly ($p < 0.05$) improve bovine sperm quality *in vitro*. It seems that n-3 PUFAs cannot be effectively introduced to conservation media as well as sperm membrane to modify sperm characteristics. Nonetheless, n-3 PUFAs might rather be supplemented to the diet of bulls in order to modify the fatty acid compositions of sperm and perform their preventive properties.

Key Words: Bovine, sperm quality, omega-3 fatty acids, freezing

0660

Field fertility results of sublethal stress treated frozen-thawed boar semen using commercial cervical insemination without ovulation induction

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Former studies reported enhanced cryosurvival of boar spermatozoa if sperm was treated with sublethal hydrostatic pressure (HP) stress before freezing (Pribenszky et al., 2007; Huang et al., 2009; Horvath et al., 2011). The present experiment aims to investigate the effect of HP treatment of fresh semen before cryopreservation on pregnancy rate, litter size, and litter characteristics. Semen was collected from four boars with 'good' freezability. Sperm rich fraction was extended with commercial extender at 35°C (BT). Diluted semen was cooled to room temperature (RT) then centrifuged at 2400 × *g* for 3 min. Pellets were re-extended with Ext II. (lactose + egg yolk), then split to treatment and control groups individually. Treatment groups were filled individually into plastic syringes closed by a plastic cap and were treated with 40 MPa HP for 80 min at RT in a programmable hydrostatic pressure machine (HHP1400, Cryo-Innovation Ltd. Hungary). After treatment, Ext III was added (Ext II. + glycerol and Equex Paste), giving a final concentration of 6% glycerol and 1 × 109 spermatozoa/ml. Semen was then loaded to 0.5 ml straws, sealed and were placed into a cooling cabinet at 15°C for 1 h. Samples were further cooled to 5°C for 2 h. Straws were then placed 4 cm above liquid nitrogen for 20 min followed by plunging and storing. Control semen was frozen without HP treatment.

Straws were thawed at 37°C for 30 s, extended with Ext. I up to 80 ml. Motility was checked by CASA (Minitube, Germany). 103 sows that came to heat at day 5 after weaning were selected and randomly assigned to the HP or control groups. Insemination was done through 10 consecutive weeks, on day 5 after weaning at 6 am and 4 pm using commercial AI catheter, without any ovulation induction, with 6 × 109 frozen-thawed semen. Pregnancy was checked by ultrasound on day 25 after AI. Piglet count and weight measurements were done after farrowing, after weaning at day 28 days and at day 42. Results were analysed by genralised mixed model. Results: 81.6% of HP and 60.8% of control sows became pregnant (OR = 2.9, $p = 0.024$). Progressive and total motility were higher in HP samples compared to controls ($p < 0.001$), however these parameters did not differ significantly between pregnant and non-pregnant sows. HP sows gave birth to significantly higher ($p = 0.008$) number of pigs compared to control sows (HP: 10.8 ± 4.5 vs. control: 8.0 ± 3.8). The ratio of stillbirth pigs did not differ between groups (HP: 13.7 ± 18.1% vs. control: 9.3 ± 10.6%). The daily gain of the HP piglets was higher compared to the control group in the fattening stage (290 g/day vs. 275 g/day), where feed was supplied *ad libitum*. Conclusion: HP treated frozen-thawed semen inseminated in the routine workflow of a commercial swine farm with commercial catheter and routine procedures resulted in pregnancy rates and litter size comparable to fresh semen insemination. Study was supported by KMOP-1.1.1.-08/1-2008-0065

Key Words: Boar, semen, cryopreservation, fertility

07. Early embryonic development:

0700

Effect of bovine oviductal epithelial cell co-culture on early cleavage kinetics of bovine IVP embryos

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The use of bovine oviduct epithelial cells (BOEC) as a co-culture system for cattle embryos has been widely used to study embryo maternal interactions and to improve embryo quality during IVP. However, the exact mechanisms of BOEC action have not been fully elucidated. We hypothesize that somatic cells could finely tune the early stages of embryo development, allowing embryos to go faster through the first cleavages, embryonic genome activation and finally reaching the blastocyst stage earlier. Therefore, the purpose of this study was to assess the protein secretion, gene expression and morphology of bovine oviductal epithelial cells according to the time of culture, and to evaluate, for the first time, the effect of BOEC co-culture on early cleavage of bovine embryos. Confluent monolayers of bovine oviductal epithelial cells (BOEC) were derived from slaughterhouse oviducts. Immature COC were aspirated from slaughterhouse ovaries. Zygotes were produced by in-vitro maturation and fertilization, and cultured in SOF medium supplemented with 10% FCS in the presence of BOEC or not. Four development groups were compared: (C) control, medium alone, (BE) BOEC during 4 days, then medium alone, (BL) medium alone for 4 days, then co-culture for the last 4 days, (B) BOEC co-culture over 8 days. Blastocyst rate was evaluated at Day 8. Cell numbers were evaluated at 96, 115, 120 and 139 h post fertilization, using Hoechst 33 342 fluorescent staining for embryos cultured in groups C and B. Oviduct protein secretion and gene expression (OSP, OVGP, C3 and GPX4) were evaluated at confluence and at the end of the culture period. Morphology of BOEC (tubulin, actin and cx43) was assessed by confocal microscopy.

Cleavage rate at 48 hpi was not significantly different between groups. Co-culture improved blastocyst rate compared to control group (C: 13%, B: 25%, $p < 0.05$). The blastocyst rate was higher in group BE compared to BL (36% vs. 25%, $p < 0.05$). The mean cell number at different times of culture did not differ significantly between treatment groups. However, the rate of embryos with 20 or more cells was significantly increased in B group as compared to C at 115 (C: 14.1%, B: 21.8%, $p < 0.05$) and 139 hpi (C: 25.9%, B: 42.2%, $p < 0.0001$). Furthermore, it was observed that co-cultured zygotes reached the 20–25 cell stage embryos (maximum at 115 hpi) sooner than the control group (maximum at 120 hpi). BOEC protein secretion, gene expression and morphological assessment suggested a functional maintenance of BOEC differentiation over the culture time. In conclusion, BOEC co-culture improved in-vitro embryo development. This BOEC effect seems more critical during early stages. Furthermore, BOEC co-culture was accelerating the kinetics of the first cleavages sequence, allowing embryos to reach faster the 20-cell stage, compared to controls. All together, these data suggest a possible role of BOEC in the regulation

of early cleavage and embryonic genome activation during early development.
Key Words: BOEC co-culture, oviductal protein secretion and gene expression, embryo development

0701

Linoleic acid incorporation and competence of bovine oocytes matured *in vitro*

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Linoleic acid (LA) is a long chain unsaturated fatty acid which increases the survival of cryopreserved bovine oocytes and embryos when added in culture media at low concentrations (0.3–9 μM). The objectives of this work were to determine the effect of different concentrations of LA on bovine oocyte maturation. We determined how LA influenced nuclear maturation, the levels of fatty acid incorporation and the lipid classes to which the LA is incorporated in bovine oocytes. Cumulus oocyte complexes (COC) aspirated from ovaries recovered after slaughter were *in vitro* matured in a chemically defined maturation medium supplemented with LA at 9, 43 and 100 μM. Nuclear maturation was determined by staining denuded oocytes with bisbenzamide Hoechst 33 342. To evaluate LA incorporation, oocytes were incubated in the presence of radiolabelled LA (0.8 μCi) and randomly separated into three groups according to incubation intervals: T0 = 0 h (n = 188), T1 = 1 h (n = 173) and T22 = 22 h (n = 252). After incubation, lipids were extracted, separated by thin layer chromatography (TLC) and the incorporation of [14C]LA into each lipid class was measured. The results show that low and medium LA concentrations (9 and 43 μM) did not affect the nuclear maturation, whereas the highest concentration (100 μM) inhibited germinal vesicle breakdown. The incorporation of radiolabelled LA to oocytes was significantly higher ($p < 0.05$) in T22 group, with an average incorporation of T0 = 1.18 ± 0.69, T1 = 12.18 ± 3.67, T22 = 83.39 ± 19.60 pmol/100 oocytes. The radioactivity associated to each lipid band isolated by TLC showed that the LA is mainly esterified in phospholipids and triglycerides ($p < 0.05$). The incorporation of an unsaturated fatty acid such as LA to oocyte phospholipids mainly phosphatidylcholine, could affect membrane physical properties such as membrane fluidity, and would explain previous reports on the effects of LA for embryo/oocyte cryopreservation. However, high levels of LA may negatively alter the physical properties of membranes and may explain their inhibitory effect on germinal vesicle breakdown, altering signaling events of the oocyte. Furthermore, triglycerides are reservoirs of energy used during oocyte maturation and early embryonic development, therefore the esterification of LA in this lipid class could impact oocyte and embryo lipid metabolism. This study was supported by grants from the National Agency of Scientific and Technical Research and National Institute of Agricultural Technology.
Key Words: Oocyte, *in vitro* maturation, linoleic incorporation

0702

Prostaglandin and progesterone production by bovine embryos and co-cultured luteal cells in two *in vitro* culture systems

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The role of prostaglandins and progesterone (P_4) in early embryo-maternal interactions is still poorly understood. The aim of this study was to evaluate the production of P_4, PGE_2 and $PGF_{2\alpha}$ in two *in vitro* culture systems of bovine embryos co-cultured with frozen-thawed bovine luteal cells (BLC). The effect of media oil overlaying on the concentrations of these lipophilic mediators was also evaluated. Enriched luteal cell populations were obtained from early (Days 2–6) bovine corpora lutea and frozen in liquid nitrogen. Day 2 (Day 0 = IVF) bovine embryos were *in vitro* cultured in SOF-aa + 5% serum in an atmosphere of 90%N_2 + 5%CO_2 + 5%O_2 until Day 7. In experiment 1, embryos (n = 900) were randomly allocated to one of four *in vitro* culture groups in 4-well dishes (25 embryos/400 μl/well): (i) embryos; (ii) embryos co-cultured with BLC; (iii) embryos, oil overlay; (iv) embryos co-cultured with BLC, oil overlay. Additionally, luteal cells were cultured with or without oil overlaying, and medium alone was used as control. Media were collected at day 7 for P_4, PGE_2 and $PGF_{2\alpha}$ measurement. In experiment 2, embryos (n = 1000) were randomly allocated to two *in vitro* culture groups in slide chambers, without oil overlay (25 embryos/400 μl/chamber): (i) embryos; (ii) embryos co-cultured with BLC. Luteal cells were also cultured alone and medium alone was used as control. Media were collected at Day 7 for P_4 and $PGF_{2\alpha}$ measurement. A RIA and an ELISA assay were used for the measurement of P_4 and prostaglandins, respectively. In experiment 1, $PGF_{2\alpha}$ and PGE_2 were produced by luteal cells (mean ± SEM: 0.18 ± 0.03 and 36.14 ± 2.51 ng/ml, respectively), embryos (0.14 ± 0.01 and 55.27 ± 8.21 ng/ml, respectively) and the co-culture of embryos plus luteal cells (0.24 ± 0.02 and 125.94 ± 32.40 ng/ml, respectively). As expected, P_4 was produced by luteal cells and its co-culture with embryos but, it was also detected in media of embryos cultured alone (0.14 ± 0.08 ng/ml). The interaction luteal cells × embryos for P_4 and $PGF_{2\alpha}$ production was not significant ($p = 0.74$ and $p = 0.99$, respectively), while for PGE_2 there was a tendency for significance ($p = 0.14$). Overlaying culture medium with oil significantly decreased the concentrations of P_4 detected in the medium ($p < 0.0001$; 50-fold decrease), tended to decrease them in the case of $PGF_{2\alpha}$ ($p = 0.11$) or had no effect ($p = 0.74$) in the case of PGE_2. In experiment 2, P_4 and $PGF_{2\alpha}$ were produced by luteal cells (283.21 ± 33.80 and 0.24 ± 0.02 ng/ml, respectively), embryos (0.47 ± 0.20 and 0.15 ± 0.01 ng/ml, respectively) and the co-culture embryos plus luteal cells (277.84 ± 37.79 and 0.32 ± 0.03 ng/ml, respectively). The interaction embryos × luteal cells was not significant ($p = 0.91$ and $p = 0.28$, respectively). In conclusion, frozen-thawed luteal cells and Day 7 bovine blastocysts produced P_4, $PGF_{2\alpha}$ and PGE_2 *in vitro*. However, except for PGE_2, this production was not enhanced by the simultaneous presence (co-culture) of embryos and luteal cells. Grant PTDC/CVT/65690/2006 and PhD fellow (Ana Torres; SFRH/BD/37666/2007) from FCT.
Key Words: Embryo, luteal cells, progesterone, prostaglandins, bovine

0703

Molecular mediators of conceptus-maternal crosstalk during bovine maternal recognition of pregnancy

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The majority of embryonic losses in cattle occur during the preimplantation stage with a significant proportion of these occurring before the day of maternal recognition of pregnancy (around Day 16 post conception). Successful growth and development of the post-hatching blastocyst and pregnancy establishment are a result of the interaction between a competent embryo and a receptive uterine environment. However, few studies examined the direct dialogue between the conceptus and the endometrial tissues. The aim of this study was to examine the global transcriptome profiles of Day 16 bovine conceptus and pregnant endometrium tissues to identify genes that contribute to the dialogue during the period of pregnancy recognition. Global transcriptome profiling of the bovine conceptus and endometrial tissues at Day 16 was carried out using RNA sequencing techniques, following the Illumina standard procedures for library preparation and genome analyzer sequencing. Following various quality control procedures, transcripts were mapped to the bovine genome. Those transcripts detected in at least four of the five replicates were screened and submitted to Ingenuity pathway analysis for functional annotations. Subsequently, the transcripts were also analyzed for differentiating tissue-specific and commonly detected transcripts. Moreover, to examine endometrial-conceptus crosstalk, ligand and receptors in both tissues were identified and interactions were analyzed using Cytoscape software. A total of 16 018 and 16 262

conceptus and endometrium transcripts were detected, respectively. Of these totals, 2261 and 2505 transcripts were conceptus- and endometrium- specific, respectively, belonging to various functional groups (cytokines, enzymes, growth factors, ion channels, receptor, transcription regulators, etc.). 13 757 (˜85%) transcripts were commonly detected, but with various intensities, between both tissue types. A total of 128 conceptus-expressed ligands with corresponding receptors on the endometrium, and 115 endometrium-expressed ligands with corresponding receptors on the conceptus, were identified, of which 87 were in commonly. This study is one of the first to provide a comprehensive list of secreted molecules in the conceptus that interact with receptors on the endometrium and vice versa during the critical window of maternal recognition of pregnancy. The identified tissue-specific genes and ligands may serve as potential candidates to study pregnancy recognition and to select potential early pregnancy markers. Supported by Science Foundation Ireland (Grant numbers: 07/SRC/B1156 and 10/IN.1/B3011)

Key Words: Conceptus, endometrium, maternal recognition, pregnancy, ligands

0704

Genomic action of thyroid hormones during bovine early embryo development *in vitro*

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The composition of culture media is a key element in the process of *in vitro* embryo production. With the development of defined culture media, many components that are present in trace amounts in serum have been excluded from the culture system. One such component is thyroid hormone (TH), which has important regulatory roles in growth, metabolism and differentiation and is known to be present in serum and the female reproductive tract. We have previously shown that TH has a beneficial effect on embryo development. The objective of this study was to explore the mechanistic pathways by which thyroid hormones exert their effect on bovine embryos. Bovine cumulus-oocyte complexes (COC) were used to produce embryos *in vitro* by standard procedures. Embryos were cultured in synthetic oviductal fluid based medium supplemented with the main THs, triiodothyronine (T3) and thyroxin (T4) to a final concentration of 50 ng/ml and no hormone in controls. COCs and embryos in different stages of development were harvested, their mRNA was extracted and reverse transcribed. Pools of cDNA were screened for thyroid hormone receptor (TR) mRNAs using qPCR. Subsequently, blastocysts were fixed in para-formaldehyde and TRs were localized by immunofluorescence using a rabbit anti goat antibody. Additionally, the gene expression profile of treated and non-treated blastocysts pools were compared using a custom made EmbryoGene oligo-array.

Our study confirmed the expression of TRα and β in COCs and in both treated and control blastocyst. Although, the level of TR mRNAs were higher in the treated embryos but the difference was not statistically significant. TR proteins were detectable in blastocysts of both groups with a difference in the distribution pattern. TH treated embryos exhibited peri-nuclear concentration of TR while it was homogenously distributed in the cytoplasm of control embryos.

Transcriptome analysis of embryonic genome in blastocyst stage showed upregulation of 870 genes in the treated group (>1.5-fold, $p < 0.05$), while 540 genes were significantly downregulated. We found significant enrichment of genes having function in developmental processes (131, $p < 0.05$) and mitochondrial activity (76, $p < 0.005$). Differences in the quantity and distribution of TR protein, TR mRNA expression and the differential regulation of a large set of genes related to cellular proliferation and energy production in embryos exposed to TH during IVC support our previous observations of a beneficial effect of TH during *in vitro* embryo development. Finally, the study highlights the significance of biological components missing from defined embryo culture media.

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Key Words: Embryo, develpoment, blastocyst, thyroid hormone, gene expressin

0705

Oleic acid counteracts the negative effect of saturated fatty acids on bovine oocyte developmental competence

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Elevated levels of free fatty acids in the blood during metabolic stress are often associated with reduced fertility. Previous findings of our group showed impaired development of oocytes after exposure to saturated fatty acids during maturation. This study shows free fatty acid changes in follicular fluid during metabolic stress and their effect on oocyte developmental competence *in vitro*. Cyclic heifers were synchronized with a CIDR® intravaginal device during 7 days and a prostaglandin injection (PG; 5 ml Enzaprost®) 1 day before CIDR® removal. Heifers were randomly allocated to a control group ($n = 6$) that received *ad libitum* grass silage or to an experimental group ($n = 6$) that was fasted during the period of superstimulation. On day 9 of the synchronized cycle (oestrus day 0) the dominant follicle of the first follicular wave was removed and the superstimulation protocol, with a CIDR® device to suppress a spontaneous LH surge, was started two days later (twice daily decreasing doses for four days, total 200 mg Folltropin-V®). A timed LH surge was induced by GnRH (1 mg Fertagyl®) at 48 h after PG. Ovaries were collected at 22 h after the LH peak by ovariectomy. Follicular fluid was collected per follicle of >8 mm and after isolation of the free fatty acid fraction and lipid extraction, fatty acids were analyzed by mass spectrometry. All procedures performed on the animals were in accordance with national regulations and established guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee. Metabolic stress increased the concentration of free fatty acids in both blood and follicular fluid, but the concentration in follicular fluid did not reach the levels in blood. Oocytes collected from slaughterhouse ovaries (4 runs, 400 per group), were *in vitro* matured without or with the most prominent fatty acids, palmitic (PA), stearic (SA) and oleic acid (OA). Concentrations were administered to the oocytes similar to those measured in follicles of control and metabolically stressed heifers. This resulted in comparable blastocyst rates at day 8 of culture after *in vitro* maturation in the absence of fatty acids or presence of fatty acids in control or metabolic stress concentrations ($31 \pm 8.7\%$, $34 \pm 7.8\%$ and $28 \pm 1.7\%$). Thus although the induced metabolic stress caused an increase in the concentration of free fatty acids in follicular fluid, the three most prominent fatty acids did not reduce the *in vitro* development competence of oocytes. Future research should investigate how this compares to metabolic stress on *in vivo* maturing bovine oocytes.

This study is supported by Pfizer Animal Health

Key Words: Oocyte, fatty acid, follicular fluid, metabolic stress, developmental competence

0706

RNA-Seq profiling of bovine preimplantation embryos

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In order to provide a full view of the transcriptome changes during the early stages of bovine development we sequenced the total RNA content of bovine oocytes, 4-cell, 8-cell and 16-cell embryos and the inner cell mass and trophectoderm envelope of expanded blastocysts on the Illumina Genome Analyzer IIx. For each experiment pools of *in vitro* matured oocytes from the German Simmental breed were fertilized using sperm of a single bull and 10 embryos per developmental stage were combined for sequencing. Synthesis of cDNA was initiated directly in the cell lysate using random priming in order to avoid loss of RNA during preparation and to capture all RNA species. Amplified cDNA and sequencing libraries were prepared using kits

from Nugen (Ovation RNA-Seq, Nugen, San Carlos, CA, USA). For the generation of three biological replicates sperm from the distant breeds Jersey (n = 2) and Brahman (n = 1) were used. This cross-breeding design allowed tracking of single sequencing reads back to the maternal or paternal genome, where breed-specific SNPs are present in the expressed transcripts. The analysis of this dataset resulted in monitoring of zygotic genome activation and imprinting of single transcripts and a catalog of splicing isoforms, novel transcripts, non-coding RNAs and differentially expressed genes between developmental stages. In total 2094 genes showed differential expression between any of the stages at a false discovery rate of 1%. Specifically, we found 581 differentially expressed genes (DEG) between immature and matured oocytes, 229 DEG between matured oocytes and 4-cell embryos, 424 DEG between the 4-cell and 8-cell stage, 362 DEG between the 8-cell and 16-cell stage, 792 DEG between the 16-cell stage and the inner cell mass and 842 DEG between the 16-cell and the trophoblast. In summary, by fully exploiting the single-nucleotide resolution of the RNA-Seq method, this dataset provides an invaluable resource for the study of zygotic genome activation, imprinting, transcript annotation and gene expression in early developmental stages of bovine embryos.

Key Words: Preimplantaion embryos, RNA-Seq, imprinting, transcriptome profiling

0707

Exogenous melatonin improves ovine embryo viability in undernourished ewes during the breeding season

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Season and nutritional condition could have significant effect on the reproductive performance in sheep, particularly in the semiarid region, where the availability of nutritional resources are highly seasonally variable. Melatonin treatment is effective inducing cyclicity and increasing ovulation and lambing rates during anoestrus. On the other hand, undernutrition increases embryo mortality and decreases pregnancy rates in ewes. The aim of this study was to investigate the effect of exogenous melatonin on ovulation rate, recovered ova and embryo viability in undernourished post-partum ewes during the reproductive season. The experimental design involved 24 adult non-cycling, post-partum ewes, with a mean (±SEM) live weight (LW) of 65.9 ± 1.8 kg and mean of body condition (BC) of 2.8 ± 0.06. At parturition (November), ewes were treated (+MEL) or not (−MEL) with a subcutaneous implant of melatonin (Melovine®, CEVA). After 45 days, both groups were synchronized with an intravaginal progestagen for 14 days and fed to provide 1.5 (Control, C) or 0.5 (Low, L) times daily maintenance requirements. Thus, experimental groups were: C^-MEL, C^+MEL, L^-MEL and L^+MEL. At oestrus (Day = 0) ewes were mated and on Day 5 embryos were recovered by mid–ventral laparotomy and classified according to their developmental stage and morphology. After embryo collection, ovaries were recovered and processed for IVF. After the period of the experimental diets, LW and BC of C^-MEL and C^+MEL ewes did not change significantly, but ewes in groups L^-MEL and L^+MEL had an average weight loss of 9.6 and 10.5 kg, respectively ($p < 0.001$). No effect of diet or melatonin treatment was observed on ovulation rate and number of recovered embryo per ewe. Number of viable embryos per corpora lutea from L^+MEL (0.50 ± 0.2) was significantly higher than L^-MEL (0.12 ± 0.1), C^+MEL (0.20 ± 0.1) and C^-MEL (0.30 ± 0.2) ($p < 0.05$). Overall, the melatonin effect was particularly evident in undernourished ewes, increasing both viability (L^+MEL: 65%; L^-MEL: 25%; $p < 0.05$) and pregnancy rates (L^+MEL: 66.6%; L^-MEL: 16.6%; $p < 0.05$). Neither nutrition and melatonin nor their interaction had a significant effect on the *in vitro* oocyte development. Melatonin tended to increase the number of cleaved oocytes in undernourished ewes (L^+MEL: 6.8 ± 1.3; L^-MEL: 3.9 ± 1.3, $p < 0.09$) but number and rate of blastocysts were similar between groups. In conclusion, this study shows that treatment with melatonin implants at lambing during the breeding season improves the viability of embryos recovered from undernourished ewes, although this effect seems not to be mediated at the oocyte competence level. Thus, use of melatonin implants even during the breeding season could be a helpful tool, particularly when embryo development is affected by negative factors as undernutrition or post-partum periods. Supported by AGL2007–63822 from CICYT and A-26 from DGA.

Key Words: Exogenous melatonin, undernutrition, embryo viability, ewe

0708

Comparison of homologous vs. heterologous fetal fibroblasts to support goat embryo attachment *in vitro*

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The study of large animal embryonic stem cells *in vitro* has implications for the understanding of lineage differentiation and transgenesis. Availability of goat embryonic stem cells may also offer an advantage over primary fibroblast lines to serve as donor cells for somatic cell nuclear transfer to create transgenic goats. However, the culture conditions for derivation of embryonic stem cells from goat embryos have not been studied extensively and attachment to suitable feeder cells is an important first step for cell line derivation. The objective of this study was to compare homologous fibroblasts derived from 30 to 35 day-old goat fetuses and a mouse embryonic fibroblast cell line (STO) for their ability to support attachment of goat embryos *in vitro*. Goat embryos were collected *in vivo* after superovulation on day 7 after breeding and shipped overnight to the lab in M199 medium + 10% fetal bovine serum (FBS) at 37°C. Feeder cells were grown to subconfluence and then mitotically arrested by treatment with mitomycin C (10 μg/ml for 2 h) prior to use (Table 1). Blastocyst stage embryos (n = 36) were randomly assigned to culture on either STO feeder or goat fetal fibroblast (gFF) feeder cell lines in Dulbecco's Modified Eagle Medium (DMEM) + 15% FBS containing growth factors (2 ng/ml hLIF, 2 ng/ml FGF, 2 ng/ml EGF). The percentage of embryo attachment and number of days for embryo attachment (day 0 is day of fertilization) were assessed for each feeder type. The results for two replicates are presented in Table 1. Embryo attachment to feeder cells is likely influenced by quality of embryos such as lipid content when put into culture. While attachment rate was variable, these results tend to indicate that goat embryos are more likely to attach to STO feeders compared to the goat fetal fibroblasts tested, yet time to attachment was not different between feeders. Further study is needed to improve attachment of goat embryos to feeder cells to promote derivation of goat ES cell lines and the factors that influence this process.

Supported by NSERC 385837-2010

Key Words: Goat, embryo attachment, feeder cells, embryonic stem cells

Table 1. Summary of feeder cells and embryo attachment on two feeder cell lines

Feeder cell line (replicate no.)	STO (1)	STO (2)	gFF (1)	gFF (2)
Feeder cell passage	12	6	1	2
No. of days feeder grown before deactivation	3	1	5	3
Feeder cell seeding density ($x10^5$ cells/well; 12 well plate)	5	5	4	7
% of attached embryos (attached/total)	67 (2/3)	100 (4/4)	43 (10/23)	0 (0/6)
Mean day of embryo attachment (day 0 is day of fertilization)	16	15	17	/

0709

Fusion of cytoplasmic fragments to porcine oocytes improves the ability for embryonic development

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We have already reported that cytoplasmic fragments can be obtained by serial centrifugations (centri-fusion) of oocytes matured surrounded with intact cumulus cells for 46 h (DO46 oocytes) and that fusion of cytoplasts (fragments without chromosomes) to low quality IVM oocytes, which were denuded of cumulus cells at 24 h and cultured for 46 h in total (DO24 oocytes), can improve the developmental ability of the fused oocytes (Viet Linh et al., *J Reprod Dev* 2011). However, two morphological types of fragments; brownish (B) and transparent (T) fragments could be distinguished. In the present study, we conducted further investigations to characterize 2 types of the fragments in terms of organelle content and their ability to support fertilization and embryonic development. The two groups of the fragments were found to be different in an appearance (color, size and quantity), rate of cytoplasts in total ooplasmic fragments, and mitochondria density; however, they were similar in ATP content. MitoTracker Red staining and transmission electron microscopic observations revealed that the B fragments had condensed mitochondria and the T fragments, on the other hand, have less number of organelles. DO24 oocytes showed higher mitochondria distribution but lower ATP content comparing with DO46 oocytes. When DO24 oocytes were fused with pairs of cytoplasts (two B, or two T, or one B and one T cytoplasts) and fertilized *in vitro*, they showed similar effects on improving male pronuclear formation resulting in normal fertilization, and also subsequent embryonic development to the blastocyst stage. The ATP contents of the fused DO24 oocytes were raised to a value similar to that of DO46 oocytes. These results suggest that B and T cytoplasts equally contribute to the improvement of fertilization and subsequent embryo production of oocytes with poor cytoplasmic maturity. On the other hand, oocytes reconstructed by fusion of a karyoplast (fragment containing metaphase chromosomes) and two cytoplasts from B fragments showed higher sperm penetration and male pronuclear formation rates than those of oocytes reconstructed with T fragments. These results indicate that, although the mitochondria in oocytes determines their fertilization ability, fusion of ooplasmic fragments can enhance the developmental ability irrespective of the presence and activity of mitochondria.

Key Words: Pig, oocyte, cytoplast, fusion, mitochondria

0710

Diffusion influences *in vitro* mammalian embryo culture: a numerical calculation

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Recent embryo culture studies suggest that microenvironment and embryo density significantly affect the development rate of blastocysts. Development of bovine blastocysts in the well-of-the-well (WOW) culture system was better than that in conventional systems. To determine the differences in the concentration of chemicals, such as growth factors and/or waste materials, between the conventional and WOW systems, we constructed a model to calculate the amount of secreted chemicals on the basis of Fick's second law of diffusion using spreadsheet software and compared the concentration of these chemicals adjacent to the embryos.
Model cells to represent concentration of secreted chemicals from embryo were defined in spreadsheet software (Microsoft Excel 2003). Medium and embryo model cells were prepared. Fick's second law of diffusion was assigned to the medium cells. Production and diffusion of secreted material from the embryo model cells occurred in an exponential manner. The time resolution for the calculation was 60 s and the total calculation time was 6000 s for 100 steps. The following were prepared for calculation 1: (i) a group culture (GC) model, (ii) a distant group culture (DGC) model, and (iii) a WOW model. The distance between adjacent embryos in GC and DGC models was 1 and 2 mm, respectively. The average concentration of the medium cells neighboring an embryo was compared between the models. In calculation 2, structural differences in the size of the wells were investigated. The concentration of the secreted chemical at 6000 s was two times higher in the WOW model than in the DGC model. From 60 to 600 s, the concentration in the WOW model was almost half that in the GC model. The calculated concentrations in the WOW and GC models were almost the same after 1000 s. The average concentration of the secreted chemical around the embryos was compared between models with well widths of 100 and 300 μm. The concentration in the model with 300-μm diameter wells was half that in the model with 100-μm diameter wells because three-dimensional diffusion is higher in the wider well model. Although the depth of the wells changed from 100 to 300 μm, the diffusion kinetics were similar and the difference in concentrations remained the same in these models. On the basis of the calculation results, we suggest that the improvements observed in the WOW system are due to an increase in facilitation of autocrine and paracrine factors and higher diffusion kinetics of waste materials in the WOW system than in conventional group systems.

Key Words: Well-of-the-well, Fick's second law of diffusion, spreadsheet calculation, secreted material

0711

Use of a mechanical vibration system to observe the effects of mechanical stimuli on *in vitro* mouse embryo development

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Mammalian embryos are transported to the uterine cavity through the fallopian tubes during development. Embryo development can be regulated not only by hormones but also by applying mechanical stimuli (MS), such as shear stress (SS), compression, and/or friction force, in the fallopian tubes before implantation. MS that mimic the physiological mechanical environment of the fallopian tubes can improve embryo culture results. To mimic the mechanical environment in *in vitro* culture, mechanical vibration systems were developed; these systems enhanced porcine and human blastocyst development. In this study, we examined embryo motion, SS induced by vibration, and blastocyst development to understand the effects of mechanical vibration on embryo development. Frozen two-cell stage embryos from ICR mice were defrosted and cultured in 50 μl of mW culture medium for 3 days. Conventional *in vitro* static cultures (control) and mechanical vibration cultures were compared for the average number of blastocysts and the rate of blastocyst development. In the mechanical vibration group, the embryos were cultured at a frequency of 74 Hz for 5 s at 15–min intervals. The rate of embryo development to the blastocyst stage was 48% (N = 235) and 51% (N = 235; $p > 0.05$) in the vibration and control culture groups, respectively. The mean number of blastocysts that developed from the vibration cultures was 98 ± 4 (N = 66), whereas that developed from the control cultures was 95 ± 4 (N = 73; $p > 0.05$). There was no significant difference in either blastocyst development rate or average cell number between the two groups. We observed vibrating motion in the mouse embryos and calculated the translation velocity (1.2 dyn/mm^2) and SS (2.4 mm/min) of the embryos. We observed that a vibrating embryo velocity of < 120 dyn/mm^2 in the microdroplet was lethal to the blastocysts within 12 h. When mechanical agitation was applied to the microdroplet medium, the medium did not move and the diffusion rate by medium motion was the same as that in a static condition; this was determined by the observation of 0.5–μm microspheres in the microdroplet. Using this culture system, we can apply SS and mechanical vibration to the rotating embryos in microdroplets to facilitate diffusion not by medium motion but by mechanical vibration of embryo. Based on previous dynamic culture results, actuation systems to mimic the physiological mechanical environment of fallopian tubes will allow convenient and practical manipulation of

chemical and mechanical microenvironments for *in vitro* embryo development. However, because there was no significant improvement with vibrations of 74 Hz, inappropriate mechanical stimuli by vibration were applied during embryo development and the vibration energy induced negative effects that reduced positive effects, such as moderate SS, and facilitated diffusion. Therefore, it is important to clarify the cellular mechanism of mammalian embryo development using mechanical vibration stimuli.

0712

Rhesus macaque embryo development and gene expression is altered by oxidative damage to the fertilizing sperm

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Oxidative stress due to reactive oxygen species (ROS) is a major contributor to sperm damage and survival following cryopreservation. Men with elevated levels of ROS in semen have been reported to be subfertile. Mechanisms for lowered embryo development and pregnancy rates from cryopreserved sperm are unclear. The goal of this study was to determine whether sperm exposed to ROS as an experimentally isolated component of cryopreservation would impair *in vitro* embryo development in a relevant animal model, the rhesus monkey. We investigated the development of rhesus macaque embryos and RNA expression after intracytoplasmic sperm injection (ICSI) using ROS-exposed sperm. Sperm were oxidatively stressed using a xanthine-xanthine oxidase (XXO) system for 2.5 h to generate high levels of ROS, particularly superoxide anion and hydrogen peroxide. The fluorescent probe BODIPY$^{581/591}$C-$_{11}$ was used to detect lipid peroxidation via flow cytometry to confirm that a significant sperm population was oxidatively stressed. This system generated 85% positive cells for lipid peroxidation and 91% positive for membrane integrity using propidium iodide. Only motile sperm were used for ICSI with superovulated M2 rhesus oocytes; fresh (control) and oxidatively stressed (treated) sperm were used. Fertilization and cleavage rates for the control and oxidative group were 68% and 79%, and 72% and 78% respectively. An observable difference was seen at cleavage to the four cell stage. Controls produced 72% cleavage to the four cell stage while the oxidative stress group decreased to 28%. Further development to the eight cell stage for the control was 29% and the oxidative stressed group did not progress further. RNAseq using an Illumina system was performed with an average of 30 million 40–50 bp reads per sample, which is sufficient to accurately identify and quantify ˜10 000 genes expressed in these samples. Differential expression analysis was performed on two cell embryos from both control and oxidatively stressed sperm using Deseq software, and determined 45 genes were differentially expressed. Gene ontology analysis revealed that among the differentially expressed genes, five are related to nucleosome and microtubule function (PLXNA1, CBLN3, CTCF, RND3, and DNAH1), three to apoptosis (SFRP4, GJA1, and LASS5), several to regulation of gene expression (GJA1, PCDH1, CTCF, LASS5, PBX1, RUNX2, and SFRP4), and one to freezing response (RUNX2). Furthermore, staining of the cytoskeleton and DNA was performed using a conjugated α-tubulin antibody with FITC and Hoechst 33342. DNA fragmentation and abnormal cytoskeleton were seen in embryos generated from ROS-exposed sperm. This study demonstrates that oxidative damage of the sperm has an effect on embryo development due to changes in gene expression.

Key Words: Oxidative stress, embryo development, gene expression, spermatozoa, Rhesus macaque

0750

Ovulatory response and embryonic development in superovulated lactating Holstein cows supplemented with *trans*-10, *cis*-12 conjugated linoleic acid in the diet

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The pregnancy in ruminants requires ovulation of competent oocytes, insemination at the correct time, an appropriate pattern of estradiol and progesterone during the follicular and luteal phases of the estrous cycle. However the ovarian follicular growth, corpora lutea (CL) function and progesterone production can be influenced by dietary fatty acids. The aim of this study was to evaluate the effect of adding trans-10, cis-12 conjugated linoleic acid (CLA – Lutrell®Pure; BASF Chemical Company) to the diet on the number of CL and collected embryo recovered from superovulated lactating Holstein cows. Fourteen lactating Holstein cows were assigned to two treatments: (i) Control group (n = 7, normal feeding) and (ii) CLA Group (n = 7, the same diet as control group plus 70 g/day of CLA), top-dressed for 45 days. The estrous cycle was synchronized with progesterone implant (PRID®alpha 1.55 g). The superovulation (SO) treatment was began on day 10 of the estrous cycle with 40 mg (1 mg/ml saline solution) of porcine follicle stimulant hormone, which was administered to each cow (Pluset®) twice a day 12 h apart for 4 days and in decreasing doses. On the fourth day of SO treatment, 5 ml of PGF2α analog (ENZAPROST®T, Dinoprost 5 mg/ml, CEVA Sante Animale) were applied in the morning. The estrus detection began 24 h after applying PGF2α then the cows were inseminated. Seven days after artificial insemination (AI), embryos were collected non-surgically in commercial flushing media (ViGROTM Complete Flush; AB Technology, Pullman, WA, USA), for evaluation. The statistical analysis was made by SPSS Program, using the Student *t*-test for independent samples. Levene's test was made to evaluate the homogeneity of variances. Superovulation response for both group of study was similar, having no statistical significance between control (CL = 58) and CLA (CL = 47) groups. Adding CLA has an effect ($p \leq 0.10$) on number of embryos in morula stage in comparison to the control group; however there was no significant difference in the blastocyst stage suggesting that adding CLA can promote a delay effect on embryonic development rate.

Key Words: CLA, lactating cows, embryo, superovulation, corpora lutea

0751

Effect of treatment of bovine blastocysts with colony stimulating factor 2 during the morula-blastocyst stages of development on growth and gene expression of trophectoderm outgrowths

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Colony stimulating factor 2 (CSF2) can enhance the competence of the bovine embryo for establishment and maintenance of pregnancy after transfer into recipients. In particular, treatment of *in vitro* produced embryos with CSF2 from day 5–7 after insemination increased conceptus survival and trophoblast elongation and interferon-tau (IFNT) expression at Day 15 of gestation, pregnancy rate at Day 35 of gestation and the proportion of pregnancies at Day 35 that go successfully to term. Here we used an *in vitro* model of trophectoderm (TE) outgrowth from Day 8 blastocysts to test the hypothesis that pretreatment with CSF2 from Day 6–8 after insemination would enhance outgrowth of cells from the blastocyst while maintaining commitment to the TE lineage. Bovine embryos were treated with 0 or 10 ng/ml BoCSF2 at Day 6 after insemination. Blastocysts were harvested at Day 8 and placed individually in wells of 96-well plates that had been coated in growth factor reduced Matrigel. Embryos were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v)

fetal bovine serum until Day 15 after insemination. Each experiment was replicated several times (r = number of replicates) with 1–12 blastocysts per treatment in each replicate. Data were recorded at Day 15 after insemination for attachment and outgrowth rate (r = 15), outgrowth surface area (r = 14), total cell number (r = 4), steady state mRNA for CDX2 (r = 6), GATA6 (r = 6) and IFNT (r = 4), IFNT antiviral activity (r = 3), immunoreactive CDX2 (by immunofluorescence) (r = 3), and percent of cells in outgrowth that were CDX2 positive (r = 4). Overall, 26% (SEM = 2.5) of embryos formed cellular outgrowths. Most measures of TE outgrowth were not affected by CSF2. The exception was for expression of CDX2, which was 1.4-fold higher for outgrowth treated with CSF2 ($p < 0.04$). There was no effect of CSF2 on the percent of cells that were CDX2 positive (95.6 ± 1.16 vs. 94.2% ± 1.1) so the increase in CDX2 expression was likely due to increased expression per cell and not to an increased proportion of cells that remained TE. Total CDX2 protein, as measured by the immunofluorescent intensity per nucleus, did not change, however (1.03 ± 0.02 vs. 1.01 ± 0.02). In conclusion, exposure to CSF2 during the morula-blastocyst stages of development can alter the developmental program of TE, as indicated by an increase in steady-state amounts of CDX2 mRNA. The functional significance of the increase in CDX2 expression is not clear because CSF2 did not affect growth or lineage commitment of cells in the outgrowth. Moreover, there was no significant increase in CDX2 protein, either because the change in transcript abundance did not result in increased protein synthesis or because the method for measuring CDX2 was not precise enough to detect a small increase. (Support: AFRI Grants 2009-65203-05732 and 2011-67015-30688 from USDA-NIFA).

Key Words: Embryo, bovine, trophectoderm, CDX2, CSF2

0752

Expression of pleuripotent marker gene Oct-4 in preimplantation buffalo (*Bubalus bubalis*) embryos produced *in vitro* using media supplemented with recombinant leukemia inhibitory factor

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The transcription factor Oct-4 is a critical factor in controlling mammalian early embryonic development because of its role in pleuripotency. Oct-4 is thought to influence several genes expressed during early embryonic development, and thus may be very important to the process of development and cell differentiation. The expression of Oct-4 is correlated with the undifferentiated cell phenotype of mouse preimplantation embryos. In bovine embryos, the Oct-4 is expressed in trophectoderm as well as in the ICM. In this study, the expression pattern of Oct-4 at the mRNA level and at protein level was investigated throughout the preimplantation stages of *in vitro* produced buffalo embryos in media supplemented with recombinant human leukemia inhibitory factor (100 ng/ml treatment group). Expression was examined by RT-PCR in immature oocytes, *in vitro* matured oocytes, developing preimplantation stage embryos at 2–4, 8–16 cells, morula and blastocyst cultured in mSOF media supplemented with recombinant LIF. Gene specific PCR primer was designed from conserved regions of bovine sequences for Oct-4. β actin was included as an endogenous control and a single 327 bp product was obtained indicating that there was no contamination by genomic DNA during RNA extraction. Both hatching rate and mean total cell numbers in blastocysts were significantly higher ($p < 0.05$) in treatment than control group. mRNA transcripts encoding Oct-4 gene was recorded from immature oocytes, matured oocytes and preimplantation stage embryos from 2 to 4 cell to blastocyst stage. Further confirmation of PCR product was done by cloning and sequencing. Strong signals of Oct-4 protein expression by immunocytochemical localization was found in oocytes and embryonic cytoplasm at the two-cell, four cell, 816 cell, morula, and blastocyst stages. Expression of Oct-4 mRNA in buffalo preimplantation embryos and oocytes indicated that the *in vitro* produced embryos in LIF supplemented media are in a state of pleuripotency. In conclusion, our data indicates that the post fertilization culture environment influences the mRNA expression in the resulting embryos. The challenge for the future is to modify the conditions of *in vitro* culture during this critical window of development in order to try and mimic the pattern of mRNA expression as it occurs *in vivo*, and in that way, produce embryos of higher quality *in vitro*.

Key Words: Buffalo, IVEP, leukemia inhibitory factor, Oct4 gene expression

0753

FGF signalling in the trophoblast precedes conceptus elongation in the pig

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Specification of the trophoblast lineage is a result of a cross-talk between the embryonic and the extraembryonic compartment in rodents. FGF4 produced by the epiblast induces an FGFr/MAPK response in the neighbouring trophoblast cells characterized by Elf5 and Cdx2 expression. The interaction between these transcription factors creates a positive-feedback loop that results in the proliferation and differentiation of the trophoblast. The aim of this study was to identify components of the trophoblast specification circuitry in pre-elongation pig embryos. Embryos from spherical to tubular stages (d11–d12 after mating) were collected by flushing, and subsequently fixed and processed for whole-mount immunofluorescence (for FGFr2, pMAPK, and FGF4) and RNA in situ hybridization (ISH) for CDX2 and ELF5. In addition, RNA was extracted and the expression of trophoblast and mesoderm markers was analyzed by RT-PCR. CDX2 mRNA detected in the trophoblast cells of ovoid embryos became extinct by the late ovoid/early tubular stage. In the epiblast, however, CDX2 was detected in the posterior end and in the primitive endoderm of all stages studied. ELF5 mRNA was not detected in the trophoblast at any stage; however, it was expressed in the epiblast of ovoid embryos, and in the epiblast and the extraembryonic mesoderm of tubular embryos. The ISH results were confirmed by RT-PCR. FGFr2 was found in the epiblast and primitive endoderm of ovoid/tubular embryos and low levels of expression were detected in the trophoblast of ovoid embryos. In contrast, in late tubular embryos, FGFr2 became highly expressed in the trophoblast. FGF4 and pMAPK were detected in the epiblast of ovoid embryos, but not in the trophoblast at this early stage. In comparison, in late tubular embryos FGF4 and pMAPK were highly expressed in the trophoblast. These studies show that factors participating in trophoblast lineage specification in rodents are not expressed in the same synchrony and the same cellular compartments during porcine pre-elongation. ELF5 mRNA is detected in the epiblast and in nascent mesoderm, but not in trophoblast, whereas CDX2 mRNA expression is confined to early trophoblast and the epiblast. The presence of active FGF signalling in the trophoblast of late tubular stage embryos was concurrent with the reduction in CDX2 in the trophoblast. Furthermore, embryos with low CDX2 and active FGF signalling in the trophoblast possessed a nascent extraembryonic mesoderm. The dynamic changes described by these experiments show that activation of FGF signalling in the trophoblast precedes the elongation of the pig conceptus and correlates with reduction in CDX2. These findings suggest that signals produced by embryonic tissues (the epiblast and extraembryonic mesoderm) may modulate the initiation of this process, thereby ensuring that the trophoblast is equipped to support the growth of the gastrulating embryo.

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Key Words: Trophoblast, extraembryonic mesoderm, FGF signalling, pig embryo, Elf5

0754

Eld's deer embryos produced *in vitro* can develop to term after transfer into recipient females

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Eld's deer is a medium sized deer (50 kg) localized to a small area in Southeast Asia. Two subspecies of Eld's deer, thamin (Rucervus eldii thamin) and siamensis (Rucervus eldii siamensis), are listed as reserved species under the Thai Wildlife Preservation and Protection Act (1992). The use of reproductive technologies has the potential to maintain genetic diversity of this small population. Objective of the study was to assess the developmental competence and viability of thamin embryos produced by *in vitro* fertilization after embryo transfer. Over two breeding seasons, oocyte donors were synchronized by inserting intravaginal progesterone sponges (CIDR-G) on day 0 and retained for 14 days and administering GnRH (8 μg, Receptal®) i.m. on day 16. In eight oocyte donors, FSH was additionally administered (four doses of 20 mg/dose, Follitropin®), at 12 h intervals before oocyte collection. On day 21, oocytes were collected after ovariectomy (non-FSH stimulation, n = 11) or laparoscopically aspirated (FSH stimulation, n = 8). The recovered ova were *in vitro* matured and fertilized with frozen-thawed thamin sperm in IVF DOF medium. Presumptive zygotes were *in vitro* cultured at 38.5°C in 5% CO_2, 5% O_2 in DSOF medium for either 8 days (oocytes from three non-FSH stimulated hinds) or transferred into synchronized (as in non-FSH stimulated oocyte donor but started later for 6 days) recipients at the 2–8-cell stage. Percentage of cleaved embryos was 63% (51/81) at 36 h post insemination (hpi), percentage of morula was 6.2% at 144 hpi and percentage of blastocyst was 5% at 192 hpi. Blastocyst cell number averaged 86.5 ± 28.2 (n = 4). The 2–8-cell stage embryos were random transferred singly into the ipsilateral oviduct of 10 recipients, one additional recipient received two embryos. Pregnancies, (4/11, 36%) were diagnosed by EIA analysis of progesterone metabolite in faeces, > 1000 ng/g. One pregnancy failed prior to 90 days of gestation and two fawns died shortly after spontaneous preterm births at days 215 and 224 of gestation, histopathological diagnosis indicated amniotic aspiration pneumonia (transferred embryos derived from FSH stimulated oocyte donors). A healthy female fawn was born unassisted with no complications at day 234 of gestation (embryos derived from non-FSH stimulated oocyte donors). This study demonstrated the first successful *in vitro* embryo production and subsequent birth of a live Eld's deer fawn. However, the development and adaptation of reproductive technologies compatible with the easily stressed Eld's deer remains a challenge.

Key Words: Eld's deer, embryo devlopment, *in vitro* fertilization, embryo transfer

0755

Role of hyaluronic acid in maturation and further early embryo development of bovine oocytes

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Hyaluronic acid (HA), an important component of the extracellular matrix, plays a crucial role for cumulus cell expansion. Genes and proteins involved in HA synthesis and its receptor CD44 are expressed in cumulus oocyte complexes (COCs) in different animal species and increase during maturation. Hyaluronidase enzymes (Hyal) degrade HA into smaller biologically active HA fragments. To investigate the effects of the molecular size and concentration of HA on oocyte maturation and further embryo development, bovine oocytes were matured *in vitro* in the presence or absence of HA, Hyal-2 or 4-Methylumbelliferone (4 μ); an HA synthesis inhibitor. The rates of oocyte nuclear maturation to metaphase II stage and development of embryos to blastocyst stage and blastocyst quality were recorded. Hyal-2 inhibited cumulus cell expansion without affecting oocyte maturation ($p < 0.01$) and further embryo development. Whereas, 4 μ at 1 mM reduced cumulus cell expansion ($p < 0.005$) and oocyte maturation ($p < 0.005$) rates. Combined treatment (HA + 4 μ) also inhibited cumulus cell expansion ($p < 0.05$), but had no effect on MII percentage compared to the control. Treatment of COCs with 1 mM 4 μ during maturation decreased cleavage rate (49 ± 4.8 vs. 76 ± 9.8; $p < 0.05$) and completely inhibited development to blastocyst stage (0 ± 0.0 vs. 31 ± 4.9; $p < 0.01$) as compared to the control. In the combined treatment, cleavage rate was not significantly different from the control, however, the blastocyst rate was significantly reduced ($p < 0.05$). These data suggest that HA production by cumulus cells during maturation is essential not only for cumulus cell expansion, but also for oocyte maturation and further embryo development. This effect is is neither influenced by HA-degradation by Hyal-2 nor is dependent on the size of the HA molecules. This study was funded by BBSRC research grant BB/G008620/1.

Key Words: HA, HYAl, blastocyst, 4-MU, oocyte maturation

0756

Rescuing the development competence of poor quality oocytes by mitochondrial transfer

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Mammalian oocytes contain extraordinarily large number of mitochondria whose dysfunction or damage can result in reduced post-fertilization embryonic development. Here, we show that morphologically poor quality pig oocytes (oocytes with < 5 layers of cumulus cells and non-homogeneously spread granular/agranular cytoplasm) had significantly lower content of mitochondrial DNA (mtDNA) and lower post-fertilization development competence than those of morphologically good quality pig oocytes (oocytes with > 5 layers of cumulus cells and homogeneously spread granular cytoplasm) ($p < 0.05$). Microinjection (10 pl) of purified mitochondria from healthy granulosa cells improved the post-fertilization *in vitro* development competence of poor quality oocytes in terms of the rate of blastocyst formation (34.4 vs. 22.3%; $p < 0.05$), hatching ability (40.7 vs. 24.2%; $p < 0.05$) and total nuclei number per blastocyst (69.7 vs. 56.3; $p < 0.05$). Unfertilized good or poor oocytes contained significantly lower content of mtDNA than those of fertilized oocytes ($p < 0.05$). However, the improvement in development competence of mitochondria-injected poor quality oocytes was not due to improvement in fertilization rate ($p > 0.05$). Improvement in development competence of mitochondria-injected poor quality oocytes was also observed following parthenogenetic activation ($p < 0.05$). Importantly, the *in vitro* development of mitochondria-injected poor quality oocytes resembled those of non-injected good quality oocytes ($p > 0.05$). Microinjection of mitochondria isolated from xenogenic cattle species did not had any beneficial or adverse effect on the development competence of the pig oocytes ($p > 0.05$). In conclusion, mitochondria transfer could rescue the development competence of poor quality oocytes and hence, may be a future therapeutic modality for infertility treatment.

Key Words: Development competence, mtDNA, mitochondria, embryo, pig

0757

Addition of caffeine on embryonic development of *Danio rerio*

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The objective was to analyze the influence of adding different concentrations of caffeine (0, 50, 150 and 200 ppm) during distinct phases of embryonic development of Zebrafish (Danio rerio), evaluat-

ing the survival rate, heart rate, length of the yolk sac, chorionic space and length of the embryo. Three repetitions were performed using two experimental trials to evaluate the correlation between caffeine levels and stages of embryonic development. 400 embryos were divided into eight experimental incubators with oxygen of about 7.4 mg/l. The first experimental trial was to add caffeine in distilled water and then package the embryos in the solution during the first 24 h post-fertilization (hpf). After this period the embryos were removed and placed in distilled water for completion of the development. In the second experimental trial, the embryos were placed in distilled water, and after 24 hpf packed in equal caffeine concentration cited above, being removed after 24 h of development and also allocated in water to complete the development. After 0, 24, 48 and 72 h of incubation, were measured the length of the yolk sac, chorionic space and the length of the embryo, with Motic Image Plus Program. Heart rate was measured after 48 and 72 hpf and the survival rate was observed at the end of the experiment. The results of the selected variables were evaluated by analysis of variance to study the effect of the interaction between caffeine concentrations and immersion times of trials I and II, and simple regression analysis according to the levels of caffeine and methods, using the SISVAR program, with 5% probability. The addition of caffeine did not influence significantly the length of the chorionic space and the survival rate, however, affect the heart rate, the length of the embryo and yolk sac. The length of the embryo on trial I had lower mean (2199.9 μ) compared to the trial II (2273.6 μ). It was observed a linear increase of the yolk sac as the concentration of caffeine increased, and the smallest size was evaluated in the experimental trial I with 50 ppm (495 μ) and the largest size was evaluated in trial II with 200 ppm (566 μ). A decrease in heart rate was observed as concentrations of caffeine increased, the lowest frequency being evaluated in trial I, with 200 ppm (130 bpm), and higher frequency with 50 ppm in trial I (153 bpm) after 72 hpf . Statistical difference between the methodologies, and the heart rate was always lower in embryos subjected to trial I. Caffeine alters the normal development of the embryo, with detrimental effects that can influence its survivability.

Key Words: Fish, embryo, coffee, zebrafish, heart rate

08. Embryo transfer:

0800

Superovulatory response of beef cows treated with a split-single injection of Folltropin-V diluted in hyaluronan after storage in the refrigerator or after freezing

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Previous studies have shown that superstimulation protocols can be simplified successfully by reducing the twice-daily FSH treatment protocol to two intramuscular (i.m.) injections of FSH in a 0.5% hyaluronan-based solution 48 h apart (split-single treatment). A study was designed to investigate the stability of the FSH-hyaluronan solution and the best method of storing unused aliquots of the diluted preparation The objective of this study was to evaluate the superovulatory response of beef cows treated with a split-single injection of FSH diluted in 0.5% hyaluronan after storage in the refrigerator or after freezing. Angus donor cows (n = 60) were assigned to one of three treatment groups to be superstimulated with 300 mg Folltropin-V (Bioniche Animal Health Inc., Belleville, ON, Canada) diluted in hyaluronan (MAP-5, 50 mg; 5 mg/ml, Bioniche). Cows in the Control group received 200 mg of Folltropin-V in MAP-5 (50 mg) as a single i.m. injection, followed 48 h later with a second i.m. injection of 100 mg of Folltropin-V in MAP-5 (50 mg); cows in Group 2 (Frozen) received the same treatment as cows in Group 1 except that the Folltropin-V and MAP-5 (50 mg) was reconstituted and frozen (−20°C) for 2 weeks before being injected; cows in Group 3 (Fridge) received the same treatment except that the Folltropin-V and MAP-5 (50 mg) was reconstituted and placed in the refrigerator (3–5°C) for 2 weeks before being injected. On Day 0 (at random stages of the estrous cycle), all cows received 5 mg estradiol-17β plus 50 mg progesterone and an intravaginal device impregnated with progesterone (Cue-Mate, Bioniche). On Day 4 (expected day of follicle wave emergence), all cows were superstimulated with 300 mg of Folltropin-V in a split-single injection protocol as described above. Folltropin-V was prepared by injecting 10 ml of MAP-5 (50 mg) into the vial and shaking vigorously. On Day 4, 5 ml of the Folltropin-V plus MAP-5 (50 mg) solution was injected i.m. and on Day 6 another 2.5 ml was injected. In the morning of Day 6, all cows received 500 μg cloprostenol (Ciclase, Syntex, Argentina) and Cue-Mates were removed in the morning of Day 7. In the morning of Day 8, cows received 12.5 mg pLH (Lutropin-V, Bioniche) and were inseminated 12 and 24 h later. Ova/embryos were collected non-surgically on Day 15 and evaluated following IETS recommendations. Data were analyzed by ANOVA. There were no significant differences among groups in number of CL, total ova/embryos, fertilized ova or transferable embryos (Control: 16.2 ± 2.5, 16.1 ± 2.7, 10.9 ± 2.3 and 7.4 ± 1.5, respectively; Frozen: 18.1 ± 2.1, 17.7 ± 2.6, 12.5 ± 2.1 and 9.3 ± 1.7, respectively; Fridge 17.2 ± 1.6, 16.0 ± 2.0, 12.4 ± 2.0 and 8.5 ± 1.4, respectively). Results confirm that following reconstitution of Folltropin-V in MAP-5 (50 mg) freezing or refrigeration for a period of 2 weeks does not adversely affect efficacy (superovulatory response) following a split-single treatment protocol.

Key Words: Bovine, superovulation, split-single injection, MAP-5, hyaluronan

0801

Survival of *in vivo*-produced bovine embryos exposed to 1.5 M ethylene glycol for different periods of time prior to conventional cryopreservation

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Ethylene glycol (EG) is the most commonly used cryoprotectant in commercial bovine embryo transfer due to its rapid permeation permitting direct transfer to the recipient's uterus after thawing, without the necessity of cryoprotectant removal. Although EG has been used for many years, its potential toxicity after exposure to embryos is controversial. Most protocols used by practitioners stress the importance of keeping embryo exposure to EG to a maximum of 5–10 min before freezing. However, there are no reports confirming the toxic effects of EG when embryos are exposed for longer periods of time. An experiment was designed to evaluate the effect of different periods of exposure of *in vivo*-produced bovine embryos to 1.5 M EG prior to freezing on survival rates after thawing. *In vivo*-produced embryos (n = 667) collected from superovulated donor cows 7 days post-insemination were used. All embryos used were IETS Grade 1 morulae and blastocysts. Within 3 h of collection, embryos were allocated at random into the treatment groups and exposed to a solution of 1.5 M EG and 0.25 M sucrose (Vigro EG Freeze with sucrose; Bioniche Animal Health, USA) and loaded in 0.25 ml plastic straws at room temperature (22–25°C). The straws were sealed and placed in a Freeze Control CL 5500 freezer (Cryologic Inc, Australia) at –6.5°C, seeded and after 10 min of equilibration, cooled at –0.6°C/min to –35°C, before plunging into liquid nitrogen. After at least 1 week storage in liquid nitrogen, embryos were thawed in a water bath at 30°C for 12 s. Embryos were then cultured at 38.8°C in SOFaa medium under mineral oil in a controlled atmosphere (5% CO2, 5% O2 and 90% N2) for 72 h to determine re-expansion and hatching. The experiments was performed in two trials, in which embryos were exposed to EG for 5 min, 10 min and 20 min (trial 1), or 10 min, 20 min and 30 min (trial 2). Re-expansion and hatching rates were compared by Chi-square test. In trial 1, re-expansion and hatching rates post-thaw did not differ ($p \leq 0.05$) among groups: 5 min (60/66, 91% and 41/66, 62%, respectively), 10 min (61/67, 91% and 41/67, 61%, respectively) and 20 min (57/67, 85% and 34/67, 51%, respectively). In trial 2, re-expansion and hatching rates also did not differ ($p \leq 0.05$) among groups: 10 min (135/152, 89% and 105/152, 69%, respectively), 20 min (132/161, 82% and 107/161, 66%, respectively) and 30 min (126/154, 82% and 101/154, 66%, respectively). In

conclusion, *in vivo*-produced bovine embryos can be safely exposed to EG for up to 30 min at room temperature prior to freezing, without adversely affecting survival rates after thawing.

Key Words: Ethylene glycol, bovine, embryo, exposure, freezing

0802

Consequences of embryo transfer using *in vitro* produced embryos for characteristics of the offspring and recipient after calving

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The objective was to determine whether embryo transfer affects characteristics of the recipient female and resultant offspring. Embryo transfer was performed either using fresh (ET–F) or vitrified (ET–V) embryos along with artificial insemination (AI). Data were analyzed from lactating Holstein cows which calved as a result of AI using non-sorted semen (n = 42) or embryo transfer using embryos produced *in vitro* with X–sorted sperm and that were transferred fresh (ET–F; n = 41) or after vitrification (ET–V; n = 38). Parturition was induced in some cows in which gestation length exceeded 280 day. Gestation length was longer ($p < 0.002$) for ET–V than for other groups (ET–F, 275.4 ± 1.0 day; ET–V, 278.8 ± 0.9 day; AI, 275.0 ± 1 day). The number of cows in which parturition was induced was greater ($p < 0.003$) for ET than AI [ET–F, 6/41 (14.6%); ET–V, 12/38 (31.6%); AI, 0/42 (0%)]. There was a tendency ($p = 0.06$) for incidence of calving difficulties to be greater for ET [ET–F, 8/41 (19.5%); ET–V, 4/38 (10.5%); AI, 1/42 (2.4%)]. There was no significant treatment effect on postpartum disease or on reproductive function as measured by days to first service, services per conception and pregnancy success at first service. Ten ET calves were crossbreds and there was one set of twins in the AI group. The percent of calves that were female was higher ($p < 0.0001$) for ET [ET–F, 38/41 (92.7%); ET–V, 35/38 (92.1%); AI, 22/43 (51.2%)]. Subsequent analysis was on female Holstein calves. There was a tendency ($p = 0.05$) for birth weights to be higher for ET–V than for other groups (ET–F, 41.2 ± 1.5 kg; ET–V, 45.3 ± 1.5 kg; AI, 42.0 ± 1.8 kg). The percent of calves that were dead at birth or died within 20 day of life tended to be higher ($p = 0.10$) for ET [ET–F, 6/31 (19.4%); ET–V, 9/32 (28.1%); AI, 1/22 (4.5%)]. Losses after day 20 tended to be higher ($p = 0.06$) for ET–F than ET–V but, overall, ET was not significantly different from AI [ET–F, 9/25 (36.0%); ET–V, 2/23 (8.7%); AI, 5/21 (23.8%)]. Heifers were inseminated when reaching farm weight and height minimums. Age at first service tended to be higher ($p = 0.06$) for ET–V than other groups (ET–F, 385.9 ± 3.7 day; ET–V, 395.7 ± 3.3 day; AI, 387.7 ± 3.7 day). First service conception rate was not different between treatments [ET–F, 5/16 (31.3%); ET–V, 8/20 (40.0%); AI, 6/16 (37.5%)]. In conclusion, embryo transfer using *in vitro* produced embryos resulted in lengthened gestation lengths, increased incidence of induced parturition, and tended to increase incidence of calving difficulty and calf loss before day 21 of life. There were no negative consequences for recipients with respect to postpartum disease or fertility.

Key Words: IVF, embryo transfer, artificial insemination, vitrification, cattle

0803

Season and follicular population on the efficiency of an embryo production program in Holstein heifers

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The evaluation of the ovarian follicular population (FP) and season can be an alternative to reduce the variation on *in vivo* dairy heifer embryo production. The objective of the study was to evaluate the effect of the population of ovarian follicles ≥3 mm in two seasons on the production of embryos. In summer (Temperature Humidity Index; THI = 74) and winter (THI = 54), nulliparous Holstein heifers (n = 80) between 12 and 18 months of age were scanned four times during each season corresponding on average to 3 weeks before superovulation, with an ultrasound machine equipped with a 5 MHz linear transducer, to determine the population of ovarian follicles ≥3 mm in diameter. The heifers were randomly assigned to one of the following treatments: T1) Low FP (≤4 follicles, n = 10), and T2) High FP (≥5 follicles, n = 9). The animals were superovulated with 300 mg of FSH, administered every 12 h for 4 day in eight decreasing doses, and inseminated with sexed semen at 12, 18 and 24 h after estrus onset; they received an injection of 100 μg of GnRH at the time of the first insemination. Embryos were collected non-surgically using standard procedures 8 day after insemination. The variables compared were total ova/embryo collected (TOEC), number of blastocysts (Bla), morule (Mor), transferable embryos (TE), degenerated embryos (Deg) and oocytes (Ooc). The data were analyzed using the CATMOD procedure of SAS (2004). The results are presented as percentages in Table 1. There were no differences ($p > 0.05$) in TOEC, Bla, Mor, TE, Deg and Ooc between groups of heifers. In conclusion, under the conditions of this study the follicular population and the season did not affect the number and types of structures collected as well as the number of transferable embryos in Holstein heifers.

Key Words: Follicular population, heat stress, embryos

Table 1. Structures collected from superovulated Holstein heifers with high (HFP) or low (LFP) follicular population during summer and winter

Variable	n	TOEC (n)	Blastocyst (%)	Morule (%)	TE* (%)	Degenerated embryos (%)	Oocytes (%)
HFP	9	60	21.7	15.0	36.7	11.7	51.7
LFP	10	71	39.4	7.0	46.5	11.3	42.3
Summer (S1)	10	83	28.9	12.0	41.0	8.4	50.6
Winter (S2)	9	48	35.4	8.3	43.8	16.7	39.6
HFP × S1	5	42	14.3	19.0	33.3	7.1	59.5
HFP × S2	4	18	38.9	5.6	44.4	22.2	33.3
LFP × S1	5	5	43.9	4.9	48.8	9.8	41.5
LFP × S2	5	30	33.3	10.0	43.3	13.3	43.3

*The sum of blastocysts and morulae.

0804

PGF2a treatment during the periovulatory period increases embryo recovery rate in superovulated buffaloes

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The low embryo recovery rate reported in buffaloes may be related to the failure of oocytes to enter the oviduct after superstimulation of follicular growth. In rabbits, the administration of subsequent doses of PGF2α during the periovulatory period stimulates contraction of oviduct smooth muscles, allowing the activation of the oviduct fimbriae to capture the oocytes (Osada et al., 1999). We hypothesized that the use of PGF2α during the periovulatory period increases embryo recovery rate in superovulated buffaloes. Buffalo donors (n = 14) were randomly divided into two groups: Control (C-G) and PGF2α (PGF-G). Follicular wave emergence was synchronized with a progesterone (P4) releasing intravaginal device (Sincrogest®; OuroFino) plus the injection of 2 mg estradiol benzoate (Sincrodiol®; OuroFino) at random stages of the estrous cycle (D0 AM). All buffaloes received twice-daily injections of FSH (200 mg; Foltropin-V®; Bioniche) in eight decreasing doses, starting on D4 AM. PGF2α

(0.53 mg sodic cloprostenol; Sincrocio®; Ouro Fino) was given on D6 PM and D7 AM, and the P4 device was removed on D7 PM. Then, 20 μg GnRH (buserelin acetate; Sincroforte®; OuroFino) was given on D8 PM. Inseminations were done 12 and 24 h after the GnRH treatment. Buffaloes from PGF-G received four extra doses of PGF2α (0.53 mg sodic cloprostenol; Sincrocio®; Ouro Fino) from D8 PM to D10 AM every 12 h (12/12 h). The ova/embryos were collected 6 days later (D14) nonsurgically. Twenty-one days after the uterine flushing the buffaloes were restarted in all treatments in a cross over design. The variables were analyzed by GLIMMIX procedure of Statistical Analysis System (SAS®). Embryo production (i.e. total ova/embryos recovered) was increased in superovulated buffaloes treated with PGF2α during the periovulatory period (PGF-G = 4.1 ± 0.8) compared to buffaloes not treated (C-G = 2.6 ± 0.7; p = 0.03). However, similar number of transferable (2.0 ± 0.7 vs. 2.9 ± 0.7; p = 0.13), freezable (2.0 ± 0.7 vs. 2.8 ± 0.7; p = 0.19) and degenerated embryos (0.6 ± 0.2 vs. 0.5 ± 0.2; p = 0.79) were found in both groups (C-G and PGF-G respectively). Moreover, buffaloes from PGF-G had more oocytes (0.7 ± 0.3) than the ones from C-G (0.0 ± 0.0; p = 0.04), which may be due to an increased motility of the oviduct when PGF2α was administered. Results are indicative that the administration of PGF2α during the periovulatory period was effective to increase embryo recovery rate (total ova/embryos recovered) in buffaloes subjected to multiple ovulations.

Key Words: Buffaloes, superovulation, embryos, PGF2α, periovulatory period

0805

Sex ratio variation of lambs born from interbreed fresh and frozen embryo transfer

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Sex ratios have been the subject of numerous investigations, particularly in studies with humans. In general, however, the literature relating to sex ratio in sheep is limited. Population studies have generally been based on an expected sex ratio of 0.5, and any factor causing marked divergence from this ratio is important. This paper examines the influence of some factors, year and season of birth, type of birth (single to triplet) and breed or sheep on the sex ratio of lambs at birth in interbreed embryo transfer programs. The data used in these analyses were collected as part of a wider study, in which interbreed fresh and frozen embryo transfers were conducted in introducing new genetics. A total of 360 fresh and frozen embryos were obtained from prolific (Romanov) and meat type (Charollais and Suffolk). During four years (2006, 2007, 2009 and 2011), three different lambing seasons (winter, spring, autumn), birth type (single, twin, triplet) and breed type were of dam studied as possible sources of variation in sex ratio at birth. Deviations from a 1:1 sex ratio were tested by binomial tests. Year of observation (2006: 41%, 2007: 55%, 2009: 53%, 2011: 67%) had no effect on the secondary sex ratio at birth. From this study, there is no evidence that breed or strain of sheep (Prolific: 48% and Meat Type: 56%) markedly affects sex ratio. There was a trend for the sex ratio (male) to be higher in lambs born as triplets than in single births (79% vs. 51%). Season of birth was found significantly ($p < 0.05$) important factor for the differential of sex ratios at births. Lambs born in winter season had higher male biased sex ratio than those born in spring (47%) and autumn (43%). It was concluded that main factors can skew sex ratio in embryo transfer programs are season of birth and birth type.

Key Words: Sex ratio, embryo transfer

0806

High efficiently reproduce mutton breeding sheep by the embryo transfer technic in the field

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In order to improve the efficiency of sheep embryo transfer (ET), key factors were investigated in the field. A total of 1109 Suffolk and Dorset sheep donors and 7664 local recipient ewes were treated in the breeding season from 2006 to 2011, at 1.5–5 years of age. Donors were superovulated with FSH (400 IU, FolltropinV, Canada,) in a declining dosage for 3.5 days, and bred with rams. Five trials were conducted as follows: (i) Before treatment, pregnancies were screened by ultrasound in all recipients ewes which had been purchased on the market. (ii) Rates of estrus in recipients were compared for synchronization methods of progesterone sponge (60 mg) + PGF2a (0.05 mg) or 2 × PGF2a (0.05 mg) injection at 10 days interval. (iii) Comparing recipient pregnancy ratea following surgical and laparoscopic ET. (iv) Comparing recipient pregnancy rates following long distance (600 km, 15 h) transport of fresh and frozen embryos. (v) Comparing donor embryo numbers for the first and second surgery.

Results: (i) The pregnancy rates in grazed herds, house-fed herds and market purchased herds were 2.4% (99/4125), 9.3% (51/548) and 16.6% (52/313) respectively before estrus synchronization treatment. (ii) Adult recipient estrus rates following progesterone sponge + PGF2a or 2X PGF2a injection at 10 days interval were 87.5 ± 3.6% (493/563) and 90.2 ± 2.8% (1043/1156, $p \geq 0.05$) respectively. (iii) The pregnancy rate following laparoscopic transfer (60.3%, 2165/3588) was higher than the surgical transfer (55.6%, 2266/4076) ($p \leq 0.05$). (iv) The pregnancy rate of frozen embryos and fresh embryos with long distance transport were 54.3% (25/46) and 52.6% (20/38) ($p \geq 0.05$) respectively. (v) The number of transferable embryos from the first surgical collection was greater than from the second collection, 11.7 ± 8.3 (8646/739) and 8.6 ± 6.2 (3182/370) ($p \leq 0.05$) respectively. The conclusions of this study were (i) Ultrasound screening of recipients eliminated pregnant recipient ewes, thus reducing pregnancy losses, unexpected pregnancies are common in recipient herds in China. (ii) The estrus rate of recipients synchronized by progesterone sponge + PGF2a was numerically higher than the other treatment, but not significantly higher. The 2× PGF2a injection was simpler and the reproductive tract of ewes was cleaner. (iii) Laparoscopic transfer saves time and is less surgically invasive, which may help increase the number of pregnancies. (iv) The pregnancy rates of frozen embryos and fresh embryos transported a long distance were similar and both resulted in acceptable pregnancy rates. (v) The transferable embryos number of donors decreased with repeated collections. The results proved that ET can be efficient an efficient tool insheep breeding following the improving key procedures of sheep ET. On average, 3.42 (4215/1232) recipient pregnancies have been obtained through a single superovulation donor treatment.

Key Words: Reproduction, sheep, embryo transfer

0807

Day of collection affects the number of embryos recovery on ewes

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There is high variability on the number of embryos recovery per ewe in response to superovulatory treatments on embryo transfer programs in this species. One of the reasons is due to ewes that respond on superovulation protocol (checked by laparoscopy CL count) but when flushed no or few (compared to CL count) embryos or non fertilized oocytes is recovered. On literature the day of embryo collection vary between Day 5 (D5) to Day 7 (D7) (D0, insemination day). The aim of this study was to evaluate if the day of collection affects the number of embryos recovery per ewe. For the present study a total of 64 embryo collections were performed on Dorper ewes (D6, n = 24; D5, n = 40). The synchronization and superovulation protocol were the same for all ewes and consisted of 14 days of progesterone (Easy Breed, CIDR®;

Pfizer, Brazil) being Day-14 the day of CIDR insertion, changed for a new one on Day-7 coinciding with a 125 μg of cloprostenol (Ciosin®; Coopers, Brazil). The superovulation consisted of eight decreasing doses (2 × 40, 2 × 30, 2 × 20, and 2 × 10 mg) of FSH (Folltropin-V; Bioniche, Canada) administered twice a day 60 h before CIDR removal. At CIDR removal a 200 IU of eCG (Novormon®; Syntex, Argentina) were given and 24 h later, coinciding with the last FSH injection, a dose GnRh (Gestran®; Tecnopec, Brazil) were administered. Inseminations were performed using frozen semen by laparoscopy at 39 (D0) and 45 h after progesterone withdrawal. Embryos were collected via laparotomy and flushing of each uterine horn. The total embryo recovery was significantly different ($p < 0.01$, Q square test), 42.86% on D6 (138 embryos and oocytes/322 CL) and 59.75% on D5 (334 embryos and oocytes/559 CL). Also the means of embryos or non fertilized oocytes per ewe were significantly different, 5.11 on D6 and 8.35 on D5, and when considered only the number of embryos, 4.78 on D6 and 7.75 on D5 ($p < 0.01$, *T*-test).
The means of hatched embryos recovered on D6 were 1.04, but only 0.03 on D5. In conclusion the day of collection affected the number of embryos recovered and more studies are necessary to determine if this finding is related with the timing of sheep embryo development.
Key Words: Ewe, embryo transfer

0808

Comparing two superovulation protocols in dromedary camels

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The number of comparative studies on superovulatory protocols in Dromedary camel is very limited. Most superovulatory protocols are based on the decreasing doses of FSH. Our working hypothesis was that a sharp rather than soft decline in decreasing doses of FSH may result in better superovulatory responses in Dromedary camels. Nine Dromedary racing camels were randomly assigned into two groups and received a single dose of eCG (900 IU; IM; Pregnecol 6000®; Bioniche, Canada) on Day 1 and twice daily injections of FSH (17.5 mg; IM; Folltropin-V®; Bioniche, Canada) for 5 days and a single injection on Day 6. Prostaglandin analogue (500 μg; IM; cloprotesnol, Vetaprost®; Aburaihan, Iran) were injected twice on Day 5 of superovulation. Group 1 females (n = 5) received FSH with relatively similar doses for the start followed by soft decline (2.5, 2, 1.5, 1.5, 1, 0.5); whereas, Group 2 females (n = 4) received FSH with the higher initial doses followed by sharp decline (3, 2.5, 1.5, 1, 0.5, 0.5). Females were mated with fertile bull 36 and 60 h after the last FSH injection. Concurrent with the first and second mating, females received GnRH analogue (25 μg; IM; Alarelin®; Aburaihan, Iran) and hCG (2000 IU; IV: Choriomon®; IBSA, Switzerland), respectively. Embryos were recovered non-surgically 8.5 days after the first mating. Data were analyzed using Student's *t*-test. The total number of transferable embryos were significantly higher in Group 2 (6.0 ± 1.59) than Group 1 (1.8 ± 0.82) females ($p < 0.05$). In conclusion, starting superovulation with high doses of FSH followed by sharp decline in FSH may provide good superovlatory response in dromedary camels.
Key Words: Superovulation, dromedary camel, FSH

0809

Factors affecting embryo collection and transfer in alpacas

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One of the main limitations of embryo collection and transfer in alpacas is the inability to manipulate the reproductive tract per rectum in small females. As a result, donor alpacas are selected based on size and/or ability to perform rectal palpation. We hypothesized that embryos could be successfully collected from the uteri of females without manipulation of the reproductive tract per rectum. Our objectives were: (i) to determine the feasibility of performing embryo collection and transfer without rectal palpation; and (ii) to determine the effects of the day of the estrous cycle on embryo collection rates and size of the embryos. Fifteen females (5–18 years) were teased daily and naturally mated by a fertile male. Follicular development and ovulation were monitored by ultrasonography. Embryo collections were performed on Days 6–8 after mating by non-surgical uterine lavage. Embryos were transferred non-surgically into recipients that were administered 1000 IU hCG IV on the same day the donor was mated. Catheterization of the cervix during embryo collection or transfer was accomplished via vaginoscopy only. Data were analyzed by Fisher's exact test or *t*-test. Data are expressed as mean ± SD and significance was set at $p < 0.05$. A total of 32 embryo collection attempts were performed including five collections post-mortem. In live females, catheterization of the cervix was complete (19/27; 70%), partial (5/27; 18%) or not achieved (1/27; 3%); information was missing for two collections. Overall embryo collection rate was 47% (15/32), and no differences in embryo collection rates were observed for attempts in live animals (41%, 11/27) or post-mortem (80%, 4/5). In addition, embryo collection rates were not affected by the day of the cycle. One or more embryos were recovered from all females in which three or more embryo collections were attempted. There were no differences in diameter between embryos collected on Day 6 (565 ± 208 μm) or Day 7 (533 ± 150 μm); however, embryos collected on Day 8 were significantly larger (1159 ± 547 μm) than those collected on Days 6 and 7. A total of 11 embryos were transferred non-surgically into 10 recipient alpacas, with one female receiving 2 embryos. Four pregnancies were detected by ultrasonography at 25 days of gestation (40%, 4/10) but three pregnancies were lost by Day 35. One female is currently pregnant at ~220 days of gestation. In summary, non-surgical embryo collection and transfer can be performed in alpacas using vaginoscopy and transcervical catheterization without manipulation of the reproductive tract per rectum. The ability to utilize small-sized females as embryo donors allows breeders to increase the pool of genetically-valuable females in an embryo transfer program. Potentially, the use of large-sized alpacas or llamas as embryo recipients may improve pregnancy rates by allowing manipulation per rectum and deposition of the embryo into the left uterine horn.
Key Words: Embryo transfer, alpaca, camelid, assisted reproduction

0810

Superovulation, embryo recovery, and pregnancy rates in seasonally anovulatory mares treated with recombinant equine FSH

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We evaluated the effect of recombinant equine FSH (reFSH) on follicular growth, multiple ovulations and embryo production in seasonally anovulatory mares. During winter 2011 (July–August in Argentina, South America) forty light breed donor mares, selected based on their reproductive records to have no more than 20% multiple ovulations per season, and presenting follicles < 10 mm in diameter and no CL at ultrasound examination, were randomly assigned (n = 10/group) to one of the following treatments: Group 1: twice daily intramuscular (IM) injections of 0.65 mg reFSH (AspenBio Pharma, CO), Group 2: once daily IM injection of 1.3 mg reFSH, Group 3: twice daily IM injection of 0.32 mg reFSH, and Group 4: once daily injection of saline (control). Treatment was administered until a follicle of 35 mm was observed or for a total period of 10 days. When the largest follicle reached ≥ 35 mm treatment was discontinued and 2500 IU hCG was injected intravenously 36 h later. Mares receiving hCG were inseminated with fresh or frozen semen every

48 h until ovulation (s) were detected. Eight days after first ovulation transcervical embryo recovery was performed and embryos grade 1 and 2 (IETS) were counted. Recovered embryos were non-surgically transferred to synchronized recipients, when available, and pregnancy diagnosed by ultrasonography 7, 14 and 21 days later. Results are summarized in Table 1. All mares receiving reFSH, but none receiving saline control, responded to the treatment with follicular growth. On average, 6.5 days of reFSH treatment were required for mares to develop follicles of ovulatory size (> 35 mm). Ovulations were detected in 80% of mares in Groups 1 and 2, 50% of mares in Group 3 and in none of Group 4 (Control). Among ovulating mares, no differences in number of ovulations, number of embryos recovered, or pregnancy rates were observed among reFSH treatments. Overall, a 59% pregnancy rate was achieved with embryos collected and transferred during the anestrus season. These results indicate that treatment with reFSH during deep anestrus results in follicular development, ovulation of fertile oocytes, and production of embryos that established viable pregnancies after transfer. Also, a single daily administration of reFSH was as effective as two daily administrations, which allows for a simplified administration regimen. In conclusion, reFSH treatment of seasonally anovulatory mares allows production of viable embryos during the winter season.

Key Words: Recombinant FSH, anovualtory mare, anestrus, superovulation, embryo transfer

Table 1. Ovulation and embryo recovery rates of anovulatory mares treated with reFSH

reFSH Dose	Days of treatment	Ovulating mares	Follicles > 35 mm	Ovulations per ovulated mare	Embryos recovered per ovulated mare	Embryos transferred	Pregnancy rate
0.65 mg B.I.D.	5.9 ± 0.6[a]	8/10	5.2 ± 0.9[a]	5.1 ± 1.0	1.9 ± 0.5	14	57%
1.3 mg S.I.D.	7.1 ± 0.8[a]	8/10	6.4 ± 0.8[a]	5.5 ± 0.8	2.6 ± 0.5	16	56%
0.32 mg B.I.D.	7.3 ± 0.7[a]	5/10	4.1 ± 1.0[a]	4.4 ± 0.9	2 ± 0.4	4	75%
Saline	10 ± 0[b]	0/10	0 ± 0[b]	–	–		

[ab]$p < 0.05$ (*T*-test). Mean ± SEM.

0811

Use of fluorescent stains to assess viability of fresh embryos from superovulated pigs

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Hormonal treatments to induce superovulation in sows, as a part of an embryo transfer program, may affect embryo quality. Numbers of both viable and transferable embryos must be quantified to compare efficiency of superovulation treatment. Common approaches to do so include: (i) subjective microscopy assessment, (ii) *in vitro* incubation to assess embryo hatching and (iii) *in vivo* transfer; the first method is less expensive and less time-consuming than the others but it is the least precise. The objective of this work was to test the use of DNA fluorescent stains to estimate the proportion of live and dead embryo cells. Sows, primiparous (n = 5), were given eCG-hCG (PG600, Intervet) to induce superovulation; four days after artificial insemination, embryos were recovered by oviduct flushing. Each embryo (in 1 ml Dulbecco's PBS) was stained by adding 10 µl of each SYBR14 (100 nM) and Propidium Iodide (12 µM), fixed after 5 min with glutaraldehyde (0.2%), mounted on a glass slide, viewed by epifluorescence microscopy (20× and 40× objectives) and photographed. Numbers of SYBR14 + ve (live) and PI + ve (dead) cells were counted from those microphotographs. Twenty six embryos were recovered, some at 4-cell development stage (n = 15) and some morulae (n = 11); blastomeres, in most of the cases, displayed bright fluorescence and were clearly distinguishable. Proportions of live cells were 100% for 4-cell embryos and from 33% to 72% for morulae. These are preliminary results; we are still testing this approach using other superovulation treatments and validating this technique by doing embryo morphology assessment and *in vitro* incubation in parallel.

Key Words: Superovulation, sow, SYBR14/PI, blastomeres, morulae

0850

Efficacy of hyaluronan as a diluent for a two-injection FSH superovulation protocol in *Bos taurus* beef cows

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Due to the rapid absorption and short half-life of FSH, superovulation of cattle has traditionally been done with seven or eight bi-daily injections of FSH plus one or two injections of PG over a four day period. Restraining beef cattle for multiple injections involves stress to the cattle and increased risk of injury to both cattle and humans. The present study involved superovulation of Bos taurus beef cattle in six commercial embryo transfer (ET) programs either on farm or housed in ET facilities. Half of the donors (controls) were superovulated with eight injections of Folltropin-V (Bioniche Animal Health, Inc.) while the other half were injected twice with the same lot no. of Folltropin-V reconstituted in a slow release formulation (SRF) diluent containing 0.5% hyualuronan (Map 5, Bioniche). The study was divided into two phases: 1-In 2010, cows were each superovulated once by six ET practitioners, in either a control (n = 63) or treatment group (n = 57); 2-In 2011, cows (n = 46) were each superovulated twice in a cross-over design by three ET practitioners. Animals were synchronized prior to the start of FSH injections by one of four protocols: (i) FSH was started on any day between days 8 and 13 of the estrous cycle; (ii) use of a CIDR and GnRH with FSH started two days later; (iii) use of a CIDR and estradiol 17β with FSH started 4 days later; (iv) insertion of a CIDR with FSH started 4 days later. Control females received eight injections of descending doses of FSH totaling 260–400 mg. On the day that FSH injections were initiated (Day 0) for all four synchronization protocols, controls received the first of 8 twice-daily descending doses of FSH, while donors in the treatment group received a dose of FSH in SRF diluent totaling 67% of the total amount of FSH given to control donors. Treatment donors received the remaining 33% of SRF FSH on day 3. Both treatment and control donors received two injections of PG on day 3 or 4 and CIDRs were pulled on day 4. All animals were inseminated at 12 and 24 h following onset of standing estrus. Donors were flushed 7 days after onset of estrus and all ova/embryos counted and graded for stage and quality. There was no effect of year on any category of ova/embryos recovered, nor was there any treatment by year interaction. The least squares means of ova/embryos for both years combined are presented in table 1. There were no significant differences (ANOVA) in the number of ova, transferable embryos, unfertilized ova (UFO) or degenerate embryos recovered from the control vs. the treated groups. This study demonstrated that Bos taurus cattle can be successfully superovulated with only two injections of FSH reconstituted in an SRF diluent containing 0.5% hyaluronan and involving a variety of synchronization protocols. Acknowledgments-We thank Chelsie Burroughs for providing the statistical analysis of the data in this study.

Key Words: Cattle, superovulation, embryos, hyaluronan, embryo transer

Table 1. Least squares means of ova/embryos recovered from control vs. treated donors

Group	Number	Ova/embryos	Embryos	UFO	Degen.
Controls	109	14.0 ± 1.0	7.9 ± 0.8	3.4 ± 0.5	2.7 ± 0.4
Treated	103	12.2 ± 1.0	6.8 ± 0.8	3.1 ± 0.5	2.4 ± 0.4

0851

Superovulatory response in hair ewes following subcutaneous osmotic pump infusion of FSH

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The objective of this study was to compare superovulatory responses in hair ewes administered FSH by twice daily intramuscular injections or by subcutaneous osmotic pump infusion. During summer-autumn, 22 mature hair ewes received intravaginal sponges containing 20 mg of fluorogestone acetate (FGA; Chronogest®; Intervet) for 14 days and were randomly assigned by liveweight (38 ± 0.80 kg.) and body condition score (three out of five) into two groups (n = 11 each): Group 1 received 200 mg of FSHp (Folltropin®; Bioniche) in decreasing, intramuscular doses over 4 days (40, 40, 30, 30, 20, 20, 10 and 10 mg) at 12 h intervals, whereas Group 2 received 200 mg of FSHp administered by subcutaneous osmotic pumps (Alzet®) and a single intramuscular dose of 60 mg of FSHp at the moment when to implant the pump. FSH treatments were initiated 11 days after sponge insertion. Intrauterine insemination with fresh semen (100×10^6 spz) was done 56 h after sponge withdrawal. Ovulation rate was observed by endoscopy 7 days after sponge withdrawal. The number of regressed CL was determined by pale almost white color of the structures at the time of endoscopy. The results were analyzed by X_2 with the statistical package Statistix 8.0. Mean ± SE (8 ± 1.6) number of ovulations (CL), was greater ($p < 0.01$) in Group 2 (9.8 ± 1.8) than in Group 1 (6.2 ± 1.5). On the contrary, numbers of regressed CL were higher (33/108) and recovery rate was lower (45.3%) in Group 2 than in Group 1 (5/68 and 65.6%, respectively; $p < 0.01$). Although superovulatory response was greater in hair ewes treated with osmotic pumps, the high rate of regressed CL observed in this group is of concern and must be studied further.

This work was supported by a project 164592-CONACYT grant.

Key Words: Hair ewes, FSH, osmotic pumps

0852

Relationship between ovarian status prior to superovulatory treatment and ovarian response in hair ewes

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The objective of the study was to determine the relationship between ovarian follicular status prior to a superovulation stimulatory treatment and ovarian response. In the spring, 48 hair ewes (2–3 age) in each type of breed (Dorper, Katahdin, Pelibuey and Blackbelly; n = 12) received intravaginal sponges containing Fluorogestone Acetate (FGA; Chronogest®; Intervet) for 14 days and two intramuscular injections of 15 mg of Luprostiol (Prosolvin®; Intervet) applied at sponge insertion and 7 days after. Starting 11 days after sponge insertion, ewes received 200 mg of FSHp (Folltropin®; Bioniche) in decreasing intramuscular doses (40, 40, 30, 30, 20, 20, 10 and 10 mg) at 12 h intervals over 4 days. All ewes received a single dose of 100 μg of GnRH (Fertagyl®; Intervet) 36 h after sponge withdrawal. The results were analyzed by the GLM process of statistical program SAS 9.2. From 48 total ewes under treatment, 41 responded with a superovulatory response. In those 41 ewes, prior to FSHp treatment, the numbers mean of follicles (2–5 mm) observed by echography (DP-6600 VET Cencen® Midray with 7.5 MHz transrectal probe) was a 12.2 ± 1.3 and a mean number of corpora lutea (CL) found by endoscopy (Karl Storz endoscopy® 7 mm) 7 days after sponge withdrawal was 7.7 ± 2.2. There was a significant positive correlation ($r = 40$; $p < 0.05$) between these two variables. Dorper ewes had the greatest ($p < 0.05$) superovulatory response (11.8 ± 1.41 CL), as compared to Black belly (9.0 ± 7.4), Katahdin (7.5 ± 1.5) and Pelibuey (5.6 ± 1.3) which did not differ. In conclusion, a positive correlation exists between ovary status prior to initiating treatment and ovarian superovulatory response. Dorper ewes would appear to be more responsive to FSH treatments than Katahdin, Black belly and Pelibuey breeds.

This work was supported by a project 164592-CONACYT grant.

Key Words: Hair ewes, FSH, FGA, GnRH, corpus luteum

0853

Influence of season on superovulatory response and embryo yield of Santa Ines ewes submitted to FSH treatment started near the time of emergence of the first follicular wave

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The study was designed to investigate the influence of season on superovulatory response and embryo yield in Santa Ines ewes submitted to FSH treatments started near the time of emergence of the first follicular wave in a progesterone treatment protocol. Day of emergence of the first follicular wave was defined in a previous study that evaluated follicular dynamics in following estrus synchronization treatments (Oliveira et al., *Acta Scientiae Veterinariae* 2011;40). Thirty Santa Ines ewes were divided into three groups according to season of treatment (Non-breeding, n = 10; Transition, n = 10 and Breeding, n = 10) and submitted to superovulatory treatments beginning at the time of emergence of the first follicular wave. All ewes received a device containing 0.3 g progesterone (CIDR®; Pfizer-New Zealand) for six, six and eight days, respectively. Thus, FSH treatment was started on Days 4, 4 and 6 of the protocol for Non-breeding, Transition and Breeding seasons, respectively (Day 0 = onset of protocol). All ewes received an injection of 37.5 μg of D-cloprostenol (Prolise®; Arsa-Argentina) on Day 0 and at CIDR removal. The superovulatory regimen consisted of eight i.m. injections of pFSH (Folltropin®; Bioniche-Canada) administrated twice daily (40, 40, 30, 30, 20, 20, 10 and 10 mg of pFSH). A single i.m. dose of 200 IU of eCG (Novormon®, Syntex-Argentina) was given concurrently the CIDR removal. Ewes were mated by a fertile ram. Embryo collections were done 7 days after CIDR withdrawal. The superovulatory response was classified in scores: (0) 4 or less CL; (1) between five and 10 CL, and (2) 11 or more CL. Data were analyzed by GLIMMIX (logistic regression) using the SAS. Most of donors (70%, 50% and 100% for Non-breeding, Transition and Breeding seasons, respectively) had a superovulatory response classified in score 2 ($p > 0.05$). There were no differences among seasons ($p > 0.05$) for ovulation rate (Non-breeding: 89.2 ± 4.1%; Transition, 90.8 ± 3.2% and Breeding, 97.9 ± 1.4%), number of ovulations (Non-breeding: 12.4 ± 0.9; Transition, 13.1 ± 2.3 and Breeding, 17.0 ± 2.3) and number of luteinized unovulated follicles (Non-breeding: 1.7 ± 0.7; Transition, 1.4 ± 0.4 and Breeding, 0.7 ± 0.5). Furthermore, there were no differences among seasons ($p > 0.05$) in proportion of ova/embryos recovered (Non-breeding: 69.9 ± 5.6%; Transition, 55.1 ± 8.5% and Breeding, 54.9 ± 5.7%), number of ova/embryos recovered (Non-breeding: 8.6 ± 1.0; Transition, 7.0 ± 1.7 and Breeding, 9.0 ± 1.4), number of viable embryos (Non-breeding: 3.2 ± 0.8; Transition, 3.9 ± 1.9 and Breeding, 3.8 ± 1.5). In conclusion, there were no differences in superovulatory response and embryo yield among seasons when FSH treatments were initiated near the emergence of the first follicular wave following progesterone treatment in Santa Ines ewes. Financial support:
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Key Words: Superovulation, emergence follicular wave, embryo, ewe

0854

Superovulatory responses and embryo recoveries in Rathi (*Bos indicus*) cattle: prospects and limitations

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Superovulatory responses of Rathi (Bos indicus) cattle have been low. The objectives of this study were to test the superovulatory responses and embryo recoveries of Rathi cows following different doses of Folltropin and the pregnancy rates after transfer of embryos to synchronized recipients. Post-partum cows were superovulated 60 days post-partum using 400, 300, 200 or 240 mg of Folltropin administered in 8 descending doses 12 h IM starting from day 8–10 of a CIDR-B. Cows were induced to estrus by IM administration of a prostaglandin (PG) (Inj Cyclix) and withdrawal of CIDR-B at the time of last injection of FSH. Cows were inseminated thrice at 12 h interval using frozen semen. Suitable recipients were synchronized by injecting a dose of PG one day before the injection of PG to donors. Embryos were recovered by non-surgical means on day 7 post insemination. Transrectal ultrasonography was performed on the day of embryo recovery to record the number of unovulated follicles and the number of corpora lutea. Estrus expression was poor in superovulated donors which posed a difficulty in precise timing of insemination. The total ovulatory responses and the total embryo recoveries, were non-significantly different among all the groups (Table 1). However, the mean number of transferable grade embryos was significantly higher in the 300 mg group compared to all other groups. Large numbers of unovulated follicles were visible ultrasonographically on the day of embryo recovery in the 400 mg group. A total of 2, 5, 3 and 2 animals yielded no embryos in 400, 300, 200 and 240 mg groups respectively and in a total of 5 animals the flushing catheter could not be inserted through the cervix. Transfer of recovered embryos to 29 recipients in the horn ipsilateral to the CL bearing ovary did not result in pregnancy (Examined by transrectal ultrasonography at day 40). It was concluded that superovulation and embryo recoveries are difficult in Rathi (Bos indicus) cows due to physiological differences and that 200–300 mg appears to be the optimum dose for induction of superovulation.

Key Words: *Bos indicus*, embryo, folltropin, Rathi, superovulation

Table 1. Superovulatory responses, total and transferable grade embryo recoveries in Rathi (Bos indicus) cows with different doses of Folltropin-V

Dose of Folltropin	Number of animals	Ovulatory response	Total embryo recovery	Transferable grade embryo recovery
400 mg	5	6.0 ± 2.15	2.2 ± 1.02	0.6 ± 0.67
300 mg	13	5.53 ± 0.99	1.86 ± 0.53	1.30 ± 0.45*
200 mg	15	7.13 ± 1.21	3.66 ± 1.38	0.6 ± 0.27
240 mg	4	8.5 ± 0.74	1.75 ± 0.96	0.5 ± 1.15

*Significant ($p < 0.05$).

09. Epidemiology, reproductive diseases & pathology I:

0900

Reproductive diseases survey in small herds assigned to set up artificial insemination programs in São Paulo State, Brazil

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Besides management facilities such as fences and corrals, implementation of reproductive technologies such as artificial insemination (AI) requires the improvement of sanitary and feeding practices to reduce the risk of low fertility. Federal regulations in Brazil have helped to control Brucellosis and Tuberculosis disease by vaccination. However, high prevalence of other reproductive diseases in cattle is frequently associated with low fertility and could compromise the success of AI programs. Therefore, the aim of this study was to monitor the reproductive disease status and performance reproductive of different herds selected to set up a regional AI program. Twenty herds, formed by 10–80 crossbreed cows, located in eight municipalities of São Paulo State, Brazil, were selected to participate in a breeding program through AI. Blood samples (n = 306) were collected from randomly chosen animals for serological diagnosis of Leptospirosis, Infectious bovine rhinotracheitis (IBR), Bovine viral diarrhoea (BVD), Enzootic bovine leukosis (EBL) and Neosporosis. Laboratory exams were carried out according to methodology recommended by the World Organization for Animal Health. During the next six months, producers were advised to implement corrective measures to disease control, including massive vaccination against Leptospirosis and IBR. At the end of this period, animals were prepared to fixed-time AI (TAI) and a second serosurvey (n = 203) was done for BVD, EBL and Neosporosis. In the first disease screening, all herds had at least one blood sample reagent to one of target diseases. Results indicated seroprevalence for Leptospira (40%), IBR (80%), BVD (90%), EBL (35%) and Neospora (60%). Three or more agents were positive in 13.1% of samples, two agents in 35.0% and one agent in 28.7%. The percentage of negative samples for the five agents was 22.8%. Prevalence of BVD, EBL and Neospora at time of AI was reduced to 59.6%, 23.1% and 46.3%, respectively. The mean conception rate after TAI was 48.5% (range from 20% to 78%) and was not associated with serological responses to diseases. It was concluded that acceptable AI pregnancy rates could be achieved in herds with high prevalence of reproductive diseases if corrective measures to eradicate them or decrease them are implanted.

Key Words: Artificial insemination, cattle, reproductive diseases, TAI, disease survey

0901

Isolation of mycoplasma species from the uterus of early postpartum dairy cows with and without endometritis and its association with dystocia

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Among various bacterial species isolated from the uterus in the cows with endometritis, some species are known to be related with the occurrence of endometritis. However, little information is available on the involvement of mycoplasma infection in cows with endometritis and calving conditions. Therefore, the objectives of this study were to clarify the isolation rates and species of mycoplasma in cows without and with endometritis and to elucidate association of mycoplasma infection with dystocia. A total of 172 Holstein-Friesian cows housed in three freestall barns in Iwate prefecture, Japan, were examined at

week 5 (W5) and week 7 (W7) postpartum. Intrauterine samples were collected using a small brush (cytobrush) technique. Following its withdrawal, swab samples were taken and placed in mycoplasma culture broth at 37°C for 72 h, and in a transport medium for the recovery of aerobic and anaerobic bacteria in a subset of the cows. A novel and rapid PCR was used to detect seven mycoplasma species (*Mycoplasma bovis*, *M. arginini*, *M. bovigenitalium*, *M. californicum*, *M. bovirhinis*, *M. alkalescens*, and *M. canadense*). The cytobrush was also rolled gently along the length of glass slide for polymorphonuclear neutrophil (PMN) count. At W5 and W7, the diagnostic criteria for cytological endometritis were ≥6% and ≥4% PMN, respectively. Mycoplasma isolation rates were compared between cows that had normal calving and cows that had dystocia, and between those with endometritis and those without by Fisher′s exact probability test. Among the seven mycoplasma species, only *M. bovigenitalium* was detected in 15 out of the 172 cows (8.7%). Five cows were diagnosed as positive at W5 only, nine were positive at W7 only, and the other cow was positive both at W5 and W7. Mycoplasma isolation rate was significantly higher ($p < 0.01$) in dystocia cows (5/13; 38.5%) compared to cows that calved spontaneously (10/159; 6.3%). However, isolation rate of other bacteria in dystocia cows was not different from that in normal-calving cows. While mycoplasma isolation rate in cows with endometritis (1/48; 2.1%) was similar to that in cows without endometritis (4/124; 3.2%) at W5, the isolation rate in cows with endometritis (6/52; 11.5%) tended to be higher ($p = 0.07$) than that in cows without endometritis (4/120; 3.3%) at W7. At W5, 6 cows were found both with dystocia and endometritis and of those 1 animal was positive to *M. bovigenitalium*. At W7, 8 cows were found both with dystocia and endometritis and of those 4 animals were positive to *M. bovigenitalium*. In conclusion, out of the seven species, only *M. bovigenitalium* was detected in the uterus of postpartum cows, and mycoplasma infection in the uterus may be associated with dystocia at the last calving in dairy cows.

Key Words: Dairy cows, dystocia, endometritis, mycoplasma, postpartum

0902

Prevalence of subclinical mastitis in dairy farms in urban and peri-urban areas of Kampala, Uganda

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It is widely recognized that subclinical mastitis (SCM) is an extensive problem in the dairy industry worldwide, causing large production losses. It is of particular concern in developing countries, where the prevalence generally is higher and the economical implications greater. Earlier research has found prevalences of 25.2–55.2% of SCM at cow level in some African developing countries. However, there are no published results from Uganda, despite the importance of the agricultural sector and dairy industry in the country. The aim of this study was to investigate the prevalence of SCM in dairy cattle in the urban and peri-urban areas of Kampala and furthermore to gain information about pathogens, antibiotic resistance patterns and possibilities of prevention. The study was conducted as a field study in 18 small-scale dairy farms. All cows at the farms were examined, and cows with signs of clinical mastitis were excluded. Cows ($n = 195$) were tested with California Mastitis Test (CMT) and udder quarters with CMT score ≥3 were milk sampled for bacteriological analysis. To allow further sub-analysis of the results, stage of lactation, parity, milk production, production type, udder hygiene and cow breed were recorded. The effects of various factors from a first Chi2-test analysis were further analyzed in a multivariate analysis using logistic models. Preliminary results indicate that 86.2% ($n = 168$) of the tested cows had SCM in one or more quarters. The most common bacteriological outcome was infection with coagulase negative staphylococci (54.7%), followed by negative growth (24.9%) and streptococci (16.2%). Factors with considerable impact on the prevalence of SCM at cow level included stage of lactation, where the prevalence increased with lactation days; parity, where multiparous cows had higher prevalence than primiparous cows; and production type, where zero grazing cows had increased prevalence compared to grazing cows. Thus, the preliminary results suggest that the prevalence of SCM in Uganda might be substantially higher than in comparable developing countries. The bacteriological pattern resembles other reports from comparable countries, but is not identical. This implies that there is a large need of improvements in terms of hygiene and management in order to reduce the prevalence of SCM. Also, further research is needed to follow up such interventions, to better map out the prevalence of SCM on national level and to identify the properties of well-functioning herds, in order to use them as role models for success given the prevailing conditions.

Key Words: Dairy cattle, subclinical mastitis, mastitis pathogens, developing countries

0903

Effect of mastitis during the first lactation on necessity to cull of Holstein cows

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The future production of a dairy herd depends largely on primiparae. Recently, survival analysis was used to assess the effects of diseases on productive life. The aim of this study was to evaluate the effect of postpartum mastitis between first calving and subsequent conception on culling of Holstein cows using survival analysis. Four commercial dairy farms milking Holstein cattle were used in this study. Herd sizes were 235, 416, 508, and 898 lactating cows and the average milk production of the herds was 8145 kg per cow per lactation on a 305-day basis. A data set of 9183 first lactation cows was used. Calving to first mastitis interval was categorized in three levels (< 10 days, 10–60 days, and more than 60 days between calving and first clinical mastitis). The length of productive life was defined as the interval from first calving to the date of death or culling on the farm (LPL) or the interval from 30 DIM after the first calving to date of death or culling on the farm (LPL30). Survival data were analyzed using the Kaplan-Meier method, and differences in the cumulative survival rates between subgroups were compared with log-rank and Wilcoxon tests. Survival statistical analysis was performed using the statistical software package JMP. Mastitis tended to increase the chance of culling ($p = 0.07$). Survival analysis using Wilcoxon test showed a significant difference between the length of productive life for cows with different intervals from calving to first mastitis ($p < 0.01$). The average of LPL was 774.5, 791.7, and 991.5 day for the < 10, 10–60, and more than 60 days between calving and first mastitis. The results demonstrated that clinical mastitis between first calving and conception increased the chance of culling.

Key Words: Dairy cows, mastitis, culling, survival analysis, first lactation

0904

Detection of *Neospora caninum* in some cases of unexplained ewe abortions in the north east of Iran

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Unexplained abortions observed in north east of Iran sheep farms were investigated to determine if there was an association with Neospora caninum infection. Ewe hoggets and ewes on three farms that exhibited a persistent abortion problem, despite vaccination against Toxoplasma and Campylobacter infections, were chosen for this study. Blood was obtained from aborting, non-aborting and selected ewes that had dead foetuses at scanning. Some ewes were euthanized and examination of their uterine contents, gross examination and histopathology were performed. Serology was conducted for *N. caninum*, Toxoplasma gondii and Leptospira interrogans serovar Pomona and Leptospira borgpetersenii serovar Hardjo antibodies. Whole selected tissues were

used in polymerase chain reactions (PCR) to detect DNA from *N. caninum* and *T. gondii*. Gross examination and histopathology was not diagnostic and no bacterial pathogens were isolated. Serology using a commercial indirect enzyme-linked immunosorbent assay (ELISA) revealed *N. caninum* antibody-positive sheep (4/70). The mean seroprevalence of *N. caninum* was 5.7%. DNA from aborted fetuses was extracted primarily from placenta and CNS tissues. Tissues were analyzed using PCR with primers Np21+ and Np6+. From the 70 ovine fetuses 8.5% were diagnosed as being infected by *N. caninum* using PCR (6/70). The results of these current investigations are suggestive of an association between *N. caninum* infection and abortion in sheep within north east of Iran.

Key Words: *Neospora caninum*, abortion, ovine, PCR, ELISA

0905

Seroreactivity to leptospirosis and reproductive failure in mares embryo recipients in Rio de Janeiro, Brazil

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There is a lack of studies regarding to the effect of leptospirosis in mares, particularly in those that are involved in embryo transfer programs. Tropical regions, such as Rio de Janeiro, have adequate climatic conditions (high temperature, rainfall, and humidity) that promote the maintenance of the agent on the environment, spreading the leptospiral infection. Since leptospirosis may lead to early embryonic death, abortion and birth of weak foals it becomes an important reproductive disease in these regions. The aim of this study was to evaluate the impact of leptospirosis on embryo transfer procedures in Rio de Janeiro (Brazil). A serological survey was conducted from August 2008 to March 2010 in nine herds from Rio de Janeiro, Brazil, with high rates of reproductive failure, as early embryonic death (>12%), abortion (>12%) and perinatal death (10%). The herds were selected under a criterion in that mares should be aged 3–13 years, be vaccinated annually against herpesvirus, and has no history of reproductive disturbance by clinical, gynecological and trans-rectal ultrasound examination. Blood samples were collected independently of the time of the embryo transfer or pregnancy moment. A survey of practitioners revealed detailed information about the losses was obtained from practitioner. A total of 357 recipient mares were tested using microscopic agglutination test (MAT), in which live antigens undergo reaction with equine serum samples to detect agglutinating antibodies, with cut-off point of 1:200. A total of 247 mares (69.2%) were seroreactive, mainly for serovars Bratislava and Icterohaemorrhagiae. Seroreactivity to leptospirosis was associated ($p < 0.001$) with reproductive failure. Additionally, a seroreactive mare is 2.88 times more likely (relative risk – RR) to present reproductive failure than a seronegative mare. Seroreactivity to leptospirosis was particularly associated to early embryonic death ($p < 0.001$/RR 6.81), abortions ($p < 0.001$/RR 203.82), and perinatal death ($p < 0.001$/RR13.23). Noteworthy that in contrast to herpesvirus infection, leptospirosis may lead to pregnancy failure at any moment during pregnancy. Although a routine post mortem of de aborted feti was not performed, this study demonstrated a significant correlation between seroreactivity to leptospirosis and reproductive failure in embryo recipients mares, leading to early embryonic death, abortion, and foals perinatal death, and therefore must be considered as an important cause of reproductive failure in mares involved in embryo transfer programs.

Key Words: Leptospirosis, reproductive failure, embryo, mares, diagnosis

0906

Leptospirosis by serovars Bratislava and Copenhageni and reproductive failures in mares in Rio de Janeiro, Brazil

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In horses, leptospirosis determines important reproductive disorders, including abortions, perinatal mortality and prematurely born foals. It has been suggested that serovar Bratislava may be adapted to horses, but there is a lack of studies regarding the real role of the infection by this serovar in the reproductive syndrome of leptospirosis in horses. Additionally, the association of each serovar with specific reproductive parameters has not yet been described in horses. From August 2008 to March 2010, 608 mares from eight herds located in Rio de Janeiro, Brazil, were screened for leptospirosis. According to the reproductive history of the mares (data from the assistant veterinarian), herds were categorized in Group A (five herds, 357 mares), with a high occurrence of reproductive problems (embryonic recovery <50%, embryonic death >10%, abortions >5% and occurrence of perinatal death), and Group B (three herds, 251 mares), where all the mares were pregnant. A total of 74 out of the 357 mares from Group A presented early embryonic death (20.7%), while 78 (21.8%) aborted and 18 (5.0%) had perinatal death of the foal, totaling 170 mares with reproductive problems in group A (47.6%). Of the 608 serum samples tested by microscopic agglutination test (MAT), 273 (44.9%) were reactive, predominantly against serovar Bratislava (170 sera, 62.3% of reactive), but also against Copenhageni (103 sera, 37.7% of reactive). In Group A, 247 out of 357 sera (69.2%) were reactive, distributed between reactivity against serovar Bratislava (160 sera, 64.8% of reactive) and Copenhageni (87 sera, 35.2% of reactive). In Group B, 26 out of 251 sera (10.3%) were reactive, against Bratislava (10 sera, 38.5% of reactive) or Copenhageni (16 sera, 61.5% of reactive). Seroreactivity against Bratislava was more associated ($p < 0.001$; OR 3.6; CI 2.5–5.3) with the occurrence of reproductive problems than seroreactivity against Copenhageni. This is valid for early embryonic death ($p < 0.001$; OR 5.3; CI 3.1–8.9), perinatal death ($p < 0.01$; OR 4.5; CI 1.6–12.5) and also for abortions ($p < 0.001$; OR 2.3; CI 1.9–3.0). Therefore, we conclude that, not only leptospirosis impairs reproduction in horses, but also that Bratislava is more associated with reproductive failure in horse than other serovars.

Key Words: Leptospirosis, reproductive failure, recipient mare, diagnosis

0907

Microbiological and molecular *Lactobacillus* spp. detection in mare vaginal samples

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Pathogenic bacteria responsible for endometritis in mares, like *Streptococcus zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa* and Klebsiella pneumoniae were isolated on other publications from vagina of mares, representing a risk factor for bacterial ascendant infection The presence of *Lactobacillus* spp. for a healthy vaginal enviroment is considered to be important in women; however, there are few studies related to the composition of the vaginal flora of healthy mares. The present work aims to determine the occurrence of *Lactobacillus* spp. in the vaginal micro-environment of mares. A total of 35 crossbred multiparous mares, aged between 4 and 12 years, with no history of reproductive problems, treatments and with anatomical normal reproductive tracts, were used. Two deep vaginal swabs were obtained from the mares at estrus for Lactobacillus isolation and PCR evaluation. Ten heathy woman, in different cycle phases, volonteered to be used as control. *Lactobacillus* spp. was isolated from vaginal

swabs in 5.7% of mares and in 90% of the samples obtained in women. *Lactobacillus* DNA was detected by PCR in 22.9% of the mares and in all of the vaginal samples from the women. In conclusion, this study showed a low occurrence of *Lactobacillus* spp. in mares, suggesting that this bacterium may not play a fundamental role in the equilibrium of the vaginal micro-environment of mares.

Key Words: Mare, vagina, *Lactobacillus*, endometritis, microbiological

0908

Prevalence of virulence factor genes of *E. coli* is associated to the different outcomes of uterine infection in the bovine and canine species

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Escherichia coli (*E. coli*) is the pathogenic bacterium most commonly isolated from the uterus during the first week post-partum in cows with puerperal metritis and in bitches with pyometra. However, this pathogen is associated with different outcomes in these species. In the cow, the presence of *E. coli* in the early puerperium favours the establishment of infection with *A. pyogenes* and gram-negative anaerobes, usually originating a non-life-threatening uterine infection associated with impaired reproductive performance. In the bitch with pyometra, the presence of *E. coli* is associated with the most severe systemic signs, originating a potentially life-threatening disease. The aim of the present study was to compare the prevalence of virulence factor (VF) genes in *E. coli* isolates from bitch pyometra (n = 20) and from cow puerperal clinical metritis (n = 20). Phylogenetic grouping was performed using a triplex PCR targeting the genes chuA, yjaA and DNA fragment TspE4-C2. Thirty VF (including adhesins, toxins, iron-acquisition systems, protectins) were screened by conventional PCR. The prevalence of VF from *E. coli* isolates of canine and bovine origin were compared using the Fisher's exact test.

Bovine isolates belonged to the phylogenetic groups A (25%) and B1 (70%), with only 1 isolate (5%) belonging to group D. In contrast, almost all canine isolates belonged to group B2 (90%), except for 2 isolates that belonged to group A (5%) and B1 (5%). From the 30 VF analyzed, significantly more VF were found in canine than in bovine isolates (22 (71%) vs. 14 (45%); $p < 0.03$). The number of isolates with 9 or more VF was significantly higher in the canine than in the bovine species (respectively, 18 (90%), range 7–15 VF vs. 3 (15%), range 5–10 VF; $p < 0.0001$). All isolates were positive for type 1 fimbriae (fimH) and curli genes (cgsA and cgsD). In 90% of the canine isolates, these 3 VF were associated with S fimbriae and siderophore yersiniabactin. Although the hlyE gene was present in all bovine isolates, the remaining VF were detected in 40% or less of the isolates. The prevalence of the following VF genes was significantly higher in canine isolates than in bovine isolates: S fimbriae (sfa, $p < 0.00001$), P fimbriae (pap, $p < 0.001$), F1C fimbriae (focG, $p \leq 0.001$), cytotoxic necrotizing factor (cnf1, $p < 0.001$; cnf2, $p \leq 0.03$), uropathogenic specific protein (usp, $p = 0.05$), toxin alpha hemolysin (hlyA, $p < 0.03$), polysaccharide capsule synthesis gene (kpsMII, $p < 0.001$), siderophore yersiniabactin (fyuA; $p < 0.002$), aerobactin (iuc, $p < 0.01$), invasion protein (ibeA, $p < 0.00001$). In conclusion, *E. coli* strains isolated from cow puerperal metritis had a low potential of virulence. In contrast, bitch pyometra *E. coli* isolates had a high virulence potential, which might be relevant in the pathogenesis of pyometra. These differences between canine and bovine *E. coli* isolates may partially explain the different outcomes of the uterine infection in the two species. Grants CIISA74 and FCT-PTDC/CVT/66587/2006. S Henriques is a FCT PhD fellow

Key Words: *E. coli*, VF genes, bitch, cow, uterus

0950

Evaluation of cumulus-oocyte complexes and follicular fluid harvested from bovine herpes positive cattle following administration of dexamethasone

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The increasing demand for farm animals with superior genetics, mainly by developing countries, is boosting the international market of animal germplasm. In Brazil, almost all of the *in vitro* embryos are produced using cumulus-oocyte complexes (COC) recovered by ovum pick-up (OPU). Therefore, sanitary precautions are necessary to prevent transmission of pathogens when utilizing *in vitro* produced embryos. The objective of this study was to investigate the presence of bovine herpes virus type 1 (BHV-1) in the follicular fluid (FF) and viable COCs, following dexamethasone administration. The presence of BHV-1 was detected via utilization of a real time PCR assay. Ten (n = 10) BHV-1 naturally infected and serologically positive cows were selected. The animals were homogeneously divided into 2 groups (G1, G2). The G1 received dexamethasone (0.1 mg/kg) i.v. every 24 h for five days (D0–D4), while G2 received saline solution according to the protocol of the G1 and all animals. On D10, G1 and G2 animals had their COC and FF collected by OPU. Before dexamethasone administration and OPU, animals had a nasal and vaginal swab samples collected for BHV-1 real-time PCR quantification and a blood sample collected for serum neutralization. On D0, titles of neutralizing antibodies (NA) against BHV-1 virus, range from 4 to 16. At this time the virus was detected in all nasal swab samples for G1 and G2 groups ($5.08 \pm 2.65 \times 10$) and four vaginal swab samples (4/10) with $2.34 \pm 1.12 \times 10$ viral DNA copies. On D10, NA titles against BHV-1 were between 32 and 128 (G1 and G2). According to real-time PCR results, on D10, viral DNA copies were detected on nasal swabs ($2.12 \pm 1.04 \times 10^4$; $3.67 \pm 2.23 \times 10^3$) and vaginal swabs ($2.28 \pm 1.19 \times 10^3$; $7.41 \pm 2.47 \times 10^2$) for G1 and G2 respectively. One sample of FF on G1 (5.25×10^2 DNA copies) and two COCs samples (1.18×10^2; 1.69×10^2 DNA copies) for G1 and G2 respectively, were positive. Comparing DO and D10, results demonstrated at least a fourfold increase of NA title and a high increase in numbers of DNA BHV-1 copies on nasal and vaginal swabs on G1 and G2. Dexamethasone administration, as well as a stress caused by the sequence of procedures can promote replication of BHV-1, confirming the possible contamination of COCs and FF in animals with native virus replication.

Acknowledgments: CNPq, FAPEMIG

Key Words: BHV, oocytes, follicular fluid, dexametasone

0951

Evaluation of bovine spermatozoa following exposure to bovine herpesvirus type 1 (BoHV-1)

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The potential for contaminated bovine semen to spread pathogens is a major concern to breeders, as well as the health authorities of all countries currently utilizing reproductive technologies. A number of viral and bacterial pathogens can be associated with semen and BoHV-1 is among those pathogens. Bovine herpes virus type-1 can be present in the semen of apparently healthy animals, facilitating the risk of its transmission. The purpose of this study was to evaluate bovine semen

following experimental exposure to BoHV-1. Bovine semen straws (0.5 ml) were obtained from the same bull, thawed for 30 s at 38°C and evaluated for viability (live sperm concentration, motility and morphology). Samples were divided in two groups: control and those exposed to 30 µl of BoHV-1 ($10^{6.5}TCID_{50}$/ml, Colorado sample). For all groups, 50 µl of diluted semen in HTF medium (Vitrocell/Embriolife) was supplemented with either 30 µl of HTF medium (control group) or 30 µl of BoHV-1, and 5% heparin. The groups were incubated at 38°C C in 5% CO_2 and 90% humidity, and evaluated after a period of 2, 4 and 24 h of exposure. A X^2 test was used for statistical evaluations, and the experiment was repeated four times. The concentration of the control group was of 7×10^5 and 2×10^5/ml, with 80% and 70% motility, respectively after 2 and 4 h, and absence of live sperm after 24 h. The concentration of the treated group was 6×10^5 and 2×10^5/ml concentrations, with 80% and 70% motility, respectively, for the first two periods. However, the 24 h evaluation of the control and the treated group had the same result with complete absence of live spermatozoa. The results of electron microscopy showed enveloped virus-like particles attached to the cytoplasmic membrane of the flagellum and the sperm middle piece after 2 h. After 4 h, structures like capsids were identified inside the sperm membrane. Nucleocapsid particles were also observed in the middle piece of spermatozoa. These structures were not found in the control group. We suggest that while there were no significant differences in tests of viability, the viral particles found on treated sperm at 2, 4 and 24 h coincide with the replication cycle of BoHV-1. These observations support the notion that sperm are a mechanical vector for the transmission of the pathogen. Support from the National Council for Scientific and Technological Development (CNPq), São Paulo Research Foundation (FAPESP) and Vitrocell/Embriolife are gratefully acknowledged.

Key Words: Bovine herpesvirus type 1, cattle, semen

0952

Factors effecting conception rate in pasture-fed dairy cows

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Conception rates of dairy cattle are declining internationally both in housed and pasture-fed and managed lactating dairy cows. The objective of this study was to identify cow-level risk factors for conception to the first breeding in lactating cows managed on pasture. In a prospective single cohort study reproduction and production data from 23 511 cows from 53 herds from 4 regions in New Zealand were collected and the probability of conception to the first artificial insemination (ConS1) of the seasonal breeding program was modelled using a general estimating equation model with adjustment for clustering of cow within herd. To assess the degree of clustering in the data the intraclass correlation (ICC) was estimated and the proportions of variance explained at the three hierarchical levels of the data (i.e. region, herd, cow) was calculated using a variance component model. ConS1 varied among regions [55.7% (SE = 1.2), 46.5% (SE = 1.5), 49.5% (SE = 1.7) and 55.7% (SE = 1.5%) for Waikato, Taranaki, Canterbury and Otago, respectively] and varied among herds (range = 34–67%; $p < 0.001$). Older cows (>8-years-old) had lower ($p < 0.05$) ConS1 than 3 and 4–8 year-old cows, with 2-year-old cows intermediate [50.5% (SE = 1.6), 53.3% (SE = 1.5), 52.4% (SE = 1.6), 47.1% (SE = 1.7) for 2, 3, 4–8 and >8-year-old cows, respectively]. Cows not in oestrus by the start of the breeding program (incidence = 9.4%) had lower ConS1 than those in oestrus [46.2% (SE = 2.9) vs. 55.5% (SE = 0.6), $p = 0.003$]. Cows with clinical mastitis (incidence = 13.3%) had lower ConS1 than those not diagnosed with mastitis [49.7% (SE = 1.7) vs. 52.1% (SE = 1.2), $p = 0.022$]. Cows in the lowest quartile for milk protein % tended ($p = 0.06$) to have a lower ConS1 than those with higher protein % [48.7% (SE = 1.4), 51.3 (SE = 1.6), 51.6% (SE = 1.7), 51.8% (SE = 1.6) for protein % of <3.48%, 3.49–3.73%, 3.74–4.04% and >4.04%, respectively]. ConS1 increased as the interval from calving to the start of the breeding increased [37.9% (SE = 1.9), 43.3% (SE = 1.8), 44.2% (SE = 1.8), 47.4% (SE = 1.6), 51.8 (SE = 1.6), 54.0% (SE = 1.8), 57.9% (SE = 1.4) for cows calved ≤4, 5, 6, 7, 8, 9, 10+ weeks, respectively]. Cow breed, milk fat %, milk fat (kg/cow/day) and milk protein (kg/cow/day) were not significantly associated with ConS1 at the univariable level. Totals of 0.3%, 1.6% and 98.1% of the variance were explained at the region, herd and cow levels, respectively. These results demonstrate that multiple factors including herd, age, detection in oestrus, mastitis, milk protein % and date of calving relative to the start of the breeding program all affect the probability of conceiving to first breeding. The great majority of the variation was explained at the cow level, rather than at herd or region levels. This suggests that cow-level factors should be addressed when trying to improve reproductive performance of herds and regions.

Key Words: Cattle, female, conception, risk factors

0953

Identification of toxic shock syndrome toxin-1 (TSST-1) gene of *Staphylococcus aureus* isolated from bovine mastitis milk in Urmia, Iran

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Staphylococcus aureus is a major causative pathogen of clinical and subclinical mastitis of dairy domestic ruminants. This agent produces a variety of extracellular toxins and virulence factors such as toxic shock syndrome toxin-1 (TSST-1) that contribute to its pathogenic potential. In this study 25 *S. aureus* isolated from four dairy herds of Urmia region, which is located in West Azerbaijan province of Iran. The tested isolates were identified on the basis of the cultural and biochemical properties as well as by amplification of the aroA gene specific to *S. aureus*. Isolates were also analysed for the presence of the TSST-1 encoding gene (tst) using polymerase chain reaction (PCR). *tst* gene was detected in 5 (20%) of the total number of 25 isolates. The present study revealed that the PCR amplification of the aroA gene could be used as a powerful tool for identification of *S. aureus* from the cases of bovine mastitis. Results from the present study also showed that the strain of *S. aureus*, which caused mastitis, could potentially produce staphylococcal toxic shock syndrome toxin-1. Overall, our results suggest that it is of special importance to follow the presence of TSST-1 producing *S. aureus* in foodstuffs, especially for protecting the consumers from toxic shock syndrome (TSS).

Key Words: *Staphylococcus aureus*, mastitis, PCR, TSST, cattle

10. Epidemiology, reproductive diseases & pathology II:

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The developmental ability of cattle oocytes is impaired in animals with liver disorder

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The objective of this study was to investigate the effect of liver disorder on the developmental potential of bovine oocytes. Ovaries were collected at slaughter houses from cattle in which the liver was classified by a veterinarian into the following categories: control group (n = 6), with normal livers; and, liver disorder (LD) group (n = 6), in which the liver was diagnosed as abnormal (e.g. hepatitis, liver degeneration and fatty liver) and was discarded. In the first experiment, oocytes were aspirated from the ovaries of both groups individually and good quality oocytes were matured in modified medium 199 (m-199), fertilized with frozen-thawed semen, and cultured in modified synthetic oviductal fluid (m-SOF). Cleavage and development to the blastocyst stage were examined at 7 days after co-incubation with frozen-thawed semen. Follicular fluids (FF) were collected, and the concentrations of glucose, non-esterified fatty acid (NEFA), β-hydroxybutyrate (BHBA) and γ-glutamyl transpeptidase (γ-GTP) were determined as indication of nutritive condition and liver function. Proportion of good quality oocytes and frequency of cleavage and blastocyst development were analysed using ANOVA

with Fisher's PLSD test. Compostion of FF were analysed using *t*-test. We found that the proportion of good quality oocytes and the frequency of development to the blastocyst stage were lower ($p < 0.05$) in the LD group (40.6% and 12.5%, respectively) than the control group (62.4% and 36.0%, respectively). The concentrations of γ-GTP and BHBA in the FF from the LD group (156.5 IU/l and 41.8 mg/l, respectively) were higher ($p < 0.05$) than the control group (25.0 IU/l and 28.1 mg/l, respctively). There was a negative correlation between the concentration of γ-GTP and BHBA and the proportion of oocytes developed to the blastocyst stage ($p < 0.05$). In a second experiment, oocytes from cattle with normal liver were pooled and matured in m-199 supplemented with various concentrations of γ-GTP and BHBA, then fertilized and cultured for 7 days. Supplementation of m-199 with γ-GTP and BHBA had no effect on the proportion of oocytes that developed to the blastocyst stage. By contrast, supplementation of m-SOF with γ-GTP and BHBA resulted in a concentration dependent reduction in the frequency of cultured oocytes developed to the blastocyst stage (control group, 43.5%; 50 IU/l γ-GTP group, 20.6%; 50 mg/l BHBA group, 20.9%). These findings indicate that quality of oocytes and their potential for development are lower in cattle with liver disorders than in healthy cattle. Our analysis indicated that one possible factor that is detrimental to the development of oocytes in LD cattle might be the high concentration of γ-GTP and/or BHBA in their follicles and/or oviductal fluid.

Key Words: Bovine, oocyte, follicular fluid, embryo development, liver disorder

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Clinical mastitis is not associated with induction of luteal regression in dairy cows

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One possible cause of low fertility in clinically mastitic cows is early regression of the corpus luteum (CL) post-AI, leading to termination of pregnancy. However, clear evidence of impaired progesterone secretion following mastitis is debatable. Our objective was to determine whether clinical mastitis induces CL regression, consequently shortening the inter-estrus interval. In Experiment 1, performed in Ohio (USA) and in Israel, progesterone profiles following clinical mastitic events were determined in cyclic, inseminated and pregnant cows. Blood samples (n = 4–5) were taken after mastitis was diagnosed (day 0) every 2–3 day for about 8–10 day. In Israel, Gram-negative *E. coli* was isolated in milk samples taken on day 0, and in Ohio and Israel, Gram-positive staphylococci, streptococci or coagulase-negative staphylococci were isolated. Samples were taken from uninfected controls. All mastitic cows, in both locations, received a commercial medication of non-steroidal anti-inflammatory drug at the time of mastitis diagnosis. None of the progesterone profiles of 12 cows infected with *E. coli* (six cyclic, two inseminated and four pregnant cows) differed from uninfected controls, and none showed early progesterone decline. Similarly, none of the 10 cows infected with Gram-positive bacteria (seven pregnant and three inseminated cows) showed any signs of early luteal regression, and their progesterone concentrations did not differ from those of uninfected controls. Thus, no association was found between clinical mastitic events and early luteal regression. In Experiment 2, an epidemiological survey was conducted in Israel. Events (n = 1357) of clinical mastitis were examined. Of these, only 201 cases (14.8%) satisfied the following criteria for analysis: only *E. coli* was isolated; at least 2 estrous cycles were noted (18–25 day) prior to the event; events occurred prior to 100 day of pregnancy, between day 5 and 15 of the cycle in cyclic cows, and at more than day 5 post-AI in inseminated cows; no other diseases were detected around the event. Timing of the event and estrus around the time of detection of mastitis were determined by Afimilk® charts. A total of 11 cows (5.5%) exhibited estrus < 8 day after the event and of these, only five cows exhibited an inter-estrus interval smaller or > 18–25 day (2.5%). The correlation coefficient between decline in milk yield and days to estrus after occurrence of an event was low ($r = 0.155$, ns). Results indicated a minimal, almost negligible association between *E. coli* mastitis and short inter-estrus interval. The near lack of association between the clinical event and premature induction of luteal regression suggests that the main cause of low fertility caused by *E. coli* mastitis post-AI is directed at the embryo rather than the CL.

Key Words: Mastitis, corpus luteum, progesterone

1002

Incidence of cytological endometritis and its effects on reproductive performance of crossbred dairy cows

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Subclinical endometritis or cytological endometritis (≥5% of neutrophils in an endometrial cytology sample) affect as many as 50% of all cows with more than 7 weeks postpartum in high-producing dairy herds. Dairy cows with subclinical endometritis had impaired subsequent reproductive performance. The aim of this study was to evaluate the incidence of cytological endometritis from 32 to 70 days in milk (DIM) and its effects on the reproductive performance of crossbred dairy cows, maintained in hot climate in Southwest of Brazil. The study was conducted from April 2010 to June 2011 in a commercial farm (Centralina, Minas Gerais, Brazil). Lactating cows (Holstein/Gir; n = 172), with no history of retained placenta, without clinical signs of uterine infection, and body condition score (BCS) above 2.00 (1 = very thin and 5 = obese) were used in the study. Ultrasound examination was performed to determine the presence of corpus luteum (CL) and uterine fluids. Vaginal discharge was evaluated by the gloved hand methods. Cytological samples were collected only in cows with clear or translucent vaginal mucus and no uterine fluids. Cytological sample of the endometrium were collected using a cytobrush adapted for cattle. Slides for cytological examination were prepared at the farm by rolling the cytobrush on a glass microscope slide and air-dried. In the lab, the cytology slides were stained with modified Wright Giemsa stain. Each slide was examined at 400 × magnification to perform the differential cell count of 200 cells (polymorphonuclear neutrophils and endometrial cells) by two observers. Cytological endometritis was defined when the proportion of neutrophils were ≥5% (Gilbert et al., 2005). Later, the cows were submitted to conventional artificial insemination (AI) or timed AI. The incidence of cytological endometritis was analyzed using PROC LOGISTIC of SAS program, including in the model calving season, BCS, presence of CL, parity and DIM. The effects of cytological endometritis on conception to first AI and pregnancy rate by 150 DIM were also analyzed using PROC LOGISTIC of SAS program. The incidence of cytological endometritis was 26% (44/172) and was not affected by parity, season of parity, presence of CL and DIM, while cows with BCS ≤ 2.50 had a higher ($p = 0.042$) incidence of cytological endometritis (31.53%; 35/111) than cows with BCS ≥ 2.75 (14.75; 9/61). Conception rate to first insemination was not influenced ($p = 0.40$) by the presence of cytological endometritis, but pregnancy rate at 150 DIM was higher (61.11% vs. 38.46%; $p = 0.044$) in cows without cytological endometritis. In conclusion, crossbred dairy cows also have cytological endometritis and it affects their reproductive performance. Supported by FAPEMIG

Key Words: Cytobrush, neutrophils, uterine infection, reproduction

1003

The effects of dystocia on production, reproduction and health of dairy cattle

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Dystocia in cattle results in many unfavorable consequences such as production losses, reduced fertility, more days open and more services per pregnancy. In addition, difficult calving lead to more frequent culling and even death of a cow.

A retrospective cohort study was designed to evaluate the effects of dystocia on days open, days to first service, pregnancy rate, milk yield at first month after parturition, culling rate, retained placenta, cystic

ovaries, metritis, clinical mastitis, abomasal displacement, obvious clinical lameness and milk fever. Records from 4561 births of two large Holstein dairy herds in Tehran suburb were evaluated from March 20th 2007 till March 20th 2009. These data were analyzed by SAS version 8.2. Chi-square test, *t*-test and logistic regression were used for analyzing the data and a p value ≤ 0.05 was considered significant. There was a significant correlation between dystocia and stillbirth. Dystocia had significant effects on days open, days to first service, pregnancy rate, and milk yield at first month after parturition and also on incidence of metritis and abomasal displacement. There was no significant effect on culling rate, retained placenta, cystic ovaries, clinical mastitis, obvious clinical lameness and milk fever.

Key Words: Holstein cows, dystocia, reproduction, health, retrospective cohort

1004

Post-operative fertility following caesarian section in cows: a 12-year retrospective study

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Post operative fertility following caesarian section in cows remains a concern for the surgeon as well as the owner. Prospectively such studies help to innovate or improve surgical technique, postoperative management and assessment of prognosis of post operative fertility. 151 cows were assessed for post operative fertility following caesarian section under field conditions. The cows were in the age group of three to ten years. In all the cases incomplete cervical dilatation was the cause of dystocia. 118 of the operated cows were nullipara (78.14%). 140 patients (92.7%) were referred by field veterinarians/paravets. 130 caesarian sections were conducted at owners' doorsteps (86.09%) and 21 surgeries (1.4%) were conducted in the hospital/polyclinic premises by employing left lower flank paramedian oblique incision.112 cows (74.17%) came into estrus within 6–8 months post surgery. 30 cows (19.86%) were observed in first estrus post surgery after 12–14 months. 9 cows (0.5%) came in first estrus post partum/surgery after 18–20 months. 17 (1.12%) of the operated cows became repeat breeders which later on became normal following treatment. One cow (0.66%) developed uterine adhesions with the body wall. 142 animals (94.03%) remained normal cyclic and breeding cows in their life span. It was concluded that time of presentation for surgery, previous handling by a vet or paravet were the main factors which affected post operative fertility following caesarian section in cows.

Key Words: Post operative, fertility, caesarian section, cows

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Relationship between cyclical changes in endometrial cytology and the diagnosis of subclinical endometritis in cows

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Increased arrival of neutrophils (PMN) to the endometrium during estrus and metestrus in the cow had been described. This higher number of PMN could affect the diagnosis of subclinical endometritis (SE) and cause false-positive diagnoses. The aim of this study was to evaluate the relationship between the changes in the endometrial cytology during estrous cycle and the diagnosis of SE with the cytobrush technique (CB). The hypothesis was that the percent PMN did not change during the estrous cycle in normal cycling cows. Postpartum Holstein cows (27–56 days postpartum, dpp; BCS ≥ 2.5) from a commercial dairy farm located in Buenos Aires province were used. Cows were first inspected for presence of fresh and/or dry external vaginal discharge. The mucus content of the vagina was evaluated (diagnosis of clinical endometritis) and also they were examined by rectal palpation and ultrasonography. Only cows with clear vaginal mucus (without pus) and with active ovaries (presence of CL) were included. Cows were treated with an Ovsynch protocol [GnRH day (d)-9, PGF d-2 and GnRH d 0]. From synchronized cows (n = 17), CB and blood samples (P4) were taken during estrus (day 0; 12 h after second GnRH), metestrus (d4), diestrus (d11) and proestrus (d18). Endometrial cytology samples were collected using a CB modified for use in cattle; it is a stainless steel device that includes two disposable parts (brush and plastic sheet) which were changed between samples. Cytology slides were stained with a quick stain (Tincion 15, Biopur Argentina) and the degree of endometrial inflammation was assessed by counting a minimum of 200 cells at X 40 and expressed as % PMN. The counting was performed blindly in triplicate by two operators. Endometrial cells were photographed and measured at 100× with image processing software (Image J; NIH, USA). Data were analyzed by GLM and MIXED procedures of SAS. Correlation of data by different evaluators was measured by Kappa coefficient. All cows were negative for SE, there were no significant differences in the percentage of PMN between different sampling days (1.51 ± 0.50, p > 0.64); and PMN counts were always below 2%. PMN counts showed high correlation [Kappa 0.81 (same operator) and 0.76 (different operators)]. Therefore, in this study, results with CB were always below the cut-off point of 5% PMN set for diagnosis of SE. In contrast, serum P4 concentrations significantly changed between the days of the estrous cycle cycle [p < 0.01; (d0, 0.42 ± 0.42; d4, 1.16 ± 0.43; d11, 4.69 ± 0.44; d18, 2.85 ± 0.46)]. The area (μ^2), perimeter (μ) and Feret diameter (μ) of endometrial cells, showed no significant variations during different days of the estrous cycle (361.16 ± 24.26 μ^2 p > 0.12; 74.4 ± 2.32 μ, p > 0.37; 27.76 ± 0.85 μ, p > 0.48). In conclusion, these results show that despite the uterine physiological cyclical changes that occur during the estrous cycle, the diagnosis of SE by CB technique is not affected and the increase in PMN counts reflect true inflammatory changes that occur in the endometrium.

Key Words: Dairy cattle, subclinical endometritis, cytobrush, estrous cycle, percent PMN

1006

A retrospective study of treatment of Trichomonosis in bulls

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Systemic treatments with nitroimidazoles, despite their challenges and risk of adverse side effects, have become more popular than the less effective topical treatments for *Tritrichomonas foetus* in the bull. However, this choice of treatment is being challenged in South Africa with the use of topical diminazene. The objective of this study was to compare the curative effect and occurence of complications between these two treatments. Retrospective clinical data on the treatment of 40 bulls was collated from academic hospital records (n = 14), therapeutic trial records (n = 13) and practitioners (n = 13). Dimetridazole powder was dissolved in water and drenched by stomach tube or bottle daily for 5 days at 50 mg/kg. Rumenotorics and 5–10 l of water was given on a daily basis and antibiotics were administered parenterally from the day before the onset of treatment. Diminazene treatment was done using a 21 mg/ml solution instilled into the preputial cavity and held inside for 15 min daily for 5 days. A paste formulation was used in five cases. For those cases with follow-up information, 73.3% (n = 22) were treated successfully. For bulls treated with dimetridazole 91.7% (n = 11) were treated successfully compared to 61.1% (n = 11) for bulls treated with topical diminazene only. The ages of bulls treated successfully and unsuccessfully did not differ (Mean, median and range 4.9, 5, and 3–7 years and 5.4, 4.5, and 4–9 years respectively). Nineteen animals experienced complications (48%). All except one were associated with dimetridazole and involved the gastrointestinal tract, including inappetance (n = 11), reduced rumen activity (n = 5), rumen stasis (n = 3), and diarrhoea (n = 4). None were fatal, but some required prolonged treatment. One bull treated topically developed mild balanoposthitis. The difficulty of treating mature bulls is significant. The restraint of bulls to enable oral administration is only possible with good handling facilities. The placement of a nose ring is advisable prior to treatment. The use of sedatives was also necessary in some cases. In contrast, topical treatment causes the animal minimal distress and they become more

tractable and amenable to treatments. Care is required to minimise the gastrointestinal side-effects of dimetridazole including feeding good-quality roughage. Rumen function must be monitored by rumen fluid analysis. The response to the administration of fresh rumen fluid was good, with no bulls requiring more than one treatment. The availability of a source of fresh rumen fluid is important. This study does not support the development of nervous signs associated with treatment with dimetridazole. In this study topical treatment was shown to have significant practical advantages. It is a more specific treatment option directed at the site of infections only, namely the preputial cavity. However, it cannot be expected to be 100% effective as *Tritrichomonas foetus* also colonises the distal urethra in some bulls. These findings support a shift in emphasis in investigation back towards topical treatments.

Key Words: Trichomonosis, treatment, dimetridazole, diminazene

1007

Aspermatogenesis and hypospermatogenesis in culled Sahel goats in Nigeria

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Bilateral testicular hypoplasia occurs among Sahel goats in northeastern Nigeria, but the testicular size associated with the condition for the prediction of aspermatogenesis or hypospermatogenesis has been uncertain. The present study was carried out to determine the testicular and epididymal sperm cell counts and histopathology of the testes of culled Sahel goats at various testicular sizes. One hundred and twenty five randomly selected Sahel goats aged between 1½ and 2½ year old were examined at slaughter from April to August 2009. Before slaughter of the goats, their body weights (BW), scrotal lengths (SL) and circumferences (SC) were measured, and their testes and epididymides were collected after slaughter. The epididymides were separated from the testes and both were weighed. The testicular weight (TW) was divided by the body weight to get the gonadosomatic index (GSI). Testicular longitudinal length (TLL) and midcircumference (TMC) were measured. The left testis and epididymis (head, body and tail) were homogenized with normal saline containing formalin and spermatozoa were counted using a hemocytometer. Tissue samples of the right testis and epididymal segments (head, body and tail) were fixed in 10% buffered formalin, processed, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin and examined for histopathological changes by light microscopy. Gonadosomatic index values were used to categorize the goats into five groups of 25 goats each having very small (VSS), small (SS), medium (MS), large (LS) and very large (VLS) testes with GSI values of 0.3–1, >1–2, >2–3, >3–4 and >4 g/kg, respectively. BW, age GSI, SL, SC, TW, TLL, TMC, epididymal weights, testicular and caudal epididymal counts positively correlated ($p < 0.05$) with one another. Caudal epididymal sperm cell count was used to sort the goats into groups with anticipated azoospermia (AZS), oligospermia (OS) and normospermia (NOS). AZS in the studied population (29.6%, 37 out of 125) occurred in majority of goats with VSS (80%, 20 out of 25) and SS (68%, 17 out of 25) testes, but did not occur in those with MS, LS and VLS testes. OS occurred at all testicular sizes (18.4%, 23 out of 125), but most (91.3%, 21 out of 23) of the condition occurred in VSS to MS testes. The histopathological changes in OS and AZS testes were related to alterations in the population of spermatogenic cells in the epithelium of the seminiferous tubules indicating maturation arrest with aspermatogenesis or hypospermatogenesis. The epididymis had incomplete differentiation of the epithelium mostly in AZS and OS testes, occurring more frequently in the caput followed by the corpus and cauda epididymides. In conclusion, aspermatogenesis or hypospermatogenesis was invariably encountered in goats with VSS, SS and MS testicular sizes and the condition could be predicted with internal or external testicular measurements.

Key Words: Aspermatogenesis, hypospermatogenesis, testicular size, testicular hypoplasia, Sahel goat

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Puncture of epididymis in boars as a procedure in diagnostics of porcine brucellosis

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The existence of porcine brucellosis has been serologically proven in the Republic of Serbia, mostly in the regions that stretch along the rivers Danube, Morava and Mlava, but the agent has only recently been isolated and confirmed as Brucella suis biotype 2. This disease is known as the 'rednja' in the Republic of Serbia and it is characterized by abortions in sows in the territory of the hamlet or village, caused by mating of sows with the same infected boar. In these regions, breeding pigs are often kept in woods or pasture, and the contact is possible with many other pigs including wild pigs. It is a well known fact that there are no pathognomonic clinical signs of the disease in affected animals. The symptoms of this disease are nonspecific and only signs of frequent abortions or orchitis in boars can be suspicious of brucellosis. A reliable diagnosis can be achieved only by isolation and identification of pathogen Brucella suis. In the literature, the most important samples for bacteriological tests are mandibular, retropharyngeal and supramammary lymph nodes. The blood is often used and pathogens can be isolated in the acute phase of illness. Also, some of the recommended samples are: parts of fetal membranes, aborted foeti, vaginal or preputial swabs, boar semen, and testicles after castration etc. However, it is not mentioned anywhere in the literature that material obtained by epididymis puncture of live animal can also be a significant sample for bacteriological examination in addition to blood sampling. Material obtained by puncture of the epididymis has semen without secretions of the accessory sex glands. This paper describes the procedure of epididymis puncture in boars suspected for porcine brucellosis as an alternative method of sampling in live animals in conditions of shortage of material for bacteriological examination. Total of five samples were tested using this methodology. Two samples were collected from animals in confirmed epizooty, three samples belonged to boars experimentally infected with Brucella suis biotype 2. The results showed positive findings of Brucella suis in all five samples. From the samples obtained from experimental infection, successful isolation of Brucella suis occured 14, 44 and 73 days after experimental infection respectively. Material from epididymis puncture of an infected animal can be recommended as safe and reliable for the isolation and identification of Brucella suis biotype 2 in situations when there are no other samples available. Isolation and identification of Brucella suis biotype 2 using this methodology is possible both before antibody formation and when the antibody titre is high. Although ejaculate is the better material for Brucella suis isolation because it contains accessory sex glands secretions, most of the time it is hard to get the sample in field conditions in comparison with epididymis puncture. The results of bacteriological examination of obtained material showed that growth of Brucella suis was not affected by other bacterial species.

Key Words: Puncture of the epididymis, bacterial isolation, diagnosis Brucella suis biotype 2

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Reproductive and productive parameters of Holstein cows presenting normal or pathological puerperium

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The objective was to evaluate a reproductive and productive survey of 103 Holstein cows (25 primiparous and 78 pluriparous from second to third lactation) presenting normal or pathological puerperium, in a cross-sectional study involving observations of a Brazilian dairy herd.

Gynecological examinations were performed weekly from calving to the end of the puerperium using rectal palpation, ultrasonography and vaginoscopy. Non parametric data were analyzed by the Fischer's exact test and reproductive and milk yield data were subjected to analysis of variance and compared between groups with *F*-test. Level of significance was 5% ($p < 0.05$) for all statistical tests. Uterine infections were classified according Sheldon et al. (2006) and the animals with retained placenta and uterine infection were treated with antibiotics and other drugs, according to protocols used on the farm. The incidence of twins, assisted births, retained placenta and uterine infections until the first service were 11.7%, 20.4%, 38.8% and 75%, respectively. The incidence of retained placenta was more frequent ($p < 0.05$) in pluriparous (44.9%, 35/78) compared to primiparous (20.0%, 5/25) cows. The incidence of acute metritis was highly significant ($p < 0.0001$) in animals presenting retained placenta (81.1%, 30/37) when compared with non retained cows (16.7%, 10/60). Cumulative frequency of primiparous and multiparous Holstein cows with uterine infection at 14, 21 and 42 days postpartum were 25.0% (6/24), 62.5% (15/24), 75.0% (18/24) and 46.6% (34/73), 50.7% (37/73) and 60.3% (44/73), respectively. Uterine involution [28.1 ± 5.7 (35/92) and 36.7 ± 12.2 (57/92) days] and first postpartum ovulation [35.0 ± 18.3 (32/79) and 49.4 ± 21.7 (47/79) days] differed ($p < 0.001$) between cows presenting or not uterine infection until 42 days postpartum, respectively. First service was, on average, at 75.9 ± 31.0 days after birth, and the pregnancy rate was 20.7%. The pregnancy rate at first service was 20.7% (19/92). Daily milk yield was, on average, 27.9 ± 7.3 liters per cow, up to 42 days after calving, and cows presenting retained placenta and uterine infection produced less ($p < 0.05$) milk (25.0 ± 6.5 l) compared to animals that did not have these conditions (30.9 ± 6.3 l). In conclusion, postpartum uterine diseases have shown a negative impact on fertility and milk production of the herd.

Key Words: Dairy cattle, postpartum, uterine infection, retained placenta, Holstein

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Factors affecting stillbirth and twin birth in Holstein dairy cattle in northeast of Iran

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The objectives of this study were to determine frequency of stillbirth and twin birth, and the individual and environmental factors affecting them in commercial dairy herds located in northeast of Iran. Parity of dam, season of calving, sex of calf, the number of calves born per calving event and survival of calf up to 24 h after birth were recorded for all calving events that occurred between 21 March 2009 and 20 March 2010 in nine industrialized Holstein dairy herds. Out of 3595 calving events, 155 (4.3%) stillbirths and 79 (2.2%) twin births were recorded. Logistic regression with herd as a random effect was used to evaluate the effects of calving season, sex of calf, parity of dam and twinning on stillbirth. Also, the effects of calving season and parity of dam on twinning was evaluated using the same technique. Season of calving and parity of dam had significant effects on twinning rate. Odds of a twin birth was greater during spring (OR = 2.39; $p = 0.033$) and autumn (OR = 2.27; $p = 0.038$) than winter calving. Multiparous cows were more likely to produce twin births compared with primiparous cows (OR = 6.17; $p < 0.001$). Frequency of stillbirth was significantly associated with sex of calf and twinning. Odds of stillbirth was greater for twin births compared with singleton births (OR = 3.13; $p = 0.004$). Male calves were more likely to be dead at birth than female calves (OR = 2.93; $p < 0.001$). The seasonality of twinning would be relevant to the season of conception. Also, some factors such as conceptus sire, nutrition, or maternal body condition have been speculated to affect the seasonal variation in twinning. Generally, our results show that individual and environmental factors affect stillbirth and twin birth rate in Holstein dairy cows.

Key Words: Stillbirth, twin birth, dairy cattle, Iran

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Incidence of cytological endometritis and its effects on reproductive performance in Nelore cows

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Fertility is a critical factor affecting profitability of beef farms. A small number of beef cows are diagnosed with cytological endometritis, which somehow undermine the subsequent reproductive performance. The aim of this study was to evaluate the incidence of cytological endometritis and its effects on reproductive performance of Nelore cows submitted to a 90-day breeding season (BS). Twelve primiparous and 37 multiparous Nelore cows between 29 and 90 days postpartum, with body condition score above 2.50, no history of retained placenta and no clinical uterine infection were used in the study. The diagnosis of cytological endometritis was made by uterine lavage technique in which the samples were harvested, prepared and then stained to be examined microscopically. Cytological endometritis was defined as the presence of ≥5% of neutrophils. After collecting the samples, the cows were submitted to a timed artificial insemination protocol. On the first day (day 0), 1 mg of estradiol cypionate was administered intramuscular (ECP®; Pfizer) and also an intravaginal device containing 1.9 g of progesterone (CIDR®; Pfizer) was inserted; on day 7, 12.5 mg of dinoprost tromethamine was administered intramuscular (Lutalyse®; Pfizer); on day 9, 1 mg of the same estradiol cypionate was administered intramuscular and the intravaginal progesterone device was removed. The calves were removed for 48 h, and on day 11, they were returned to the cows and all these cows were inseminated with frozen semen from a Nelore bull. The use of timed artificial insemination was an excellent method of increasing pregnancy rate and to avoid contamination which may exist for a natural breeding. The incidence of cytological endometritis, conception rate to first insemination and pregnancy rate at the end of BS were analyzed by the program PROC LOGISTIC of SAS including in the model the effect of parity (primiparous vs. multiparous). The effect of the incidence of cytological endometritis on conception rate at first artificial insemination and pregnancy rate at the end of BS were analyzed by the same program. The overall incidence of cytological endometritis was 22% and was not affected by parity (primiparous: 25.0% vs. multiparous 21.6%; $p = 0.808$). Similarly, conception rate at first artificial insemination were not affected by parity (primiparous: 50.0% vs. multiparous: 70.3%; $p = 0.206$). However, pregnancy rate at the end of the BS was higher ($p = 0.024$) in multiparous (83.8%) than in primiparous cows (50.0%). Conception rate at first artificial insemination and pregnancy rate at the end of the BS were not affected ($p > 0.05$) by the presence of cytological endometritis. The conception at first artificial insemination and pregnancy rate at the end of the BS in cows with cytological endometritis were 73.7% and 63.2%, respectively, and in cows with no cytological endometritis were 81.8% and 72.7%, respectively). These results suggested that cytological endometritis seems to have a small relevance in fertility performance of Nelore beef cows.

Key Words: Reproduction, neutrophils, uterine lavage, protocol

11. Estrus synchronization:

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Pregnancy rates in *Bos indicus* crossbred suckled cows treated with a progesterone releasing device, estradiol and inseminated at 48 or 54 h after device removal

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Progesterone releasing devices and estradiol are extensively used during synchronization protocols for fixed-time AI (FTAI) of beef cattle in South-America. Although most protocols are similar, it has been recently shown that fertility can be compromised when cows are treated with low dose progesterone releasing devices, estradiol cypionate and inseminated 54 h after device removal or later. An experiment was designed to determine the effect of the time of FTAI (48 vs. 54 h after device removal) on pregnancy rates in suckled Bos indicus cows. Suckled Braford cows (n = 200) that were between 60 and 90 days postpartum, in a growing plane of nutrition, and had a mean body condition score of 2.6 ± 0.3 (1–5 scale) were used. On Day 0, all cows received an intravaginal progesterone releasing device with 0.5 g of progesterone (P4; DIB 0.5, Syntex SA, Argentina) and 2 mg estradiol benzoate (EB; Syntex SA) i.m. On Day 8, DIB devices were removed and all cows received 500 μg cloprostenol (Cyclase DL, Syntex), 400 IU eCG (Novormon, Syntex) and 0.5 mg estradiol cypionate (Cipiosyn, Syntex) i.m. at the same time. Cows were then randomly assigned to be inseminated at 48 ± 1 or 54 ± 1 h after device removal. Transrectal ultrasonography (Chison 500 Vet, 5.0 MHz) was performed to determine the presence of a corpus luteum (CL) and pregnancy status on Day 0 and 42, respectively. Proportions were compared using Chi-square test. Overall, 23% of the cows had a CL on Day 0. In addition, pregnancy rates did not differ between the two insemination times (p = 0.9; Table 1). It was concluded that cows treated with P4 releasing devices containing 0.5 g of P4 and estradiol cypionate can be inseminated between 48 and 54 h after device removal, without adversely affecting pregnancy rates. These results have important practical implications because it allows for effectively inseminating large groups of cows managed in extensive conditions.

Key Words: FTAI, intravaginal device, ovulation

Table 1. Pregnancy rates in Bos indicus crossbred suckled cows treated with a progesterone releasing device, estradiol and inseminated at 48 or 54 h after device removal

Group	n	%
FTAI at 48 h	100	43
FTAI at 54 h	100	44

p = 0.9.

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Effect of progesterone content in a releasing device on the time of ovulation in *Bos indicus* crossbred suckled cows

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Treatments with intravaginal progesterone (P4) releasing devices and estradiol are extensively used in South-America for fixed-time AI (FTAI) in beef cattle. Although most treatments are similar, there are devices impregnated with different amounts of P4 in the market that may influence the time of ovulation. An experiment was designed to determine the time of ovulation in suckled Bos indicus crossbred cows treated with intravaginal devices with two different concentrations of P4. Crossbred suckled beef cows (n = 18) that were more than 90 days postpartum and a body condition score between 2.5 and 3 (1–5 scale) were used. On Day 0, all cows received 2 mg estradiol benzoate (EB; Syntex SA, Argentina) IM and were randomly assigned to 1 of 2 treatment groups (9 each) to receive an intravaginal device containing 0.5 or 1 g of P4 (DIB 0.5 and DIB, respectively; Syntex SA). On Day 8, devices were removed and all cows received 500 μg of cloprostenol (Cyclase DL, Syntex SA) IM, 1 mg estradiol cypionate (Cipiosyn, Syntex SA) IM and 400 IU eCG (Novormon, Syntex SA) IM. Transrectal ultrasonography (Mindray DP 2200, 5 MHz) was performed every 8 h from Day 10 (i.e. 48 h after DIB removal) until ovulation to determine size of the preovulatory follicle (POF) and ovulation time. Means were compared between groups by Student *t*-test. There were no significant differences between groups in the size of POF or time of ovulation (p > 0.05; Table 1). We conclude that the amount of P4 contained in the device, does not affect the time of ovulation.

Key Words: FTAI, ovulation, *Bos indicus*, P4

Table 1. Size of preovulatory follicle (POF) and time of ovulation in Bos indicus crossbred suckled cows treated with an intravaginal device containing either 0.5 (DIB 0.5) or 1 (DIB) g of progesterone

Group	n	Size of POF (mm)	Time of ovulation (h)
DIB 0.5	9	11.8 ± 0.3	68.4 ± 2.9
DIB	9	11.2 ± 0.3	62.7 ± 1.8

p > 0.1.

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Serum progesterone concentrations, corpus luteum development and follicular dynamics after administration of equine chorionic gonadotrophin (eCG) at the end of a synchronization protocol for fixed-time AI in anestrous beef cattle

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The mechanism by which eCG, given at the end of a progesterone/estradiol-based synchronization protocol, increases pregnancy rates to fixed-time AI (FTAI) in Bos taurus cattle is not clear. This experiment was carried out to evaluate the effect of eCG administration associated to FTAI, on follicular dynamics, ovulation, corpus luteum development (CL) and serum progesterone (P4) concentrations. Multiparous suckled Hereford cows (n = 46) 60–75 days postpartum (dpp), with a body condition score (BCS) of 3.5 ± 0.1 (mean ± SE, 1–8 scale) and without CL detected by daily ultrasound for 10 days were used. Cows received an intravaginal device during 8 days containing 0.5 g of progesterone (DIB 0.5, Syntex, Buenos Aires, Argentina) and 2 mg of estradiol benzoate (Benzoato de Estradiol, Syntex) i.m. One dose of 500 μg of cloprostenol (Ciclase DL, Syntex) and 0.5 mg of estradiol cypionate (Cipiosyn, Syntex) was administered i.m. at device removal and FTAI was performed 52–56 h after DIB removal. Nose plates to all calves were placed in order to prevent suckling from the day of DIB insertion until insemination. At the time of DIB removal, cows were divided into 2 homogeneous groups (according to BCS and dpp). One group received 400 IU of eCG i.m. (n = 23; Novormon, Syntex) while control group did not receive eCG (n = 23). Follicular dynamics, ovulatory follicle development and the CL size were monitored daily by transrectal ultrasonography (7.5 MHz, Well D, WED 9618, China) from DIB removal until 14 days after FTAI. Blood samples were collected daily during the same period from the coccygeal vein to determine serum P4 concentrations. Progesterone concentrations were determined in duplicate by a direct solid-phase radioimmunoassay (Diagnostic Product Co., LA, CA, USA). The ovulation rate (cows

ovulated/synchronized) was compared by Chi square test, in the other variables, all means were compared using Student's *t*-test. Results are shown in Table 1. According to the results, eCG treatment increased preovulatory follicular growth, ovulation rate, subsequent CL area and serum P4 concentrations. This explains the higher pregnancy rate when eCG is given at device removal in Bos taurus cattle.

Key Words: Equine chorionic gonadotrophin, progesterone, fixed-time artificial insemination, anestrous cows

Table 1. Ovarian response after eCG (400 IU) given at P4-device removal in anestrous suckled Hereford cows

	Ovulation rate (%)	Follicle at ovulation (mm)	Follicular growth rate* (mm/day)	CL area† (cm^2)	P4 in ovulated cows‡ (ng/ml)
ECG	65.2%(15/23)[a]	14.5 ± 0.4[a]	1.4 ± 0.2[c]	3.4 ± 0.3[a]	3.6 ± 0.6[a]
No eCG	34.8%(8/23)[b]	12.9 ± 0.6[b]	0.9 ± 0.2[d]	2.6 ± 0.2[b]	1.4 ± 0.6[b]

For a same column, a vs. b ($p < 0.05$); c vs. d ($p < 0.1$) *From DIB removal to ovulation †From days 6 to 14 after FTAI ‡Mean from day of FTAI to 14 days later.

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Effect of testosterone on dominant follicle growth and luteal dynamics in beef heifers

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The study was designed to determine the effects of testosterone on ovarian function in cattle. We tested the hypothesis that administration of testosterone will shorten the life-span of the dominant follicle resulting in early emergence of a new follicular wave. Experiment 1 was conducted to determine which form of testosterone (free or esterified) will result in a rapid increase in circulating testosterone concentrations and last for ≥ 24 h. The results indicated that with 200 mg unconjugated testosterone (free testosterone; $n = 4$) in oil increased circulating testosterone concentrations more than 10-fold (measured with DPC RIA Kit) after 1 h in treated cows compared to testosterone enanthate ($p < 0.01$; $n = 4$). Therefore, unconjugated testosterone in oil was selected for Experiment 2. Beef heifers were treated intramuscularly with 200 mg of unconjugated testosterone in 4 ml canola oil twice at 12 h intervals on the Days 1 (T1), 3 (T3) or 6 (T6) after ovulation (Day 0) of the first follicular wave ($n = 6$ per group), or canola oil without testosterone (controls; $n = 6$). Ovarian structures were monitored daily by ultrasonography (MyLabTM5 scanner with a 7.5 MHz transducer). The dominant follicle grew slower over the next 5 days in T1 ($p = 0.05$) and T3 ($p = 0.06$) groups compared to the control, while the dominant follicle in the T6 group regressed more slowly ($p = 0.02$). The diameter profile of the dominant follicle of the post-treatment wave (Wave 2) and the ovulatory wave did not differ between treatment groups and the control group. Overall, testosterone treatment (T1, T3 and T6 combined) extended the interwave interval of existing (Wave 1; $p \leq 0.001$) and the post-treatment waves (Wave 2; $p = 0.02$) compared with the control. The proportion of 2-wave cycles tended to be higher ($p = 0.08$) after testosterone treatment (T1, T3 and T6 combined; 64.7%) compared to the control group (16.7%). Mean LH concentrations (Day 0–5 of treatment) were lower in T1 and T3 compared to the control group, while LH concentrations were not affected in T6. Testosterone treatment did not affect circulating FSH concentrations or number of follicles (4–5, 6–8 or ≥ 9 mm) among the treatment groups within the first follicular wave. The diameter of CL did not differ between treatment and control groups during the first 12 days of the treatment cycle, but CL diameter was smaller in testosterone groups during the regression phase (Days 15–18; $p < 0.05$). Results did not support our hypothesis. Treatment with testosterone resulted in slower growth of the dominant follicle when treatment was initiated at or before the time of dominant follicle selection, and slower regression of the dominant follicle when treatment was initiated during the static phase. In addition, treatment with testosterone delayed the emergence of the next follicular wave irrespective of the status of the dominant follicle at the time of treatment.

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Key Words: Testosterone, follicle development, cattle, follicular and luteal dynamics, FSH and LH

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Addition of eCG on day 5 instead of day 7 of modified HeatSynch protocol might improve the ovarian follicular response of the anestrus suckled Hereford cows

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Numerous hormonal protocols have been described to overcome prolonged postpartum anestrus in suckled beef cows. Many of them utilize eCG that has both FSH and LH like activity and stimulates follicular growth and ovulation in cattle (Newcomb et al., *J Reprod Fertil* 1979;56:113–118, Duffy et al., *Theriogenology* 2004;61:725–734). Unpublished observations by our group indicate that maximum follicular size was obtained four days after administration of eCG to anestrus cows without producing double ovulations, therefore our objective was to evaluate the ovarian response after a modified HeatSynch protocol in suckled beef cows, after administering eCG at Day 5 or Day 7 (beginning of treatment = Day 0). Twenty anestrus multiparous Hereford cows between 60 and 90 days postpartum were enrolle dinto one of two treatments (eCG Day 5 or eCG Day 7). Modified HeatSynch protocol consisted in: Day 0: 0.008 mg of Buserelin (GnRH, Laboratorio Rio de Janeiro, Santa Fe, Argentina) and insertion of a progesterone releasing intravaginal device (IVD) containing 500 mg of natural progesterone (Cronipress, Biogenesis Bago, Montevideo, Uruguay); Day 7: 0.500 mg of D-Cloprostenol, (Prostaglandina, Laboratorio Rio de Janeiro, Santa Fe, Argentina) and removal of the IVD; Day 8: 1 mg of Estradiol 17beta (Laboratorio Rio de Janeiro, Santa Fe, Argentina). Cows received 400 IU of eCG (Biogon, Biogenesis, Montevideo, Uruguay) at Day 5 (Treated, $n = 8$) or 7 (Control, $n = 9$); US was performed at Days 5, 7, 8, and 9. Results on ovarian follicular dynamics were analyzed by PROC MIXED (SAS), diameter at Day 8 by PROC GLM, and ovulation by PROC FREQ. Results are summarized in Table 1. Administration of eCG at Day 5 instead of Day 7 of the modified HeatSynch protocol increased size of the dominant follicle (DF) at progesterone source removal at Day 8, and resulted in more ovulations within 48 h after estradiol administration. No double ovulations were recorded. Results are promising; howerer additional studies are necessary to evaluate the pregnancy outcomes of this protocol.

Key Words: Beef cattle, postpartum anestrus, eCG, Heatsynch

Table 1. Dominant follicular growth, size at Day 8, and ovulation in suckling anestrus beef cows after administration of eCG at Day 5 of 7 of a modified HeatSynch protocol

Parameter	eCG Day 5	eCG Day 7	p
DF1 growth Days 5–8 (mm)	2.15 ± 0.23	1.14 ± 0.25	0.05
DF diameter at Day 8 (mm)	18.2 ± 1.11	14.3 ± 1.17	0.03
Ovulation Day 9	77.8% (7/9)	37.5% (3/8)	0.09

1DF: dominant follicle.

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Mutations in genes involved in the oestrous cycle associated with expression of oestrus in cattle

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Heat detection is a key factor in profitability of dairy herds, but is increasingly difficult in the modern dairy cow with shorter duration and less intense oestrus. Concurrent with the unfavourable correlation between milk yield and fertility, oestrous detection rates have declined to <50%. A number of mutations have been identified in genes associated with fertility and production traits, but, to date, no single nucleotide polymorphism (SNP) has been associated with oestrous expression. Therefore, the objective of this study was to investigate SNPs previously linked to fertility for their associations with oestrous expression.
Blood was collected from 205 Holstein Friesian dairy cows and genotyped at 41 loci of 18 genes chosen for their roles in the oestrous cycle and milk production. SNPs were then examined for correlations with activity increases at oestrus, recorded via activity monitors, using generalised linear models. Activity increased at oestrus between two and fourfold. Larger increases were associated with mutant alleles in oestrogen receptor-α and gonadotrophin releasing hormone receptor genes ($p < 0.10$) and in the STAT5A gene ($p < 0.05$); smaller increases were associated with mutant alleles of the activin receptor type IIB and prolactin receptor genes ($p < 0.10$). Plausible explanations for these associations with oestrus expression are the influence of these genes on oestradiol production, timing of oestradiol exposure, and threshold concentrations to elicit oestrus. Influences on oestradiol, which can affect the intensity of oestrus, can be either direct, or indirect; through metabolic and milk production pathways. In conclusion, alleles in these 5 genes provide the opportunity for selection of animals displaying stronger oestrus activity which could aid reversal of the decline in oestrous detection and thereby contribute to sustainability of the dairy industry worldwide.
Key Words: Oestrus, single nucleotide polymorphism, dairy cow

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Efficiency of combined protocol for re-insemination of open cows after pregnancy diagnosis on day 20 post insemination and its effect on fertility

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Delayed pregnancy diagnosis and re-insemination of non-pregnant cows lead to high economic losses in modern dairies. The objective of the present field study was to establish the benefit of re-insemination of cows diagnosed as non-pregnant by ultrasonography 20 days after artificial insemination. A total of 245 Japanese Black cows were artificially inseminated and early pregnancy diagnosis (EPD) was performed on 92 cows by ultrasonography 20 days after insemination (Tringa linear, Esaote piemedical, Netherlands) equipped with a 5 MH linear transducer. Non-pregnant cows were divided into two groups randomly; first group (n = 17) received GnRH analogue (10 μg, Buserelin, Teikoku Zoki Co. Ltd, Japan) and timed artificial insemination (TAI) 16–20 h later (GnRH-TAI), while the second group (n = 16) was artificially inseminated when estrus detected (Estrus-AI). The other 153 cows were considered as negative controls in which routine rectal palpation was done for pregnancy diagnosis 45–55 days post-insemination. The statistical analysis was performed using chi square test. Eleven of 92 cows (12%) in the EPD group were diagnosed as infertile due to ovarian abnormalities and were excluded from the experiment. Forty-eight cows (48/81, 59%) were considered as pregnant by EPD, while the remainder (33/81, 41%) were considered as non-pregnant. Of the 33 cows diagnosed as non-pregnant, 17 were timed-inseminated and 16 were estrus detected and 13 (81%) were inseminated. Conception Rate was 35% and 38% in the two groups, respectively ($p > 0.05$), and the total pregnancy rate, within this study, for the EPD group (60/81, 74%) was increased significantly ($p < 0.01$), when compared to the pregnancy rate in negative control cows (82/153, 54%) within the same period. In conclusion, this field study showed that re-insemination of non-pregnant cows, following EPD, is highly efficacious in improving fertility through reducing inter-insemination and inter-calving intervals, in addition to early detection of ovarian disorders.
Key Words: Early pregnancy diagnosis, estrus detection, re-insemination protocol, GnRH

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Comparison of the proportion of *Bos indicus* heifers pregnant following synchronisation of ovulation and fixed-time AI or oestrous detection before and after a single prostaglandin $F_{2\alpha}$ treatment

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Ovulation synchronisation and fixed-time AI (FTAI) was compared to oestrus detection and AI (ODAI) in Brahman-cross heifers (n = 553; 75% Bos indicus) managed on a commercial beef cattle property in central Queensland, Australia, to determine which protocol resulted in the highest proportion pregnant after a single AI. All heifers were weighed, body condition scored (BCS; 1–5) and examined for CL presence by ultrasound. Heifers averaged 327 kg (280–418 kg), BCS 2.5% and 46% had a CL present. Heifers were then alternately allocated to either one of two FTAI groups which commenced treatment to synchronise ovulation on Day 0 of the experiment (n = 139) and Day 8 (n = 141), or one ODAI group (n = 273). The FTAI heifers had a low-dose intravaginal progesterone (P_4) releasing device (IPRD; Cue-Mate®; one P_4 impregnated pod; 0.78 g P_4; Bioniche Animal Health Aust/Asia) inserted and received 1 mg of oestradiol benzoate (ODB; Bomerol™, Bayer Australia) i.m.; 8 days later the IPRD was removed and the heifers received 500 μg cloprostenol ($PGF_{2\alpha}$; Ovuprost™, Bayer Australia) i.m. and 300 IU equine chorionic gonadotrophin (Pregnecol™, Bioniche Animal Health Aust/Asia) i.m.; 24 hrs later all heifers received 1 mg of ODB i.m.; FTAI occurred 54 hrs post IPRD removal. All heifers in the ODAI group initially received no treatment, but were observed twice daily (~12 hourly) from Day 4 to 9 of the experiment and AI after detection of oestrus (AM/PM rule). On Day 9 any heifer that had not been detected in oestrus, regardless of CL presence, was treated with 500 μg $PGF_{2\alpha}$ i.m. and subsequently observed and AI until Day 13. The data was analysed using a GLM with binomial distribution and logit link. Results for FTAI heifers were combined as the pregnancy rates (PR; proportion pregnant of entire group) of the heifers which commenced ovulation synchronisation treatment on either Day 0 or Day 8 did not significantly differ ($p = 0.696$). The FTAI heifers achieved a greater PR (34.6%) than the ODAI heifers (23.2%; $P = 0.003$), however the conception rate (proportion pregnant of heifers inseminated) did not significantly differ between FTAI (34.6%) or ODAI (44.1%; $p = 0.059$) heifers. A CL presence by treatment interaction demonstrated that heifers without a CL had a significantly higher PR in the FTAI groups than the heifers in the ODAI group (36.3% vs. 6.0%; $p < 0.001$). These results support the use of FTAI technology to improve the genetic merit of extensively managed beef cattle in northern Australia.
The authors would like to acknowledge Bioniche Animal Health Aust/Asia as the major contributor and thank Bayer Australia and Meat and Livestock Australia for their support.
Key Words: Oestrous synchronisation, artificial insemination, progesterone, ovulation synchronisation, *Bos indicus*

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Development of the 'sMartwin' synchronization protocol for beef cattle twinning

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Equine chorionic gonadotrophin (eCG) has been successfully used in cattle synchronization protocols. We reported multiple ovulation in heifers with doses ranging from 300 to 1000 IU eCG given at progesterone device removal. A series of experiments was designed to determine the effect of strategic use of eCG during a synchronization protocol for inducing double ovulation and higher fertility in beef cattle. In Trial 1, 27 beef cows received a CIDR device (1.38 g progesterone; Eazi-Breed, Pfizer Animal Health, NZ) and 100 μg GnRH (Ovurelin; Bomac Laboratories Ltd, NZ, USA) i.m. on Day 0 (protocol start), 500 μg cloprostenol (PGF, Schering-Plough Inc, NZ) i.m. on Day 7 (CIDR removal) and 100 μg GnRH i.m. on Day 9 (56 h after PGF). Cows randomly received 400 IU eCG (Pregnecol, Bomac Laboratories Ltd) on Day 2 or 7, or no treatment. In Trial 2, 18 Black Angus cows similarly received GnRH and CIDR on Day 0, but CIDR removal and PGF treatment occurred on Day 6 and 100 μg GnRH i.m. was given on Day 8 (56 h after PGF). Cows were randomly assigned to receive 800 IU eCG i.m. on Day 3 of the protocol (eCGD3) or no treatment (Control). Artificial insemination (AI) was done concurrently with the second GnRH treatment using frozen/thawed semen from one proven bull. In Trial 3, 72 Angus-cross suckled cows were randomly assigned to two groups to receive the same treatments and AI as in Trial 2. Ultrasonography was performed using an Aloka-SSD900 system with a 7.5 MHz linear probe (Aloka Co. Ltd., Tokyo, Japan) to monitor follicular dynamics in Trials 1 and 2, and to detect pregnancy 30 and 40 days after AI in Trials 2 and 3, respectively. Data were analyzed using SAS procedures (v. 9.1; Cary, NC, USA) by Student's *t*-test or Wilcoxon rank-sum tests. Binomial data were analyzed by Chi-square/Fisher's Exact test or logistic regression. In Trial 1, 4 eCG-treated cows had a second follicular wave, emerging on Days 8 (two cows), 9 and 11 and a delayed ovulation (>2 SD from the mean PGF-to-ovulation interval). No double ovulations were induced. In Trial 2, mean number of ovulations tended ($p < 0.07$) to be higher in the eCGD3 (1.33 ± 0.2) than in the Control group (1.0 ± 0.0), while overall pregnancy rate ($p < 0.05$) and embryo number ($p < 0.04$) were higher in the eCGD3 (88.9%; 1.11) than in the Control group (33.3%; 0.33). In Trial 3, overall pregnancy rate was higher ($p < 0.0001$; odds ratio = 3.5, confidence interval = 1.2–10.2) in the eCGD3 (37/51; 72.6%) than in the Control (9/21; 42.9%) group. Out of 37 cows scanned for twins, 11 (34.4%) and 6 (19%) eCGD3 cows had double ovulation and carried twins compared to none of 9 pregnant cows scanned in the Control group. The use of eCG clearly improved conception to timed AI and successfully induced double ovulation and twinning in beef cattle when given strategically during a CIDR-based protocol. The high conception rate achieved makes this protocol suitable for synchronization for fixed-time artificial insemination in cattle.

Key Words: beef cattle, synchronization, twinning, ovulation, GnRH

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Follicular wave synchronization with estradiol benzoate in Gyr (*Bos indicus*) cattle

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Bos indicus cows can be very sensitive to hormones. The objective was to compare the usual dose of 2 mg of estradiol benzoate (EB) with a reduced dose (1 mg), for synchronizing emergence of a new follicular wave at the beginning of an FTAI protocol. Furthermore, as a secondary objective, we also evaluated the effect of EB during follicle deviation and dominance. The experiment was performed in Monte Verde Farm, in Uberaba, MG, Brazil. Twenty two cyclic cattle (n = 10 heifers and n = 12 nonlactating cows) underwent an estrous synchronization protocol with a progesterone-releasing intravaginal device (Sincrogest®; Ouro Fino Animal Health, São Paulo, Brazil) and received 1 or 2 mg of EB (Sincrodiol®; Ouro Fino Animal Health), on a random day (designated D0). Follicular atresia was monitored, once daily with ultrasonography, from D0 to D4, and was defined when negative difference of follicle diameter were observed in consecutive evaluations. On D8, the device was removed and 250 μg of cloprostenol (Sincrocio®; Ouro Fino Animal Health) was given. On D9, 0.01 mg of GnRH (Sincroforte®; Ouro Fino Animal Health) was given to induce ovulation, with daily monitoring of follicular dynamics until ovulation. Regarding the secondary objective, females that ovulated were randomly allocated in two groups, and they received 2 mg of EB on days 3 (GD3, n = 4) or 5 (GD5, n = 5) after ovulation. Following these treatments, follicular development was monitored for 6 days. Independent of animal category, both doses of EB (1 and 2 mg) induced follicular atresia in 100% (22/22) of animals, with ovulation rates of 6/11 vs. 4/11, respectively ($p > 0.05$), with average diameters (mean ± SD) of preovulatory follicles of 11.3 ± 1.2 and 9.8 ± 2.0 mm. Giving EB at follicle deviation (GD3) caused immediate follicular atresia (2.0 ± 1.4 days) and emergence of a new follicular wave in 3.8 ± 1.0 days. However, when EB was given during follicle dominance (GD5), one cow ovulated, whereas follicles from the others underwent delayed atresia (4.0 ± 0.8 days), based on numerical observation. In addition, the emergence of a new follicular wave was not observed until five days after treatment. In conclusion, in Gyr females, a reduced dose of EB (1 mg) at the beginning of the protocol with progesterone-releasing device effectively induced follicular atresia. In the absence of exogenous progesterone, EB more effectively synchronized wave emergence when given during follicle deviation.

Key Words: *Bos indicus*, follicular wave synchronization, estradiol benzoate

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Size of the dominant follicle at prostaglandins application in onset of estrus and fertility of Holstein cows

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The objective of the study was to evaluate the effect of dominant follicle size at the time of PGF2 application on the interval to onset of estrus and fertility of Holstein cows. Over two years, the diameter of the dominant follicle of 241 cows was determined with an ultrasound equipped with a 7.5 MHz linear probe (Sonovet 600) at the time of 500 μg of cloprostenol (PGF; Celosil, Schering-Plough) administration. The interval to onset of estrus, days in milk, number of calving, and season of the year were also registered for each cow-case. Pregnancy diagnosis was conducted by ultrasonography 45 days after AI for cows that did not return to estrus. Sizes of the dominant follicle were analyzed by the MIXED procedure and fertility was analyzed with the CATMOD procedure of SAS. The interval to onset of estrus after PGF administration was affected ($p < 0.05$) by the size of the dominant follicle and season of the year. Cows with large dominant follicles (14–16 mm, n = 132) at the time of PGF administration showed estrus 27.8 h earlier ($p < 0.05$) than cows with small follicles (10–13 mm, n = 109). The interval to onset of estrus after PGF administration was similar for fall and winter months, but 38.6 h shorter ($p < 0.05$) than during spring and summer months. Cow fertility was affected ($p < 0.05$) by season and size of the dominant follicle, with lower conception rates (29%) during spring months than during summer, fall or winter months (47.8, 45.6 and 53.3%, respectively). Cows with small dominant follicles had higher conception rates ($p < 0.05$) than cows with larger dominant follicles (48.4 vs. 35.2%). In conclusions, large dominant follicles present at the time of administration of PGF will result in a shorter interval to estrus, but lower fertility following AI than smaller dominant follicles. In addition the fertility was lowest during the spring months.

Key Words: Prostaglandins, dominant follicle, estrus

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Comparison of three estrus synchronization methods on estrus response and conception rates in buffaloes

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Reproduction in buffaloes is marred by delayed puberty, difficulties in estrus detection, summer anestrous, seasonal breeding and lowered fertility with frozen semen. There is an increasing trend of setting up commercial dairy farming in Pakistan which requires more efficient reproduction from buffaloes. Therefore, the objective of this study was to determine efficacy of three standard synchronization methods on estrus response and conception rates in buffaloes. Buffaloes with average body condition of 2.5 ± 0.5 and mean parity of 3.5 ± 0.5, located at four farms were assigned throughout the year to receive a single treatment of PGF2α (0.150 mg, Dalmazin TM, Fatro Co., Italy; n = 90) i.m., or Ovsynch protocol (GnRH, 50 μg of a GnRH analogue, Dalmarelin TM, Fatro Co., i.m., PGF2α, and GnRH; n = 110) or an intravaginal device containing 1.38 g progesterone (CIDR, Pfizer, New Zealand; n = 250) for 7 days with PGF2α 1 day before CIDR removal. Estrus detection was carried out by visual observation; twice daily for at least 30 min. Buffaloes in PGF2α group were inseminated with frozen/thawed semen once ˜12 h after of obvious signs of estrus. Whereas those in Ovsynch and CIDR group were timed-AI (TAI) 24 and 36 h after second GnRH and 48 and 60 h after CIDR removal, respectively. Pregnancy status was determined by ultrasonography (Aloka SSD 900, with 7.5 MHz probe) between days 30 and 40 post AI. Estrus response and conception rate were analyzed using Chi-square test. Conception rate was determined by number pregnant/number AI × 100. The results revealed significant differences ($p < 0.05$) in estrus response among treatments. Estrus rate was 50% (45/90) in PGF2α, 70% (77/110) in Ovsynch and 90% (225/250) in CIDR treated buffaloes. However, conception rates did not differ among the three groups 40 (18/45), 35 (27/77) and 45% (101/225) for PGF2α, Ovsynch and CIDR treatment, respectively. Based on the better estrus response, CIDR synchronization protocol may be suitable for enhancing reproductive management in buffaloes.

Key Words: Synchronization methods, fertility, buffalo

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Reproductive performance of Egyptian Baladi goats following induction of estrus using prostaglandin F2α

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The objective of this study was to investigate the occurrence of estrus and fertility following treatment with PGF2α in 60 normal cycling Baladi goats. The animals were assigned into one of two groups to receive either PGF2α (8 mg of dinoprost, Lutalyse, Upjohn Co; n = 30) i.m. or saline treatment (Control; n = 30) i.m. The treatment were repeated 11 days later. The incidence of estrus and the interval from the second treatment to the appearance of estrus sign was reported. Bucks (one every 10 females) with harness were used for detecting estrus and breeding the does. They stayed with the does till mating occurred. Pregnancy diagnosis was performed 50 days post breeding using ultrasonography (B mode real time equiped with a 7.5 MHz probe; Pie Medical, Holland). The parturient does, type of birth, gestation length and time of placental drop were also recorded. Data were analyzed by Chi-square and Student *t*-test. Statistical analysis was carried out with SPSS sofware. All the PGF2α-treated does were in estrus within 48 h after the second PGF2α, 80.0% of them proved to be pregnant. All the pregnant does kidded normally. Three does gave single kid, 18 gave twin kids and the remainder three kidded triplet kids. In contrast, in saline-treated goats two (6.7%), one (3.3%), and three (10.0%) does were in estrus at 6, 24, 36 h after treatment, respectively. Furthermore, other six (20.0%) does showed estrus 48 h following treatment with saline. Overall, four (13.3%) does become pregnant at day 50 post breeding. The pregnant does kidded normally. Two gave single kid, and 2 gave twin kids. Estrus response and pregnancy rate between treatment groups were statically significant at $p < 0.05$. The average (mean ± SD) gestation length was 148.4 ± 3.5 and 147.6 ± 3.1 days in the PGF2α and Control group respectively. The placenta dropped after 180.58 ± 7.5 and 200.35 ± 9.1 min from the expulsion of the last kid in the PGF2α and Control animals respectively. Finally, it could be suggested that the use of PFG2α during the breeding season was effective for estrus synchronization without any adverse effect on the reproductive performance of Egyptian Baladi goats.

Key Words: Baladi, goat, estrus, PGF2, fertility

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Comparison of two commercially available progestagen intravaginal inserts in a short-term protocol for estrus synchronization in cyclic ewes

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Proved protocols for estrus synchronization in the ewe consist of an intravaginal treatment with progestagens for 12–14 days. At the end of a long-term treatment subluteal blood progestagen concentrations are measured leading to increased LH pulse frequency and to prolonged persistence and aging of the ovulatory follicles which may negatively affect fertility. To overcome these problems progestagen administration can be shortened to 5 or 6 days in combination with injection of prostaglandin F2α at the time of insert removal. The aim of this study was to compare the efficacy of two different short-term progestagen treatments for estrus synchronization in cyclic ewes. A total of 292 ewes from 3 farms (A, B, C) were randomly assigned to 2 groups and treated for 6 days with Eazi-breed™ CIDR® G (n = 145) or Chronogest® CR (n = 147) vaginal inserts in combination with 0.125 mg Cloprostenol and 300 IU eCG at device removal. Blood samples were taken for progesterone (P4) determination at insert application and removal as well as 14 days later. One day after insert removal rams fitted with marking harnesses were joined to ewes for 35 days and maked ewes recorded daily. Lambing data were recorded and fertility to first service period and overall assessed. Results show that 24–96 h after insert removal 94.4% of the ewes were in estrus. Estrus response was not affected by the type of progestagen treatment and was lower ($p < 0.05$) in nulli- (82.1%) than in pluriparous (97.5%) ewes. Type of progestagen treatment did not affect fertility, but farm and parity influenced the percentage of ewes that lambed as well as the lambing rate. Overall more ewes lambed on farm A compared to farms B and C (93.0 vs. 72.5 and 59.1%). In nulliparous animals the percentage of ewes that lambed was higher to the first service period but overall lower compared to pluriparous animals (51.5 vs. 49.3% and 64.7 vs. 84.2%). Lambing rates were higher on farm A than on farms B and C to first service period and overall (1.3 ± 1.4, 0.9 ± 1.0, 0.7 ± 1.0 and 1.9 ± 1.1, 1.3 ± 0.9, 1.0 ± 1.0) and in nulliparous overall lower than in pluriparous ewes (1.1 ± 1.1 and 1.6 ± 1.1). Serum P4 concentrations measured 14 days after insert removal were higher in the Chronogest® CR than in the Eazi-breed™ CIDR® G group (6.8 ± 4.1 vs. 5.7 ± 3.3 ng/ml). Ewes of farm A had higher P4 values compared to ewes of farms B and C (8.3 ± 4.1 vs. 4.5 ± 1.5 ng/ml and 3.8 ± 1.7 ng/ml) and nulliparous lower P4 concentrations than pluriparous ewes (4.5 ± 2.2 vs. 6.7 ± 4.0 ng/ml). In conclusion, a 6-day treatment with Chronogest® CR and Eazi-breed™ CIDR® G combined with prostaglandin and eCG at insert removal resulted in similar estrus response and fertility in cyclic nulli- and pluriparous ewes.

Key Words: Ewe, fertility, progestagen, synchronization

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Short and long protocols for estrus synchronization and timed-AI in Dorper and White Dorper ewes

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The aim of this work was to compare pregnancy rate among three estrus synchronization protocols with different progesterone primings and timed artificial insemination (TAI) in purebred Dorper (D) and White Dorper (WD) sheep. Of these animals, 126 were yearling lambs between 8 and 14 months old (D = 57; WD = 69) and 121 ewes (D = 83; WD = 38). In November 2011, sheep received an intravaginal insert containing 330 mg progesterone (Eazi-breed CIDR, Pfizer, Brazil) for 14 (CIDR14; n = 169), 9 (CIDR9; n = 39) or 6 days (CIDR6; n = 39). At CIDR withdrawal, all the animals received 400 IU of eCG (Folligon, Intervet, Brazil) and, in adition, 10 mg of Dinoprost (Lutalyse, Pfizer, Brazil) were given in those in CIDR9 and CIDR6 groups. Teasers were used for sexual stimulation, but estrous behavior was not recorded. Fourty-eight hours after CIDR withdrawal inseminations were performed by laparoscopy with 0.4 ml of fresh semen diluted in PBS (1:5), with an average concentration of 150×10^9 spermatozoa per uterine horn. All inseminations of the group CIDR9 and CIDR6 were performed 3 days prior to inseminations of the CIDR14 to prevent the variability on the moment of TAI among groups. Pregnancy was detected by ultrasonography (Honda HS1500 scanner with a 5 MHz linear array probe, Japan) 41 days afther TAI. Data were analyzed by logistic regression (PROC LOGISTIC from the SAS software, 2010), with $\alpha = 0.05$. Statistical model included the effect of treatment (CIDR14 vs. CIDR9 vs. CIDR6), animal category (yearling lambs vs. ewes), breeds (D vs. WD), and their interactions on pregnancy rate. Average pregnancy rate was 70% (173/247). Despite a larger number of animals in CIDR14, no effect of duration of progesterone priming on pregnancy rate was observed (68.6, 79.5 and 66.7% for CIDR14, CIDR9 and CIDR6, respectively; $p > 0.05$). There was no difference in pregnancy rate between yearling lambs and ewes (73.1 vs. 66.9%, respectively; $p > 0.05$) or between breeds (D = 69.3% vs. WD = 71.1%). Within White Dorper sheep, the pregnancy rate was higher in yearling lambs compared to ewes (78.3 vs. 57.9%, respectively; $p = 0.02$) whereas within Dorper sheep, ewes had higher pregnancy rates than yearling lambs (71.1 vs. 66.7%, respectively; $p = 0.02$). High fertility rates obtained for yearling lambs (73.1%) support the use of TAI by laparoscopy in this animal category due to the lower fertility rates following vaginal insemination. Compared to the 14-day protocol, both short protocols resulted in adequate pregnancy rates, thus becoming an alternative for TAI programs using fresh semen by laparoscopy in Dorper and White Dorper sheep. Acknowledgements to Dorper Campo Verde.

Key Words: CIDR, estrous synchronization, timed-AI, sheep, pregnancy rate

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Human chorionic gonadotropin and gonadotropin releasing factor agonist induce ovulation in the mare with the same high efficiency

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Human chorionic gonadotropin (hCG) exists as five independent molecules that vary in structure and biological function. Two of them, hCG and sulfated hCG, produced by placental syncytiotrophoblast and pituitary gonadotrope cells, respectively, bind a joint receptor, common to luteinizing hormone (LH), the hCG/LH receptor. Gonadotropin-Releasing Hormone (GnRH) acts mainly on a specific receptor in the cell membranes in the anterior pituitary, where it triggers the synthesis of the same α and the two separate β-chains of the follicle-stimulating hormone (FSH) and LH, which induces ovulation. Both hCG and GnRH, have been used extensively by equine practioners to synchronize ovulation for artificial insemination. The present study evaluated the efficacy of hCG and a GnRH agonist, deslorelin acetate, given intramuscularly, to induce ovulation in mares of the Brazilian breed, Mangalarga Marchador. Animals (n = 120; body condition score 5–6, in a 0–9 scale) were maintened in pasture (*Cynodon* spp.), and supplemented with mineral salt *ad libitum*. Mare's estrous cycles (n = 462) were followed and analyzed in an equine embryo transfer unit located in Lavras, Brazil (21°14′S; 45°00′W; 918 m altitude), during the 2009–2010 breeding season. For ovarian scanning and follicle development assessment a Pie Medical Falco-100 Esaote ultrasound with a 6.0 MHz probe was used. Among all observed estrous cycles, 306 showed a dominant follicle measuring at least 35.0 mm, and this parameter was established as the time to induce ovulation. Human chorionic gonadotropin (Vetecor® 5000 IU, Hertape Calier) was used in 95 cycles (2500 IU/mare) and in the other 211 deslorelin acetate (Deslorelina, FG Veterinária) was used (100 µg/mare). Treatments were done at random. Data were analyzed by the Chi-square analysis using the R software at a significance level of 5%. There was no difference between groups in the percentage of mares that ovulated ($p > 0.05$). From 95 estrous cycles in which hCG was used, ovulation ocurred in 80 (84.2%), and when deslorelin acetate was used, ovulation ocurred in 186 of 211 estrous cycles (88.1%). The interval from treatment to ovulation was 49.8 ± 33.13 h for hCG and 49.6 ± 33.22 h for GnRH. Pregnancy rates were 71.87%. In conclusion, pharmacological induction of ovulation in the mare can be achieved using either hCG or GnRH agonist with the same efficacy. Ovulation rates of at least 86% should be expected when these hormones are used at a follicle size of 35 mm. Therefore, cost benefits should be considered when choosing the most suitable hormone to be used for the induction of ovulation in equine breeding management.

Key Words: Estrous synchronization, equine, hCG, GnRH agonist

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Evaluation of novel protocols for ovulation induction in the mare

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Ovulation inducing agents are routinely used in the mare, especially when reproductive technologies are applied. This study aimed to evaluate the effect of novel treatment protocols on pre-ovulatory follicular diameter, endometrial edema scores and synchrony of ovulation in the mare. A total of 135 Campolina breed mares, aged 3–14 years, from two Studs in C. Lafaiete – Minas Gerais, Brazil (Latitude 20°40′S and Longitude 43°48′W) were used. An ultrasound scanner equipped with a 5–MHz linear – array transrectal transducer was used (CTS – 3300 V, SIUI, Guangdong, China). Follicle diameter was estimated from the average of height and width of the antrum from two frozen images. A scoring system (0–5) was used for the degree of endometrial edema; 0 was given to diestrus mares with no edema and 5 to mares with maximal edema. The following products were administered to induce ovulation: estradiol cypionate intramuscularly (ECP – E.C.P.®; Pfizer Saúde Animal, São Paulo, Brazil), human chorionic gonadotrophin intravenously (hCG – Vetecor®; Hertape Calier, Minas Gerais, Brazil) and deslorelin acetate intramuscularly (DES – produced in our laboratory). Mares with a ≥35 mm follicle and at least grade 2 endometrial edema were randomly assigned to the following groups: Group 1 – 1.0 mg DES, n = 20); Group 2 – 0.5 mg DES, n = 20); Group 3 – 2.000 IU hCG, n = 20); Group 4 – 750 IU hCG, n = 20); Group 5 – 1.0 mg DES and 2.000 IU hCG, n = 15); Group 6 – 0.5 mg DES and 750 IU hCG; n = 10); Group 7 – 10.0 mg ECP, n = 10); or Group 8 – no further treatment, Control, n = 20). Ultrasonography was performed daily until ovulation or 120 h after treatment, whichever occurred first. Data were analyzed using ANOVA and Fisher's exact test. Ovulation rate between 24 and 48 h after treatment did not differ ($p > 0.05$) among Groups 1–6 (Groups 1, 2 and 4 – 80%; Group 3 –

75%; and Groups 5 and 6 – 100%), but were higher ($p < 0.01$) than Groups 7 and 8 (20%). Pre-ovulatory follicular diameters in Groups 1–5 (38.5 ± 2.03, 39.1 ± 2.68, 38.6 ± 3.02, 38.6 ± 2.94, 39.1 ± 1.84, respectively) were lower ($p < 0.01$) than in Groups 7 (38.9 ± 2.38) and 8 (41.4 ± 3.60), whereas Group 6 (38.9 ± 2.38) was intermediate, but tended ($p < 0.09$) to be lower than Groups 7 and 8. On the day of ovulation, mean endometrial edema scores in Groups 1–6 (0.78 ± 0.46, 0.87 ± 0.64, 0.79 ± 0.65, 0.90 ± 0.64, 0.73 ± 0.56, 0.95 ± 0.37, respectively) were higher ($p < 0.01$) than Group 8 (0.42 ± 0.47) with Group 7 (0.71 ± 0.57) intermediate. In conclusion, the administration of hCG or DES, either alone or in combination, with a reduced or recommended dose, efficiently induced and synchronized ovulation, whereas ECP did not. Mares treated with either hCG or DES ovulated with higher endometrial edema scores and lower pre-ovulatory follicular diameter than in control mares.

Key Words: Mare, ovulation, hCG, deslorelin, ECP

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Use of a deslorelin implant to induce estrus in coyotes (*Canis latrans*)

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Subcutaneous deslorelin implants have been used in wolves (Canis lupus) and domestic dogs (C. l. familiaris) to induce estrus, which is often necessary when planning artificial inseminations. An induction protocol can also serve to synchronize estrus in females, if more than one is to be inseminated. The objective of this study was to determine if a subcutaneous, time-released deslorelin implant would induce estrus in coyotes (C. latrans). Deslorelin implants (2.1 mg, Ovuplant®; Peptech Animal Health Pty Limited, NSW, Australia) were placed subcutaneously in the inguinal region of six, sexually mature female coyotes located at the National Wildlife Research Center's Predator Research Facility in Utah, USA, on January 3, 2012. Six female coyotes housed at the same facility did not receive implants and were monitored as controls. Sixteen days later, serum progesterone concentrations, measured by chemiluminescent microparticle immunoassay, were elevated above baseline (>2.0 ng/ml; range of 1.0–40.5 ng/ml, median = 12.3 ng/ml) in five of six treated females compared with an elevation in 1 of 6 females in the control group (range of 0.3–3.8 ng/ml, median = 0.9 ng/ml). Serum progesterone concentrations were log-transformed and analyzed by two-sample *t*-test, binary data (elevated v. not elevated) were analyzed by fisher's exact test. Coyotes receiving implants had higher serum progesterone concentrations than control coyotes ($p < 0.05$) and treated animals had elevated serum progesterone levels over baseline compared to control animals ($p = 0.08$). Vaginal cytological evaluation in the treated females revealed cells consistent with estrogen influence. To our knowledge, this is the first report of induction and synchronization of estrus in coyotes. This technique may be valuable in breeding management, especially using semen from males not located at the same facility.

Key Words: Estrous induction, deslorelin, coyote, estrous synchronization

1151

Integral reproduction program on captive bottlenose dolphins with assisted reproduction techniques developed in Dolphinaris

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As capture and import of dolphins has been forbidden in Mexico since 2003, Dolphinaris began a reproductive program divided into three phases: frozen semen bank, reproductive management to achieve natural pregnancies and artificial insemination (AI).

Semen was obtained from the three species of captive dolphins present in Mexico (Atlantic, Pacific and Indopacific Bottlenose). In the process to frozen was used a fructose-based extender, mixed with raw semen in proportion 1:1, or until a concentration of 800 millions cell/ml, after this the temperature was lowered to 4°C at a rate of 0.1°C/min; and a second lowered temperature at 1.5°C/s until reaching −80°C, finally, stored in liquid nitrogen for further use. To achieve the increase of number of calves, we followed the ovarian activities with trans-abdominal ultrasound (SONOSITE 180 PLUS®, 2.5–10 MHz convex probe), we also observed the natural copulas and with ultrasound we checked ovulation. 7 weeks after, was confirmed pregnancy with embryo sac. In one female of 20 years old, that had not ovarian activity since 1999, we used hormonal management to induce ovulation. The treatment was based on 25 mg of Lutalyse® and 1200 IU of PG600®, 7 days after. 20 days after the first injection, follicle growth was observed, and ovulation occurred 14 days later. Pregnancy diagnosis was performed at 50 days after natural copulation, when we observed an embryo sac, with ultrasound. Since 2006, two techniques were developed for AI with limited success. The first one involved the use of a fiber-endoscope of 1.6 mts trans-vaginal via, to accomplish AI when a > 20 mm follicular was detected; AI was done twice with 3 ml of thawed semen (of our sperm bank) deposited in the pseudocervix. Another Four attempts with the same technique endoscopic were made, but with deposit of semen in the uterus body, and no pregnancies were achieved. In 2008, we used a sterile conical vaginoscope to do AI, to see the entrance of cervix and with a catheter of polypropylene of 50 cm of long and 3 mm of wide, to introduce 12.5 ml of thawed semen (800 millions sperm/ml; 75% motility, 80% live cell, status 4) at 36°C and capacitated with McCoy 5A modified (Sigma Aldrich®), in the uterus body, after ovulation was detected by ultrasonography. As a result of this approach, the first dolphin calf by AI was obtained with frozen semen in Mexico. In June 2011, the same protocol was used on a female of 17 year old, also resulting in a pregnancy. As a result of the 2008 reproductive program, five calves were born in 2009; three were from natural ovulation, one was from induced ovulation, these 4 calves were by natural copula, and one male calf born product of our vaginoscope technique AI. Calves were weaned after 1.5 years of lactation and ovarian activity of mothers was monitored by ultrasound. Two weeks after weaning, ovaries showed follicular activity, resulting in a fertile ovulation at 1 month of weaning. Currently, all these females are again pregnant, 4 by natural breeding, and 1 by artificial insemination.

Key Words: Dolphin, artificial insemination, frozen semen, ovulation induction

1152

Timed artificial insemination with sex-sorted semen in synchronized and re-synchronized estrous cycles in Nelore cows

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In the last decade, the use of sex-sorted bovine sperm has been incorporated widely in commercial beef and dairy operations. The possibility of increasing the production of a specific calf gender has important economic implications. The objective of this study was to evaluate the interval to timed-AI (TAI) with sex-sorted semen in a ovulation synchronization protocol and after resynchronization in Nelore cows. In the first replicate, 339 suckled, multiparous Nelore

cows were used and in the second replicate, 88 cows were used. The protocol was initiated between 30 and 60 days post-partum. On Day 0, cows received an intravaginal device containing 1.0 g of progesterone (Sincrogest®; Ouro Fino) plus an i.m. injection of 2.0 mg estradiol benzoate (EB; Sincrodiol®; Ouro Fino). On Day 8, the device was removed and 0.25 mg cloprostenol sodium (Sincrocio®; Ouro Fino) and 300 IU eCG (Folligon®; Intervet-Shering Plough) were administered i.m. On Day 9, 1.0 mg EB was administered to induce ovulation. Cows were assigned to TAI using sex-sorted semen from a single sire (2.1 million sperm) at 36 (n = 86), 48 (n = 130) or 60 (n = 123) h after device removal. Ovarian ultrasonographic examinations (7.5 MHz, CTS-3300 V, SIUI, China) were done twice daily for 96 h after device removal to determine the interval from TAI to ovulation. For resynchronization, cows received an intravaginal device and 0.105 mg i.m. of buserelin acetate (Sincroforte®; Ourofino) on Day 19. The device was removed and ultrasonographic examinations were done for pregnancy diagnosis on Day 27. Non-pregnant cows received 0.25 mg cloprostenol sodium at that time, and on Day 28, 1.0 mg EB to induce ovulation. Cows received TAI using sex-sorted or non-sorted semen from a single sire 60 h after device removal (Day 29.5). Data were analyzed using the SAS program. Pregnancy rate was higher ($p < 0.001$) when the TAI was done at 60 h (30.9%; 38/123) than at 48 h (20.8%; 27/130) which was higher than at 36 h (5.8%; 5/86). Pregnancy rates were higher when TAI was done 0–12 h (37.9%; 35/95) than 12.1–24 h (19.4%; 21/108; $p = 0.05$) or >24 h (5.8%; 5/87; $p = 0.0001$) before ovulation. Pregnancy rate in resynchronized cows was lower ($p < 0.05$) following the use of sex-sorted semen (7.3%; 3/41) than nonsorted semen (14.9%; 7/47). In conclusion, pregnancy rate with sex-sorted semen was highest when TAI was done close to the time of ovulation in suckled Nelore cows, and following re-synchronization, was lower than with nonsorted semen.

Key Words: Sex-sorted sperm, synchronization, resynchronization, ovulation, cows

12. Female reproductive tract – ruminants:

1200

Evaluation of uterotonic effects of Dinoprost in pyometric and clinically healthy lactating dairy cows

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The aim of present field trial was to investigate the uterotonic effects of a PGF2α product (Dinoprost, Enzaprost, Ceva Santé Animale) in healthy and pyometric dairy cows with or without active corpus luteum. At days 40 ± 5 postpartum, 50 out of 1250 cows were ultrasonographically diagnosed as pyometric and randomly assigned to treatment groups: Pyometric + Dinoprost (PD) cows (n = 25) were treated with 25 mg of dinoprost, while Pyometric + Saline (PS) cows (n = 25) were treated with saline. Pyometra was defined as accumulation of mixed echo-density fluid in the uterine lumen and distension of the uterus with thin walls and without uterine tone. Fifty healthy cows were assigned randomly to control groups: Healthy + Dinoprost (HD) cows (n = 25) were treated with 25 mg of dinoprost, while Healthy + Saline (HS) cows (n = 25) were treated with saline. All experimental cows ranged from parity 2–8. Ultrasonography was performed at the time of examination and 7 days later to evaluate changes in uterine diameter. Measurements were performed ~10 cm from the bifurcation of the uterus from perimetrium to perimetrium to obtain the gross diameter of the uterine horn, from the uterine vascular layer to endometrial epithelium (mucosa) to obtain the endometrial thickness and from mucosa to mucosa to obtain lumen diameter. Data were analyzed using the GLM procedure of the SAS system. Two time point differences before and after treatments as well as total differences were analyzed. The gross uterine and luminal diameters were different ($p < 0.05$) between pyometric and healthy cows at the beginning of the experiment. The mean uterine diameter (least square mean ± SEM) for group PD, PS, HD and HS at the time of treatment and 7 days later were (61 ± 2.28; 35 ± 2.20 mm), (49 ± 2.35; 52 ± 2.26 mm), (39 ± 2.48; 30 ± 2.39 mm) and (37 ± 2.41; 31 ± 2.33 mm) respectively. The mean luminal diameter for PD and PS and endometrial diameter for HD and HS at the time of treatment and 1 week later were (46 ± 3.05; 8 ± 2.50 mm), (33 ± 3.15; 40 ± 2.58 mm), (16 ± 3.32; 8 ± 2.72 mm) and (16 ± 3.23; 10 ± 2.65 mm) respectively. The mean plasma progesterone (P4) concentration for PD, PS, HD and HS at the time of treatment and 1 week later were (4.98 ± 1.02; 2.73 ± 0.87 ng/ml), (7.43 ± 1.05; 5.21 ± 0.9 ng/ml), (4.34 ± 1.11; 2.31 ± 0.95 ng/ml) and (4.01 ± 1.08; 4.62 ± 0.92 ng/ml) respectively. Treatment with dinoprost significantly ($p < 0.05$) reduced gross uterine diameter in PD in comparison to PS group in the presence or absence (P4 concentrations <1 ng/ml) of active corpus luteum. Dinoprost treatment did not decrease ($p > 0.05$) gross uterine diameter in groups HD and HS. Luminal diameter was significantly smaller ($p < 0.05$) in the PD than in the PS group. The plasma P4 concentration between PD and PS and also HD and HS groups were not different ($p > 0.05$). It was concluded that injection of 25 mg of Dinoprost to pyometric cows would have uterotonic effects and may help to involution of uterus independent of luteolysis.

Key Words: Dinoprost, uterine diameters

1201

The Sensa cow, a useful step between two dimensional pictures plus slaughterhouse material and *in vivo* clinical practice on transrectal exploration

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Training on rectal palpation (RE) of the cow's reproductive tract is difficult for veterinary students and instructors because neither of them can clearly identify which anatomic structure is being palpated by the student. The first training attempt is demanding because it only consists of a general orientation on the genital tract. The 'Sensa cow' system was developed for training tool on RE. A silicone reproductive tract in a box is equipped with touch sensors connected to a computer, which allows for visualization of parts of the reproductive tract that are actually palpated. The Sensa cow differs from other systems (Kinnison et al., *Anat Sci Educ* 2009;2:280–285) in that RE feedback is possible via screen or a peer. Sensa also meets animal welfare standards. The objective was to evaluate whether a training session using Sensa leads to a better skills and a faster recognition of the different parts of the genital tract in the live cow. Two groups of veterinary students that have not previously been exposed to RE participated in the study. The Sensa group (n = 23) started with a training session using the Sensa cow before performing RE on a cow. The Control group (n = 20) started RE training on a cow. Both groups performed two RE sessions in a cow 2 days apart consisting of localization of the cervix, horn bifurcation, a uterine horn and ipsilateral ovary, secondly the contralateral uterine horn and the ipsilateral ovary. Time required for identification of these structures was recorded. After the two RE sessions in a cow, students filled a standardized questionnaire about their subjective experience using both systems. Students felt more comfortable and certain at palpation during their first RE in a cow in the Sensa group (61%) than in the Control group (40%), although not significant ($p = 0.28$; Chisq-test). The amount of the mean time taken to identify structures at RE did not differ ($p > 0.05$; Kaplan-Meier test) between groups. In conclusion, Sensa cow is a useful and practical training tool for RE in cows.

Key Words: Cattle, education, transrectal exploration, computer training tool, skillslab

1202

Selected amino acid transporters are altered in the bovine endometrium and conceptus during the pre-implantation period of pregnancy and are modulated by altered progesterone concentrations *in vivo*

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The aim of this study was (i) to characterise the expression of selected amino acid (AA) transporters in the bovine endometrium and compare them to expression levels in the conceptus during the pre-implantation period of pregnancy and (ii) to determine whether these transporters are modulated in models of increased and delayed post-ovulatory rise in progesterone (P4) concentrations *in vivo*. Following estrus synchronization cross-bred beef heifers detected in standing heat (estrus = -Day 0) were randomly assigned to be either inseminated (n = 59) or non-inseminated cyclic controls (n = 24). Endometrium from the ipsilateral uterine horn was recovered from cyclic heifers on Day 7, 10, 13 or 16 and from pregnant heifers (confirmed by the presence of a conceptus) on Day 7, 10, 13, 16 or 19. Quantitative real-time PCR was performed for selected solute carrier family members from the literature (SLC) 1A1, 1A3, 1A4, 1A5 (Acidic AA transporters): 38A11, 38A2, 38A4, 38A7, 43A2, 6A14, 7A1, 7A5 and 7A8 (Neutral AA transporters) and 7A4, 7A6, 7A7, [Cationic amino acid (AA) transporters]. A comparison of the expression of these SLCs was made with RNA sequencing data derived from conceptus tissue. During the pre-implantation period, a temporal increase in the expression of SLC1A5, SLC38A2, SLC7A1 and SLC7A5 in the endometrium was coordinate with decreased expression in the conceptus ($p < 0.05$). In contrast, expression of SLC43A2 and SLC1A4 increased in both endometrial and conceptus tissues as pregnancy progressed. SLC7A4, SLC7A7 and SLC7A8 expression decreased in the endometrium and increased in the conceptus during elongation ($p < 0.05$). An early increase in P4 concentrations (due to P4 supplementation) lead to increased endometrial expression of SLC1A5, SLC38A7, SLC6A14, SLC7A1 on Day 7 post-estrus, with an early (Day 7) and sustained increase in expression to Day 13 for SLC38A4, SLC7A5 and SLC7A7 compared to controls ($p < 0.05$). In contrast, heifers with a delay in the post-ovulatory increase in P4, displayed a lower increase in expression of SLC43A2, SLC6A14, SLC7A6 and SLC7A7 throughout the estrous cycle ($p < 0.05$). In conclusion, amino acid transporters are temporally regulated in a tissue specific manner in the endometrium and conceptus during the pre-implantation period of pregnancy. Moreover their expression in the endometrium *in vivo* under conditions where conceptus elongation is increased (elevated P4) or decreased (low P4) following transfer may alter the transport of acidic, neutral and cationic AAs into the uterine lumen. We propose that transport of AAs into the uterine lumen contribute to the elongation capacity of the conceptus during the pre-implantation period of pregnancy.

Key Words: Pregnancy, progesterone, gene expression, uterus, solute carrier family

1203

Inhibin alpha subunit induces apoptosis of bovine granulosa cells via Bcl-2 family

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Many researchers have proved that inhibin alpha subunit plays an important role in granulosa cell growth and proliferation. Our previous studies have detected that the apoptosis rate of bovine granulosa cells over-expressing inhibin alpha subunit was significantly higher than that of the control group. However, the mechanisms of apoptosis induced by inhibin alpha subunit are not exactly elucidated. Therefore, bovine granulosa cells were transfected with eukaryotic expression plasmid encoding inhibin alpha subunit. The expression of inhibin alpha subunit and green fluorescent protein was confirmed in Hela cells by quantitative real time PCR and semi-quantitative western blotting using inhibin alpha subunit monoclonal antibody (Epitomics, Inc., 863 Mitten Road, Suite 103 Burlingame, CA), respectively. The mRNA of apoptosis-related genes Bax, Bcl-2, BclXL was extracted and detected by RT-PCR 48 h after transfection. Similarly the proteins for Bax, Bcl-2, BclXL were analyzed by western blot analysis. The results of RT-PCR identified that the mRNA of proapoptotic gene Bax was significantly elevated (3.45 ± 0.29, $p < 0.05$), while the anti-apoptotic genes Bcl-2, BclXL were down-regulated (0.77 ± 0.18, 0.67 ± 0.06, $p > 0.05$). Western-blot showed that the pro-apoptotic proteins Bax were significantly elevated (1.45 ± 0.18, $p < 0.05$), while the anti-apoptotic proteins Bcl-2 and BclXL were down regulated (0.38 ± 0.07, 0.67 ± 0.16, $p < 0.05$). Therefore, these results showed that inhibin induces apoptosis of bovine granulosa cells via Bcl-2 family to activate the mitochondrial pathway.

Key Words: Inhibin, apoptosis, bovine, granulosa cells

1204

Low insulin-like growth factor-1 concentrations before parturition are associated with the development of cytological endometritis in multiparous dairy cows

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Cytological endometritis (CE) is characterized by extensive leukocytic infiltration of the endometrium and is associated with longer intervals to conception and a greater probability of culling in dairy cows. Information about the metabolic and inflammatory status of cows in the prepartum period, with the association to the development of CE is scarce. The objective of this study was to evaluate whether prepartum plasma concentrations of insulin-like growth factor-1 (IGF1), insulin, non-esterified fatty acids (NEFA) and haptoglobin (Hp) were associated with the development of CE. Blood was collected from 119 multiparous Estonian Holstein dairy cows from a single 1200 cow free-stall dairy herd and analysed for concentrations of IGF1, insulin, NEFA and Hp on day 14 before calving. Uterine cytology samples were collected at 40 days in milk (DIM) using endometrial brush which was fastened onto a stainless steel device for use in cows. The cytological criterion was set at >8% of neutrophils as the threshold indicator of CE. Concentrations of the plasma variables were dichotomized using receiver operating characteristic (ROC) curve analysis, and the optimal threshold for each parameter was based on the highest sum of sensitivity and specificity for predicting the occurrence of CE. Plasma variables with area under the ROC curve (AUC) >0.6 (computed using the trapezoid rule) were submitted to univariate and multivariate logistic regression models as predictors for CE. The prevalence of cytological endometritis was 30.3% (36/119). ROC curve analyses showed optimal threshold concentrations of 74.62 ng/ml (AUC = 0.67; p = 0.003), 0.075 g/l (AUC = 0.65; p = 0.009), and 107.50 μEq/l (AUC = 0.63; p = 0.027) for IGF1, NEFA and Hp for the identification of positive CE status, respectively. Plasma insulin concentrations were not associated with CE (AUC = 0.51; p = 0.84). Univariate logistic regression analysis showed that the odds of developing CE increased by a factor of 4.10 ($p < 0.001$) when plasma IGF1 was <74.62 ng/ml, by a factor of 3.65 (p = 0.011) when plasma Hp was >0.075 g/l, and by a factor of 3.38 (p = 0.005) when plasma NEFA was >107.50 μEq/l 14 days pre-calving. When IGF1, NEFA and Hp were submitted to a multivariate logistic regression model, only IGF1 remained a significant predictor for CE. IGF1 also remained a significant predictor for CE (odds ratio = 2.90; 95% CI 1.06–7.92; p = 0.040) after including calving and transition related conditions and disorders (analysed together as one variable), prepartum body condition score, lactation, and first 45 day milk yield as covariates. In conclusion, multiparous dairy cows were at greater risk of experiencing CE at 40 DIM if they showed low plasma IGF1 concentrations 14 days before parturition.

Key Words: Endometritis, cytology, cow, insulin-like growth factor 1

1205

Functional and structural changes in bovine corpus luteum during partial luteolysis

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Prostaglandin F2α (PGF2α) does not always cause CL regression. Sometimes luteolysis does not occur but a drop of plasma progesterone (P4) concentration to values near to 1 ng/ml is observed in the first 12–24 h (h) after PGF2α treatment followed by a rebound to the initial values. Partial luteolysis is poorly understood and there are no reports regarding the changes observed in the CL volume and luteal blood flow during this critical period, which is the objective of this study. Ovulation of 18 adult clinically healthy Caracu cows was synchronized [Day-9: GnRH (lecireline; Gestran Plus®Tecnopec, SP, Brazil) + P4 intravaginal device (Primer®Tecnopec, SP, Brazil); Day-2.5: PGF2α (sodium cloprostenol; Sincrocio®Ouro Fino, SP, Brazil); Day-2: PGF2α + P4 intravaginal device withdraw; D0: GnRH]. All cows ovulated 24–32 h after GnRH. On Day 6 cows were randomly divided in three experimental groups: Saline (n = 6; 2 ml, i.m.); 2× PGF (n = 6; two treatments i.m. of 500 μg sodium cloprostenol 2 h apart) and 1/6PGF (n = 6; 83.3 μg sodium cloprostenol, i.m. once). Beginning immediately before the treatments and 24, 32 and 48 h after, blood sampling for plasmatic P4 determination and CL volume (three cross-sectional images with maximal areas were frozen and recorded – using B-mode grayscale). Power-Doppler mode was used for subjective estimation of percentage of CL area with blood flow (0–100%) signals, color signals of blood flow during a real-time imaging exam using (Sonovet Pico, Medison Co., Ltd equipped with a 5–9 MHz, linear-array transducer) were performed. The data were analyzed using Repeated Measures ANOVA/TUKEY test and results are shown in Table 1. Based on these results we conclude that during the phenomenon of partial luteolysis, besides the temporary impairment of the P4 secretion ability, there is also a temporary reduction of luteal volume.

Key Words: Cattle, luteolysis, corpus luteum, prostaglandim F2 alpha, partial luteolysis

Table 1. Mean ± SEM changes after treatment with saline or PGF2α

Hour	P4 (ng/ml)			CL blood flow (%)[a]			CL volume (%)[a]		
	Saline	2×PGF	1/6PGF	Saline	2×PGF	1/6PGF	Saline	2XPGF	1/6PGF
0	2.3 ± 0.4	1.6 ± 0.2	2.0 ± 0.2	100	100	100	100	100	100
24	3.4 ± 0.5	0.6 ± 0.1[b]	1.7 ± 0.1[b]	95 ± 13.3	76.6 ± 8.1	73.2 ± 9.1	123.1 ± 19.6	55.8 ± 10.8[b]	64.5 ± 10.5[b]
32	4.3 ± 0.3	0.5 ± 0.1[b]	1.8 ± 0.2	110 ± 18.3	65 ± 8.1[b]	98.8 ± 17.4	135.7 ± 20.7	47.4 ± 8.1[b]	89.2 ± 8.9
48	3.1 ± 0.2	0.3 ± 0.1[b]	2.1 ± 0.1	95 ± 18.7	43.3 ± 7.7[b]	100.5 ± 16.5	140.5 ± 14.5	40.7 ± 11[b]	92.5 ± 12.7

[a]Percentage change from 0 h.
[b]Effect of treatment from 0 h ($p \leq 0.05$).

1206

Effects of mating, artificial insemination or intravaginal deposition of raw semen or seminal plasma on vaginal and uterine blood flow in German Holstein cows

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Transrectal color Doppler sonography is a useful technique for the evaluation of uterine and ovarian blood flow in cattle and horses. In horses, the effects of raw semen, seminal plasma and semen extender on blood flow in the uterine and ovarian arteries has been investigated. However, no current research has been reported in cows. The objective was to characterize and compare the perfusion of vaginal and uterine arteries after challenging the reproductive tract of dairy cows via natural mating, conventional artificial insemination (AI), or intravaginal deposition of semen, seminal plasma or a placebo. In a double-blind study, 25 mg PGF2α (dinoprost, Dinolytic®; Pfizer, Karlsruhe, Germany) was administered to six German Holstein cows during diestrus and followed by 10 μg GnRH (buserelin, Receptal®, Intervet, Unterschleissheim, Germany) 48 h later. Intravaginal or intrauterine treatments were carried out 12 h after GnRH. Animals served as their own controls, using a change-over design with an interval of 14 days between experiments. Corresponding time intervals were chosen for the untreated control estrus. All animals displayed estrus and ovulated between 30 and 48 h after GnRH. Blood flow volume in the uterine (uBFV) and vaginal arteries (vBFV) ipsilateral to the ovary bearing the preovulatory follicle was estimated by using transrectal Doppler sonography (Toshiba SSA 370 A Version K instrument with a 7.0 MHz microconvex probe; Tokyo, Japan). Statistical analysis was carried out using ANOVA for repeated measurements. Transient rises ($p < 0.05$) in vBFV were observed between 3 and 12 h following mating as well as 3–9 h after deposition of raw semen and seminal plasma. The most distinct rise of vBFV values was seen 6 h after mating. At this time vBFV values were 199% higher compared to values immediately before mating (0 h). No changes ($p > 0.05$) in vBFV values were noticed after AI and after deposition of a placebo into the vagina. Mating as well as deposition of either raw semen or seminal plasma induced also a rise in uBFV values ($p < 0.05$), but in contrast to vaginal perfusion, a rise in uBFV values could also be observed after AI ($p < 0.05$). Similar to vaginal perfusion the highest change in uBFV values was also noticed after mating. Maximum uBFV values were observed 9 h after mating. At this time point uBFV was 79% higher ($p < 0.05$) than those at 0 h. Natural mating, semen, seminal plasma or AI affects vaginal and uterine blood flow as measured by Doppler. This may have a role for the establishment of pregnancy.

Key Words: Blood flow, vagina, uterus, raw semen, artificial insemination

1207

Oxylipidomics of the cow uterus: effects of the peri-ovulatory endocrine milieu of the estrous cycle

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In beef cattle, a large diameter of the pre-ovulatory follicle (POF) and the resulting high progesterone (P4) concentrations during diestrus increase odds of pregnancy. Metabolic pathways in the uterus (i.e. metabolism of fatty acids) may explain such an association; however, it is poorly understood. Oxylipids are derived from the metabolism of the unsaturated fatty acids arachidonic acid (AA), linoleic acid (LA), alpha linolenic acid (ALA), dihomo-gamma-linolenic acid (DGLA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and function as local mediators of immune response and inflammation. Only a limited number of oxylipids (i.e. prostaglandins F and E series) are known to have a function in the regulation of reproduction in cattle. The objective was to measure individual concentrations of oxylipids derived from AA (n = 41), LA (n = 12), ALA (n = 3), DGLA (n = 5), EPA (n = 15) and DHA (n = 11) present in the uterus of Nelore beef cows. Cows had their ovulation synchronized to generate groups of cows with high (HP; N = 11) or low (LP; N = 11) post-ovulatory P4. Seven days after ovulation induction, cows were slaughtered and PBS flushings of the ipsilateral uterine horn and endometrium samples were collected. Maximum diameter of the POF (mean ± SEM; 12.8 ± 0.4 mm vs. 11.1 ± 0.4 mm) was greater in

HP vs. LP ($p < 0.01$). P4 concentration on D7 (4.5 ± 0.3 vs. 3.4 ± 0.3 ng/ml; $p < 0.05$) and rate of increase in P4 concentrations from D1 to D7 were higher in the HP than in the LP group (53.6 ± 8.8-fold vs. 15.3 ± 1.9-fold; $p < 0.01$). Concentrations of the 87 different oxylipids were measured simultaneously using liquid chromatography mass spectrometry (LC-MS) in both flushings and endometrium. Data were analyzed by summing the log2-transformed concentrations of individual oxylipids according to their precursor compound. The sum of all compounds within each precursor group was compared between HP and LP. In addition, the ratio of summed concentrations of compounds was compared between HP and LP groups. Whereas total concentration per precursor was not different in flushings, ratios between total concentration of DHGLA:EPA, DHGLA:ALA and DHGLA:AA tended to be reduced in HP vs. LP (0.1 ± 0.03 vs. 0.23 ± 0.07, 0.13 ± 0.04 vs. 0.3 ± 0.11, and 0.008 ± 0.002 vs. 0.01 ± 0.002, respectively; $p < 0.08$). No differences were observed in the oxylipid profile of endometrial tissue. Manipulation of POF growth and consequent changes in the endocrine milieu during the first week of diestrus influences the oxylipid profile of uterine flushings, which may be associated with pregnancy outcome.

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Key Words: Cattle, uterus, oxylipids

1208

Gene expression of glucose transporters in cattle endometrium in response to manipulations of the pre-ovulatory follicle size

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In beef cattle, changes in the peri-ovulatory endocrine milieu are associated with conceptus growth and fertility. A large size of the pre-ovulatory follicle (POF) and resultant high progesterone (P4) concentrations during diestrus affect pregnancy rates positively. Our hypothesis is that modulation of POF size and diestrus P4 concentrations regulates nutrient availability in the uterus. Specifically, optimal glucose concentrations in the histotroph are required for adequate embryo growth during early gestation. The objective was to determine if POF size and resulting P4 concentrations during the first week of diestrus influence gene expression of Solute Carrier Protein (SLC) families that are related to glucose transport. Cyclic, non-lactating Nelore cows received two injections of cloprostenol (PGF; 0.5 mg; i.m.) 14 days apart. Ten days later (day 10; D-10), cows received a P4-releasing device along with estradiol benzoate (2 mg; i.m.). To modulate the growth of the POF and alter post-ovulatory P4 production, on D-10 animals received PGF (high post-ovulatory P4 group; HP; $n = 15$) or not (low post-ovulatory P4 group; LP; $n = 15$). The P4-releasing devices were removed and PGF injected on D-2.5 in cows of the HP group and on D-1.5 in the LP group. Ovulation was induced with buserelin (GnRH; 10 μg; i.m.) on D0. Diameter of POF and ovulation were assessed by ultrasonography since D-2. From D1 to D7, plasma was obtained for measurement of P4 concentration. On D7, cows that ovulated were slaughtered (HP, $n = 11$ and LP, $n = 11$) and endometrium was dissected. Differences between group means were determined by student's *t*-test. Maximum diameter of the POF (mean ± SEM; 12.8 ± 0.4 vs. 11.1 ± 0.4 mm) was greater in HP vs. LP ($p < 0.01$). Progesterone concentration on D7 was larger on the HP group (4.5 ± 1.0 and 3.3 ± 1.1 ng/ml; $p < 0.05$). Relative concentrations of transcripts coding for facilitative sugar transporters (SLC2A1, SLC2A3, SLC2A4 and SLC2A5), a sodium-dependent glucose co-transporter (SLC5A1) and other transporters related to glucose uptake (ATP1A2, ATP1B2, SLC37A4) were determined by qPCR, using cyclophilin as the endogenous control gene. There were no significant differences in expression of SLC2A1 (mean ± SEM;0.91 ± 0.04 vs. 1.02 ± 0.07), SLC2A3 (1.14 ± 0.16 vs. 1.05 ± 0.1), SLC2A4 (1.20 ± 0.14 vs. 1.01 ± 0.05), SLC2A5 (0.95 ± 0.12 vs. 1.04 ± 0.12), SLC5A1 (1.35 ± 0.25 vs. 1.49 ± 0.44), ATP1A2 (1.29 ± 0.17 vs. 1.03 ± 0.1), ATP1B2 (1.20 ± 0.11 VS. 1.06 ± 0.1), SLC37A4 (0.37 ± 0.04 vs. 0.31 ± 0.04), between HP and LP, respectively ($p > 0.05$). In conclusion, modulation of POF size and diestrus P4 concentrations did not affect the expression of glucose transporter genes. It is possible that activity of SLC proteins, rather than gene expression, or alternatively, expression and function of genes related to carbohydrate metabolism, are regulated by the peri-ovulatory endocrine milieu in cows. Financial support from CNPq (481087/2010 – 9 to MB), FAPESP (2011/03226 – 4 to MB and 2010/01284 – 4 to FSM) and CAPES to MRF; Ourofino, CCPS-USP and Deoxi Biotecnologia.

Key Words: Cattle, uterus, solute carrier proteins

1209

The peri-ovulatory endocrine millieu regulates the expression of polyamine synthetic pathway in the bovine endometrium

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In beef cattle, beneficial effects of a large size pre-ovulatory follicle (POF) and high progesterone (P4) concentration on embryo development have been reported. However, molecular mechanisms involved in this positive relationship are not entirely known. Polyamines are regulators of cell proliferation and differentiation, processes that occur in the uterus during early pregnancy. Therefore, polyamines may be play an important role in the endocrine environment at that time. The objective was to evaluate the expression of genes in the polyamine synthetic pathway in the endometrium of cows that ovulated follicles of different size. Thirty cycling, non-lactating Nelore cows received a intravaginal P4-releasing device along with estradiol benzoate on day 10 (D-10). Animals were divided to receive PGF (high post-ovulatory P4 group; HP; $N = 11$) or not (low post-ovulatory P4 group; LP; $N = 11$) on D-10. Devices were removed and PGF injected on D-2.5 in cows of the HP group and on D-1.5 in the LP group. Ovulation was induced with GnRH on D0. Blood samples were collected daily after the second GnRH injection for P4 measurement and endometrial tissue was collected on day 7. Expression of the following polyamine synthetic pathway molecules was measured by qPCR: ornithine decarboxylase (ODC), adenosylmethionine decarboxylase 1 (AMD1), spermidine/spermine N1-acetyltransferase 1 (SAT1), spermidine synthase (SRM), antizyme inhibitor 1 (AZIN1) and polyamine oxidase (PAOX). Expression of a transmembrane transporter (solute carrier protein, SLC3A2) and of a cell proliferation marker (proliferating cell nuclear antigen; PCNA) was also measured. Differences between group means were determined by student's *t*-test. Maximum diameter of the POF (mean ± SEM; 12.8 ± 0.4 vs. 11.1 ± 0.4) was larger in HP vs. LP ($p < 0.01$). The rate of increase in P4 concentrations from D1 to D7 was greater on the HP group (53.6 ± 27.7- vs. 15.3 ± 6.4-fold, respectively; $p < 0.05$). There was no difference on the mRNA expression for ODC1, SAT1, PAOX, SRM and SLC3A2 ($p > 0.05$), however mRNA expression of AMD1 ($p = 0.09$) and AZIN1 ($p = 0.06$) was less in the HP group, while expression of PCNA ($p < 0.05$) was greater in the HP group. Considering the rate of P4 concentration increase from D1 to D4 for each animal, regardless of the experimental group, a quadratic effect of the rate of P4 increase was observed for the relative expression of AMD1 (adj. R2: 36.77; $p < 0.01$). Our results suggest that P4 decreases polyamine synthesis, not by recycling via acetylation pathway or oxidation of acetylated products, but by decreasing the de novo synthesis through the regulation of the antizyme system that acts to degrade ODC protein without changing ODC gene expression.

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Key Words: Cattle, uterus, polyamines

1210

Prostaglandin E1 analogue (PGE1) and follicle stimulating hormone (FSH) increase the expression of FSH receptor in the cervix of goats (*Capra hircus*) during the estrous cycle

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During the peri-ovulatory period the circulating FSH concentrations increase. Administration of FSH or PGE1 analogue to induce the cervical relaxation was investigated. The objective was to determine the effect of FSH or a PGE1 analogue on the expression of FSH receptor (FSHr) protein in the goat cervix. During the breeding season, goats (n = 20) were synchronized by using an intravaginal sponge containing 30 mg of flugestone acetate (Progestagen; Intervet Ltd.; Thailand) and 250 IU eCG (Folligon; Intervet Ltd.; Bangkok, Thailand) given at sponge removal. Goats were equally assigned to 4 groups to receive no treatment (Group 1, Control), 2 mg FSH (2 mg; Ovagen; ICPbio Limited, New Zealand) 24 h after sponge removal (Group 2), 2 mg FSH at 24 h and 1 mg PGE1 (Misoprostol; Sigma-Aldrich; Germany) at 48 h after sponge removal) or 1 mg PGE1 48 h after sponge removal. All treatments were administered intracervically. Goats were sacrificed, cervices were collected 54 h after sponge removal and divided transversely into 6 sections, while alternate sections were fixed in formalin, embedded in wax and sliced in 7 μm sections. The expression of FSHr was determined by immunohistochemistry in five cervical layers (epithelium, stroma, and circular; longitudinal and transverse muscle) and in three regions (vaginal, mid and uterine regions). Data were analyzed by a univariate ANOVA and means were compared by Sidak's test. Prostaglandin E1, FSH and PGE1 or FSH increased the FSHr expression in goat cervix at 54 h after pessaries removal. The expression of FSHr was greatest in the cervix of those administrated by PGE1 ($p < 0.05$). FSHr expression was lowest in the cervix of goats given FSH ($p < 0.05$). The combination of PGE1 and FSH decreased ($p < 0.05$; compared with control group) the expression of FSHr. The expression of FSHr was greater ($p < 0.05$) in the uterine end than in the mid region or vaginal end. There was no significant difference ($p > 0.05$) in FSHr expression between the mid region and vaginal end. The PGE1 analogue and FSH increased the expression of FSHr in the goat cervix. Goats were synchronized with a progestagen sponge and eCG and this may have additionally caused cervix relaxation and increase in FSHr expression. Therefore the further studies are required to investigate whether the intracervical application of PGE1 analogue or FSH affects the expression of FSHr in a control group without treatments for synchronization (a progestagen sponge or eCG).

Key Words: Goat, cervix, FSHR, PGE1

1211

Intracervical administration of follicle stimulating hormone (FSH) or prostaglandin E1 (PGE1) induces expression of EP1 and EP2 receptors in the cervix of Thai goats (*Capra hircus*) during oestrus

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The success of intrauterine artificial insemination by transcervical technique can be limited by the complexity of the goat cervix. In order to facilitate cervical penetrability, stimulation of cervical relaxation is required. During the peri-ovulatory period the circulating FSH concentrations increase and it induces the synthesis of prostaglandin in the cervix. This study was designed to determine the expression of EP1 and EP2 receptors after treatment with FSH and/or PGE1 analogue. During the breeding season, goats (n = 20) were synchronized by using an intravaginal sponge containing 30 mg of flugestone acetate (Progestagen; Intervet Ltd.; Thailand) and 250 IU eCG (Folligon; Intervet Ltd.; Bangkok, Thailand) given at sponge removal. Goats were equally assigned to 4 groups to receive no treatment (Group 1, Control), 2 mg FSH (2 mg; Ovagen; ICPbio Limited, New Zealand) 24 h after sponge removal (Group 2), 2 mg FSH at 24 h and 1 mg PGE1 (Misoprostol; Sigma-Aldrich; Germany) at 48 h after sponge removal) or 1 mg PGE1 48 h after sponge removal. All treatments were administered intracervically. Goats were sacrificed, cervices were collected 54 h after sponge removal and divided transversely into 6 sections, while alternate sections were fixed in formalin, embedded in wax and sliced in 7 μm sections. The expression of EP1 (EP1r) and EP2 (EP2r) receptors was determined by immunohistochemistry in five cervical layers (epithelium, stroma, and circular; longitudinal and transverse muscle) and in three regions (vaginal, mid and uterine regions). Data were analyzed by a univariate ANOVA and means were compared by Sidak's test. Expression of EP1r was greater in the cervix of goats treated with FSH or PGE1 than the Control group ($p < 0.05$). Expression of EP1r was lowest in cervix of goats treated with FSH plus PGE1, which, in turn, was lower than the Control group ($p < 0.05$). The mid region of the cervix had greater expression of EP1r than in the vaginal or uterine ends. Expression of EP2r decreased in the cervix of goats treated with PGE1 plus FSH. Expression of EP2r was greatest in the cervix of the Control goats and lowest in that of goats treated with PGE1 plus FSH. The expression of EP2r was higher in cervix of goats treated with PGE1 than in goats treated with FSH only ($p < 0.05$). EP1 and EP2 receptors seem to have an important role in cervical relaxation. PGE1 increased the expression of EP1r. Cervical treated by PGE1 or FSH decreased the expression of EP2r. Combination of PGE1 and FSH given significantly suppressed the expression of EP2r. Treatment with the combination of FSH and PGE1 decreased the expression of the EP1r which may indicate that are antagonists. A reduction in EP1r leads to the decrease in smooth muscle contractions. Expression of EP1r and EP2r indicates an involvement of PGE1 in the physiology of cervical relaxation or contraction.

Key Words: EP1 receptor, EP 2 receptor, cervix, FSH, misoprostol

1212

Transient post-mating uterine inflammation in the alpacas

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Deposition of semen in the alpaca is intrauterine. A post-coital bacterial endometritis was reported by Vilca et al. (1977. V Congreso Nacional de Biología Cusco, Perú), causing early embryonic mortality, as well as Velez (1996. Bachelor thesis degree. Universidad Católica de Santa María, Arequipa, Perú.), but no explanation about the cause was given. In horses, a post-coital transient uterine inflammation is a normal physiological response to the presence of semen, and assists to remove the excessive spermatozoa, seminal plasma and contaminants before the embryo enters the uterus, ˜5.5 days after ovulation. This study was designed to determine the probable transient post-mating uterine inflammation in alpacas from day 1 to 7 post-mating. Adult females (n = 21) were selected from a herd under natural high altitude pastures at La Raya Research Station, Cusco, Perú; when a follicle at least 7 mm in diameter was detected by ultrasonography (ALoka SSD-500, with 7.5 MHz linear probe). Alpacas were then mated for 20, 40 or 60 min (n = 7 at each time). Three alpacas were euthanized daily on each of 1–7 days post-mating and the reproductive organs removed. Sections of both uterine horns were taken and placed in a buffered formol solution, and sent to the histological laboratory for preparation of tissue sections. Histological results are pending. Occurrence of ovulation, presence, size and color of the CL, number and size of follicles were recorded. Inflammation was apparent on gross examination of all uterine horns, and was more intense in the groups mated for 40 and 60 min compared to the 20 min group. At 1 day post-mating, the inflammation was intense, and gradually diminished with time post-mating until it had almost disappeared around 6–7 days post-mating. In a few cases, inflammation was still prominent at 7 days post-mating, particularly in the 40- and 60- min mating groups. In conclusion, 20 min of mating induced mild inflammation in both uterine horns (i.e. petechiae in uterine body and cervix) that disappeared almost completely by 6 days post-mating, which is the time of embryo enters the uterus in alpacas; however, long mating times (40–60 min), induced very intense inflammation (i.e. intense redness, petechiae and presence of blood in luminal fluid) that lasted more than 6 days. Longer mating time was associated with prolongation of the period of transient post-mating endometrial inflammation, and may be responsible for infertility or early embryonic loss.

Key Words: Alpaca, uterus, inflammation

1213

Fate of the unfertilized ova in alpacas

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Betteridge and Mitchell (*J Reprod Fertil* 1974;39:148) found that in the mare, the unfertilized ova remain in the oviduct for months, never entering the uterus. Bourke et al. (*Theriogenology* 1995;44:255–268) hypothesized that unfertilized llama ova remain in the oviduct, similar to what occurs in the mare. Two experiments were designed to determine the fate of unfertilized alpaca ova. Experiment 1 included 10 female alpacas randomly distributed into five groups (two females per group). Alpacas were given 1 ml of GnRH (Cystorelin, Merial 50 μg/ml of gonadorelin diacetate tetrahydrate, IM) for induction of ovulation when a follicle at least 7 mm was present. Alpacas in the respective groups were sacrificed at 1, 2, 3, 5 and 6 days post-ovulation. Ovulation was detected by transrectal ultrasonography every 4 h, starting 24 h after GnRH treatment. Using a solution of PBS and surfactant, the oviduct ipsilateral to the side of ovulation was flushed. In alpacas sacrificed on days 5 and 6, the uterine horns were also flushed. The solution was collected in petri dishes; ova identification was performed under a 4 and 10× stereomicroscope. A single ovum was collected from each female on each day (i.e. n = 2 ova per collection day) except for day 6. At 1 day post-ovulation, the ova had normal morphology and were surrounded by cumulus oophorus cells. At day 2 post-ovulation, the ova had a clear, shrunken, dissociated cytoplasm as well as unequal cell division. At day 3 post-ovulation, one ovum had cumulus oophorus cells similar to that observed on day 1 post-ovulation, and the other had a completely disorganized and very clear cytoplasm. At day 5 post-ovulation, one ovum was shrunken with broken zona pellucida, and the other had round vesicles around a small black cytoplasm. At day 6 post-ovulation, an empty, broken zona pellucida and many cumulus oophorus cells were flushed from the uterine horn ipsilateral to the side of ovulation. In Experiment 2, 2 alpacas without dominant follicles were selected for superovulation. On day 1, alpacas were given a single dose of 400 IU of eCG (Folligon, Intervet, IM). Alpacas were monitored by transrectal ultrasonography to identify follicular development, on day 2, 4, 6 and 7 post-eCG. When follicles reached at least 6 mm, alpacas were given 1.5 ml of GnRH (Cystorelin, Merial, 50 μg/ml of gonadorelin diacetate tetrahydrate, IM) to induce ovulation. Animals were sacrificed on day 14 post-eCG, at which time the oviducts and uterine horns were flushed. A total of seven ova were collected from the uterus of the two alpacas. Three ova were surrounded by cumulus oophorus cells, and the rest were degenerated with small dark cytoplasm. Varying degrees of degeneration were detected, perhaps as a result of different ovulation times. In conclusion, unfertilized ova are not retained in the oviduct of the alpaca.

Key Words: alpacas, ova, oviduct

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Interaction between neurotrophin 4 and gonadotrophins in bovine ovary

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Hormonal factors secreted by embryos and reproductive tracts are essential for successful development of preimplantation embryos. We have shown that neurotrophin 4 (NT4) is a protein immunolocalized in bovine follicle granulosa cells (GCs; Sun et al., 2011., *Asian-Australas J Anim Sci* 24:336–343). The objective of this study was to confirm the interaction between NT4 and gonadotrophins in the bovine ovary by exploring (i) the effect of LH and FSH on NT4 mRNA expression in GCs and (ii) the effect of NT4 and K252α (kinase inhibitor) on FSHR and LHR mRNA in GCs. In experiment 1, GCs were cultured for 48 h without serum and divided into four groups to continue incubation in serum-free medium for additional 8, 16 and 24 h with different concentration of LH or FSH (0, 10, 20, 40, and 60 μg/ml), respectively. Supernatants were collected after centrifugation at the end of culture. GCs were collected by digestion with 0.25% trypsin and total RNA from GCs were extracted using a RNAprep Pure Micro Kit (Tiangen Co., Beijing, China), according to the manufacturer′s instructions. The expression levels of NT4 mRNAs and proteins were evaluated by quantitative real-time PCR and ELISA, respectively. In experiment 2, GCs were cultured, allocated into groups as above and treated with NT4 or K252α (0, 10, 30, 60, 100 μg/l) for the same times as in the experiment 1. GCs collection and total RNA extraction were the same as experiment 1. All experiments and measures were repeated three times. Statistical analysis was performed using one-way ANOVA (SPSS 13.0) followed by Dunnett′s multiple range test. In experiment 1, the expression levels of NT4 mRNA were increased ($p < 0.05$) after adding LH or FSH in groups treated with 10 and 20 μg/ml for 8 and 16 h, respectively; and in the group treated with 20 μg/ml for 24 h. However, there was no significant difference in NT4 protein expression levels measured by ELISA ($p > 0.05$). In experiment 2, expression levels of LHR and FSHR mRNAs in GCs treated with NT4 at different concentrations for different time were higher ($p < 0.05$) than those of the control; the expression of LHR and FSHR mRNA was highest at 30 and 100 μg/ml for all time groups, respectively. However, expression of all gonadotrophin receptors was blocked by treatment with K252α, and LHR and FSHR mRNA expression lower than those of the control. We concluded that NT4 may have a role in regulating the function of bovine ovary by interacting with gonadotrophins. This work was supported by the State Key Development Program of Basic Research (‘973’ Program) of China (No. 2011CB944203).

Key Words: Neurotrophin 4, tyrosine kinase B, gonadotrophins, ovary, bovine

1251

Bovine seminal plasma synchronizes ovulation in heifers

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Ovulation-inducing factor (OIF) is a protein that has been detected in the seminal plasma of both induced and spontaneously ovulating species. In camelids (induced ovulators), OIF induces ovulation by triggering LH release. The function of OIF in cattle (spontaneous ovulators) is unknown. Bovine seminal plasma induced ovulation in llamas but not in heifers; however, it appeared to be luteotrophic in cattle (Tanco et al. *Theriogenology*, in press). The present study was designed to determine if bovine seminal plasma influences the subsequent corpus luteum (CL) development in heifers. Seminal plasma was pooled from 160 ejaculates (n = 160 bulls). The OIF concentration in the pooled seminal plasma was measured by RIA, and the treatment volume (12 ml) was adjusted to a dose of 250 μg of OIF (Tanco et al., *Bio Reproduct* 85:452–456). Beef heifers (n = 29; 362.4 ± 8.2 kg; mean ± SEM) were examined ultrasonographically (MyLab5, 5–8 MHz linear-array probe) daily for at least 3 days to confirm the presence of a CL and a growing follicle of 9–11 mm in diameter. A 25 mg dinoprost dose (PGF, Lutalyse, Pfizer Animal Health Inc., Canada) was given i.m., followed 12 h later by 25 mg pLH (Lutropin, Bioniche Animal Health, Canada) to synchronize and standardize LH exposure among animals (pre-empt endogenous LH release). Heifers were assigned randomly to three groups and given no further treatment (Control, n = 10), bovine seminal plasma i.m. 12 h after LH treatment (n = 10), or bovine seminal plasma i.m. within 4 h after ovulation (n = 9). Ovulation was monitored by ultrasonography every 4 h starting at 22 h after LH treatment, while luteal development was monitored daily until next ovulation. All other end points were compared among groups by analyses of variance, except for the comparison between the interval to ovulation in control and post-ovulation seminal plasma-treated heifers (combined) vs. pre-ovulation seminal plasma-treated heifers (*t*-test). All heifers ovulated, and the largest diameter of the ovulatory follicle did not differ among groups. The interval to ovulation was longer ($p < 0.05$) in heifers treated with seminal plasma before ovulation compared to the others (33.2 ± 0.53 vs. 31.3 ± 1.02; mean ± SEM). All ovulations in the pre-ovulation treatment group occurred within a 4 h period, while the range for others was 22 h. Greater synchrony in the pre-ovulation treatment

group compared to others was reflected in analysis of the absolute values of the residuals of interval to ovulation (1.9 ± 0.05 vs. 3.0 ± 0.75; <0.0001). No difference was detected among groups (pre-ovulatory treatment, post-ovulatory treatment, and controls, respectively) in maximum CL diameter (17.5 ± 0.88, 18.6 ± 1.07 and 19.2 ± 1.22), the day of maximum CL diameter (7.9 ± 0.78, 6.4 ± 0.75, 6.0 ± 0.37), or the day the CL was first detected (1.9 ± 0.28, 2.1 ± 0.26, 1.4 ± 0.24). In conclusion, bull seminal plasma had no apparent effect on CL development (results of plasma progesterone assay pending), but was associated with a more synchronous interval to ovulation. Research supported by the Natural Sciences and Engineering Research Council of Canada.

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Effect of semen quality on cow uterine artery resistance index by color Doppler ultrasound after artificial insemination

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Cattle may develop endometritis after natural mating or artificial insemination (AI) with the consequently associated hyperemia. Semen quality, which may influence the degree of inflammation, has been assessed by several techniques. A novel technique that uses fluorescent probes to assess acrosome and plasma membrane integrity and mitochondrial function (PIAIC) is able to assess the semen fertilizing capacity. The objective of this study was to determine the effect of AI using semen of different degrees of quality on the uterine vascularization. Fifty- to 70-day postpartum Nellore cows (n = 182) had their reproductive tract examined and were divided into 3 groups to be inseminated using semen with high (44.5%, n = 68), medium (23.0%, n = 56) or low (8.5%, n = 58) proportion of viable sperm based on PIAIC. Blood flow was evaluated by ultrasound scanning (M5vet, Mindray Medical International Ltd., São Paulo; with Doppler technology, spectral mode and equipped with a 6.5 MHz linear probe) of the right (AR) and left (AL) uterine arteries at 30 h prior to AI, 4 and 24 h after AI. The resistance index (RI, ranging from 0 = high to 1 = low perfusion) of the arteries was used to evaluate vascularization. The average RI of the two arteries over time was analyzed by PROC MIXED (SAS 9.2, SAS Institute Inc., Cary, NC, USA, 2010) in ANOVA for repeated measurements. There was no effect of treatment ($p > 0.05$) on the RI of the uterine arteries. The RI values for the High group were 0.75 ± 0.02 (30 h before AI), 0.62 ± 0.01 (4 h after AI) and 0.66 ± 0.01 (24 h after AI). The RI values for the Medium group were 0.74 ± 0.01 (30 h before AI), 0.64 ± 0.01 (4 h after AI) and 0.69 ± 0.02 (24 h after AI). The RI values for the Low group were 0.77 ± 0.02 (30 h before AI), 0.64 ± 0.01 (4 h after AI) and 0.67 ± 0.01 (24 h after AI). However, there was an effect of time ($p < 0.05$). RI values at 4 (0.63 ± 0.01) and 24 h (0.67 ± 0.01) after AI were lower than those at 30 h prior to IA (0.75 ± 0.01). Artificial insemination of Nellore cows with semen of different qualities has not resulted in marked changes in uterine blood flow, but there was a marked effect on RI values within 24 h after AI, as evaluated by spectral Doppler sonography.

Key Words: Vascularization, inflammatory response, fluoroscent probes, semen quality, cattle

13. Female reproductive tract – nonruminants:

1300

Influence of the level and duration of local inflammatory reaction of mares' uterus to the systemic plasma concentrations of acute phase proteins

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The aim of this study was to investigate the clinical relevance of measuring blood concentrations of serum amyloid A (SAA), haptoglobin (Hp) and fibrinogen (Fib) in equine reproductive management, and their dynamic changes in response to insemination with frozen-thawed semen. Clinically healthy mares were investigated during one oestrous cycle, with insemination of frozen-thawed semen immediately after detection of ovulation. Inflammation of the endometrium was evaluated by uterine cytology and presence of ultrasonically visible fluid during oestrus and 24 h after insemination. Transrectal ultrasonography was performed every 12 h for detection of intrauterine fluid and growth of primary follicle. Uterine cytology was performed every 48 h, if primary follicle growth was detected in barren mares and from day 9 in postpartum mares, until ovulation and once 24 h after insemination in all mares. Blood samples were collected at same time as endometrial cytology was performed and was continued until 72 h after insemination with 48 h interval from 23 standardbred mares. The frozen semen originated from two stallions in different batches were used. Mares were classified in three groups. Problem-mares (n = 5) had polymorphnuclear neutrophils (PMNs) in cytological and/or bacterial growth in endometrial sample before insemination. Mares having no signs of endometritis before ovulation, but accumulated intrauterine fluid and had elevated number of PMNs in endometrial sample 24 h post-insemination were classified as mares with prolonged inflammatory reaction, thus 13 mares had no sign of endometritis at any observed time-point or diagnostic method (group of normal mares). Changes in concentrations of SAA, Hp and Fib were compared between and within groups using linear mixed models with polynomial time effects. The changes in SAA plasma concentrations around insemination within PIR group were significant peaking 3 days before ovulation (time effect; $p = 0.003$ and quadratic time effect; $p < 0.001$). This plasma SAA time trend in the PIR-group mares was significantly different compared to normal and problem mares ($p = 0.002$ and $p = 0.004$ respectively). The plasma concentrations of Fib within the PIR group were close to significant (time effect; $p = 0.032$ and quadratic time effect; $p = 0.089$). The trend over time of plasma Fib was significantly different between PIR-mares and from problem mares ($p = 0.021$). There were no changes of concentration of Hp over time within or between the groups of mares. The local inflammatory reaction to frozen-thawed semen in the uterus of mares, detected by normal clinical procedures, did not influence the plasma concentrations of SAA, Hp and Fib above clinically relevant concentration limits, measured 72 h. However, there where some different trends in changes of SAA and Fib in mares with different endometrial status during oestrus.This study was supported by ETF Grant No. 7539.

Key Words: Mare, APP, uterus, artificial insemination

1301

Diestrus oxytocin administration prolongs luteal function and decreases endometrial cyclooxygenase-2 expression in mares

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Prior research has shown that late diestrus administration of oxytocin prevents luteolysis, thereby prolonging the luteal phase of the estrous cycle in mares. The objective of this study was to evaluate the ability of different intervals of oxytocin administration to prevent luteolysis in

mares and to examine changes in the expression of cyclooxygenase-1 (COX1) and cyclooxygenase-2 (COX2) in the endometrium after oxytocin treatment. Mares were examined with transrectal ultrasonography to determine day of ovulation (day 0) and daily from day 8 through the duration of the study. Daily blood samples were taken for determination of progesterone concentrations. In Exp. 1, mares were randomly assigned to one of three treatment groups receiving 60 IU of oxytocin daily as an intramuscular injection on days 8–10 (n = 7), days 8–12 (n = 7), or days 8–14 (n = 7). An additional control group received an intramuscular injection of sterile saline daily on days 8–12 (n = 7). Prolongation of the luteal phase was defined by elevation of serum progesterone >1 ng/ml through day 30 postovulation as determined by an enzyme-linked immunosorbent assay. Proportional data were compared using Fisher's exact test. Quantitative RT-PCR data were compared using a Wilcoxon rank sum test, and immunohistochemistry data were analyzed using logistic regression. Oxytocin administration on days 8–10, 8–12, and 8–14 prolonged luteal maintenance in 3/7, 4/7, and 6/7 mares respectively, compared to 0/7 control mares ($p < 0.05$). In Exp. 2, endometrial biopsies of mares treated with oxytocin from days 8–14 (n = 6) demonstrated reduced COX2 but not COX1 expression as determined by quantitative RT-PCR and immunohistochemistry when compared with control mares (n = 6) at day 14 postovulation ($p < 0.05$). In conclusion, oxytocin administration prolonged luteal maintenance in mares with an increasing number of mares responding to treatment as the number of days of oxytocin administration was increased beyond day 8 postovulation. Luteal maintenance in mares that received oxytocin was also associated with a reduction in endometrial COX2 expression. Supported by the Equine Drug Research Council, Hughes Fellowship and Clay Endowment.

Key Words: Oxytocin, luteolysis, cyclooxygenase-2, equine

1302

Estrogen alpha and progesterone receptors in endometrial immune cells of mares with endometritis, effect of immunomodulation

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Our objective was to evaluate Estrogen alpha (ERα) and progesterone receptors (PR) expression in endometrial immune cells from mares with endometritis, treated with an immunomodulator (Mycobacterial Cell wall-DNA Complex Sterile Suspension (MCC-V) at estrous by intrauterine route. Twenty four Criollo type mares, susceptible to endometritis were experimentally infected at the beginning of estrus with Streptococcus zooepidemicus. The infection was confirmed by endometrial culture, cytology, and presence of intrauterine fluid by ultrasonography. All mares included developed endometritis and were assigned during estrus to three MCC-V treatment groups and a Placebo group of 6 mares each. Treatments with 1.5 ml of MCC-V at different concentrations were: T1: 4500 μ, T2: 1500 μ, and T3: 500 μ. Placebo (P) consisted in 1.5 ml of sterile water for injection. Treatments and placebo were administrated in final amount of 25 ml of Kenney extender. Biopsy samples were taken from each mare at ovulation and on day 7 post-ovulation respectively. An immune peroxidase staining technique was used to visualize ERα and PR immune-reactivity in the polymorphonuclear neutrophiles (PMN), and in mononuclear cells (lymphocytes and monocytes, L&M). The data were analyzed by ANOVA test and the model included the effect of MCC-V doses, cell types, day of estrous cycle and the interactions between them. A $p \leq 0.05$ was considered statistically significant. Total immune cells (marked and unmarked) was significantly affected by: MCC-V treatments ($p = 0.04$, for ERα and $p = 0.01$ for PR), day of the estrous cycle ($p \leq 0.0001$) and there was an interaction between them ($p \leq 0.0001$).There were no differences in the amount of L&M marked for ERα or PR for any MCC-V doses or day.The number of PR marked PMN cells was higher in T2 than in P group at ovulation (1 ± 0.22 vs. 0.37 ± 0.19, $p \leq 0.001$), and no differences were found among T1, T3 or P group (0.36 ± 0.16, 0.24 ± 0.17, 0.37 ± 0.19 for T1, T3 and P group respectively). All MCC-V treatment doses decreased the number of PMN marked for ERα with respect to the P group on day 7 pos-ovulation (2.34 ± 0.32, 0.60 ± 0.26, 0.38 ± 0.32 and 0.41 ± 0.31 for P, T1, T2 and T3 group respectively, $p \leq 0.01$). The presence of ERα and PR in PMN, M and L, suggest that steroid hormones are involved in the immune response regulation. The changes in PMN marked for ERα and PR, caused by MCC-V indicates that steroid receptors may be involved in the long term reduction of inflammation obtained after the immunomodulator treatment.

Key Words: Horses, endometrium, iflammation, immunomodulator, steroids hormone receptors

1303

Endometrial steroid hormone receptors, proliferating cell nuclear antigen and progesterone profiles in mares resistant to endometritis

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In order to evaluate some molecular endocrine patterns of normality in mare endometrium, estrogen alpha (ERα), progesterone receptor (PR), proliferating cell nuclear antigen (PCNA) and plasmatic progesterone profiles (P4) were investigated in seven mares resistant to endometritis. Animals were selected as resistant to endometritis according to Brinsko, 2003. Endometrial biopsies and blood samples were repeatedly taken in four moments of the estrous cycle as follows: at the beginning of estrous with ovarian follicles >30 mm, endometrial folds and edema, at 24 h after estrous detection, at 24 h post ovulation and at day 7 post ovulation. Blood samples were taken to determine plasmatic P4 concentrations by radioimmunoassay. An immunoperoxidase staining technique was used to visualize ERα, PR and PCNA immunoreactivity in each endometrial biopsy in different tissue compartments (luminal epithelium, LE; superficial stroma, SS; glandular epithelium, GE and interglandular stroma, IS). Data were evaluated by analysis of variance for repeated measurements using the mixed model procedure of Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). P4 concentration on day 7 post ovulation was higher than in all other moments of the estrous cycle ($p < 0.0001$). Regarding the immunoreactivity to ERα, PR and PCNA, differences were found only in two of the four studied compartments; the luminal epithelium and the interglandular stroma at diestrous exhibited lower immunoreactivity than in estrus. For ERα: 0.9 vs. 1.34, ($p < 0.05$) in LE and 1.20 vs. 1.70 ($p < 0.01$) in IS. For PR: 1.39 vs. 1.86, ($p < 0.01$) in LE and 0.82 vs. 1.25, ($p < 0.01$) in IS. For PCNA 0.62 vs. 1.23, ($p < 0.01$) in LE and 0.82 vs. 1.25, ($p < 0.01$) in IS. No differences were detected in the GE or SS along the study for ERα, PR or PCNA immunoreactivity ($p > 0.05$). The lower immunoreactivity to ERα, PR and PCNA found at day 7 post ovulation, compared to 24 h from the beginning of estrus would suggest an inhibitory effect of high levels of plasmatic P4 found on this day. The similar immunoreactivity to ERα, PR and PCNA between the beginning of estrus and day 7 post ovulation, found in LE and SS, would suggest that P4 levels at luteal phase, maintain an inhibitory effect on ERα, PR and PCNA expression at the beginning of the following follicular phase. Overall, synchrony was found between the expressions of both receptors and proliferating cell nuclear antigen, suggesting that cell proliferation and endometrial remodeling may be mediated through steroid receptors. In conclusion, our results provide information regarding characterization of endometrial molecular endocrinology in mares resistant to endometritis.

Key Words: Horses, steroid hormone receptors, PCNA, endometrium

1304

Nodal and receptors Alk4 and Alk7 in equine corpus luteum: mRNA quantification and functional role

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The role of Nodal – a transforming growth factor beta superfamily member – in equine corpus luteum (CL) is not known. The aim of the present work was to evaluate (i) the transcription level of Nodal and the receptors Alk4 and Alk7, and (ii) the modulation of prostaglandin (PG) E2 secretion by Nodal, in the mare CL, throughout the luteal phase. Luteal tissue and jugular venous blood were collected post mortem at the local abattoir from randomly designated cyclic Lusitano mares. Further on, based on plasma progesterone (P4) concentration and type of structures present in the ovaries, CL were classified into early luteal phase (presence of a corpus hemorrhagicum and P4 > 1 ng/ml, early CL; n = 5), mid luteal phase (CL associated with follicles 15–20 mm in diameter and P4 > 6 ng/ml, mid CL; n = 5) and late luteal phase (CL associated with preovulatory follicle 30–35 mm in diameter and P4 between 1 and 2 ng/ml, late CL; n = 5). Specific primers for target genes (Nodal, Alk4 and Alk7), and for housekeeping gene (B2MG), were designed and tested by conventional PCR. Further on, enzymatically isolated luteal cells from all stages of luteal phase were incubated with: (i) no treatment (control), (ii) Nodal at different concentrations (0.1, 1, 10 and 50 ng/ml), and (iii) equine Luteinizing Hormone (LH 10 ng/ml; positive control), for 6, 24 and 48 h, at 37°C in a 5% CO_2 atmosphere. Relative quantification of mRNA transcription was accomplished by Real Time PCR. Nodal mRNA level was increased in early CL and decreased in late CL ($p < 0.05$), while Alk4 showed the opposite profile, with the highest mRNA level in late CL ($p < 0.05$). Alk7 did not change. Considering Nodal effect on PGE2 secretion, after 24 h treatment, Nodal (1 and 10 ng/ml) decreased PGE2 secretion from late CL ($p < 0.05$). After 48 h treatment, Nodal (10 ng/ml) decreased PGE2 secretion from mid and late CL ($p < 0.05$). The positive control LH increased PGE2 production after 24 h incubation of mid CL ($p < 0.05$) and after 48 h treatment of early and mid CL ($p < 0.05$). In conclusion, the stage specific transcription profile of Nodal and its receptors in the equine CL was shown, throughout the luteal phase, as well as the putative role of Nodal on CL secretory function in the mare. The present findings suggest the participation of Nodal in the physiologic regulation of equine CL.

Key Words: Corpus luteum, mare, nodal

1305

Influence of endometrial condition of mares on expression of lactoferrin

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Lactoferrin (Lf), one of the iron-binding proteins, is known as multiple functional substance found in colostrum, saliva, tear and mucous membrane in mammals, and has important role in the regulation of body defense system in the mucous membrane. In stallions and mares, expression of Lf was also shown in the various reproductive tracts (Kolm et al., 2006), but little is known about the relationship between Lf expression and uterine condition of mares. In this study, we investigated the effects of reproductive status, infection condition and degree of degenerative changes on the endometrial expression of Lf in mares. Fifty-nine barren Thoroughbred mares, aged 4–21 years old, were used in this study. After general reproductive examinations, ultrasonograpy per rectum were performed to check the existence of corpus luteum (CL). Bacteria or fungi in recovered uterine fluid were checked by culture. Uterine biopsy sample was collected from the border of uterine horns with endoscopic aid. The formalin-fixed endometrial biopsy tissues were used to diagnose the existence of fibrotic glandular nest (nest), and presence of Lf by immunohistochemical technique using rabbit anti-horse Lf antibody (Kolm et al., 2006). Lf expression in the uterine glandular epithelium, glandular lumina, and endometrial epithelium was categorized into five grades on the basis of the intensity of the positive staining. Data were analyzed using the StatView 5.0 software (1998; SAS Institute Japan, Tokyo, Japan). Significant differences between each group (microbial infections or not infections, existence of CL or absence of CL, normal uterine gland or nest gland in the same sections) were analyzed with the Mann–Whitney *U*-test. A p-value <0.05 was considered significant. Positive staining of Lf in endometrium was observed in 56 out of 59 mares. Strong Lf expression in endometrium was clearly found in the uterine glandular epithelium, glandular lumina and endometrial epithelium. Expression of Lf in the glandular lumina was more intense in mares without CL than those with CL. In case of nine mares with bacteria or fungi infection in the uterus, Lf expression in the endometrial epithelium was significantly stronger than those in normal mares ($p < 0.01$). In 27 mares with nest, immunohistochemical analysis showed less staining of Lf in glandular epithelium of fibrotic glandular nest than in normal gland in the same sections ($p < 0.05$). The present study demonstrated enhanced expression of Lf in the endometrium of mares with positive bacteria; /fungal culture test. This result supports the previous results in mRNA expression of Lf in mares' endometrium with mating-induced endometritis. Immunohistochemical analysis also revealed different staining between nest and normal glandular epithelium, probably caused by chronic degenerative changes. These results suggest that Lf might play an important role to prevent microbial infections in mares' uterus.

Key Words: Mare, lactoferrin, infection

1306

Sow oviduct and uterus myoelectrical activity during early pregnancy in pigs using telemetry implants

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During the estrous cycle uterine and oviductal motility are of vital importance for the transport of gametes and embryos. The aim of the present study was to record oviduct myoelectric activity in sows during early pregnancy. The studies were carried out on 16 Polish landrace sows (95–115 kg body weight). The silicone base silver bipolar electrodes were adapted to be sutured precisely in the oviduct and uterus. Pigs were surgically fitted with TL10M3-D70-EEE (DSI, St. Paul, MN, USA) implants positioned between the abdominal muscles, and three silicone electrodes sutured on the left or right oviduct (bulb and isthmus) and the corresponding uterine horn. The myoelectric activity was recorded using the DL10 analog output (DSI) coupled with PowerLab (ADInstruments, Melbourne, Australia) and PC computer. Recording speed was set at 40 points/s, and signal band (high cut-off 50 Hz, low-cut 10 Hz) was used for analysis. Blood was withdrawn for plasma P4 and LH during the cycle and early pregnancy. Pigs synchronized with eCG and hCG (Werfaser, Fa. Alvetra, Austria) were inseminated 24 and 48 h after treatment with pooled semen processed on-site. Electromyography was continued for 18 days after insemination. After that period the sows were slaughtered, electrodes were checked for their position and the reproductive system gently flushed for embryo collection. Embryos were examined estimated for their quality and vitality. Examination of the reproductive tract post mortem indicated that the telemetry recording system and the material used (bipolar electrodes) did not have a negative effect on physiological processes in the pig. Analysis of oviduct and uterus EMG activity in sows in various stages of the estrous cycle as well as early stages of pregnancy shows for the first time that telemetry enables to assess myoelectrical and oviductal activity *in vivo*.

Key Words: Uterus, mioelectrical activity, pregnancy, pigs, telemetry implants

1307

Interactions of spermatozoa and uterine epithelial cells in the pig: a cell culture study

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In the pig, post insemination a large number of vital sperm undergo a transient binding with the uterine epithelium, influencing the expression of genes relevant for the maternal immune response, which may eventually impact reproductive success. We established a cell culture model from primary porcine uterine epithelial cells (UEC) to examine the binding patterns and to characterize participating surface molecules on the sperm as well as the endometrium. Since interactions between sperm and oviduct epithelium are lectin-mediated, we hypothesised that the binding of sperm to UEC is due to lectin-ligand interactions, too. The sperm rich fraction of four German Landrace boars was collected and extended in cell culture medium with 20% heat inactivated foetal bovine serum to 100×10^6 sperm/ml. Sperm suspension (500 µl) was released onto a UEC monolayer and incubated for up to 10 min at 37°C. Subsequently the UEC were washed with PBS to remove loose sperm and observed under a phase contrast microscope. As a control the same procedure was undertaken with porcine foetal fibroblasts. Images (2 repeats/boar) were divided into fields of 61.6 μm^2 and the area with and without sperm was counted. For statistical analysis a Mann–Whitney Rank Sum Test was carried out. Sperm binding density (μm^2) was significantly ($p = 0.002$) higher in UEC (15923.6 ± 2657.9) than in fibroblasts (3018.4 ± 638.1). For UEC sperm binding could be observed within < 5 min. It was noted that while clusters of sperm attached to single UEC along the complete perimeter of the cell, other cells were not populated by the sperm at all. Binding occurred at the sperm head. Bound sperm remained motile. To validate the hypothesis that the observed sperm binding is mediated via lectins, we tested 21 lectins for their binding properties to porcine sperm and evaluated the fluorescence intensity flowcytometrically. Strong binding was observed for WGA/sWGA, ConA, RCA120 and SJA which correspond to *N*-acetylglucosamine/sialic acid, Galactose/*N*-acetylgalactosamine, Mannose and Galactose respectively. These results are comparable to previous studies. It is shown that the binding behaviour of sperm is specific for epithelial cells from the reproductive tract. With regard to the binding mechanism, it could be established that sperm carry sugar moieties on their surface enabling lectin binding. Investigations concerning the expression of sugars on epithelial cells as well as blocking experiments are underway, in order to establish which binding partner provides lectin and sugar respectively. The results will provide valuable insight into events in the uterus early after sperm disposal, which might help to optimize current inseminations procedures.

Key Words: Pig, endometrium, cell culture, sperm binding, lectin

Table 1. Mean fluorescence intensity of various lectins tested

Lectin	Intensity	Lectin	Intensity	Lectin	Intensity
WGA	1044.6	GSL I	85.8	PHA-E	29.9
SWGA	576.7	GSL II	84.7	PNA	23.8
ConA	264.0	SBA	69.0	UEA I	18.6
RCA120	179.6	VVA	46.1	DSL	15.7
SJA	135.0	AIL	44.2	DBA	13.0
LCA	116.1	ECL	41.3	LEL	12.7
PSA	98.4	PHA-L	39.9	STL	11.6

1308

Expression and localization of key elements of microRNA processing machinery in the porcine endometrium during early pregnancy

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MicroRNAs (miRNAs) are a class of small RNAs that silence gene expression. Function of most of them remains unknown, however, they are believed to play crucial role in regulation of a variety of biological processes, including embryo implantation and pregnancy. Thus, objective of this study was to evaluate expression and localize key elements of miRNA processing machinery in the porcine endometrium. Nine mRNAs involved in the miRNA biogenesis: DROSHA, DiGeorge syndrome critical region gene 8 (DGCR8), Exportin-5 (XPO5), DICER, TAR RNA-binding protein 2 (TARBP2) and eukaryotic translation initiation factor 2C, 1–4 (EIF2C1-4) were quantified in the porcine endometrium during the estrous cycle ($n = 31$) and early pregnancy ($n = 37$). Two main proteins of RNA-induced silencing complex (RISC), DICER and EIF2C2, were immunolocalized in the endometrial tissues. Two-way ANOVA followed by Bonferroni *post hoc* test was applied. DROSHA mRNA expression was maintained higher on D12 and D20 of the estrous cycle when compared to pregnancy ($p < 0.01$ and $p < 0.001$, respectively). DGCR8 mRNA levels were affected by the status ($p < 0.01$), since increased expression was indicated on D16 of pregnancy in comparison to respective day of the estrous cycle ($p < 0.01$). XPO5 gene expression was maintained at almost constant levels during the estrous cycle and early pregnancy, except D20 of the estrous cycle (vs. D10; $p < 0.01$), when the highest levels were indicated. Expression of DICER mRNA on D16 retained slightly higher in pregnant and non-pregnant animals (vs. D10-11, $p < 0.01$). The lowest expression of TARBP2 was detected during pregnancy on D12 and 20 (vs. D10; $p < 0.01$). EIF2C1 gene expression was maintained at almost constant levels during the estrous cycle and early pregnancy, except D11 and D20 of pregnancy (vs. D10; $p < 0.05$ and $p < 0.001$, respectively), when its expression was maintained low and D20 of the estrous cycle (vs. D10; $p < 0.001$) when the expression was high. Increased levels of EIF2C2 were indicated on D16 pregnancy (vs. D10-11 an D16; $p < 0.05$). EIF2C3 mRNA levels were only elevated on D16 in both pregnant and non-pregnant animals (vs. D12; $p < 0.001$ and $p < 0.05$, respectively). EIF2C4 was affected by the status ($p < 0.0005$) as a consequence of elevated expression on D16 of pregnancy in comparison to respective day of the estrous cycle ($p < 0.01$). Prominent glandular and luminal epithelium immunostaining for DICER and EIF2C2 was observed in the uterine sections from all days of the estrous cycle and pregnancy examined. The expression data revealed altered profiles of mRNAs coding elements crucial to miRNA biogenesis, suggesting status-dependent miRNA synthesis in the porcine endometrium. Since changes in mRNA expression of most investigated genes markedly coincide with initiation of maternal recognition of pregnancy and embryo implantation in the pig, it seems likely that biosynthesis of miRNA in the porcine endometrium can be under the influence of molecules participating in early embryo-maternal dialog.

Key Words: miRNA biogenesis, endometrium, estrous cycle, pregnancy, pig

1309

Is insulin a signal molecule regulating corpus luteum life span in the bitch?

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Mechanisms controlling non-pregnant canine luteal function remain still obscure. Although luteal function is mainly regulated by prolactin, metabolic signals can further modify corpus luteum (CL) physiology. Insulin is often proposed as signal molecule between metabolism and fertility. Interestingly, the increasing insulin levels are accompanied by increasing 17 β-estradiol (E2) concentrations and decreasing glucose transporter (GLUT) 4 expression at mid diestrus (Sousa et al. 2008), predisposing bitches to insulin resistance in this period. Insulin acts through its receptor (IR), the expression of which has notbeen investigated in the dog. IR, GLUT 4, interleukin (IL)-6 and nuclear factor kappa B (NF-κB) expression was assessed in CL of non-pregnant dogs (n = 28; 4 per group) during the course of the diestrus (days 10, 20, 30, 40, 50, 60, 70 after ovulation; p.o.) and tested by immunohistochemistry, western blotting and/or Real Time PCR. Glycaemia was determined by colorimetric assay, while insulin, progesterone (P4) and E2 plasma profiles by RIA. Data were analyzed by One-way analysis of variance (ANOVA) followed by Tukey–Kramer Comparison test. Correlations were done using Pearsonâ€™s Correlation test. Differences were considered significant when P ï‚£ 0.05. Hormonal analysis showed highest values for P4 on day 20 (22.96 ng/ml) and for E2 (27.34 pg/ml) and insulin (3.7 μU/ml) on day 40 p.o. Glycaemia was constant over the studied period, but insulin/glucose ratio showed a time dependent variation reaching maximal values on day 40 p.o (6.58). IR was detected on all days investigated in luteal cells cytoplasm and the signals were strongest in the first half of diestrus (days 10–40 p.o.), with intensity decreasing afterwards. RT-PCR revealed that IR expression was upregulated at days 20 and 70 p.o. and downregulated at day 40 p.o. ($p < 0.05$). This expression was negatively associated with insulin levels (r = -0.69; p = 0.006) and positively correlated with GLUT4 (r = 0.89; p = 0.01) over diestrus and with IL-6 expression (r = 0.96; $p < 0.0001$) in the first half of diestrus (days 10–40 p.o.). Furthermore, IR expression was negatively correlated with NF-κB expression (r = -0.57; $p < 0.05$) at mid diestrus (days 30, 40 and 50 p.o.). No correlation could be observed between IR expression and P4 and E2 concentrations. In summary, the data presented suggest that the insulin action in the CL may be regulated at the IR followed downstream by GLUT 4 and further point towards a stimulatory role of insulin and IL-6 on luteal glucose availability in the bitch.

Key Words: Bitch, corpus luteum, insulin, diestrus, GLUT4

1310

Expression of oviductin mRNA in the canine oviduct during the periovulatory period

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The oviduct is of particular importance in canine reproduction as it supports oocyte maturation, sperm capacitation and storage, fertilization and embryo development to the morula or blastocyst stage until 8–10 days after ovulation. The major estrogen-dependent, oviduct-specific glycoprotein (OVGP), also called oviductin, may interact with gametes and embryos to promote these events, yet its secretion during the estrous cycle and its potential roles in the bitch are still unknown. The objective of this study was to investigate the mRNA expression of oviductin in the canine oviduct at precise times around ovulation. Beagle bitches were ovariectomized at six stages (six bitches per stage): anestrus, before the LH peak (pre-LH), after the LH peak but before ovulation (pre-ov), 1 day (Day 1), 4 days (Day 4) and 7 days (Day 7) after ovulation. Three oviductal regions were collected i.e. ampulla, isthmus and utero-tubal junction (UTJ). Total RNA was extracted from the whole oviductal regions and then reverse transcribed using oligodT and random primers. The mRNA amount of oviductin was assessed in duplicate by real-time quantitative PCR (LightCycler 480; Roche Diagnostics) using canine-specific primers and normalized using two reference genes (RPS19 and GAPDH). Oviductin mRNA was specifically expressed in the three oviductal regions at all stages examined. Moreover, the expression of oviductin mRNA varied significantly with both the oviductal region and the stage of the estrous cycle. Considering all stages together, the amounts of oviductin mRNA were 7- and 1.5-fold higher in the isthmus and in the UTJ, respectively, than in the ampulla. In all the oviductal regions, the lowest oviductin mRNA levels were detected at anestrus and on Day 7 while the highest levels were recorded at Day 1 in the ampulla, Day 1 and Day 4 in the isthmus, and Day 4 in the UTJ. At these stages, the amounts of oviductin mRNA were 40-, 16- and 70-fold higher in the ampulla, isthmus and UTJ, respectively, compared with anestrus, and 10-, 5- and 28- fold higher than at Day 7. In conclusion, this is the first report of oviductin expression in the bitch oviduct. The region-specific higher expression of oviductin mRNA at Day 1 and Day 4 post-ovulation, compared with other stages of the periovulatory period, suggests a biological role of this glycoprotein in gamete maturation and fertilization in the bitch.

Key Words: Oviductin, oviduct, bitch

1311

Novel links between shell gland functions and phytoestrogen

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Shell quality decreases as laying hens age and the aim of the present study was to investigate how a supplement of daidzein, a natural phytoestrogen in soya, affects key factors in the shell gland and eggshell quality in late-stage laying hens of two genotypes. Eggshell formation depends on a sufficient supply of calcium by the blood and carbonate ions formed by hydration of CO_2, catalyzed by carbonic anhydrase (CA). Estrogen is important for calcium metabolism in layers and estrogen receptor-α (ERα), present in the shell gland, decreases in late-stage hens. Thiry-two Lohmann Selected Leghorn (LSL) and 32 Lohmann Brown (LB), in furnished 8-hen cages, were randomly allocated to either a daidzein diet (50 mg/kg feed) or a control diet from 60 to 72 weeks of age. Both diets were soya free. Eggs were collected at 61, 66 and 71 weeks for shell quality measurements. All birds were sacrificed at 72 weeks. Tissue from the shell gland was processed for histochemical localization of CA and immunohistochemical detection of ERα and ERβ. Slides were coded for evaluation. Analyses of variance were conducted using the mixed model procedure of SAS. In the LB hybrids the number of capillaries with CA activity was higher in the birds supplemented with daidzein compared to the LB control (p = 0.028), while the LSL hybrids were unaffected, indicating a higher sensitivity to daidzein in LB birds. However, the total number of shell gland capillaries/mm^2 was higher in the LSL compared to LB hybrid (p = 0.0071). The localization of ERα was in agreement with previous studies and there were no effects of hybrid or diet. The localization of ERβ in the shell gland of laying hen is shown for the first time. Strong nuclear staining was found in the surface epithelium and endothelium and moderate staining in tubular gland and smooth muscle cells in both hybrids. Cytosolic staining for ERβ was found in the surface epithelium and some tubular gland cells, being stronger in half of the LB hybrids and one LSL bird and unrelated to diet. Other investigations show that daidzein is more powerful in stimulating ERβ than ERα mediated processes in chicken cells. The hybrids used in this study reacted differently to daidzein but if this can be explained by the difference in ERβ noted between hybrids, remains to be clarified. Interestingly, endothelium contained only ERβ and since estrogen regulation of CA is well known, the presence of an endothelial ER provides one possible route for the increase in CA positive capillaries found in LB hybrids. Although CA is crucial for shell formation shell quality was unaffected by daidzein.

If this is due to dose, exposure time or hybrid needs further investigation. In conclusion: Data from this experiment provides novel links between shell gland function and a phytoestrogen found in soya. Since soya is used routinely in the feed to laying hens these findings prompt further investigation to clarify how the potency of phytoestrogens may be utilized in the best possible way.

Key Words: Daidzein, estrogen receptor, carbonic anhydrase, eggshell, domestic hen

1350

Uterine cellular immune response after artificial insemination in mares

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Breeding-induced endometritis (BIE) is a physiologic inflammation which plays an important role in the elimination process of semen excess from the female reproductive tract. However, when this inflammatory response takes the persistent form, it becomes one of the most frequent causes of sub-fertility in mares. Polymorphoneutrophil leukocyte (PMNs) is the major cell type of the uterine defense mechanism. This study aimed at checking the uterine PMNs response at 8 and 24 h after artificial insemination (AI) by evaluation of percentage, uterine fluid concentration and oxidative metabolism of neutrophils. To conduct this study, fifteen mares classified as susceptible and fifteen as resistant to persistent BIE were used. All mares were inseminated with 1 billion of spermatozoa, around 80% of motility, diluted in a skimmed milk extender. Samples were collected 8 and 24 h after AI. Uterine cytology samples were acquired using a guarded cytobrush with rolling and pushing motion into the uterus and smears were prepared, dried and stained with panotic dye. Uterine secretions were collected using a cotton tampon inserted inside the uterine lumen. The recovered uterine fluid was submitted to neutrophil concentration determination using a Neubauer chamber and to nitroblue tetrazolium (NBT) reduction test according manufacturer's manual (Sigma-aldrich). Data were analyzed by ANCOVA. The results obtained for uterine exfoliative cytology did not differ ($p > 0.05$) between resistant and susceptible mares at 8 h (79.1 ± 1.5% vs. 72.7 ± 1.9%) and 24 h (48.2 ± 2.7% vs. 40.2 ± 3.8%) after AI. At both time points, the concentration of neutrophils in uterine fluid from resistant mares was significantly lower when compared to susceptible animals ($p < 0.0001$). Resistant mares showed 22.6 ± 2.7 and 2.6 ± 1.5 millions/ml of neutrophils, whereas susceptible mares exhibited 7228.2 ± 1692.6 and 1718.7 ± 1860.2 millions/ml of neutrophils at 8 and 24 h, respectively. No difference ($p > 0.05$) on uterine PMNs functionality (NBT test) was verified when comparing resistant (8 h: 81.8 ± 8.8% and 24 h: 79.8 ± 9.7%) and susceptible mares (8 h: 82.4 ± 10.1% and 24 h: 81.5 ± 9.8%). We can conclude that uterine cellular immune response in susceptible mares was more intensive than in resistant mares, since they showed higher uterine neutrophil concentration and no difference was detected in the neutrophil phagocytic capacity between these animals.

Key Words: Breeding induced endometritis, uterine response, neutrophils, mares

1351

Mares with intense uterine degenerative changes have lower luteal vascularity and plasma progesterone concentration during the early gestation

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The corpus luteum (CL) is a dynamic structure that requires rapid development and regression of an extensive vascular system. In pregnant mares, an uninterrupted and complete contact between the embryonic vesicle and uterus is necessary to prolongation of the CL beyond its normal cyclical lifespan and the continued secretion of progesterone (P4). Consequently, endometrial degenerative changes could affect the embryo-maternal interaction and, posteriorly, the functional status of the CL. The purpose of this study was to describe the hemodynamics and secretory function of the CL of mares with intense endometrial pathological changes during early gestation. A total of 12 pregnant mares were assigned into two groups ($n = 6$ mares/group) according to the histological classification of the uterus: healthy endometrium group (absent of endometrial changes) or intense endometrial degeneration group (widespread, diffuse and severe inflammatory and fibrotic endometrial changes). Mares with intermediary histological classification (slight to moderate inflammatory process with fibrotic changes in individual gland branches) were not considered in this experiment. Blood samples and Doppler ultrasonography exam of the CL were performed every day during the first 20 days of gestation. Power-flow Doppler ultrasound instrument (Sonovet Pico; Medison Co., Ltd) equipped with a linear-array tranducer (LV5-9CDn, 60 nn) was used for transrectal scanning. CL vascularity was estimated subjectivley using the extent of luteal tissue (0–100%) with color signals Doppler during a real-time imaging exam. Plasma P4 concentration was measured using radioimunoassay. Day of ovulation was considered D0. The data were analyzed by two-way repeated measure and differences between two means were evaluated by Tukey's test. In both groups, a progressive increase on luteal vascularity and P4 concentration were observed ($p < 0.001$) between D0 and D6. Luteal vascularity and P4 concentration were greater from D7 ($p < 0.0001$), independently of the histological classification of the uterus. Mean luteal vascularity and P4 concentration between D7 and D20 were greater ($p < 0.001$) in mares with healthy endometrium (62.5 ± 0.4% and 11.06 ± 1.3 ng/ml, respectively) than in mares with intense endometrial degeneration (54.8 ± 0.4% and 7.04 ± 0.7 ng/ml, respectively). Early embryonic death was not detected during the experiment. This is the first report regarding the relationship between plasma P4 concentration, luteal hemodynamics and uterine degenerative changes of pregnant mares. An intimate contact between embryonic vesicle and endometrium through the entire uterus is required for the adequate maternal recognition of pregnancy in mares. The secretory function of the endometrium modulates the CL development and may be affected by the degree of endometrial pathological changes. The reasons for the lower luteal vascularity and decreased P4 concentration in mares with intense endometrial degeneration during early gestation are under investigation.

Key Words: Doppler ultrasonography, corpus luteum, endometrial degeneration, pregnancy, mare

1352

Identification of interstitial Cajal-like cells in the sow uterus and oviduct

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Interstitial cells of Cajal (ICC) are the pacemaker cells of gut motor activity and have been suspected to play similar function in other smooth muscle organs. They have been found inside the wall of the gastrointestinal tract of several species including human, where their major function is discharging slow triggering waves. ICC present on the surface specific Kit protein called type III tyrosine kinase receptor (c-kit), which was utilized in this study as a major criterions for their identification. Presence of c-kit positive cells (interstitial Cajal-like cells – ICLC) has been also reported in the myocardium, blood vessel wall, urinary tract as well as the human uterus and oviduct. The aim of this study was to localize and identify the interstitial Cajal-like cells in the porcine reproductive tract during the estrous cycle. Samples were taken post mortem from 12 mature sows, while stage of estrous cycle was confirmed by morphological examination of the ovaries and uterus. Afterwards the collected material was stained with hemotoxylin-eosin, or labeled with primary (CD117) and fluorescent secondary antibodies for imaging under light and confocal microscopy. Initial scanning cytometry (SCAN®) analyses were also performed in the whole cross-sections of the examined organs. Cells with morphologic and immu-

nological phenotypes similar to the ICCs of the gastrointestinal tract were identified inside the oviductal and uterine walls by both, histological and immunofluorescence techniques. The c-kit positive cells were localized in the muscle layers as fusiform with dendritic processes forming a cellular network. In all studied samples we found accumulations of ICLC in the isthmus of the oviduct and the passage from isthmus to the uterine horn. The density of ICLC in the uterine horn and body was low when compared to other regions. We found predominant localization of ICLCs in the vicinity of small blood vessels. In summary, we confirmed localization of the ICLC in swine reproductive tract. ICLC can be identified by their specific morphology and c-kit positive labeling by light and confocal microscopy. Scanning cytometry provided initial informations confirming localization of ICLCs in the functional networks within muscle layers of reproductive tract. Localization of the ICLC in the vicinity of blood vessels suggests that ICLCs might participate in the uptake and transmission of hormonal signals from the blood. This hypothesis will be confirmed in further studies with the use of specific labeling against hormonal receptors. Our far-fetched hypothesis is that specific ICLC accumulation in the isthmus might be considered as the primary pacemaker which initiates slow waves in the myomertrium and oviduct muscle layers.

Key Words: Cajal-like cells, pacemaker cells, motor activity, uterus and oviduct, pigs

14. Folliculogenesis, oogenesis, ovulation – ruminants:

1400

Equine chorionic gonadotropin increases prolactin receptor expression in the bovine corpus luteum

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Prolactin (PRL) is a multifunctional hormone involved in the regulation of reproduction. Several genes important for corpus luteum (CL) formation, survival, and function have been identified as direct targets of PRL. It was also reported that prolactin increases progesterone production. The signaling mechanism of PRL in the CL occurs by binding to its receptors (PRLR), long prolactin receptor (PRLRL) and short prolactin receptor (PRLRS), derived by alternative splicing. Superovulation and stimulation of the dominant follicle which are treatments that use eCG have been shown to promote important morphological and functional changes in bovine CL, such as increase on CL volume and progesterone production. Therefore, the objective of this study was to investigate PRLR (PRLRL; PRLRS) gene and protein expression in the bovine CL after treatments with eCG. For that, Nellore cross bread cows (n = 18) were divided into control (n = 5), stimulated (n = 6) and superovulated groups (n = 7). All animals were submitted to estrous cycle synchronization using progesterone devices and estradiol benzoate. The stimulated and superovulated groups received 400 and 2000 IU eCG on day 8 and 4 after the beginning of the protocol, respectively. On day 6 after ovulation, blood samples and CLs were collected after slaughter. Prolactin receptor expression changes were detected by Microarray (Affymetrix GeneChip Bovine Genome Array) and validated by Real Time PCR and Western Blotting. Progesterone concentration were higher in stimulated (5.94 ± 1.24 ng/ml) than in control (3.69 ± 1.78 ng/ml) and in superovulated (2.11 ± 2.0 ng/ml/CL, $p = 0.03$) groups. The PRLR expression analyzed by microarray was 2.4-fold higher in stimulated than in control group ($p < 0.05$). RT-PCR revealed that PRLRS and PRLRL mRNA expression was 3.77- and 3.21-fold higher in stimulated than in control group, respectively ($p < 0.05$). Furthermore, the protein expression of both receptors was elevated in stimulated animals. In superovulated animals only PRLRS protein expression was greater related to control group ($p < 0.05$). It has been reported that PRLRS acts on endothelial cells stimulating angiogenesis, whereas PRLRL mediates the effect of prolactin on luteal progesterone production. In conclusion, the results presented point towards a possible involvement of eCG in the regulation of PRLR expression contributing to the CL development and increase of progesterone synthesis.

Key Words: Ovary, prolactin, corpus luteum, superovulation, progesterone

1401

Three-dimensional modeling of color Doppler ultrasound images: a new approach to the evaluation of follicular vascularization in cattle

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The blood supply is an important predictor of ovarian follicle health and, consequently, of oocyte competence. Color Doppler ultrasonography is a powerful tool to assess blood flow in ovarian follicles. In most studies, however, the evaluation of this parameter is qualitative (presence or absence of color signal) and quantification of blood flow remains a challenge. The aim of the present study was to first describe the use of a three-dimensional (3D) modeling of color Doppler images to quantitatively measure the vascularization of ovarian follicles. Follicular wave was synchronized (D0) in Holstein heifers (n = 6) with 2 mg estradiol benzoate (Sincrodiol, Ouro Fino, Brazil) and an intravaginal implant of progesterone (Sincrogest). After wave emergence, follicular dynamics was evaluated every 12 h using a portable ultrasound device equipped with a 7.5 MHz transducer (MyLab Vet Gold 30, Esaote, Italy) until the establishment of follicular dominance (D8). During each sonogram, the color flow mode (PRF 0.7 KHz) was activated to assess follicular vascularization of the dominant follicle and a video sequence (approximately 20 s) was recorded. Images of the dominant follicle before divergence were selected by the retrospective analysis of follicular growth data. At the three-dimensional Modeling Laboratory of National Institute of Technology, the video was decomposed into frames, and custom-built software (Mimics 12, Materialise, Belgium) was used to isolate the Doppler signal, to generate the 3D model, and thereafter to calculate the volume of vascularization. Results are presented as mean ± SEM. Follicular emergence and follicle deviation occurred 3.7 ± 0.2 and 5.9 ± 0.2 days after synchronization, respectively, and the size of the dominant follicle at deviation was 8.7 ± 0.1 mm. The Doppler signal was identified in all dominant follicles since their first imaging at 4.7 ± 0.2 mm. The number of frames used to generate the 3D models increased (from 58.3 ± 8.4 to 114.2 ± 23.2) with the growth in follicular diameter. The volume of vascularization of the dominant follicle increased exponentially from D5 to D8 ($y = 24.61 + 0.12x + 0.006x^2$, $R2 = 0.28$, $p < 0.05$), ranging from 25.6 ± 4.8 to 41.8 ± 5.3 mm^3, and was 27.5 ± 4.1 at deviation. This variation is coherent with the expected increase in the metabolic requirements of the dominant follicle after deviation. With this new approach it was also possible to visualize the distribution of blood vessels along the follicular wall. These preliminary results demonstrate the potential of 3D modeling of color Doppler images to measure follicular blood flow and to describe vascular architecture in ovarian follicles, providing a new tool for *in vivo* studies of folliculogenesis. CNPq and Fapemig (CVZ APQ 02863/09).

Key Words: Ovarian follicle, color Doppler, blood flow, three-dimesional modeling

1402

Ovulatory follicle in lactating dairy cows: size matters?

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Premature ovulation of a dominant follicle induced by exogenous GnRH administration reportedly reduced ovulatory follicle diameter (OVFD) and decreased fertility in beef cattle. Objectives were to investigate factors associated with OVFD and the association between OVFD and pregnancy per AI (P/AI) in lactating dairy cows subjected to timed-AI (TAI). We utilized 1048 ovulations from 1576 breeding information of cows, presynchronized or not and subjected to a 5- or 7-days Ovsynch protocol with or without progesterone (P4) supplementation (PRID containing 1.55 g of P4) between initial GnRH and PGF2α of Ovsynch. Ultrasonography was performed to measure OVFD at TAI and to determine cyclicity, ovulation and pregnancy status. The effect of parity (1 or >1), presynchronization (yes or no), ovulatory response (yes or no) after initial GnRH, cyclicity status (yes or no) at onset of treatment, ovulation synchronization treatment length (5 or 7 days), P4 supplementation (yes or no) and their interactions on OVFD were analyzed by two-way ANOVA in the General Linear Model (GLM) procedure of SAS. Predicted probabilities of pregnancy were computed using the LOGISTIC procedure. The effect of OVFD on P/AI at 32 and 60 days after TAI were determined. First-order (linear) and second-order (quadratic) continuous effects of explanatory variables were modeled. The average (mean ± SEM) OVFD was 16.4 ± 0.07 (range 11–25 mm). The most common OVFDs were 15 (n = 170), 16 (n = 169) and 17 mm (n = 170). The OVFD was affected by parity and ovulation synchronization treatment length with cows with parity >1 and those subjected to a 7-days Ovsynch having greater ($p < 0.05$) OVFD. Interactions between ovulatory response after initial GnRH and parity and between ovulatory response after initial GnRH and ovulation synchronization treatment length were also found significant ($p < 0.05$). Cows with >1 parity that did not ovulate after initial GnRH had the largest OVFD (16.9 ± 0.3 mm) and cows subjected to a 7-days Ovsynch that ovulated after initial GnRH had the smallest OVFD (15.7 ± 0.3 mm). The association between OVFD and P/AI at 32 and 60 days after TAI were not significant (overall, 483/1048 and 439/1048, respectively). In conclusion, OVFD was affected by interactions between ovulatory response after initial GnRH and parity and ovulation synchronization treatment length. However, OVFD was not identified as a predictor of pregnancy 32 and 60 days after TAI in lactating dairy cows.

Key Words: Ovulatory follicle, dairy cows, pregnancy status, GnRH

1403

Evaluation of ovarian follicle wave dynamics in the post partum period and until the second ovulation occurrence in Sarda cows

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Suckled beef production in the harsh mountain range of Sardinia traditionally use a small-frame population (Sarda cows) that graze upland pastures and forests all year around. The duration of post partum anoestrus is a major factor affecting reproductive and productive efficiency in beef cows. The present study sought to examine ovarian follicle wave dynamics in Sarda cows (n = 28) as affected by pre and post partum nutrition and suckling technique. The experiment was a 2 × 2 × 2 factorial design, in which the factors were body condition score (BCS) at calving (low = 2 units or moderate = 3 units), feeding level after calving (600 or 1000 kJ ME/days/kg M0.75), and either restricted calf presence with cows isolated from their calves except for a once-a-day suckling period or ad libitum calf access to their dam. Follicle growth was monitored daily from Day 21 post partum until second ovulation using transrectal ovarian ultrasonography. Calf isolation and once-a-day suckling began on the second day of dominance (dominant follicle (DF) >2 mm larger than any other follicle) during the fourth follicular wave post partum (at about Day 30). Reproductive efficiency was negatively affected by pre partum nutrition. Cows of low BCS at calving compared to those of moderate BCS showed longer interval from parturition to first and second ovulation ($p < 0.05$ and $p < 0.01$, respectively), greater number of follicular waves ($p < 0.05$), lower number of follicles at wave emergence ($p < 0.05$), decreased DF maximum diameter and growth rate ($p < 0.01$). A statistically significant influence of post partum feeding was not detected. Suckling treatment had no effect on follicular parameters except for the interval from calving to first and second ovulation. The anoestrus period was shorter for cows suckling once daily than those with unrestricted suckling ($p < 0.05$). BCS at calving is confirmed to be a good indicator for estimating the earliness of post partum resumption of cyclicity in beef cattle. Additionally, Sarda breed can benefit from the implementation of suckling restriction.

Key Words: Sarda cow, postpartum anoestrus, ovarian dynamics, body condition score, suckling frequency

1404

Repeatability of dominant ovulatory follicle and corpus luteum diameters in beef cattle

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Transrectal ultrasonography has been used to study the repeatability of several ovarian characteristics, such as the number of follicles and follicular wave patterns per estrous cycle. However, there is a lack of information on the repeatability of the diameter of the preovulatory follicle as well as the diameter of the subsequent corpus luteum among consecutive estrous cycles in cattle. The aims of the present study were to investigate the repeatability of the diameter of the dominant ovulatory follicle (DOF) and corpus luteum (CL) among consecutive estrous cycles within animals. In addition, the repeatability of the maximum diameter of the dominant follicle (DF) from anovulatory and ovulatory waves and its correlation with the subsequent CL maximum diameter was evaluated. Non-lactating beef cows (n = 11; 4 years old) of Aberdeen Angus breed were used from May to December in the Northern Hemisphere. Diameters of the largest follicle and corpus luteum were measured daily by B-mode transrectal ultrasonography using a 6.5 MHz linear-array transducer (Mindray M5Vet; Nanshan, China). The same operator performed all scans. Repeatability (R, range 0–1, where 1 = maximum) is defined as the proportion of the total variance that could be attributed to animal or day of estrous cycle variance. Variance components obtained from a PROC MIXED model or one-way ANOVA were used to calculate repeatability. Correlations (r) between consecutive estrous cycles and specific end points were carried out by the Spearman test and used as an indication of repeatability and association between two variables. A total of 72 estrous cycles were studied with an average of 6.5 ± 0.4 estrous cycles (range, 5–9) per animal. The number of estrous cycles with 2-, 3-, and 4-wave patterns were 54 (75%), 17 (24%) and 1 (1%), respectively. The overall repeatability ($R = 0.8$) and correlation ($r = 0.9$, $p < 0.05$) from Day -6 to -1 (Day 0 = ovulation) for DOF diameter among consecutive estrous cycles within animals ranged from $R = 0.6$ to 0.9 and $r = 0.7$ to 1.0, respectively. In this regard, high repeatability ($R \geq 0.7$) and correlation ($r \geq 0.7$, $p < 0.05$) were observed in 82% (9/11) and 100% (11/11) of the animals, respectively. The mean maximum diameter of the DF was greater ($p < 0.0001$) in ovulatory (14.4 ± 0.2 mm) compared to anovulatory (13.4 ± 0.2 mm) waves; and the repeatability among cycles for each wave was high ($R = 0.6$). The overall repeatability ($R = 0.7$) and correlation ($r = 0.7$, $p < 0.05$) for CL diameter among consecutive estrous cycles within animals ranged from $R = 0.6$ to 0.9 and $r = 0.5$ to 0.9, respectively. Moreover, 82% (9/11) and 64% (7/11) of all animals had high repeatability ($R \geq 0.7$) and correlation ($r = 0.7$, $p < 0.02$) for CL diameter. The diameters of the DOF and CL from Day -6 to 5 were highly correlated ($r = 0.7$; $p < 0.0001$). In conclusion, the results of the present study demonstrated, for the first time, that DOF and CL diameters were highly repeatable among estrous cycles within cows, and that CL diameter was directly influenced by DOF diameter.

Key Words: Corpus luteum, cow, follicle, estrous cycle, repeatability

1405

Changes in expression and localization of cellular flice-like inhibitory protein, an anti-apoptotic factor, in corpora lutea during estrus cycle and pregnancy in Thai swamp buffalo (*Bubalus bubalis*)

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In female mammals, luteal cells rapidly proliferate and form corpora lutea (CL) after ovulation. The CL plays crucial roles in establishing and maintaining pregnancy. To gain further insights on the role of cFLIP, we examined the expression in corpora lutea of swamp buffalos during the early, mid and late phases of the estrous cycle and pregnancy. cFLIP short form and long form ($cFLIP_S$ and $cFLIP_L$) mRNA and protein levels were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) and western blotting, respectively. Immunohistochemical staining was performed to demonstrate the location of cFLIP protein. $cFLIP_L$ mRNA was highly expressed in CL of pregnancy and was decreased on the late stage. $cFLIP_S$ mRNA level was low in the late stage, but increasing during pregnancy ($p < 0.05$). Higher levels of $cFLIP_L$ protein were demonstrated in CLs during pregnancy and lower levels were shown in CLs in early stages of the cycle ($p < 0.05$). The level of $cFLIP_S$ protein was high in CLs during pregnancy stage and lower levels were noted in mid stages. Strong positive staining for $cFLIP_{S/L}$ proteins were demonstrated in luteal cells of CLs of pregnancy. The present findings explained that cFLIP was at the highest level in CLs during pregnancy and this may act as a dominant survival anti-apoptotic factor by inhibiting intracellular apoptosis signal transduction in luteal cells of CLs during pregnancy period.

Key Words: Swamp buffalo, corpora lutea, cFLIP

1406

Predicting the values of oocyte reserves by antral follicle count in buffalo ovaries

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In buffalo, major dilemma of embryo transfer is poor super ovulation response. The super ovulation response is predominantly dependent on available oocyte reserves. The methods to predict the oocytes reserves could improve super ovulation response by screening donor buffaloes with better potential. Herein, we tested the hypothesis that ultrasound monitored antral follicular count (AFC) could predict the ovarian oocyte reserves. In this study, ovaries of twenty-five healthy Nili-Ravi buffaloes, 4–6 years of age, were scanned for AFC. The animals having luteal tissue on either ovary were declared as cyclic animals. There was no influence of estrous cyclicity on AFC; 16.1 ± 2.1 follicles in cyclic and 20.8 ± 2.3 follicles in non cyclic buffaloes ($p > 0.05$). The animals were further identified as low (<15), medium (15 ≥ 20) and high (>20) follicular count groups. Ovarian parameters (wet weight, volume) were smaller in animals with low AFC compared with high AFC ($p < 0.05$). At slaughtering, ovaries of the buffaloes were processed for oocyte count through histological analysis. Average oocyte reserves per buffalo were counted 24550 ± 1516.75. The oocyte reserves in low, medium and high follicular count groups were 21710 ± 702.92, 26314.29 ± 1665.96, and 31875 ± 2594 oocytes respectively. These values positively correlate ($p < 0.05$) with AFC ($r = 0.644$). Small follicles (2 > 6 mm) being 89.74% of the total follicular population truly represents the ovarian efficiency. So, it is concluded that the potential oocyte reserves of an individual buffalo could be assessed positively on basis of its AFC and perhaps it could help us to predict the buffaloes with better embryo donation potential.

Key Words: Antral follicle count, ovarian reserve, Nili-Ravi buffalo, oocytes number

1407

Ultrastructural evaluation of buffalo ovarian follicles

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The aim of the present study was to characterize buffalo (*Bubalus bubalis*) ovarian follicles. Fragments of ovarian cortex, collected from buffalo females in slaughterhouses, were randomly cut using a scalpel blade and processed for transmission electron microscopy. Of the five ovaries processed, only five follicles were analyzed, which were identified as initial secondary follicles and those in transition to the tertiary stage. However, this assessment was significant because it provided important information about the cell biology of oocytes and follicular cells. The secondary follicles consisted of an oocyte surrounded by at least two layers of cuboidal cells. It was possible to identify the beginning of deposition of zona pellucida. The oocyte maintained close contact with granulosa cells, showing structures similar to zonula adherens and many microvilli. The oocyte nucleus occupied a central position and exhibited granular aspect. The cytoplasmic organelles were distributed evenly throughout the ooplasm and the predominant structures were mitochondria and vesicles. Mitochondrial granules were not seen. Other organelles were present in small amounts, such as Golgi apparatus and smooth and rough endoplasmic reticulum. The granulosa cells had large nuclei and contained mitochondria, rough and smooth endoplasmic reticulum and Golgi apparatus. Between the granulosa cells, structures similar to zonula adherens and gap junctions were identified. Two distinct populations of granulosa cells were observed, one darker that had abundant endoplasmic reticulum and few mitochondria and Golgi apparatus, and a clearer population with increased number of mitochondria and Golgi apparatus and little amount of endoplasmic reticulum. This finding confirms that the granulosa cells of secondary follicles are equipped for hormone production. In those follicles in transition to the tertiary stage the secretion of fluid in the central region and spaces between granulosa cells was observed, due to antrum development. Ultrastructural characterization of bubaline follicles showed a differentiated cell apparatus for substrate metabolization, energy production and hormone synthesis. In general, the ultrastructure of buffalo follicles analyzed was similar to that reported for other species, but differences were identified, such as the greater amount of cytoplasmic vesicles, shape and inner content of mitochondria and presence of oocyte-granulosa cell specific junctions in secondary follicles, which was not previously reported for buffaloes. The knowledge of particular characteristics, such as the large number of vesicles in the ooplasm, allows us to understand why the buffalo oocyte has lower ability to be fertilized *in vitro* and directs new research in this area.

Key Words: Ovary, follicles, granulosa cell, cytoplasmic organelles, buffalo

1408

Identification of the FecL fecundity major gene controlling prolificacy in the Lacaune sheep breed

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In the meat strain of the Lacaune sheep breed, large variation in litter size has been observed and genetic studies explained this variation by the segregation of a fecundity major gene influencing ovulation rate

(OR), and named FecL. The influence of the prolific allele $FecL^L$ on OR is additive with one copy increasing OR by about 1.5 and two copies by about 3.0. Previous work on genetic mapping localized FecL on sheep chromosome 11 within an interval of 1.1 megabases encompassing 20 genes. With the aim to identify the FecL gene, we developed a high throughput sequencing strategy (Roche 454 GS FLX) of long-range PCR fragments spanning the 1.1 Mb interval to identify potential informative markers from an heterozygous animal. This approach led to define a 190 kb minimal interval for the FecL locus containing only two genes, insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) and beta-1,4-*N*-acetyl-galactosaminyl transferase 2 (B4GALNT2). The full sequencing of the 190 kb interval of a homozygous $FecL^L/FecL^L$ ewe and a non-carrier ewe identified only one SNP in the intron 7 of B4GALNT2 in complete linkage disequilibrium with $FecL^L$. Moreover, the $FecL^L$ mutation is associated with the ectopic expression of B4GALNT2 mRNA in the ovarian follicles of $FecL^L/FecL^L$ Lacaune ewes (1000-fold increased compared to wild-type). Thus, B4GALNT2 appears as the best positional and expressional candidate for FecL. Since it is implicated in glycosylation pathway, we localized the B4GALNT2 activity by specific lectin-histochemistry in granulosa cells of ovarian follicles only in hyperprolific $FecL^L$ carrier ewes. This was confirmed by the results of lectin-precipitation of follicular fluid and subsequent western blot experiments. The identification of the lectin-labeled proteins from $FecL^L$ and wild-type follicular fluids by mass spectrometry revealed around 30 proteins suspected to be glycosylated by B4GALNT2 only in $FecL^L$ ovaries. Among those proteins, the versican proteoglycan, inhibin alpha and betaA subunits, and bone morphogenetic protein 1 are good candidates for further studies to understand the physiological pathway used to increase ovulation rate. The identification of the FecL gene as B4GALNT2 implicated in glyscosylation leads to the discovery of a new pathway involved in folliculogenesis and the regulation of ovulation rate. Now, the question is, does the altered expression of B4GALNT2 affect the transforming growth factor beta/bone morphogenetic protein pathways, known to be affected in other ovine prolific breeds?

Key Words: Ovine, major gene, ovulation rate, B4GALNT2, glycosylation

1409

Determination of anti-Müllerian hormone plasma concentration can help to select donor sheep for laparoscopic ovum pick-up

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Anti-Müllerian hormone (AMH) is produced by granulosa cells of small antral follicles able to respond to gonadotropins. Recent results indicate that AMH represents a very interesting endocrine predictor of the ovarian response to hormonal treatments. In sheep, besides the limited efficiency of the LOPU and IVP of embryos, the expensive cost and variable individual response to FSH treatments remain important limits to the development of embryo biotechnologies. The aim of the present study was to determine AMH plasma concentrations in sheep and to relate them with the number of follicles (≥3 mm) available for oocyte puncture after FSH treatment in a LOPU-IVP program. With this objective, 16 adult Rasa aragonesa ewes were subjected to two consecutive LOPU sessions 1 week apart. Ewes received 32 mg of porcine FSH administered in decreasing doses: 8 mg (60 h), 8 mg (48 h), 6 mg (36 h), 6 mg (24 h) and 4 mg (12 h) prior to each session, and a blood sample was taken from each ewe coinciding with the first injection of each session. Plasma concentrations of AMH were determined using AMH GENII ELISA Kit (Beckman Coulter). AMH plasma concentration was positively correlated with the number of punctured follicles ($r = 0.65$; $p < 0.0001$), the number of recovered cumulus-oocyte complexes (COCs, $r = 0.41$; $p < 0.05$), and the number of oocytes suitable for *in vitro* maturation (IVM, $r = 0.39$; $p < 0.05$) at each LOPU session. AMH and the number of follicles at the first session were highly correlated with AMH and follicle number found at the second session, respectively ($r = 0.75$; $p < 0.005$ and $r = 0.55$; $p < 0.05$). AMH plasma concentration was about two fold higher in ewes with a high follicular response to FSH compared with low-responding ewes (Table 1). In conclusion, AMH plasma concentrations could be used to improve the output of the LOPU-IVP technique in sheep. AMH measurement in a single blood sample taken before FSH treatment can predict the number of follicles available for puncture per donor ewe, with a low variability between sessions. Thus circulating AMH measurement is a useful tool to select the best oocyte donors. Financed by INIA (RTA2011-128 project and fellowship for B. Lahoz) and INRA (PREDICTOV project).

Key Words: AMH, COCs, ewe, follicle, IVP

Table 1. Anti-Müllerian hormone plasma concentration before FSH treatment and numbers of follicles, recovered COCs, and COCs suitable for IVM per ewe and session (Mean ± SE), for low or high-responding groups of ewes

	n	AMH (pg/ml)	Follicle number	Recovered COCs	IVM_COCs
<20 follicles	18	97.5 ± 12.2[a]	14.8 ± 0.7[a]	9.2 ± 0.7[a]	8.2 ± 0.8[a]
≥20 follicles	14	176.7 ± 21.0[b]	23.9 ± 1.0[b]	14.4 ± 1.0[b]	13.0 ± 0.9[b]

Within a column, means differ at [a,b]$p < 0.0001$.

1410

Screening of functional shRNA molecules to knock down the expression of ovine BMPR-1B gene

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Bone morphogenetic protein 1B receptor (BMPR-1B) is one of the most important genes in regulating ovine ovulation rate and litter size. The $FecB^B$, which is Q249R mutation in BMPR-1B and leads to a higher ovulation rate, has also been supposed as a partial 'loss of function' of BMPR-IB (Fabre, 2006). Thus, we hypothesized that a decreased BMPR-IB expression leads to an increasing in ovine ovulation rate. In this study, we attempt to screen the functional shRNA molecules to knock down the expression of ovine BMPR-1B gene. Based on the cDNA sequence of ovine BMPR-1B (NM_001009431), Thermo's web siRNA design software (http://www.dharmacon.com/designcenter/DesignCenterPage.aspx) was used to select suitable siRNA targeting sites at the whole cDNA of ovine BMPR-1B (sh749 in transforming growth factor beta type I GS-motif, sh803 and sh1306 in catalytic domain of protein kinases, sh1475 in protein kinase domain, sh1685, sh2567 and sh2745 in 3′UTR). In experiment 1, BMPR-1B gene, which was derived from ovine ovarian tissue containing 1515 bp coding region sequence, was overexpressed in HEK293 cells and the HEK293 cells stably transfected with BMPR-1B were used in the following experiment. Then these 7 shRNAs were transferred to the HEK293 cells, respectively. All of seven shRNAs can reduce the expression level of exogenous ovine BMPR-IB gene in HEK293 cells from 68.3% to 99.86% (sh749/68.3%, $p < 0.05$; sh803/71.2%, $p < 0.05$; sh2745/80.5%, $p < 0.05$; sh1685/94.37%, $p < 0.01$; sh2567/96.41%, $p < 0.01$ sh1475/99.75%, $p < 0.01$ and sh1306/99.86%, $p < 0.01$) detected by qRT-PCR, and this result was confirmed by Western blot. In experiment 2, these seven shRNA molecules were then verified to knock down the expression of endogenous BMPR-1B gene in ovine granulosa cells which were cultured in DMEM/F12 containing 10% FCS. After transfected, the new DMEM/F12 containing 1% FCS was added. The result showed that all of 7 shRNAs can decrease the mRNA level of BMPR-IB gene from 54.21% to 98.14% (sh1475/54.21%, $p > 0.05$; sh2745/63.31%, $p > 0.05$; sh2567/76.78%, $p < 0.05$; sh749/80.2%, $p < 0.05$; sh1685/90.23%, $p < 0.01$; sh803/96.69%, $p < 0.01$ and sh1306/98.14%, $p < 0.01$) in ovine granulosa cells by qRT-PCR. In summary, both experiments showed that the sh1306 produced the most efficient inhibition among 7 shRNA molecules to knock down the expression of

BMPR-IB gene. Coincidently, the targeting site of sh1306 is in catalytic domain of protein kinases as the same region as the $FecB^B$ mutation in BMPR-1B gene.

Key Words: shRNA, BMPR-1B, sheep, ovulation

1411

Anti-Mullerian hormone as an age-related predictor of the antral follicle count and the response to exogenous FSH ovarian reserve test in sheep

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Inter-individual variability in the response to exogenous ovarian stimulation remains as the main limiting factor for embryo production in sheep, like in other species. Embryo outputs are related to the number of gonadotrophin-responsive follicles (i.e.: the ovarian reserve) and highly repeatable in consecutive stimulations of the same females. Thus, predictive tools for early selection of high-responder females are of critical importance in embryo programmes. Currently, anti-Mullerian hormone (AMH) is recognized, in human medicine, as a good marker of ovarian reserve status and represents a good predictor of ovarian response to ovarian hyperstimulation. The aim of this study was to assess age-related changes in AMH and its relationship with antral follicle count (AFC) as predictors of the response to an exogenous FSH ovarian reserve test (EFORT) in pre- and post-pubertal stages in sheep. Thus, plasma AMH concentrations and AFC evaluated by ovarian ultrasonography, prior and after the administration of one shot-dose of 60 mg of FSH (FolltropinTM; Bioniche Animal Health, Bio 98, Milano, Italy), were determined in a total of 15 Sarda sheep at 40, 110 and 210 days-old. The results indicate a significant decrease in AMH concentrations ($p < 0.05$) and the number of follicles with 1–2 mm in diameter with age ($p < 0.0005$), but a significant increase in the number of gonadotrophin-dependant follicles ≥4 mm ($p < 0.01$). Blood AMH levels were related to total AFC in pre-pubertal stages (40 and 110 days-old; $p < 0.01$) and, mainly, to the number of follicles with 1–2 mm in diameter ($p < 0.001$). Evaluation of AMH prior to EFORT was predictive of the growth of these follicles with 1–2 mm to larger sizes in response to the test at these pre-pubertal stages ($p < 0.005$). Thus, on the basis of the highly-repeatable response to exogenous gonadotrophin stimulation in the same individuals, the measurement of the hormone in pre-pubertal stages may be useful for selection of future donors in embryo programmes. Funded by Regione Sardegna progetti di ricerca di base legge regionale 7/2007 – bando 2008.

Key Words: Folliculogenesis, ovarian-stimulation, sheep, AMH

1412

Ovarian follicular dynamics around estrus in Beetal and Teddy goats

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Goats, in Pakistan are mostly kept by small holder farmers with the Beetal and Teddy breeds offering a high potential for meat, milk production and higher litter size respectively. Due to higher litter size over Beetal goats, it is assumed that Teddy gaots have higher ovarian activity (follicular population and Ovulation rate). Therefore, it is imperative to study the ovarian physiology around estrus in these goats. The current study tested the hypothesis that ovarian follicular population (number of small (1–3 mm), medium (>3–5 mm) and large follicles (>5 mm), size of the ovulatory follicle, and ovulation rate (No. of CL/No of ovulated goats), using transrectal ultrasonography are different between Beetal and Teddy goats of Pakistani origin. Beetal (n = 6) and Teddy (n = 8) does were synchronized using double PGF2α injections 10 days apart and were scanned on Days -2, 0 (estrus) and +2. The onset of estrus was assessed by aproned bucks. The different variables between breeds were compared and analyzed by student's *t* test. Mean number of small follicles were higher ($p < 0.05$) in Beetal goats, compared to Teddy goats, on Days -2, estrus and +2 (Table 1). However, no significant difference ($p > 0.05$) was detected in the medium and large follicles on the days of examination. The ovulatory diameter (7.3 ± 0.3 mm vs. 6.9 ± 0.5 mm), and ovulation rate (1.8 ± 0.7 vs. 1.8 ± 0.4) were found non-significant ($p > 0.05$) between the Beetal and Teddy goats, respectively. It is concluded that Beetal goats have greater population of small follicles around estrus compared to Teddy goats. The lower litter size in Beetal goats might be due to higher follicular atresia and low recruitment of follicles. Future studies can be based on the follicle recruitment and atretic factors in Beetal goats to enhance the production.

Key Words: Ultrasonography, Beetal, Teddy, follicle

Table 1. No of small, medium and large follicles around estrus in Beetal and Teddy goats

Day	Small	Follicles (1–3 mm)	Medium	Follicles (>3–5 mm)	Large	Follicle (>5 mm)
	Beetal	Teddy	Beetal	Teddy	Beetal	Teddy
-2	6.7 ± 2.0a	3.7 ± 0.8b	1.3 ± 0.6	1.0 ± 0.8	0	0
0	6.0 ± 0.9a	2.7 ± 0.5b	0.8 ± 0.5	1.5 ± 0.8	1.0 ± 0.6	1.5 ± 0.8
+2	4.0 ± 2.8a	0.1 ± 0.6b	0	1.0 ± 0.6	2.5 ± 0.7	2.5 ± 0.7

Different superscripts letters (ab) in the same row indicate a significant difference ($p < 0.05$).

1413

Removal of the pineal gland modifies follicle and corpus luteum growth dynamics in sheep

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The role of melatonin in modulating mammalian reproduction is of particular interest. Currently, many aspects of the *in vivo* actions of melatonin on the ovary still remain to be characterized. The aim of this study was to assess the effect of pinealectomy on follicle and CL growth dynamics in sheep. All experimental procedures were carried out during sheep breeding season (October–November; latitude 40°43′N). Animals were randomly divided in two groups: (A) pinealectomized group (n = 6); (B) sham-operated (n = 5). All the animals underwent a left parietooccipital craniectomy. The completeness of the gland removal was confirmed by the serum dosage of melatonin 60 days and 12 months after the surgery. Reproductive cycle and ovulation was synchronized in all the ewes with two doses of 125 μg of a prostaglandin analogue, 10 days apart. Follicle and corpus luteum growth dynamics were assessed daily during the induced oestrus cycle by transrectal ultrasonography using a real-time B-mode scanner (Aloka SSD 500; Aloka Co., Tokyo, Japan) fitted to a 7.5 MHz linear-array probe. The time length of the induced oestrus cycle (17 ± 1.6 vs. 19.2 ± 1.5 days in control and pinealectomized ewes, respectively) and the ovulation rate (1.4 ± 0.2 vs. 1.1 ± 0.2 in control and pinealectomized ewes, respectively) did not differ between the two experimental groups. The ultrasonographic study indicated that both the pinealectomized and control ovaries showed a well-defined wave-like pattern of follicle dynamics; however, there were significant differences between groups in the characteristics of the follicular waves. Melatonin deficiency caused less waves during the oestrus cycle (4.3 ± 0.2 vs. 5 ± 0.2) because waves were 1 day longer when compared with the controls (7.2 ± 0.3 vs. 6.1 ± 0.3). No differences were found in dominant follicle growth and regression rates. Significant differences were also found in CLs growth dynamics. The mean area of the corpora lutea was significantly higher in control compared with pinealectomized ewes (125.9 ± 5.8 vs. 72.9 ± 5.6 mm^2, respectively); significant differences in CL area were

also observed at the end of the luteal phase when data where analyzed on a day by day basis. Taken together results of the present study indicate that melatonin deficiency alters both follicle and CL growth dynamics, leading to a decrease in the number of follicular waves/cycle and in the mean area of CLs. These data suggest that melatonin has a key role not only in the synchronization of seasonal reproductive fluctuations but also in the functionality of the ovaries (Supported by RAS-Special Project Biodiversity).

Key Words: Pinealectomy, follicle, corpus luteum, oestrus cycle, melatonin

1414

Relationship between pre-ovulatory follicle diameter and corpus luteum size and function after ovulation induction with GnRH or ovulation-inducing factor in llamas

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Ovulation–inducing factor (OIF) is a protein present in llama seminal plasma that induces high rates of ovulation after i.m. administration in this species. As well, OIF appears to have luteotrophic properties. A positive relationship between the diameter of the ovulatory follicle and subsequent CL size and plasma progesterone concentration has been reported in cattle, but has not been investigated in camelids. To determine the respective roles of OIF and follicular diameter on subsequent CL form and function, a 2-by-2 design was used involving treatment with OIF vs. GnRH, and two categories of pre–ovulatory follicle diameter. Mature female llamas were assigned randomly to the four groups (n = 12–19 per group) and given an intramuscular dose of 1 mg purified OIF or 50 μg of GnRH when a growing follicle of either 7–10 mm or >10 mm was detected by daily transrectal ultrasonography. Llamas were examined by ultrasonography every 12 h from treatment to Day 2 (Day 0 = treatment) to detect ovulation, and again on Day 8 to determine CL diameter. Blood samples for progesterone measurement were taken on Day 8 (n = 8–9 llamas per group). Ovulation rates were compared among groups by Chi-square, and CL diameter and plasma progesterone concentration were examined by two-way ANOVA to determine main effects of treatment and follicle size, and their interaction. Ovulation rates did not differ among groups (Table 1). The diameter of the CL on Day 8 was greater when treatment was given when the follicle was >10 mm than when 7–10 mm, but was not affected by type of treatment (treatment effect, $p = 0.17$; follicle size, $p = 0.052$; interaction, $p = 0.9$). Plasma progesterone concentration was greater in the OIF- than in the GnRH-treated groups (treatment effect, $p < 0.05$; follicle size, $p = 0.5$; interaction, $p = 0.4$; Table 1). We conclude that pre-ovulatory follicle diameter is positively related to subsequent CL diameter, and that OIF treatment induces greater circulating progesterone concentrations than GnRH, regardless of follicle diameter; i.e., OIF is luteotrophic in llamas. Research supported by the Natural Sciences and Engineering Research Council of Canada and Chilean Research Council, Fondecyt Regular 1120518.

Key Words: Ovulation-inducing factor, corpus luteum, progesterone, llamas

Table 1. Effect of treatment with OIF vs. GnRH and follicle size at the time of treatment (7–10 mm vs. >10 mm) on ovarian function in llamas

End point	OIF/7–10 mm	GnRH/ 7–10 mm	OIF/>10 mm	GnRH/ >10 mm
Ovulation (%)	12/14 (86)	13/15 (87)	11/12 (92)	16/19 (84)
CL diameter (mm)*	12.6 ± 0.4	13.0 ± 0.2	13.3 ± 0.5	13.8 ± 0.3
Progesterone (ng/ml)	6.3 ± 0.6[a]	4.6 ± 0.5[b]	5.7 ± 0.7[a]	4.9 ± 0.6[b]

[ab]Values with no common superscript are different ($p < 0.05$). *Effect of follicle size ($p = 0.052$).

1450

Histological characteristics of luteal structures in dairy cows

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A corpus luteum (CL) with a cavity was detected ultrasonographically in 209 of 1789 (11.7%) of Hungarian (primarily Holstein Friesian) dairy cows from 40 to 60 days post partum, with a similar incidence in inseminated cows (529 of 4309, 12.3%), although the incidence was much lower in pregnant cows (45 of 1747, 2.6%). Based on the most common classification, these structures were allocated into two groups: a cavity <2 cm and wall thickness >1 cm was designated a CL with a small cavity (CLscav), whereas a cavity >2 cm and wall thickness <1 cm was designated a CL with a large cavity (CLlcav). The objective was to compare histological characteristics among CLscav, CLlcav, and a solid CL (no cavity) in dairy cows. Ovaries were collected from 90 slaughtered dairy cows within 4 h after death. Overall, there were six CLscav (6.7%), four CLlcav (4.4%), and six solid CL (control); all of these were clearly ovulatory structures. After physical and ultrasonographic examinations, digital images were recorded and ovaries were fixed in 4% formaldehyde solution, routinely embedded in paraffin, and histological slides were prepared and stained with hematoxylin-eosin, Azan blue and Gömöri silver impregnation. Using conventional bright-field light microscopy and specialized image-analysis software (CellD, Soft Imaging GmbH), the number of large and small luteal cells, fibroblasts/fibrocytes and pyknotic cells were counted, and the thickness of connective tissue layer was measured (at 20 locations in 10 microscopic fields). In CLscav vs. CLlcav, the connective tissue layer around the cavity tended to be thinner (mean 153.9 vs. 295.4 μm, respectively; $p = 0.09$); proportion of large luteal cells tended to be higher (26.0 vs. 19.8%, $p = 0.07$); and there were more ($p < 0.05$) fibroblasts/fibrocytes (16.6 vs. 9.4% and pyknotic cells (2.8% vs. 1.3%). Solid CL had a higher ($p < 0.01$) proportion (42.7%) of large luteal cells than both classes of CL with a cavity, and a lower ($p = 0.03$) proportion of small luteal cells (38.2%) than in CLlcav. Despite substantial differences in overall cavity size, histological differences were more subtle. Tendencies for differences for increases in number of large luteal cells and thickness of connective tissue, and significant increases in fibroblasts and pyknotic cells in CLscav vs. CLlcav were attributed to the transition of CLlcav into CLscav, with eventual obliteration of the cavity over time in most CL, manifested by the low incidence in pregnant cows (as previously described).

Key Words: Ovary, CL, histology, cow

1451

Effect of peptidoglycan on steroid production in bovine theca cells

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After parturition, uterine bacterial infection is associated with infertility by disrupting uterine and ovarian function. Arcanobacterium pyogenes, gram-positive bacteria, is the most prevalent bacteria isolated from the uterine lumen of cattle with uterine disease. Peptidoglycan (PGN) is cellular wall component of gram-positive bacteria. To examine the impact of PGN on ovarian function, we examined the effect of PGN on steroid production in bovine theca cells. Paired ovaries were obtained from cows and heifers just after slaughter at a nearby slaughterhouse. Bovine theca cells from large follicles were cultured with PGN (0, 0.1, 1, 10, 50 μg/ml). The gene expression and production of androstenedione and progesterone in the PGN treated bovine theca cells were tested for significant differences using ANOVA, followed by the Tukey–Kramer test as a multiple comparison test. PGN significantly decreased production of androstenedione ($p < 0.05$) and progesterone ($p < 0.05$), and the expres-

sion of CYP17 and StAR mRNAs ($p < 0.05$) compared with control in theca cells cultured with LH alone or LH plus E2. Furthermore, PGN suppressed expression of LH receptor mRNA in the same culture condition. The results of the present study indicate that PGN suppressed production of androstenedione and progesterone in theca cells. Thus, our data suggests that not only lipopolysaccharide but also peptidoglycan is related to the ovarian dysfunction in cow with uterine bacterial infection disease such as endometritis.

Key Words: Cow, ovary, theca cell, steroid hormone, peptidoglycan

1452

Study of gene expression in granulosa cells from different follicle growth phases in relation to bovine oocyte quality

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It is known that one in three follicles contains a competent oocyte, but to this day the features of those particular follicles have not been characterized. The objective of this study was to build the transcriptomic profile of granulosa cells originating from follicles in a range of 6–9 mm diameter in dairy cattle using microarrays and validating by qPCR. GCs were collected from slaughterhouse ovaries by dissection. Half of each sample was used for RNA extraction while the other half was analyzed by flow cytometry to characterize its originating follicle in one of three stages of antral growth: growing (G), plateau (P) and atresia (A), using DNA content. The G and A conditions were each hybridized against the P condition as a reference in order to understand the specific biological mechanisms underway in this class of follicles. The latter is believed to hold oocytes with the best capacities to produce an offspring following in-vitro assisted reproduction technologies such as maturation (MIV) and fertilisation (FIV). Two thousand and nine hundred fourty-two genes were differentially expressed ($p < 0.05$) in G vs. P and 1,974 in P vs. A, according to the microarray. Ingenuity Pathway Analysis (IPA) software was used to study the regulation pattern of genes and functions predicted to be enriched in the dataset. Cytometry was efficient in categorizing the follicles in the different phases, as determined by the down- regulation of steroidogenesis genes: cytochrome P450, subfamily XIX (CYP19A1, i.e. aromatase), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta- isomerase 1 (HSD3B1) and cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) in the granulosa cells of P follicles relative to G and probably in response to the decreasing FSH level. On the other hand, the P vs. A contrast showed up-regulation of multiple transcripts associated to apoptosis: TGM2, SH3RF1, DAB2, DSG2, DR5, CASP4, RASSF1, CCT2, IRF5. Both growth and atretic gene markers as well as key pathways genes specific to each condition remain to be validated by qPCR. According to microarray results solely, functions such as cell death, secretion of lipids and ploidy of cells, were predicted to be increased in P-follicle-granulosa compared to G-follicles, while functions such as growth of cells were predicted to be decreased in P relative to G. Although the genes more expressed in the A condition do not correspond to common atretic markers, they are likely to be indicators differentiating between the late atresia status of the follicle and early atresia, a hallmark characteristic of plateau follicles. This study will offers multiple candidate genes to be further studied at a biochemical level in order to elucidate their role in the modulation of oocyte competence.

Key Words: Granulosa, folliculogenesis, plateau, microarray, atresia

1453

MicroRNA expression during ovarian follicular differentiation

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Little is known about the involvement of microRNAs (miRNAs) in the follicular-to-luteal transition. In a previous study (McBride et al., 2012), sequencing of small RNA libraries from ovarian tissues collected at defined stages of the ovine oestrous cycle identified seventeen miRNAs that were differentially expressed between ovulatory-size follicles and corpora lutea. The aim of the present study was to characterize in bovine the expression of some of those miRNAs using RT-qPCR, specifically, miRNAs identified in sheep as differentially decreasing (miR-125b, miR-145) or increasing (miR-21 and miR-34a) during the follicle-to-luteal transition. Bovine ovaries were collected from an abattoir and used either for ex-vivo tissue analyses or cell culture experiments. Data were analysed using the General Linear Models procedure to determine main effects (Developmental stage or Day and Treatment) and Tukeys test were used for pair-wise comparison of multiple means. The expression of miR-125b and miR-145 was lower ($p < 0.05$) in early developing corpora lutea relative to healthy (Estradiol/Progesterone ratio > 1) ovulatory-size (15–17 mm) follicles (1.8-fold and 3.5-fold lower for miR-125b and miR-145, respectively) whereas the expression of miR-21 and miR-34a increased between follicles and corpora lutea (3.8-fold and 2.0-fold higher, respectively). Furthermore, in bovine follicles, higher levels of each of these miRNAs ($p < 0.04$) were detected in theca than in granulosa (8.0-fold, 63.4-fold, 1.9-fold and 3.1-fold higher for miR-125b, miR-145, miR-21 and miR-34a, respectively). To determine whether changes in miRNA levels during luteinization *in vitro* replicated the changes observed in ex-vivo tissues, bovine granulosa and theca cells were separately cultured and either treated with Forskolin (10 μM), FCS (10%) and Insulin (1 μg/ml) (FFI) for 3 days to induce differentiation, or left untreated. Levels of miR-125b and miR-145 in granulosa cells significantly ($p < 0.05$) decreased in response to FFI treatment, whereas in theca cells the levels of the two miRNAs decreased during culture regardless of whether cells had been treated with FFI or left untreated. Expression of both miR-21 and miR-34a increased during culture of granulosa cells and theca cells, although these effects were independent of treatment with FFI, indicating an effect of culture-induced luteinisation on miRNA expression. Finally, experimentally validated protein-coding genes targeted by these miRNAs in other cell types were identified using MirTarBase. Twenty-two different targets were identified, a majority of which represented genes involved in cell proliferation and differentiation, and many known to be actively involved in follicular differentiation in vivo. In conclusion, this is the first study to characterise changes in miRNA expression during the follicle-to-luteal transition in a domestic species. These results identify miR-125b, miR-145, miR-21 and miR-34a as potentially important regulators of the follicular-to-luteal transition.

Key Words: MicroRNA, follicle, corpus luteum, luteinization, post-transcriptional regulation

1454

Ovarian follicle count and plasma AMH concentration on Nelore and Angus heifers

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The anti-Mullerian hormone (AMH) is a TGFβ family member produced by the granulosa cells of pre-antral and antral follicles. Because AMH is produced by the growing follicle the concentration of AMH can be related to the number of follicles developing in each wave of the estrous cycle and be used as a biomarker for follicle population in bovines (Ireland et al., *Biol Reprod.* 2008; 79:1219–25). However, different breeds have different follicle recruitment profiles. Our objective was to determine if there is a positive relation between number of follicles and plasma AMH concentration in Nelore (*Bos indicus*) and Angus (*Bos taurus*) heifers. Thirty Angus and twenty Nelore heifers (24 months old approximately) were kept in Brachiaria brizantha grass and were supplemented with a mix of grains and had mineral and water ad libitum. Estrous cycle was synchronized with two doses of PGF2α 11 days apart. To determine the number of follicles for each animal a ultrasound device (US; Mindray Vet DPS 2200, Säo Paulo, Brazil) equipped with a 7.5-MHz probe was used to perform three exams one day after ovulation of subsequent cycles. Blood was also drawn during US. Plasma AMH concentrations were determined using the human AMH/MIS Gen II enzyme linked immunosorbent assay (ELISA) kit (Beckman Coulter, Brea, CA, USA). The kit was first validated for bovine using plasma of high and low follicle count cows, a castrated bull and a pool of follicular fluid. Results were analyzed using the Proc GLM procedure of SAS (SAS 9.2). Follicle LSmean and low (LFC) or high (HFC) follicle count groups within breed were higher ($p \leq 0.05$) in Nelore heifers (32 ± 3.1; $16 < 52$) when compared with Angus heifers (20 ± 2.6; $10 < 27$). Plasma AMH concentration was also higher ($p \leq 0.05$) for Nelore (102.3 ± 6.4 pg/ml) when compared with Angus

(78 ± 5.1 pg/ml) heifers. When animals were separated per breed into low (LFC) or high (HFC) follicle count groups plasma AMH concentrations continued to be higher ($p \leq 0.05$) on the HFC groups when compared with LFC groups (82 ± 6 pg/ml vs. 72 ± 6.9 pg/ml for the Angus heifers and 127 ± 8.8 pg/ml vs. 84 ± 7.5 pg/ml for Nelore heifers, HFC and LFC respectively). Intra and inter assay were 2.11% and 2.47%, respectively. In conclusion, plasma AMH concentration can possibly be used as a predictive biomarker for follicle count in bovine, however care should be taken with discriminatory categorical values for each breed.

Key Words: Bovine, follicle, AMH, Nelore, Angus

15. Folliculogenesis, oogenesis, ovulation – nonruminants:

1500

Granulosa cell mRNA expression of hormone receptors and enzymes and follicle blood flow in mares

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Follicular growth and atresia are driven by the complex endocrine, paracrine and autocrine mechanisms of hormones and growth factors. Follicle blood supply is an important limiting factor in different phases of folliculogenesis, such as during follicular selection and ovulation. Color-Doppler ultrasonography has been used in mares to demonstrate blood-flow changes between the future dominant and subordinate follicles, as well as in the preovulatory follicle. The aim of this study was to evaluate granulosa cell mRNA expression of hormone receptors and enzymes and follicle blood-flow changes during different phases of an expected ovulatory wave. Non-lactating mares (n = 16) were used during the reproductive season. Diameters of all follicles ≥6 mm were measured daily by transrectal ultrasonography after day 10 of the estrous cycle. The largest growing follicle was distributed in one of 6 groups (n = 8/group) according to diameter (15.0–19.0; 20.0–25.0; 26.0–30.0; 31.0–35.0; 36.0–45.0) or presence of an impending ovulation follicle (IOF) in which ultrasound scanning was performed every 6–12 h. Follicle wall blood flow was measured using color-Doppler ultrasonography immediately before ultrasound-guided transvaginal follicle aspiration and flushing for granulosa cell harvesting. Real-time polymerase chain reaction (qPCR) was conducted to analyze mRNA expression for FSH (FSH-R), LH (LH-R), estrogen (E-R) and progesterone (P-R) receptors and Caspase 3, StAR and aromatase genes. Follicle blood flow increased ($p < 0.0001$) along with follicular development until the 36.0–45.0-mm group. The relative mRNA expression of FSH-R was steady during follicle growth but decreased ($p < 0.04$) during impending ovulation. LH-R expression increased ($p < 0.0001$) in the 26–30-mm follicle group and decreased ($p < 0.0001$) in the IOF group. Aromatase expression was lower ($p < 0.001$) in the 15–19-mm group (before follicle selection), increased up to the 31–35-mm group, and decreased ($p < 0.001$) in the IOF group. A positive correlation was observed between aromatase mRNA expression and FSH-R ($r = 0.54$; $p < 0.0001$) and LH-R ($r = 0.75$; $p < 0.0001$) expression. E-R and P-R expression did not differ among follicle groups. StAR expression was higher ($p < 0.03$) in the IOF group than in the 20–25-mm and 26–30-mm follicle groups, but was similar among the other groups. Regarding follicular apoptosis, Caspase 3 mRNA expression was lower ($p < 0.01$) in the IOF group and negatively correlated with blood flow ($r = -0.30$; $p < 0.05$). Significant correlations were not observed between blood flow and other enzymes or hormone receptors. In conclusion, the results of this study demonstrated that: (I) follicle blood flow, LH-R and aromatase mRNA expression increased with follicular development but then decreased as ovulation approached, (ii) FSH-R and LH-R expression did not change significantly before follicle selection (≂23 mm), but decreased as ovulation approached, and (iii) mRNA expression of StAR increased and Caspase 3 decreased close to ovulation.

Key Words: Follicle, blood flow, granulosa cell, mRNA expression, mares

1501

Morphologic development and morphometry of the germinative cells of equine foetal ovaries

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Morphological development and size of germinative cells of the equine foetal ovary (EFO), has been the subject of little research. The aim of this study was to study the morphological development stages of EFOs; identifying and measuring germinative cells of the EFO at three different gestational age groups. The work was conducted using ovaries taken from 15 fetuses at a slaughterhouse located in São Gabriel-RS, Brazil. All the mares used in this study were bred between latitudes 27o10 23.89′S and 33o46 31.76′S. Ovaries were processed for histology and stained with hematoxilin-eosin. Fetal age was estimated through the occipital-cocigeal axis lenght measurement. Fetuses were distributed in three groups according to their gestational age. Germinative cells of EFO were evaluated and identified according to their gestational age, and morphometry was performed. Group 1 included fetuses (n = 4) with gestational age between 80 and 105 days showing different developmental stages of oogonia. The upper ovarian cortex showed a great number of mitotic cells and on the lower part of the cortex had a great number of meiotic cells. Oogonia at different developmental stages were present on both portions of the cortex in similar number (n = 946). Signs indicative of meiotic and mitotic division were observed on 45.7% and 54.3% of the oogonia, respectively. Group 2, composed by fetuses (n = 5) with gestation length between 117 and 135 days, presented oogonia and primordial follicles. Distribution of oogonia along the cortical layer was similar to the one observed on Group 1. Primordial follicles and oogonia represented 2% and 98% of all evaluated cells (n = 684), respectively. Forty five per cent of the oogonia showed characteristics of mitosis and 53% of meiosis. Ovaries of Group 3 fetuses (n = 6, between 150 and 216 days of gestational age) showed 22% of oogonia with signs suggestive of being undergoing mitotic division and 63% undergoing meiotic division 12% of primordial follicles and 3% primary follicles along the cortical layer. The diameter of the germinative cells was analysed by ANOVA and *T*-test showed no variation among the different gestational ages ($p \geq 0.05$). The diameter of mitotic nuclei of oogonia ranged between 6 and 10 μm and those of oogonia with meiotic multiplication characteristics ranged between 8 and 14 μm. The diameter of the nuclei of oocyte of primordial follicles varied from 11 to 17 μm. Morphological and morphometric development data the germinative cells of equine fetuses may have a great importance to understand the reproductive physiology.

Key Words: Horse, ovary, oogonia, foetal, histology

1502

Anti-Mullerian hormone: expression and possible roles in the porcine ovary

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Anti-Mullerian Hormone (AMH) is expressed by the granulosa cells of early developing follicles and plays an inhibiting role on the initial and cyclic processes of follicular recruitment in some species. We investigated the expression of AMH in the ovary of pre-pubertal and adult female pigs. Ovaries of 80-days old gilts (n = 12) and multiparous sows (n = 4) where collected and fixed in a 4% paraformaldehyde in 0.2 M phosphate buffer solution (pH 7.2–7.4) at 4°C for 24 h and embedded in paraffin. Immunohistochemistry analysis was performed

using the peroxidase block and avidine-biotine complex with a primary polyclonal antibody. The ovarian sections were scored semi quantitatively for extent of AMH expression by two independent observers. Arbitrary scores were given as follows: 0, no expression; 1, mild expression; 2, moderate expression; 3, strong expression. The final score was the average values between the two observers. AMH expression was observed in the pre-granulosa cells of primordial follicles and in the granulosa cells of follicles of different stages of development (primary, pre-antral and pre-ovulatory follicles) as well as in the oocyte. In pre-pubertal gilts, primordial follicles showed the weakest pattern of staining (mean score: 1.1 ± 0.01; 1.7 ± 0.01 and 1.6 ± 0.01, respectively for primordial, primary and pre-antral follicles; $p \leq 0.01$), but no difference in staining was observed between primary and pre-antral follicles. Additionally, the pattern of AMH expression was similar among primordial, primary and pre-antral follicles in multiparous sows, however pre-ovulatory follicles showed the strongest staining (mean score: 2.2 ± 0.2; 2.5 ± 0.2; 2.4 ± 0.2 and 3.6 ± 0.2, respectively for primordial, primary, pre-antral and pre-ovulatory follicles; $p \leq 0.01$). The corpora lutea also expressed AMH, and no staining was observed in atretic follicles. In general, follicles in sow ovaries showed stronger AMH expression than in gilts (primordial: 1.1 ± 0.1 vs. 2.2 ± 0.1; primary: 1.7 ± 0.1 vs. 2.5 ± 0.1, and pre-antral: 1.6 ± 0.1 vs. 2.4 ± 0.1, respectively for gilts and sows; $p \leq 0.01$). These results show, for the first time, that AMH expression in the porcine ovary is different from other species, like rodents and human. This suggests that AMH might play a different role in folliculogenesis, and may not be a follicular recruitment inhibitor, as in other species.

Key Words: AMH, female pigs, follicular expression, immunohistochemistry

1503

Prolactin, andostenedione and IGF1 serum concentrations during induced follicular growth by eCG administration in the bitch

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Although several studies were done to describe the hormonal changes during the canine estrous cycle, to our knowledge none was done to describe the hormonal changes during induced follicular growth after the administration of eCG. Therefore, the aim was to study prolactin (PRL), insulin-like growth factor (IGF1) and androstenedione (ANDR) serum concentrations during induced follicular growth by a single dose of eCG administered to late anestrous bitches. Six healthy, intact mixed breed bitches were used in a completely randomized design. The day of anestrus was confirmed based on serum progesterone (< 1 ng/ml) and vaginal cytology (VC). The first day of the treatment (TRT) each bitch received eCG (50 IU/kg, im). Sexual behavior and clinical signs of estrus (SB + CSE; 1–2), and VC (VC; 1–3) samples were obtained before eCG administration and every day during 10 days or until first day of cytological diestrus (FDCD). Blood samples were taken before eCG administration and twice a day until FDCD. Prolactin, P4, ANDR and IGF1 were measured by RIA. The SB + CSE and VC scores were analyzed by the GENMOD procedure and the hormonal concentrations by the MIXED procedures of SAS. Data are presented as LSM ± SEM. Significance was defined as $p < 0.05$. Before eCG administration, anestrus was confirmed by SB + CSE and VC mean scores (1; $p < 0.01$; 1, $p < 0.01$; respectively) and mean serum concentrations of P4 (0.23 ± 0.08 ng/ml). All bitches responded to treatment by coming into clinical estrus (SB + CSE, 1.9, $p < 0.01$; VC, 1.9, $p < 0.01$) within 2–7 days after eCG administration (3.3 ± 0.94 days). Based upon P4 concentrations on first day of cytological diestrus (SB + CSE, 1.0, $p < 0.01$; VC, 3.0, $p < 0.01$), three bitches failed to have an LH peak and ovulation (FAIL) and three had an LH peak and ovulation (OVUL; 2.80 vs. 85.33 ± 6.18 ng/ml; $p < 0.01$). Conversely, P4 concentrations at the time eCG administration were similar among all bitches (0.23 ± 0.08 ng/ml; $p > 0.37$). Although the interval from eCG to estrus was similar in all bitches (3.3 ± 0.94 days), the interval from estrus to FDCD and the interval from eCG to FDCD was shorter in the FAIL group compared to OVUL group (5.00 vs. 10.00 ± 1.31 days, $p < 0.05$; 7.66 vs. 14.66 ± 0.78 days, $p < 0.01$). PRL and ANDR concentrations were lower before than after eCG TRT (4.3 ± 1.8 vs. 6.5 ± 1.6, $p < 0.05$; 0.08 ± 0.2 vs. 0.42 ± 0.16, $p < 0.05$). Conversely, IGF1 concentrations were similar before and after eCG TRT (286.0 ± 32.2, $p > 0.53$). Prolactin concentrations were similar before estrus compared to during estrus and diestrus (6.9 ± 1.7, $p > 0.19$). Furthermore, IGF1 concentrations were higher before and during estrus compared to diestrus (286.1 ± 29.8 vs. 200.4 ± 29.2, $p < 0.01$. On the contrary, ANDR concentrations were lower before and during estrus compared to diestrus (0.35 ± 0.17 and 0.38 ± 0.15 vs.0.68 ± 0.17, $p < 0.05$. These results show that 50 IU/kg of eCG successfully induced changes in follicular growth which were paralleled with changes in PRL, IGF1 and ANDR serum concentration similar to dose occurring during a normal occurring estrous cycle.

Key Words: Bitch, follicular growth, prolactin, androstenedione, insulin-like growth factor

1504

Ovarian activity during the estrous cycle and pregnancy in Asian elephants (*Elephas maximus*)

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Elephants show many interesting and unexplained features in their estrous cycle and pregnancy- the longest described for any mammal known today- including: why they possess multiple corpora lutea (CL) despite being uniparous; why they express two LH peaks during the follicular phase of the estrous cycle timed 19–21 days apart; how they maintain their extraordinarily long pregnancy and what happens meanwhile on the ovaries. The present study combined both ultrasound and endocrine techniques in order to find consensus on some of these unexplained observations during the cycle and the pregnancy. Cycling and pregnant Asian elephants were examined using a portable 4D ultrasound device (Voluson i; GE Healthcare). Serum hormone concentrations of progestagen, prolactin (PRL), inhibin and LH were measured. The main results show that 1–11 accessory corpora lutea (acCLs) form in every cycle from luteinized unruptured follicles (LUF), induced by the first of two successive LH peaks. We found a significant relationship between LUF diameter and serum inhibin concentration ($r^2 = 0.82$; $p < 0.001$) indicating that accessory CLs seem to be responsible for inhibin secretion. Therefore, they appear crucial for dominant follicle deviation in elephants, because only after the second LH peak, a single follicle ovulates (diameter 20.2 ± 0.8 mm, n = 11 elephants) and forms the largest CL on the site of the ovary bearing the larger number of acCLs. In contrast to previous speculations, the evidence presented here demonstrates that there is no additional CL formation in pregnant elephants. The initial set of CLs formed during the cycle was maintained throughout the entire gestation. However, after week 7, all pregnancy CLs (mean diameter ± SD: 28.6 ± 4.9 mm) increased in size and grew significantly larger than in a non-conceptive luteal phase (mean ± SD: 22.4 ± 2.4 mm; $p = 0.0389$). This phenomenon was reflected by increased progestagen concentrations. Serum PRL rose 4–6 months into pregnancy and remained elevated until after parturition. This result, in conjunction with the finding from a different study that the elephant trophoblast secretes placental lactogen (elPL), let us to conclude that PRL/elPL act as luteotrophic factors. They may promote CL maintenance and progestagen secretion throughout the entire pregnancy in elephants.

Key Words: progesterone, corpus luteum, luteinized follicle, luteinizing hormone, ovulation

1550

In vitro culture of equine preantral follicles obtained via Biopsy Pick-Up method

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The equine ovary contains thousands of oocytes, most of which are enclosed in preantral follicles. *In vitro* culture systems have been established for several species allowing for the growth and development of preantral follicles and their respective oocytes. The aim of this study was to test the efficacy of two media on the *in vitro* culture of equine preantral follicles. Ovarian cortical strips were obtained from 5- to 16-year-old mares (n = 10) via Biopsy Pick-Up method (n = 10 procedures) during the breeding season. Ovarian tissue was immediately submitted to histological analysis (control; D0) or cultured *in situ* for 1 (D1) or 7 (D7) days in either α-MEM or TCM-199 and submitted to histological analysis, generating five treatment groups: control, α-MEM:D1, TCM-199:D1, α-MEM:D7, and TCM-199:D7. Two biopsy fragments with similar sizes were distributed in each treatment. End points evaluated were: follicle class and morphology (normal vs. abnormal) and follicle and oocyte diameter. Proportions for follicle development and follicle morphology were compared among and within treatments using Fisher's exact test and follicle and oocyte diameters were compared using one-way ANOVA. A total of 142 preantral follicles were analyzed in five replicates. No follicles were present in the TCM-199:D7 treatment. The proportion of primordial follicles was higher in the control ($p < 0.03$) and TCM-199:D1 ($p < 0.007$) treatments compared to the α-MEM:D7 treatment. The proportion of primary follicles was higher ($p < 0.04$) in the α-MEM:D7 treatment compared to the control. The proportion of developing follicles (transitional, primary, secondary) was higher in the α-MEM:D7 treatment compared to the control ($p < 0.03$) and TCM-199:D1 ($p < 0.007$) treatments. There was no difference ($p > 0.05$) in the percentages of normal primordial and primary follicles among treatments. A higher percentage of normal transitional follicles was observed in the α-MEM:D1 treatment compared to the TCM-199:D1 ($p < 0.005$) and α-MEM:D7 ($p < 0.004$) treatments. Overall, the percentage of normal follicles was higher in the control ($p < 0.02$) and α-MEM:D1 ($p < 0.0001$) treatments compared to the α-MEM:D7 treatment. The percentage of normal developing follicles was higher in the α-MEM:D1 treatment compared to the TCM-199:D1 ($p < 0.004$) and α-MEM:D7 ($p < 0.0002$) treatments. Mean follicle diameter was greater ($p < 0.04$) in the α-MEM:D7 treatment (40.6 ± 1.1 μm) compared to the control (37.3 ± 0.7 μm). Mean oocyte diameter was greater in the α-MEM:D1 (31.0 ± 0.7 μm; $p < 0.006$), TCM-199:D1 (30.7 ± 1.8 μm; $p < 0.006$), and α-MEM:D7 (33.2 ± 1.8 μm; $p < 0.006$) treatments compared to the control (27.4 ± 0.9 μm). In conclusion, *in vitro* culture of equine preantral follicles in α-MEM for 7 days promoted follicle development and follicle and oocyte growth, with some follicles maintaining morphological normality throughout the culture period. *In vitro* culture systems for preantral follicles could be utilized in the future to enable the use of numerous oocytes present in the equine ovary for large-scale embryo production or for preservation of genetic material.

Key Words: Equine, mare, folliculogenesis, *in vitro* culture, preantral follicle

1551

Intrafollicular haemorrhage induced by meloxicam and phenylbutazone administration in mares: preliminary data

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Studies of follicular dynamics have been carried out in order to better understand ovarian function and enable estrous cycle manipulation in mares. The enzyme cyclooxygenase 2 (COX-2) is involved in follicular production of prostaglandins (PGs) E2 and F2α, which occurs approximately 10–12 h before ovulation. These PGs induce increased blood flow into the follicle, increase intrafollicular pressure and initiate the synthesis of enzymes involved in the rupture of the follicular wall, causing the expulsion of the oocyte. Nonsteroidal antiinflammatory (NSAIDs) drugs, as meloxicam and phenylbutazone, are used for the treatment of many disorders in mares and could interfere in reproductive activity by inhibiting COX-2 and consequently, inhibition of PG formation. The aim of this study was to evaluate the effects of administration of NSAIDs in developing pre-ovulatory follicles in mares. Four mares were used in the study during three consecutive cycles and ultrasonographic examination was performed each 12 h. When follicles of 32 mm diameter were detected, buserelin (0.042 mg) was administrated to induce ovulation. The first cycle was used as control and they received the same dose of buserelin without NSAID administration. Each mare was treated in the second cycle with 0.6 mg/kg of meloxicam, and with 4.4 mg/kg of phenylbutazone in the third cycle, both administered once a day until the moment of ovulation or beginning of follicular haemorrhage. In the control group, all mares ovulated between 36 and 48 h after induction. During the two cycles of treatment with NSAIDs, it was observed that three mares (75%) ovulated between 96 and 120 h after induction with buserelin, and intrafollicular hyperechoic spots were observed in ultrasound images, consistent with hemorrhagic follicles. One mare from each treatment (a different mare each cycle) failed to ovulate. These data demonstrate that NSAID treatment caused a delay in ovulation and luteinization of anovulatory follicles.

Key Words: Nonsteroidal anti-inflammatory, pre-ovulatory follicles, ovulation, ultrassonography, mares

1552

Bcl-2 members Bid and Bax are involved in granulosa cell apoptosis in saws

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More than 99% of follicles undergo atresia, and atresia is predominantly regulated by granulosa cell apoptosis. However, the intracellular signaling pathway of apoptosis in granulosa cells has not been revealed. We examined changes in the expression of Bcl-2 members, Bid and Bax, which are considered to promote the cell death ligand and receptor mediated process in mitochondrion dependent type II apoptosis, in pig granulosa cells during atresia. mRNA and protein levels of Bid and Bax were determined by RT-PCR and Western blotting techniques, respectively. Levels of Bid and Bax mRNA and protein were increase in granulosa cells of early atretic follicles compared to those of healthy follicles. *In situ* hybridization and immunohistochemical staining revealed that mRNA and protein of them were present in the granulosa cells though only negative or traces were found in healthy follicles, but strong staining were noted in atretic follicles. Then, to confirm the proapoptotic activity of Bid and Bax in granulosa cells, we suppressed Bid and Bax mRNA expression in the cells using small interfering RNA (siRNA). When Bid or Bax mRNA was suppressed, a significant decrease in the apoptotic cell rate was noted. The present results indicate that Bid and Bax appear to be signal transduction factors in granulosa cells during atresia and to play proapoptotic roles, and confirm that garanulosa cell is type II apoptotic cell.

Key Words: Follicular atresia, apoptosis, granulosa cell, Bcl-2 member, pig ovary

1553

Morphological and molecular assessment of capuchin monkey ovarian tissue following exposure to vitrification solutions: trolox supplementation improves follicular survival

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Our aim was to determine whether addition of antioxidants (selenium or Trolox) can positively affect the quality of preantral follicles exposed to a vitrification solution (VS) containing 4 M ethylene glycol (EG) and 0.5 M sucrose, and whether relative expression of genes encoding an superoxide dismutase 1 (SOD1), heat-shock protein 70 (HSp70) and endoplasmic reticulum proteins 29 and 60 (ERp29 and ERp60, respectively) are affected in ovarian tissue obtained from capuchin monkeys (n = 4). From each of eight ovaries, nine cortical fragments were recovered. One (control) fragment was immediately subjected to histology and qRT-PCR. The remaining (test) eight fragments were exposed to tissue culture medium (TCM) only, or to a VS, with or without selenium (2.5, 5.0 or 10 ng/ml) or Trolox (25, 50 or 100 μM), and then subjected to morphological and qRT-PCR analysis. Data were analyzed with Prism4 software (GraphPad). For morphological data, p values were calculated using one-way ANOVA and unpaired *t*-test. For statistical analysis of differential gene expression, an empirical Bayes method was used to moderate the standard errors of the log-fold changes. A Benjamini-Hochberg (BH) correction was applied to the p values of the log fold changes to correct for multiple testing. Differences were considered significant when $p < 0.05$. Ovarian tissue exposed to TCM only or to VS with 50 μM Trolox exhibited similar ($p > 0.05$) percentages (59% and 76%, respectively) of normal preantral follicles when compared to control (81%). When ovarian tissue was exposed to VS alone, a decrease ($p < 0.05$) in follicular survival to 28% morphologically normal follicles was observed. qRT-PCR was used to assess the relative abundance of SOD1, HSp70, ERp29 and ERp60 in the ovarian tissue from control and treated groups. Up-regulation of SOD1 (nine fold) was observed in ovarian tissue exposed to TCM and VS with 50 μM Trolox. Exposure to VS alone or with 10 ng/ml selenium led to down-regulation of SOD1 (two fold and 5.1-fold, respectively) and up-regulation of ERp29 (five fold and three fold, respectively). Expression of genes encoding HSp70 and ERp60 was unaltered. These results indicate that supplementation of VS with 50 μM Trolox may be the most suitable method tested here to avoid follicular degeneration in ovarian tissue from capuchin monkeys after exposure to a vitrification solution.

Key Words: Vitrification, ovarian tissue, capuchin monkeys, antioxidant, gene expression

1554

Follicular event study of owl monkey (*Aotus azarai infulatus*) by ultrasound

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Twelve adult cycling female owl monkeys (*Aotus azarai infulatus*), six multiparous, four primiparous and two nulliparous were conditioned to ultrasound examination to assess the feasibility of ovarian cycle monitoring in order to obtain basic data on the follicular activity of this species. The animals were born in captivity and belonged to the breeding colony of the National Primate Center (CENP), located in the municipality of Ananindeua, in the State of Pará, Brazil. The females were analyzed for three consecutive months every 48 h by B-mode ultrasound using a 6–18 MHz linear transducer. During this period, all females showed regular estrous cycles, with mean interval of 17 ± 1.13 (mean ± SE) days, which was estimated by observing ovarian activity and visualization of preovulatory follicles. Females had two to five cycles each, for a total of 44 ovulatory events, with two or three follicular waves in 22 of these events (50%) the other 22 presented one wave, with one or two follicles in coincident development per wave. The mean diameter of follicles was 3.7 mm, the smallest diameter follicle was 2 mm and preovulatory follicles were 8 mm, with 4.9 ± 1.1 mm (mean ± #SD). The follicles presented a homogeneous, hypoechoic or anechoic aspect, circular format, occupying the ovarian stroma and in general not protruding beyond the organ edges. The corpus luteum was not visible by ultrasound, and a large follicle that became undetectable from one scan to the next was considered to be preovulatory. One dominant follicle developed and ovulated for each event. Ultrasound proved to be an useful technique for monitoring the ovarian cycle in owl monkeys.

Key Words: *Aotus* sp., ovary, follicles, ultrasonography, estrous

1555

In vitro development of preantral follicles from capuchin monkeys (*Cebus apella*)

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The aim of the present study was to develop a short-term *in vitro* culture (IVC) system for the activation and growth of preantral follicles from capuchin monkeys. Cortical tissue from each ovary of four healthy mature females were collected via ovarian biopsy and divided into nine ovarian cortical pieces of 1 mm^3 (four repetitions with two replicates). One ovarian fragment (control) was immediately divided in two pieces, which were subjected to viability analysis or to qRT-PCR. The remaining eight fragments were separately cultured *in vitro* in a medium consisting of Tissue Culture Medium supplemented with 100 ng/ml Epidermal Growth Factor (T1), either or not supplemented with 10 μM *β*-mercaptoethanol (BME) (T2), 100 ng/ml Bone Morphogenetic Protein 4 (BMP4) (T3), 25 IU Pregnant Mare Serum Gonadotrophin (PMSG) (T4), 10 μM BME and 100 ng/ml BMP4 (T5), 10 μM BME, 25 IU PMSG (T6), 100 ng/ml BMP4, 25 IU PMSG (T7) or 10 μM BME, 100 ng/ml BMP4, 25 IU PMSG (T8). After IVC, all fragments were submitted to viability analysis and qRT-PCR. Follicles were classified according to their developmental phase in primordial, primary or secondary. Follicular viability was scored using fluorescent markers (propidium iodide and Hoechst). qRT-PCR was used to evaluate the expression of hormones and growth factors. IVC of the ovarian fragments resulted in similar percentages of viable preantral follicles being present therein, which were similar ($p > 0.05$) to control values (89%). The rate of primordial follicle activation, i.e. transition of primordial to primary follicle, were not affected by IVC, but the rate of secondary follicle formation was increased in medium supplemented with BME, BMP4 and PMSG. In this latter medium, contrary to other media, anti-müllerian hormone-mRNA expression in ovarian tissue was up-regulated, while that of Growth Differentiation Factor-9 was maintained. Bmp4-mRNA expression, however, appeared down-regulated in all cultured tissues when compared to uncultured control. In conclusion, our findings show a favorable effect of BME, BMP4 and PMSG on the *in vitro* formation of secondary follicles from capuchin monkeys.

Key Words: Capuchin monkeys, *in vitro* culture, ovarian tissue, preantral follicles, gene expression

16. Genetic improvement:

1600

Genetic improving of fertility performance of Holstein dairy cow

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In the past decades, more attention has been placed on milk production in selection programs worldwide, which has caused a decline in female fertility due to the antagonistic genetic relationship between milk production and fertility. Fertility is a complex trait and different related measurements may be recorded in dairy herds, e.g. days from calving to first service (DFS), number of inseminations to conception (INS), calving interval (CI), days open (DO), interval between first and last insemination (IFL), pregnancy rate (PR), and success to first insemination (SF). The objective of this study was to construct fertility index (FI) for genetic improving of fertility performance. Breeding objective included INS and DFS. INS and DFS are traits that related to different aspect of fertility performance. Among the trait in breeding objective INS has larger economic value (-73$/cow/year). In this study genetic parameters that reported by ghiasi et al. (*Livest Sci*, 39:2011) were used. According to breeding objective eight different FI were constructed based on combination of different fertility measurements (INS, DFS, IFL and DO). For each FI genetic gains per generation for profit and traits in breeding objective were computed. The FI1 that included INS and DFS and the FI5 that included DFS, had the largest and lowest genetic gain for profit, respectively (Table 1). Animal's ability to become pregnant can improve more by using FI7 (included only INS) and animal's ability to re-cycle after calving can be improve more than other cow's ability by using FI5 (included only DFS). Genetic gain for profit and traits in breeding objective in the FI4 that included DO was little difference with FI1; if fertility record are not available, DO that are recorded in milk recording system can be used to improve fertility performance. If fertility record is available FI1 was the best fertility index for genetic improving of fertility performance.

Key Words: Fertility index, genetic gains

Table 1. Fertility indices and expected genetic gains

Fertility index	INS	Expected genetic gain per generation	Profit($)
		DFS (days)	
FI1 = -0.72 × INS-2.08 × DFS	-0.39	-7.4	4.3
FI2 = -54.52 × INS-1.22 × IFL	-0.37	-2.6	3.2
F3 = -1.79 × IFL-1.52 × DFS	-0.18	-8.9	3.2
FI4 = -1.69 × DO	-0.25	-8.6	3.5
FI5 = -2.3 × DFS	-0.03	-11	2.5
FI6 = -2.39 × IFL	-0.25	-4.6	2.8
FI7 = -77.07 × INS	-0.40	-0.97	3
FI8 = -30.77 × INS-1.32 × DO	-0.32	-6.83	3.7

1601

Evidence that total number of ovarian follicles ≥3 mm in diameter is moderately heritable in dairy cows

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The total number of morphologically healthy follicles and oocytes in ovaries (ovarian reserve), established during gestation, is positively associated with a number of indirect measures of fertility in monovulatory species, including humans and cattle. For example, young age-matched cattle with low (≤15) vs. high (≥25) numbers of ovarian follicles ≥3 mm in diameter (antral follicle count, AFC) display many phenotypic characteristics typically associated with poor fertility, while lactating dairy cows with a low AFC (≤15) had poorer reproductive performance than cows with intermediate (16–24) and high AFC (≥25). Therefore, the objective of this study was to determine if AFC, a physiological ovarian characteristic associated with fertility, is heritable. Transrectal ovarian ultrasonography was performed on a cohort of 445 Holstein–Friesian dairy cows. The total numbers of follicles ≥3 mm in diameter on both ovaries were counted and the mean total number for both ovaries per animal was calculated. Where available, the pedigree of each of the 445 cows was traced back at least four generations. The pedigree file consisted of 1935 non-founder animals. Phenotypic and genetic variance components for AFC were estimated using an animal linear mixed model. The dependent variable was the natural logarithm of AFC. Fixed effects included in the model were cow lactation number and the contemporary group of year-month of calving; cow was included as a random effect. The heritability estimate for AFC (±SE) was 0.31 ± 0.14. In conclusion, this study demonstrates for the first time that AFC is heritable in dairy cows. Further work is necessary to determine the genetic variation in AFC, and the potential benefit of selecting for AFC on herd reproductive performance.

Key Words: Heritability, ovary, follicles, bovine

1602

Polymorphisms of four microsatellite loci and the association with caprine litter size

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FecB gene (BMPR-IB mutant) is a major gene responsible for high prolificacy in many kinds of sheep. However, none polymorphism of FecB was found in the six goat flocks 550 individuals, which indicated that it was not associated with caprine prolificity. Therefore, the objective of the present study is to identify the polymorphisms of four microsatellite loci (BMS2508, OARHH35, BM143 and BM1329) linked with FecB in the goat and the association with caprine litter size. A total of 361 Boer adult females individuals were examined in this study. The effective numbers of alleles (Ne), average heterozygosity (H) and polymorphism information content (PIC) were analyzed by POPGENE softwareï¼download for free from the website http://www.seekbio.com/DownloadShow.asp?id=1059ï¼‰ and DISPAN (download for free from the website http://www.softpedia.com/get/Science-CAD/DISPAN.shtml) software. The effect of genotypes on the litter size was analyzed by GLM procedure of SAS. The results showed that there were abundant polymorphisms in four microsatellite loci in Boer goat. The number of allele for BMS2508, OARHH35, BM143 and BM1329 was 4, 8, 9 and 9, respectively. The BM1329 locus has the highest average heterozygosity (H), effective numbers of alleles (Ne) and polymorphism information content (PIC) while the BMS2508 had the lowest H, Ne and PIC. The caprine mean litter size differed significantly among genotypes ($p < 0.05$). The individuals of 121 bp/137 bp genotype at OARHH35 have the best performance as the mean litter size 2.49. However, the individuals of 206 bp/184 bp genotype at BM1329 were the poorest as 1.35. These results suggested that these microsatellite loci were abundant polymorphisms and associated with caprine litter size though FecB was not responsible for caprine fecundity. It indicated that BMPR-IB were possibly the major gene controlling caprine litter size. There may be other loci linked with caprine litter size traits excluding FecB loci.

Key Words: Microsatellite loci, polymorphism, goat, litter size

1603

Association of osteopontin polymorphisms with placental efficiency and reproductive performance in Lezhi Black goat

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To test the involvement of osteopontin gene (OPN) in placental efficiency (PE) and the reproductive performance in goat, placental traits data was collected from 30 Lezhi Black does and three known OPN single nucleotide polymorphisms (SNPs), namely 5′ promoter (P1 Locus), exon 6 (P2 Locus) and exon 7 (P3 Locus) were genotyped by using PCR-SSCP technique in 70 goat individuals. The statistical software program of SPSS 17.0 was used for data analyses. Differences between the means of placental and reproductive traits were tested for significance by the Duncan's new multiple range tests. The association of the OPN gene with placental and reproductive traits was achieved using Proc GLM of SAS. Correlations and differences with $p < 0.05$ were considered as significant. The results of this study showed that the average litter weight and PE of Lezhi Black goat were 4682.40 ± 3022.91 g and 10.45 ± 3.51, respectively. PE was very significantly different between first parity and multiparous does ($p < 0.01$). There were two genotypes (nominated AA and AB) detected by three primer pairs. In 5′ promoter, exon 6 and exon 7 of OPN gene in Lezhi Black goat, frequencies of AA genotype were 0.200, 0.899 and 0.243; frequencies of AB genotype were 0.800, 0.101 and 0.757, respectively. Least squares means of litter size for genotype AA was 0.54 ($p < 0.05$) more than those for genotype AB detected in P1 locus of the ONP gene in Lezhi Black goat. Fragments detected in exon 6 of the ONP gene had no significant effect ($p > 0.05$) on PE or on reproduction traits. In exon 7 of the ONP gene, our findings demonstrate that goats with AA genotype have higher placental weight ($p < 0.01$), cotyledon weight ($p < 0.05$) and lower cotyledon density ($p < 0.05$) than those with genotype AB. The other traits showed no relationship to the genotypes examined. These data suggest that OPN genetic variations have a role in placental traits and litter size in goat.

Key Words: Goat, osteopontin gene, placental efficiency, single nucleotide polymorphisms

1650

Simulated economic efficiency in a complete cycle production system of beef cattle bulls using *in vitro* production of embryos

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Economic efficiency was simulated in two complete cycle systems aimed at the production of beef cattle bulls. The systems differed in using or not *in vitro* production of embryos (IVP) with male sexed semen. The study was conducted using data of a farm located at the northeast region of Brazil, which used a complete cycle system (CCS) with a weaning rate of 84% and an average of 3792 heads and 1510 dams, of which, 210 belonged to the Tabapuã breed (*Bos indicus*), aimed at the production of genetics, and 1300 were for meat production purposes. The evaluation period was 36 months. The IVP scenario included 20 donors and four oocytes collection procedures during each annual breeding season. The number of synchronized recipients considered per follicular puncture procedure of each donor was eight, considering that 75% of them were used for embryo transfer and that the final conception rate was 45%. Three hundred and twenty two heifers were used as recipient per year and 172 male and 30 females were born. Considering 1% post-weaning mortality per year, in the IVP scenario, 510 bulls and 89 heifers (Tabapuã breed) directed to genetics production and 2251 heads directed to meat production were sold in 3 years. Economic values of the studied years were indexed based on the General Price Index of the Getulio Vargas Foundation, Brazil. The local currency was transformed in US Dollars. The additional cost of the IVP was US$40 838.92 for an additional income of US$447 784.15, considering U$3248.00/head and U$1624.00/head for Tabapuã bulls and heifers, respectively. The annual average income was US$1 362 373.09 and US$1 657 608.69 for the CCS without and with IVP, respectively. The annual average income per head was US$1434.08 and US$1744.85 for the CCS without and with IVP, respectively. The average income increase was due to the genetics improvement in the beef cattle production system. Total operating cost was US$771 796.80 and US$812 635.72 for the CCS without and with IVP, respectively. Total operating income was US$590 576.30 and US$844 972.97 for the CCS without and with IVP, respectively. The annual return on invested capital was 16.70 and 20.22% for the CCS without and with IVP, respectively. It can be concluded that the introduction of the IVP biotechnology improved the real economic efficiency scenario of a complete cycle system of beef bull production.

Key Words: Economic efficiency, *in vitro* embryo production, complete cycle production system, beef cattle

Economic evaluation of a complete cycle system (CCS) for the production of beef cattle without and with the use of *in vitro* embryo production (IVP)

Economic values	CCS with no IVP – US$/year	CCS with IVP – US$/year
Variable operating cost	415 290.11	415 290.11
Operating cost	356 506.69	356 506.69
IVP aditional cost	–	40 838.92
Total operating cost	771 796.80	812 635.72
Total income	1 362 373.09	1 657 608.69
Operating profit	590 576.30	844 972.97
Operating profit/hectare	201.84	288.78

1651

Detection of polymorphism in Booroola gene (FecB) and its association with litter size in Zel sheep breed in Iran

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Zel sheep breed, including 68 adult ewes in North – Iran, were screened with forced PCR RFLP method for detection of FecB gene and its effects on litter size. Genomic DNA was extracted from the blood of 68 matured ewes with age varing between 3 and 6 years and with litter size of 1–2 lambs per ewe lambing. A PCR was conducted to amplify a 190 bp fragment of FecB gene. The primers were designed as follows: Forward primer: 5′-CCA GAG GAC AAT AGC AAA GCA AA-3′; Reverse primer: 5′-CAA GAT GTT TTC ATG CCT CAT CAA CAG GTC-3′.The reverse primer deliberately introduced by a point mutation which would create an AvaII restriction site (G|GACC) in PCR products from FecB carrier sheep, whereas PCR products from the non carriers lacked this site. Digestion of FecB gene 190 bas pair with AvaII restriction enzyme resulted in two different RFLP patterns: ++ or wild type of polymorphism with a 190 bp band in 67 (98.5%) animals and B+ or heterozygote type of polymorphism with 160 and 190 bp bands in only 1 (1.5%) ewe. Non of the animals in this study had genotype of BB. A contingency table analysis with Fisher exact test was applied on litter size trait within the different genotype of Zel sheep and the results showed that the mean litter size of ewes with genotype B+ were 2, whereas ewes with genotype ++ had lower mean (1.73) with one and two litter size. The results of the present study show that there is no polymorphism of FecB gene in Zel breed.

Key Words: Zel sheep, FecB gene, litter size, PCR-RFLP

17. Genetics, genomics and epigenomics:

1700

Extensive alterations in the quantitative proteome profile during early embryonic development in cattle

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Early embryonic development in mammals is morphologically well characterized, but limited knowledge is available about the underlying molecular processes, especially at the protein level. Being interested in the dynamics of protein expression during early embryogenesis, we performed a differential quantitative proteome analysis comparing bovine morulae and blastocysts. Six biologically independent replicates, each containing 25 embryos in the morula and blastocyst stage, respectively, were generated from *in vitro* matured and fertilized bovine GV oocytes. Total protein extracts were hydrolyzed by trypsin and labeled with the iTRAQ four-plex reagents to facilitate quantification. Multiplexed peptides were subjected to cation exchange chromatography to give six fractions, each of which was analyzed by nano-LC-MS/MS on an Orbitrap XL instrument. Applying stringent criteria for identification and quantification (Scaffold Q+ software, minimal fold change >1.5, $p < 0.05$), 50 proteins were shown to be differentially abundant in morulae and blastocysts. In a second complementary approach, ultrasensitive 2D DIGE saturation labeling was used to analyze a second set of six biological replicates of morulae and blastocysts. 34 spots from differentially abundant proteins were identified (DeCyder 6.0 software, $p < 0.01$, modified Bonferroni FDR correction enabled), seven of which had also been detected by the iTRAQ approach. Our proteomic data reflect a set of crucial physiological and biochemical processes activated during morula to blastocyst transition. Several differentially abundant proteins are involved in first cell differentiation events, and a subset of proteins revealed a clear switch of metabolic activity between the two embryonic stages. Expression levels of proteins involved in catabolic processes as well as ubiquitinylation associated proteins decreased during development. In contrast, ATP synthesizing proteins and other enzymes catalyzing biosynthetic processes increased in abundance. Therefore, proteome analysis clearly evidences a switch from catabolic to anabolic metabolism between morula stage and blastocyst formation. Our results reflect, on the protein level, the gradual degradation of maternally inherited messages and the activation of the embryonic genome between the 8-cell- and the 16-cell-stage: the majority of differentially abundant proteins is increased in blastocysts, and a set of eight proteins demonstrates the buildup of the translational machinery and the on-going translational activity in blastocysts.

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Key Words: Proteome, mass spectrometry, iTRAQ, 2D DIGE, blastocyst

1701

MiR-218 modulates early lineage segregation in the bovine blastocyst by targeting CDH2 and NANOG

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Mammalian blastocyst formation is characterized by two lineage segregations resulting in the formation of the trophectoderm, the hypoblast and the epiblast cell lineages. Cell fate determination during these early lineage segregations is associated with changes in the expression of specific transcription factors such as POU5F1 (better known as OCT4), SOX2, CDX2, NANOG and GATA6. But transcription factors are not the exclusive answer to the question how pluripotency and differentiation are regulated and it is more and more presumed that microRNAs (miRNAs) play an important role in the posttranscriptional regulation of differentiation events. A combined analysis of matching miRNA and mRNA expression data in early, *in vitro* produced bovine blastocysts (day 7 post insemination) and hatched, *in vitro* produced bovine blastocysts (day 8 post insemination) revealed that miR-218 was downregulated in hatched blastocyst whereas theoretically predicted miR-218 target genes CDH2 and NANOG were upregulated in hatched blastocysts. The aim of this study was to test the hypothesized interaction between the miR-218 and its candidate targets using *in vitro* luciferase assays. Luciferase reporter constructs were engineered that contain either the wild-type 3′UTR (WT) of CHD2 resp. NANOG or mutant 3′ UTRs (MUT) with 4 bp mutations in the miR-218 target seed. HEK293T cells were co-transfected with the luciferase reporter constructs and the miRNA precursor (pre-miR-218) or a scrambled pre-miR negative control to control for non-specific effects. The scrambled pre-miR negative control did not affect the luciferase activities. Pre-miR-218 significantly reduced the luciferase activities of the WT CDH2 and NANOG reporters with 40% and 43% respectively, compared to the scrambled negative control (paired, two-tailed *t*-test, CDH2 $p = 0.0003$; NANOG $p = 1.35E\text{-}05$). However, mutant reporters of CDH2 and NANOG were not repressed by pre-miR-218, which confirms that the target site directly mediates the repression. We experimentally confirmed two novel target genes for miR-218, namely CDH2 and NANOG. The common regulation of both the epiblast marker NANOG and the adherence junction component CDH2 by miR-218 puts miR-218 in the spotlight as a central regulator of epiblast formation. Functional analyses, by knocking down or enhancing the expression of miR-218, will be performed in bovine blastocysts to test this hypothesis and to investigate the precise role of miR-218 in bovine blastocyst formation.

Key Words: Bovine, blastocyst, microRNA, epiblast

1702

TET gene family transcript profiles during bovine embryo pre-implantation stages

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The ten-eleven-translocation (TET) genes are a family of nuclear hydroxylases involved in regulating 5-methylcytosine hydroxylation, an important epigenetic component for controlling gene expression in mammalian genomes. This covalent modification has recently been established as an essential modification present in bovine early embryos that regulates expression of important developmental genes such as OCT4 and NANOG. Establishment of TET family mRNA expression levels is necessary for determining roles that TET genes play in early bovine embryo development. Bovine embryos were matured, fertilized and cultured *in vitro* according to standard laboratory procedures. RNA from pooled ova, zygotes, 2-cell, 8-cell, morula and blastocyst stage embryos (n = 10–20/group) were extracted using an RNeasy® Mini kit (Qiagen, USA). cDNA was generated using qScript™ reagents (Quanta Biosciences, USA). Quantitative Real Time Polymerase Chain Reaction (qPCR) was performed using Perfecta SYBR (Quanta Biosciences, USA) to determine TET 1, 2 and 3 transcript levels in three biological replicate samples, each measured in triplicate, and analyzed using the comparative C_t method normalized to the geometric mean of three endogenous control genes (GAPDH, SDHA, YWHAZ). As a control, data for ova were compared individually to endogenous GAPDH to determine relative mRNA quantity. Data were analyzed via paired *t*-test comparing ova mRNA levels with all other samples. In ova, TET3 was the most predominantly expressed of the three genes and was similar to GAPDH ($p > 0.05$), while TET1 and TET2 had transcript levels 277- and 25-fold less than GAPDH ($p < 0.05$). When comparing other embryo stages to ova mRNA levels, an upregulation (121 ± 21-fold; $p < 0.01$) was seen in TET1 at the 8-cell stage that continued through morula and blastocyst stages (209 ± 60- and 135 ± 35-fold, respectively; $p < 0.01$). TET2 transcript levels remained steady through all stages until a downregulation at the blastocyst stage (0.12 ± 0.01-fold below ova levels; $p < 0.01$). TET3 displayed a

similar transcript level pattern to TET2 with downregulation at the blastocyst stage (0.02 ± 0.005-fold; $p < 0.02$). The massive upregulation of TET1 upon embryo transcriptome activation may imply a role in regulating and promoting cell differentiation as compared to the relatively high transcript level of TET3 throughout early development, which implies an influence on paternal and maternal cytosine hydroxymethylation patterns both before and after fertilization. TET proteins are emerging as central players in mammalian developmental programming. Improved understanding of the role these proteins perform during early development will aid in understanding early embryo developmental programming and cellular identity establishment.

Key Words: TET family, 5-hydroxymethylcytosine, bovine, embryo

1703

Identification and analysis of beef heifers with superior capacity for fertility

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Cattle production is highly dependent upon reproductive efficiency. Early pregnancy loss, estimated to be at least 25% in beef cattle and 45% in dairy cattle, is economically detrimental to both the beef and dairy industries. The majority of embryonic loss occurs between days 8 and 16 of gestation. To identify heifers of contrasting fertility, serial synchronized artificial insemination (AI) of pubertal beef heifers (1/4 *Bos indicus*, 3/4 *Bos taurus*, n = 201) was conducted. Pregnancy was determined on day 35 post-timed AI. Heifers were ranked from highest to lowest fertility based on number of pregnancies in the four timed AI opportunities. Thirty-nine heifers were classified as having high fertility (pregnant 4/4, n = 14) or low fertility (pregnant 1/4, n = 17; pregnant 0/4, n = 8). The selected heifers were superovulated and flushed; embryos were graded and one embryo transferred per synchronized recipient (n = 73). There was no effect of fertility classification on quantity of embryos recovered per flush ($p = 0.72$) or quality ($p = 0.37$). Pregnancy rates of recipient cows receiving embryos from either high fertility (54%) or low fertility (44%) donors were not different ($p = 0.45$). Two embryos (stage 4 or 5, grade 1 or 2) were then transferred into the heifers on day 7 after observation of estrus, and pregnancy determined by ultrasonography approximately 25 days later. Pregnancy rates were higher ($p = 0.03$) in high fertility (69%) than low fertility (33%) heifers. Circulating levels of progesterone were not different in the heifers during the estrous cycle ($p = 0.85$) or at embryo transfer ($p = 0.42$). Statistical analysis included PROC GLM, PROC MIXED and Chi-Square analysis for embryo and progesterone levels at embryo transfer, progesterone levels during the estrous cycle, and pregnancy outcomes, respectively. Genome-wide association studies of DNA from the heifers using the Illumina 770 K SNP BovineHD Genotyping BeadChip detected 7 ($p < 2.0 \times 10^{-5}$) associations with fertility that were present on six different chromosomes. The uteri from the selected heifers were collected on Day 14 of the estrous cycle. No obvious histological differences were present in the endometrium of the high and low fertility heifers. These results indicate ovarian function and uterine histoarchitecture are not the reason for contrasting fertility observed in these heifers. Further analysis of endometrial gene expression and secretions from these animals will be used to elucidate physiological and genetic markers associated with fertility in cattle.

Key Words: Early pregnancy, early embryonic loss, cattle, SNP

1704

Histone deacetylation inhibition decreases transcription of imprinted genes during early embryo development in cattle

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Control of imprinted genes during early embryo development involves epigenetic mechanisms, including histone deacetylation (HDAC). It has been reported that mechanisms regulating imprinting are affected by *in vitro* culture. *In vivo* produced blastocysts have low expression of imprinted genes and low expression of XIST that controls X chromosome inactivation (XCI). In the present study, we aimed to assess the effect of a non-toxic, cell-permeable hydroxamic acid-containing HDAC inhibitor, scriptaid (SCR; S7187-Sigma, Spain), during *in vitro* preimplantation embryo development. We tested the hypothesis that SCR would increase histone acetylation levels, increase embryonic genome activation rates, and regulate imprinting and XCI in *in vitro* produced bovine embryos. Oocytes from ovaries of slaughtered heifers were matured for 24 h in TCM-199 supplemented with 10% fetal calf serum (FCS) and 10 ng/ml epidermal growth factor and then inseminated with frozen/thawed BoviPure (Nidacon, Sweeden) separated bull sperm at 39°C under an atmosphere of 5% CO_2 in air. At approximately 20 h post insemination, presumptive zygotes (n = 5184) were denuded and cultured in groups of 20–25 in 25 µl of synthetic oviduct fluid supplemented with 5% of FCS (C:control) as a basal medium or supplemented with 100 nM SCR from day 1 to day 7 (H1-7), from day 3 to day 7 (H3-7) or from day 5 to day 7 (H5-7) at 39°C under an atmosphere of 5% CO_2 and 5% O_2 in air. Cleavage rate and blastocyst yield were measured at 48 h and on days 7 and 8 of culture, respectively (day 0 = day of fertilization). A representative number of blastocysts on day 7 from each experimental group were used for evaluating embryo quality through: (i) differential cell count, and (ii) gene expression analysis by mean of quantitative real-time PCR. Data were analyzed by one-way repeated-measures of ANOVA and students *t*-test. Blastocyst yield on day 7 was not different between control and experimental groups (C:31.59 ± 2.52%; H5-7:29.91 ± 2.40%; H3-7: 31.88 ± 2.95%; and H1-7:30.40 ± 1.95%). In terms of total cell number (range from 144.45 ± 3.65 to 157.35 ± 4.74) or trophectoderm cell number (range from 104.03 ± 3.20 to 113.88 ± 4.01) no significant difference was found between groups. However, quantification of two genes involved in the mechanism of imprinting (IGF2R, GRB10) and XCI showed a down-regulation in the presence of SCR compared with the control. IGF1R transcript abundance was not affected by SCR in the culture medium. It is possible that SCR prevented deacetylation of promoters for both, XIST and imprinting genes. We conclude that gradual exposure of embryos to SCR in the culture media, does not affect embryo development and total cell number. However, HDAC inhibition down regulated the early expression of some imprinted genes and decreased XIST early expression. Whether or not decreased XIST and imprinting expression levels are beneficial to post implantation development remains to be elucidated.

Key Words: embryo development, bovine, scriptaid, imprinted genes

1705

Trichostatin-A increases levels of cytosine 5-hydroxymethylation in bovine cells

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In the last years several groups reported abnormal development of embryos obtained by reproductive technologies, such as *in vitro* culture or nuclear transfer. Currently such abnormalities have been attributed to epigenetic modifications of chromatin leading to aberrant patterns of gene expression. One of the strategies recently employed to alter and investigate the epigenetic mechanism driving development is the use of chromatin modifying agents such trichostatin A (TSA). TSA inhibits histone deacetylases leading to increased levels of histone acetylation and indirectly decreases DNA methylation. Nonetheless, this TSA effect

on the novel cytosine modification recently discovered, the 5-hydroxymethylcytosine (5-hmC) is still unclear. In order to evaluate the effects of TSA on this novel epigenetic mechanism, non-confluent bovine fibroblasts were exposed to different concentrations of TSA – 0 (control), 0.05, 0.25, 1.25 and 6.25 nM during 8, 12 and 20 h. Three fibroblast cell lines were stained for single color analysis to assess the global 5-hmC status by flow cytometry. Briefly, the cells were harvest, fixed in 0.25% of paraformaldehyde, permeabilized and DNA was denatured. The cells were incubated with a polyclonal antibody against 5-hmC sites. The samples were analyzed by flow cytometer and a least 10 000 events were acquired. The geometric mean of fluorescence intensity and percentage of positive gated cells for each treatment was recorded and used for the statistical analysis. Data were submitted to analysis of variance and mean compared by student's *t*-test with significance level of 5%. Results indicated increased levels of 5-hmC in fibroblasts exposed to 6.25 nm of TSA (8.38 ± 2.47 of mean fluorescence intensity – MFI) compared to 1.25 nm; 0.25 nm; 0.05 nm and control (7.71 ± 1.42; 7.10 ± 1.15; 6.09 ± 3.54 and 6.39 ± 1.77 MFI, respectively) regardless of TSA exposure time. The percentage of cells positive for 5-hmC staining were higher after 12 h of exposure to TSA (4.99 ± 3.46%) compared to 8 h or 20 h (2.09 ± 1.99% and 1.54 ± 1.70%, respectively). Increased levels of 5-hmC after TSA exposure suggest a linking mechanism between histone acetylation and DNA demethylation via cytosine hydroxymethylation. Since 5-hmC enrichment correlates with pluripotent cell state, we hypothesize that cells with higher 5-hmC levels could be more prone to be reprogrammed after somatic cell nuclear transfer or pluripotency induction. The development of a methodology to assess 5-hmC levels trough flow cytometric analysis will allow further studies to unravel the roles of 5-hmC in nuclear reprogramming. Moreover, the results of this project will contribute to the understanding of epigenetic mechanisms, especially those related to the dynamics of epigenetic changes during embryonic and fetal development. Financial support FAPESP 2010/19768-8.

Key Words: Hydroxymethylation, trichostatin A, bovine

1706

Across-species analysis of gene expression changes in the endometrium during the pre-implantation phase

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Different strategies evolved in the various mammalian groups to achieve recognition, establishment and maintenance of pregnancy. The biological importance of these processes is reflected in the high percentage of embryonic losses during this critical period in many mammals. To identify genes that play a conserved role in these processes, Illumina RNA sequencing (RNA-Seq) was used for analysis of gene expression changes during the pre-implantation phase in bovine (day 18), porcine (day 14) and equine (day 16) endometrium. Endometrial tissue samples from 4 pregnant and 4 non-pregnant control animals were analyzed, respectively. Obtained sequences were mapped to the genome and to known transcript sequences. The different quality of the genome assemblies and the gene annotation was identified as a main problem for data analysis. Therefore, a database was build that contains gene annotation from Entrez Gene and from Ensembl, and cross-references to human genes based on BLAST alignments of transcript sequences with human RefSeq RNAs to provide a basis for the comparison of gene expression data between different species. The obtained human genes were used as unique gene identifier. Gene Set Enrichment Analysis (GSEA) was performed based on human gene symbols for an initial characterization of the obtained data sets. GSEA results showed moderate but distinct overlaps between these three species. The overlap was highest between bovine and porcine endometrium. The comparison to published data from studies in other mammals including humans revealed e.g. a considerable overlap with genes up-regulated during the window of implantation in human endometrium, particularly for the horse. In addition to GSEA, a comparison of the identified differentially expressed genes (DEG) (analysis with DESeq, FDR 1%) was performed. This comparison revealed a relatively low number of genes that showed similar expression changes in all three species. Functional classification of these genes mainly assigned immune-related functions, suggesting a particular role of immune-related processes for implantation. More DEG with similar changes were observed for bovine compared to porcine endometrium. In conclusion, many species-specific gene expression changes were found, which corresponds to the different pregnancy recognition signals and differences in the process of embryo implantation. However, this study also identified a number of conserved gene expression changes indicating the existence of basic regulatory principles during this phase of pregnancy.

Key Words: Transcriptome, fertility, cattle, pig, horse

1707

In silico study of pattern of CpG islands in *Bos taurus* genome

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CpG islands are regions of genome characterized by presenting a high concentration of guanine + cytosine (GC). These sequences are important point of control of gene expression, regulated by addition of radical methyl. Besides, they are particularly important in early embryonic development. After fertilization, two highly specialized haploid genomes are combined in a single cell, which will be integrated and reprogrammed, allowing embryonic development. For this, the embryo must undergo a series of epigenetic changes, including a complex process of demethylation and remethylation, culminating in the first cell differentiation into inner cell mass and trophoblast cells. Interestingly, some studies have shown that methylation of CpG islands can be changed in some conditions, such as *in vitro* culture of embryos, justifying the study of these regions. To detect these sequences in the *Bos taurus* genome, we used an algorithm (cpgi130) that uses as input data all genomic contigs that assemble the 29 autosomal chromosomes, plus the sexual X chromosome (version 4.2 NCBI). We set the following parameters for the search of CpG islands: (i) the minimum percentage of 55% GC content, (ii) extension of sequences greater than or equal to 500 bp, (iii) a statistical support at least 0.6 (ratio of observed CpGs/expected CpGs). To deepen our analysis, two ad hoc PERL scripts were developed and tools do Shell/Unix were used to identify the islands through the genes they flanked or are inserted. In general, 36713 CpG islands were mapped. Among the chromosomes analyzed, the number 18 had the largest number of these (2169) and the 28 the lowest (374). After the execution of ad hoc PERL scripts, we classified CpG islands according to the following categories: Promoter region (2 kb upstream to the first exon), Intergenic (islands not associated with genes), Gene (island inserted into genes), which is divided into two subcategories, Intron (islands located in introns) and Exon (islands located in exons). The Promoter region hosted a significant amount of these elements, about 30% (10955) of all CpG islands were found in this group, showing a strong association between these sequences and the promoter regions. Most sequences, however, was classified as Intergenic 37.7% (13885). Interestingly, all the CpG islands in chromosome 26 (911) were located in Intergenic regions, which did not happen for the rest of the chromosomes. In the Gene group, 11873 CpG islands were found, which corresponds to 32% of the total. The most part of these elements 94.8% (11353) were associated with intron and only 5.2% (520) with exon. In this work was realized in silico mapping in large-scale of CpG islands. The identification of these elements is important, because it opens perspectives to conduct analysis in large-scale, of the influence of *in vitro* fertilization in the pattern of methylation in embryo cattle.

Key Words: Epigenetic, CpG island, in silico, bovine

1708

Copy number variation of testis specific protein Y-encoded gene is associated with fertility in bulls

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Large DNA sequences (>1000 base pairs) including both coding and non-coding regions that vary from individual to individual are referred to as copy number variants (CNV) and have been associated with diverse physiological and pathological states. In mammals, the Y chromosome is unique to males and contains relatively few genes (encoding <20 proteins), many of which are present in multiple copies and CNV. These genes are often associated with fertility, reproduction and reproductive function and tend to be preferentially expressed in the male reproductive tract. Testis-specific protein, Y-encoded (TSPY) is present in varying gene copy number in both human (20–76 copies) and cattle (37–200 copies) and some studies have linked this variation to semen quality in men although studies in other species have not been reported. The purpose of this study was to determine if TSPY copy number is related to fertility in bulls as assessed by adjusted non-return rates (SOL), a commonly used measure of field fertility in Canadian bulls. In addition, we investigated the associations between TSPY copy number and its transcript expression in testis as measured by qPCR as well as specific semen parameters such as average sperm concentration, sperm count, ejaculate volume and motility. In two independent trials involving 64 bulls, TSPY copy number was shown to be positively correlated to adjusted non-return rates (trial #1: Spearman $r = 0.34$, $p < 0.05$; trial #2: Spearman $r = 0.77$, $p < 0.01$). Furthermore, TSPY copy number was inversely correlated to TSPY mRNA expression in testis samples ($n = 26$, Pearson $r = -0.71$, $p < 0.0001$). There were no correlations of TSPY copy number or expression with the semen parameters measured. It was concluded that CNV of Y chromosome linked genes can influence fertility and that TSPY copy number represents a potential marker of bull fertility but its mechanism does not appear to be directly related to the semen characteristics analyzed as part of this study (Funded by NSERC, CRC program and L'Alliance Boviteq Inc.).

Key Words: Copy number variation, TSPY, bovine bull fertility, gene, Y chromosome

1709

Effects of lactation on gene expression of endometrium of Holstein cows at day 17 of the estrous cycle or pregnancy

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Objectives were to determine effects of lactation on endometrial gene expression on day 17 of the estrous cycle and pregnancy. Heifers ($n = 33$) were assigned randomly after parturition to lactating (L, $n = 17$) or non-lactating (NL, $n = 16$) groups. Cows were subjected to an ovulation synchronization program for a timed artificial insemination (TAI); 10 cows in L and 12 in NL were inseminated. Slaughter occurred 17 days after the day equivalent to TAI, and intercaruncular endometrial tissues were collected. The RNA was extracted using trizol and then purified and checked for RNA concentrations and integrity. Gene expression was determined by DNA microarray analysis, using the Affymetrix Bovine genome array, for pregnant (L, $n = 8$; NL, $n = 6$) and non-inseminated cyclic (L, $n = 7$; NL, $n = 4$) cows. Differentially expressed genes were selected with p-value <0.01 and absolute expression >40. There were 210 genes differentially regulated by lactation (136 down-regulated and 74 up-regulated). The GO analyses of up- and down-regulated genes of lactating cows revealed terms related to immunoglobulin-like fold, immune response, COMM domain and non-membrane bounded organelle. A number of upregulated genes by lactation such as IGHG1, IGLL1, IGK, and TRD were all related to immune function, particularly for B cells and $\gamma\delta$T cells. Developmental genes related to limb and neural development and glucose homeostasis (DKK1, RELN, PDK4) were down-regulated by lactation. The stated genes associated with immune function and developmental genes expressed in the endometrium impacted by lactational state are possible candidate genes for interventions to improve fertility of lactating dairy cows, South Korea.

Key Words: Endometrium, gene expression, lactation

1710

Two novel mutations in the bone morphogenetic protein 15 gene are associated with increased ovulation rate, but no sterility, in the French Grivette and the Polish Olkuska breeds of sheep

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It is believed that prolificacy and ovulation rate (OR) are associated with a particular gene(s) in Grivette (a French Massif Central breed) and Olkuska (a south Poland breed) sheep populations, respectively. The objective of this study was to localize and identify those genes in the two breeds through a genome-wide association study (GWAS) using the 54 K SNP array (OvineSNP50 DNA Analysis Kit) from Illumina. In both protocols, association analyses were performed by comparison of allelic frequencies via a Fisher test in a case (high prolificacy/OR) vs. control (normal prolificacy/OR) design. In both Grivette (27 cases vs. 11 controls) and Olkuska (28 cases vs. 35 controls) populations, the most significant association was evidenced with SNPs on the X chromosome very close to the BMP15 location. By sequencing the BMP15 gene (then considered as a candidate gene) in all cases and controls, two new polymorphisms leading to non conservative amino acid substitutions in BMP15 were identified, one in each population. These two polymorphisms are associated with the hyperprolific phenotype suggesting their potential causality. In Grivette, the 950 C>T mutation in the cDNA causes a T317I substitution in the protein. When looking at the distribution of this putative causal mutation in 203 animals combining the 38 ewes used for the GWAS and 165 stochastic additional ewes of the Grivette population, 37 were C/C with a mean prolificacy of 1.86, 82 were C/T with a mean prolificacy of 1.97 and 84 were T/T exhibiting a significantly increased mean prolificacy of 2.62 ($p < 0.001$, T/T vs. C/T or C/C, one-way ANOVA). When tested functionally by transient transfection using a BMP-responsive luciferase test in COV434 granulosa cells, the T317I mutation impaired drastically the BMP15 signaling activity. In Olkuska, the 1009 A>C mutation in the cDNA causes a N337H substitution in the protein. Looking at the distribution of this second putative causal mutation only in the GWAS analyzed population, 17 were A/A with a mean OR of 1.61, 28 were A/C with an mean OR of 2.32 ($p < 0.01$ A/A vs. A/C), and 18 were C/C with a mean OR of 4.38 ($p < 0.001$, C/C vs. A/A or A/C, one-way ANOVA). Thus, we have identified 2 novel mutations in BMP15 associated with significant increased prolificacy and OR in sheep. In striking contrast with all other known BMP15 mutations, homozygous $BMP15^{T317I}$ Grivette and homozygous $BMP15^{N337H}$ Olkuska ewes are hyperprolific but not sterile.

Key Words: prolificacy, ovulation rate, major gene, BMP15, ovine

1711

Evidence for a single gene affecting embryo/fetal survival in the Davisdale flock

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The number of lambs born per ewe (lambing rate) is a key driver of profitability. However, as farmers increasingly select for higher lambing rates, the number of ewes having triplets increases, which can reduce farm profitability due to the lower performance of triplet lambs. Increasing embryo/fetal survival, when coupled with appropriate ovulation rates, can increase the lambing rate through increasing the percentage of ewes with twins. However, heritability of embryo/fetal survival is low making this trait difficult to improve through traditional selection approaches. The aim of this study was to determine if there is evidence for a single gene with a major effect on embryo/fetal survival in the Davisdale flock. For all progeny tests (PT), all sires were crossed with non-Davisdale ewes and embryo survival measurements were recorded for 1–5 years in the resulting progeny (21–56 per sire). Each year, ovulation rate was determined for each ewe and numbers of lambs were determined at ultrasound scanning (PT 1) or lambing (PT 2 and 3). For each breeding cycle, for each ewe, the embryo/fetal survival score was calculated as the number of lambs minus the average number of lambs for all ewes with that ovulation rate for the trial. For total loss, ewes failing to lamb in the first lambing cycle were considered to have 0 lambs. Partial loss was calculated by examining only those ewes that lambed in the first cycle and had an ovulation rate of two or three. In PT 1 records from daughters of three sires, including two full-siblings, from Davisdale ewes were examined. Both total loss and partial loss significantly differed in progeny of the two full-sibling sires (mixed model analyses; $p < 0.01$, difference of 0.44 and 0.34 lamb given equal ovulation rates) with the third sire being intermediate. In PT 2, a single Davisdale sire was used to generate three full-sibling sons whose mother was also from the Davisdale line and a fourth son from a non-Davisdale ewe. Total and partial loss differed among the full-sibling sons with daughters of one sire clearly having lower loss than the daughters of his full-sibling ($p < 0.05$, difference of 0.32 and 0.17 lamb). PT 3 involved 12 sires with the same Davisdale sire as those tested in PT 2. The dams of these sires were also from the Davisdale line. Significant differences in embryo/fetal survival were also observed in the daughters of these sires ($p < 0.05$, difference of 0.58 and 0.32 lamb for total and partial loss in daughters of two of the extreme sires). Collectively, these data are supportive of a single gene affecting embryo/fetal survival segregating in the Davisdale line. Furthermore, this single gene appears to be enhancing embryo/fetal survival as the number of lambs born for pregnant ewes from PT 2 and 3, given an average ovulation rate of 2.1, is 0.09 lambs higher than predicted from other New Zealand flock data.

Key Words: embryo/fetal survival, sheep, putative single gene

1712

Methylation change of imprinted genes (IGF2 and H19) in serial recloned piglet

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A cloned pig plays an important role as a source of alternative organ for human. Cloning of animals has been successfully achieved by somatic cell nuclear transfer (SCNT). The SCNT has opened a new door for making targeted gene modification. Although the technical development of SCNT is improved consistently, the success rate of usable piglets is very low because of pathological changes in the fetal and placental phenotype. Recent studies show that the aberrant epigenetic reprogramming of the transferred donor nucleus is causative of the abnormalities of cloned animal. However, the mechanisms of nuclear remodeling and reprogramming remain unclear and the genetic status of offspring by repeated SCNT is unknown yet. Therefore we performed primary cloning of pig by using normal pig somatic cell (F0) which was from ear skin fibroblast (ESF). We got an offspring (F1) but the newborn piglet was dead in a day. Secondary cloning was conducted using ESF of primary offspring and we got an offspring (F2) which was not dead but the size of piglet was smaller than normal. So we performed tertiary cloning using donor cell derived secondary offspring and gain several tertiary offspring (F3). However, all of tertiary offspring were dead in a day. Due to the lack of offspring, we could not conduct the statistical analysis. To compare the genetic modification of serial recloned piglets, we isolated the ESF of three generations and primary donor pig and analyzed the DNA methylation pattern of IGF2-H19 differentially methylated regions (DMRs) using bisulfite sequencing. As a result, the methylation rate of each generation was F0 (89.3%), F1 (88.1%), F2 (82.1%) and F3 (73.8%) in the IGF2 upstream region (208 bp). However, the IGF2 downstream region (217 bp) showed opposite pattern which was F0 (62.8%), F1 (85.7%), F2 (91.2%) and F3 (92.3%), respectively. Meanwhile, the methylation rate of H19 had shown dramatic change which were F0 (62.5%), F1 (55.2%), F2 (23.7%) and F3 (15.1%) in DMR1 region (308 bp). In H19 DMR2 region (224 bp), the pattern of methylation was changeless, but DMR3 region (207 bp) was consistently changed again as F0 (95.2%), F1 (83.3%), F2 (77.3%) and F3 (53.5%), respectively. Our data show that the genomic DNA methylation was changed consistently over the generation. Briefly, IGF2 upstream and H19 DMR regions became de-methylated toward the later generation, but IGF2 downstream showed the other way. To demonstrate whether the methylation rate could regulate the gene expression, we analyzed the gene expression levels of IGF2 and H19 on ESF of the primary donor (F0) and the last generation (F3). Using Real-time PCR analysis, we confirmed that both IGF2 and H19 expression levels were significantly reduced three fold ($p < 0.001$). Our results suggest that DNA methylation has a critical role in the regulation of gene expression and a cloned animal by SCNT has aberrant genetic status. In other words, abnormal reprogramming may inhibit the essential gene expression, and the genetic abnormalities of cloned pigs get worse during repeated SCNT.

Key Words: DNA methylation, epigenetics, IGF2 gene, H19 gene, SCNT

1713

Partial reprogramming of imprinted gene expression during post-implantation development of mouse embryos exposed to TSA but not 5AzaC during pre-implantation

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The success rate of producing cloned animals is very low, and in many cases is associated with oversized fetuses, enlarged placentas (placentomegaly) and organs (organomegaly) of several tissues. Epigenetic modifications to imprinted genes such as the abnormal resetting of DNA methylation and/or histone acethylation patterns during fetal development are thought to play an important role in development anomalies. Although previous studies have shown that brief exposure of embryos to drugs that induce DNA demethylation (5 Aza-Cytidine; 5AzaC) and histone hyperacetylation (Trichostatin A; TSA) alter the expression of imprinted genes at pre-implantation embryonic stages (Baqir & Smith, BOR 2001, 64; 1, 280), little is known of the ability of treated embryos to reprogram imprinted gene expression after implantation and to term. Therefore, our objective was to evaluate the long-term effects of treating pre-implantation 8-cell compact embryos for 12 h with either TSA (100 ng/ml) or 5AzaC (0.1 μM) on the development outcome and reprogramming of imprinted gene expression in mouse post-implantation fetuses and placenta. Day 17–18 whole fetuses, liver and placenta were weighed and total RNA was extracted, reversed transcribed to evaluate the expression patterns of imprinted genes (p57, H19, Igf2r, Igf2, Peg1, Mash2, Ipl), non-imprinted placental-specific genes (Dlx3, Esx1, Tpbp) and a housekeeping gene (Gapdh) by qRT-PCR. Samples were standardized with an exogenous control (α-Globin RNA) and expressed as fold changes in relation to *in vivo* produced embryos. Differences of gene expression

at $p \leq 0.05$ were considered significant. Our results show that while the weight of the normal fetus and placenta from the TSA treated embryos were similar to the *in vitro* control, the 5AzaC group displayed a reduction of 35% in fetal weight and an increase of 68% in placental weight. Moreover, TSA treatment did not affect the imprinted genes p57, H19, Igf2r, Igf2, Mash2, Ipl (1.1, 0.9, 1.1, 0.86, 1.12, 1.3 fold respectively) and placenta-specific genes Dlx3 and Esx1 (0.93 and 0.9 fold respectively) when compared to the *in vitro* controls. Conversely, embryos exposed to 5AzaC displayed an aberrant expression of imprinted p57, H19, Igf2r, Igf2, Mash2, Ipl genes (0.4, 2.4, 0.4, 0.4, 1.8, 0.35 fold respectively) and placenta specific Esx1 and Tpbp genes (0.4 and 1.9 fold respectively), suggesting a potential relationship to the reduced fetal growth and increased placental and liver weights in this group. These results indicate that while the 5AzaC group displayed an aberrant fetal and placental weight and irregular expression of imprinted and placenta specific genes, successful fetal and placental growth can be obtained after treatment of pre-implantation mouse embryos with TSA. Together, these results suggest that, in contrast to DNA methylation, epigenetic errors created by histone hyperacetylation during pre-implantation are reset during post-implantation stages of development.

Key Words: Imprinting, epigenetics, mouse fetus, placenta, gene expression

1714

Chimeric RNA is an important mode of gene transcription in mammal

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Chimeric RNAs are a kind of fusion transcripts of gene expression and produced by trans-splicing of two or more distinct transcripts. They have been found and identified in some normal cells in budding yeast, fruit fly, mouse and human. However, there are no reports of Chimeric RNAs to find in cattle and pig. The objective of this study was to find some chimeric RNAs in domestic animals and reveal the molecular mechanism of the formation of chimeric RNAs by the technology of bioinformatics. We conducted a large-scale search for chimeric RNAs in cattle and pig by the technology of bioinformatics. Firstly, we downloaded genome sequences and mRNAs/Refseqs/ESTs of cattle and pig from UCSC database. EST library information and EST annotation were also downloaded from NCBI database. Secondly, the transcriptional sequences were mapped to their corresponding genomic sequences using BLAT with the default parameters. Thirdly, we parsed the raw BLAT results with a series of Perl scripts. Finally, those chimeric RNAs from two or more different ESTs library were identified according to available EST annotation. Based on the above search, we found 9811 chimeric RNAs in cattle and 30 060 in pig, including 7106 chimeric RNAs which annotated in specific gene locus in cattle and 13 186 in pig. There were mainly two types of chimeric RNAs. One was the intragenic chimeric RNAs. The other was the intergenic chimeric RNAs. For the former, the 3′ and 5′ end of chimeric RNA either came from different strands of the same locus or had an exon/intron order from genomic DNA. For the latter, the 3′ and 5′ end of chimeric RNA mainly came from two genes in different chromosome. In addition, we picked some chimeric transcripts containing the follicle stimulating hormone receptor (FSHR) gene order in cattle to analyze their sequence structure by biological information technology. The results showed that there were 10 chimeric ESTs containing FSHR gene order. They were AV605399, AV611870, AV608719, AV604550, AV601236, AV602765, AV601138, AV608357, AV605546 and AV605093, respectively. Besides the AV608357 was the type of intragenic chimeric EST, the others were the type of intergenic chimeric ESTs. Furthermore, we analyzed the sequence structure of AV605399 with BLAT tool. The BLAT result showed that 1–53 nt of AV605399 was a part of the intron sequence of FSHR gene and 99–520 nt of AV605399 was a part of the intron sequence of PDZ domain containing 1 (PDZK1) gene. The results showed that AV605399 was a chimeric EST containing a certain part of FSHR gene and PDZK1 gene. The bioinformatics analysis suggested that chimeric RNA was an important mode of gene transcription in mammal. This research was supported by National Natural Science Foundation of China (No. 31060299), Guangxi Natural Science Foundation (2011GXNSFA018109) and Guangxi Science and Technology Project (Gui-Ke-Gong 0992013-1). We thank Dr. Xin Li for the help on data analysis.

Key Words: Chimeric RNA, transcription, mammal

1715

RNAseq analysis identifies global gene expression changes during rhesus macaque (*Macaca mulatta*) oocyte maturation

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The objectives of this study were to characterize the transcriptome profiles of rhesus macaque oocytes and to determine differences in transcript abundance during the oocyte maturation process. GV and MII oocytes were collected from superovulated females. Oocytes were classified as GV or MII based on morphological characteristics. Individual MII ($n = 3$) and GV ($n = 2$) oocytes were snap frozen in liquid nitrogen and stored at -80°C until processing. The whole cell lysate was processed with the SMARTer Ultra Low Input RNA for Illumina Sequencing kit (Clontech, CA, USA) for reverse transcription (RT) and amplification. Sequencing libraries were prepared using the TruSeq DNA sample preparation kit (Illumina, CA, USA). Libraries bearing unique indexes per sample were pooled and sequenced in a single lane of a HiSeq 2000 apparatus (Illumina) by a single run of 50 bp. Rhesus Macaque genome sequences and annotation (Mmu_1.2) were obtained from NCBI. Sequence analysis was performed using CLC Genomics Workbench (CLC bio, Denmark) and differential expression by DeSeq analysis. On average, 30 861 613 good quality reads were produced from each single oocyte. Using the RNASeq algorithms, 67% of reads mapped to annotated transcripts. Among the 32 015 annotated genes, we detected more genes expressed (reads per kilobase gene model per million mapped reads (RPKM) > 0.3) in GV than in MII oocytes ($10\,981 \pm 104$ and 9359 ± 190 respectively; $p < 0.01$). The lower number of genes expressed in MII oocytes is consistent with the reported transcript degradation that follows oocyte maturation in other species. The correlation of RPKM values for all the genes analyzed between GV oocytes or between any pair of MII oocytes was > 0.95. On the other hand the average correlation between all possible GV and MII oocytes pairs was 0.82. These results indicate a high repeatability of single oocyte transcriptome analysis with sufficient sensitivity to detect different stages of oocyte development at the global transcriptome level. Also, genes known to be almost exclusively expressed in oocytes were detected in the analysis, including FIGLA, OOSP1, STELLA, VASA, GDF9, BMP15, ZP1, ZP2, ZP3, ZP4, MOS, POU5F1, NPM2, and H1FO among others. DeSeq analysis between GV and MII oocytes indicated that 2670 genes were differentially expressed (adjusted $p < 0.01$ and $FC > 2$). Biological functions overrepresented among genes overexpressed in GV oocytes ($n = 1508$) included ribosome biogenesis, regulation of protein ubiquitination, RNA processing, and cellular respiration. Among genes overexpressed in MII oocytes ($n = 1162$) gene ontology analysis indicated that the most overrepresented biological processes were cell cycle, DNA repair, and RNA processing. In conclusion, this study demonstrates the feasibility of performing RNASeq analysis from single oocytes and shows that extensive differences in transcript abundance exist between GV and MII rhesus macaque oocytes.

Key Words: RNASeq, oocyte, *Macaca mulatta*, expression profiling, transcriptome

1750

Microarray analysis of single bovine embryo

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Microarrays have become a valuable high throughput technology that efficiently describes transcripts abundance of samples. However, such limitations like the large amounts of RNA needed, have impeded the use of microarray in bovine embryos. To solve this problem of the small RNA amount of embryos (2.3 ng), linear RNA amplification techniques have been used to produce micrograms of RNA. The aim of the present study was to establish a microarray methodology analysis using a single bovine embryo. Slaughterhouse ovaries were used to obtain oocytes (N = 180) which were matured and fertilized *in vitro* (Day 0). Presumptive zygotes were divided in two culture media: with low (SOFaa with 0.5% BSA and 2.5% FCS) or high (SOFaa with 0.5% BSA and 10% FCS) FCS concentration. Embryo development was evaluated after 7 days under standard culture conditions (at 38.5°C in atmosphere of 5% O_2, 5% CO_2 and 90% N_2). The produced blastocysts were placed in PBS solution and washed five times. A single blastocyst was frozen in a minimal volume of PBS and stored at -80°C until RNA extraction. Total RNA extraction of a single blastocyst was performed using the PicoPure RNA isolation Kit (Arcturus – Applied Biosystems®). Following extraction, RNA samples were DNAse treated (Qiagen®) and RiboAmp RNA Amplification Kit (Applied Biosystems®) was used to linearly amplify the mRNA fraction of total RNA using cDNA as template in a T7 RNA polymerase-catalysed amplification reaction. The aRNA output was evaluated through NanoDrop ND-1000 (NanoDrop Technologies®) and 2100-Bioanalyzer (Agilent Technologies®). A biotin-labelled cRNA and fragmented cRNA were obtained through 3'IVT Express Kit (Affymetrix®) to perform the hybridization using GeneChip Bovine Genome Array (Affymetrix®). Following hybridization, probe arrays were washed, stained and scanned. Microarray data analysis was performed in the software FlexArray 1.6.1.1. Genes with a fold change of at least 1.5 and a probability of $p \leq 0.05$ were considered differentially expressed. Blastocyst production was 41.8 ± 2.4 and 47.2 ± 2.8, respectively for low and high FCS concentration. The mean value of aRNA produced from a single bovine embryo after amplification was 2.2 ± 0.3 and 2.7 ± 0.9 μg (respectively for low and high FCS concentration) with a distribution size of 200–1500 nucleotides and A260:A280 ratio of 1.8–2.1. A total of 40 genes were differentially expressed between low and high FCS concentration. A total of 28 genes were annotated, with 37 genes upregulated and 3 genes downregulated by high FCS concentration. Therefore, the microarray analysis of single bovine blastocyst was possible and represents a powerful tool for the study of transcriptomics of embryos during pre-implantation development.

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Key Words: Microarray, embryo, bovine, fetal calf serun

1751

Accelerated genetic gain by embryo genotyping with the Illumina Bovine SNP50 Beadchip

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The Bovine SNP50 BeadChip which contains more than 40 000 SNPs provides to the breeders a new way to select the elite animals to improve the genetics of their herd. Today, hairs are sent after calving for genomic analysis and the best animals are selected for breeding. However, being able to do this selection at the embryonic level would accelerate the genetic gain by transferring only the best embryos to recipients. Embryo biopsies containing 8–12 cells were taken by microblade dissection from *in vivo* or *in vitro* compacted morulas or blastocysts (trophectoderm cells only). Because the SNP50 BeadChip hybridization requires micrograms of DNA, a whole genome amplification (WGA) step was required before hybridization. WGA was done using the Genomiphi V2 amplification kit that uses the phi29 DNA polymerase. DNA was extracted by alkaline cell lysis and directly used for a 2 h isothermal amplification at 30°C which produced typically 2–4 μg of DNA. A proprietary imputation algorithm using parental information was developed to determine missing SNPs due to an incomplete WGA. Comparison of the genotypes obtained from amplified biopsies to cell lines obtained from the culture of the remaining embryos confirmed the accuracy and efficiency of the WGA and imputation methods. Less than 2% inconsistencies were found between biopsies and unamplified DNA obtained from the cell lines after imputation. A second validation step was performed comparing amplified biopsies to their corresponding foetus recovered at day 40 (n = 6). After imputation, the mean percentage of SNP inconsistencies between biopsies and their corresponding foetus was 1.6% and the mean difference in direct genomic value between the amplified biopsy and the corresponding foetus was only 35 ± 34. This genotyping accuracy gives the possibility to breeders to decide of the destiny of an embryo based on the info obtained from an amplified biopsy. A third validation study was then performed where more than 400 embryos were biopsied and transferred to recipients or frozen. The mean call rate (% of SNP that produced a positive signal) of those amplified biopsies was 90.65 ± 0.05% before imputation. This quality of amplification was comparable to the results obtained in the preliminary experiments which gave accurate genomic diagnosis. Final comparison will be done in this ongoing field trial with the genotypes obtained from hairs of the corresponding calves at birth. In conclusion, an efficient method was developed to genotype bovine embryos with the Bovine SNP50 BeadChip. Our preliminary results show that this technology offers huge benefits to improve selection in crosses where a large genetic variation is observed among different embryos. Combined to *in vitro* embryo production, this technology allows breeders to multiply different crosses in a very short period of time while having access to the genomic value of the future animals produced. Thus, very powerful strategies can be established by breeders to enhance very quickly the genetic of their herd. NSERC, MAPAQ and CQRL supported this work.

Key Words: Embryo, genotyping, genomic, SNP, whole genome amplification

1752

A comprehensive survey of genetic variation of Italian sheep breeds at 50 K SNP loci: implications for breed management and conservation

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The objective of the study was to perform a comprehensive survey of genetic variation in Italian sheep breeds through high-throughput SNP genotyping in order to provide population genetics data that may be useful in orienteering management and conservation actions. A set of 496 animals, representative of 20 sheep breeds/populations evenly distributed all along the Italian peninsula were genotyped at the Ovine SNP50 BeadChip®. The most widespread dairy and meat breeds as well as some of the most endangered breeds were included in the study. SNP loci resulted to be a very powerful tool to finely reconstruct both known and hidden breed relationships, also allowing differentiating among ancient and more recently admixed population groups through sharing analysis of varying window size haplotypes. The Multi Dimensional Scaling (MDS) analysis generally highlighted a clear and continuous geographical gradient in the genetic diversity pattern; this result suggests a major role for short/medium-range admixture in shaping modern-day breed genetic background, consistently with historically extensive sheep farming practices and seasonal transhumance movements. A major exception was represented by the introgression of Bergamasca (Northern Italy) into Appenninica (Central Italy), as clearly highlighted by SNP data. Analyses are still under way in order to detect possible selection signatures. Once completed, the study will provide a better understanding on amount, distribution and functional significance of genetic variation in Italian sheep breeds that may contribute to more efficient management, conservation and valorisation of Italian sheep genetic resources.

Key Words: Single nucleotide polymorphisms, sheep, genetic diversity, conservation genetics

1753

Using metabolomics to non-invasively assess bovine embryo viability *in vitro*

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Early embryonic mortality contributes upwards of 75–80% of all embryonic and fetal deaths. Currently, *in vitro* embryo assessment of mammalian embryos, including bovine, relies on morphology and cleavage rates to non-invasively assess the health of an embryo. However, despite the importance of morphological evaluation, it is a poor predictor of embryo viability as it is inherently subjective, nor does it relay information regarding the genetic status of the embryo. Adjunctive technologies, such as metabolomics, have aimed at exploring the health of a cell, based on its secreted/uptaken metabolite constituents. Proton nuclear magnetic resonance (^{1}H NMR) is a robust analytical tool that simultaneously measures all metabolites in a given sample with minimal sample preparation and little chemical bias, making it ideal for the analysis of the spent culture media. We hypothesize that embryos developing at different rates differ in their metabolomic signature. The objective of this study was to determine the metabolomic signatures of fast (FG) and slow (SG) growing embryos at timed stages of development. Standard *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) protocols were used on oocytes aspirated from abattoir obtained ovaries. Presumptive zygotes were placed individually in 40 μl synthetic oviductal fluid (SOF) culture drops. Media from FG embryos was collected at 2-cell (30 h post fertilization or hpf), 4-cell (42 hpf), 8-cell (49 hpf), 16-cell (90 hpf), morula (144 hpf) and blastocyst (168 hpf) and from SG embryos 12- to 24 h later to reach equivalent embryo stage. Analysis was performed on a 600 MHz Bruker NMR spectrometer. Metabolites identified and analyzed included energy substrates, lactate and pyruvate; and amino acids, alanine, glutamate, leucine, isoleucine, and valine. Changes in metabolite levels were analyzed using a Two-Way Analysis of Variance and Principal Component Analysis. Data indicates distinct differences between the 4-cell SG and FG embryos for pyruvate ($p < 0.05$, $n = 9$). Production and consumption also differed considerably for each metabolite pre and post maternal to embryonic transition (MET), for example, consumption was high during the 2- to 4-cell stage of glutamate and alanine, followed by a significant level of production ($p < 0.05$, $n = 9$) at the morula and blastocyst stage for FG and SG groups. On average, FG embryos consumed more amino acid (valine, leucine, isoleucine, glutamate, and alanine) than SG groups, although not statistically significant. To our knowledge, this is the first study of its kind to characterize the metabolomic profiles of SG and FG bovine embryos produced *in vitro*. To conclude, ^{1}H NMR provides a sensitive method for detecting metabolomic differences between SG and FG embryos, providing evidence towards the use of metabolomics as a tool for non-invasively assessing an embryo's developmental potential. Furthermore, finding biomarkers of viability will improve the selection procedure for IVP cattle embryos and improve implantation rates.

Key Words: Metabolomics, non-invasive assessment, bovine preimplantation embryo, nuclear magnetic resonance, early embryonic mortality

1754

Transcriptome changes during bovine blastocyst formation in response to culture environment

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Identification of transcripts involved in blastocyst formation and analysis of their expression patterns in response to culture conditions may help us to understand the mechanisms that control blastocyst formation and to understand why certain embryos fail to reach this stage. Therefore, we aimed to examine the effect of alternative *in vivo* and *in vitro* culture conditions during the time of blastocyst formation on the transcriptome profile of bovine blastocysts. With the advent of non-surgical transvaginal endoscopic technology, two different blastocyst groups were produced. The first group (Vitro_morula) was matured, fertilized and cultured *in vitro* (using CR1 medium supplemented with 10% OCS) until morula stage (Day 5 post fertilization) then transferred to synchronized recipients and blastocysts were collected at day 7 by uterine flushing. The second group (Vivo_morula) was matured, fertilized and cultured *in vivo* until morula stage (Day 5) then flushed out and cultured *in vitro* until day 7. Complete *in vitro* (IVP) and *in vivo* blastocysts were produced and used as controls. Gene expression pattern between each blastocyst group and *in vivo* blastocyst control group were compared using EmbryoGENE's bovine microarray over six replicates of each group. Interestingly, all blastocyst groups showed high number of differentially expressed genes (DEGs) compared to *in vivo* control group. Vivo_morula, Vitro_morula and IVP groups showed 773, 842 and 841 DEGs, respectively compared to control group ($FC \geq 2$, $FDR \leq 0.05$). Ontological classification of DEGs indicating that cell death was the most significant function in all groups with up-regulation of most of the DEGs involved in this function compared to *in vivo* control group. In addition, a clear significant pattern of lipid metabolism related genes was found *in vitro*_morula and IVP groups, but not in

Vivo_morula group, with down-regulation of most of lipid metabolism related genes compared to control group. Pathway analysis revealed that integrin signalling and NRF2-mediated oxidative stress pathways were the dominant pathways *in vivo*_morula group. However, TNRF1 signalling pathway was the dominant *in vitro*_morula and IVP blastocyst groups. A total of 183 transcripts found to be commonly expressed *in vitro*_morula and Vivo_morula groups and gave the same pattern of expression in both groups with high abundance of cell death related genes. In conclusion, *in vitro* culture conditions critically determining embryo quality, measured in terms of gene expression patterns, during the time of blastocyst formation. Moreover, here we identified genes/pathways that are influenced by *in vitro* conditions. This will be helpful in order to modify culture conditions at this critical stage of development to enhance the development of competent blastocyst.

Key Words: Bovine, blastocysts, embryo culture

18. IVF/ICSI/IVP:

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Developmental rates of IVP bovine embryos using minigradient of Percoll®, Isolate®and Optiprep®

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The sperm selection method is one important step of embryo IVP systems and may influence the embryonic development rates. The objective of this study was to compare the efficiency of different sperm selection methods used in bovine IVP systems in terms of embryo development. A total of 2455 oocytes obtained from ovaries of *Bos taurus* cattle collected in a slaughterhouse were used in this experiment (n = 11 replicates). After *in vitro* maturation oocytes were distributed and inseminated according to four treatments: (i) Conventional Percoll group (90 and 45%) – 4 ml, centrifuged at 700× *g* for 20 min, (ii) Minipercoll group (90 and 45%) – 800 μL, centrifuged at 700× *g* for 5 min, (iii) Miniisolate group (90 and 45%) – 800 μL, centrifuged at 700× *g* for 5 min; and (iv) Minioptiprep group (30.28 and 26%) – 1.2 ml, centrifuged at 900× *g* for 15 min. Developmental rates at D2 (cleaved/oocytes inseminated) and at D8 (blastocyst/oocytes inseminated) of culture were compared among treatments. Data were analysed by using chi-squre test. The cleavage rates were similar among the Conventional Percoll® and Miniisolate® groups (70.4% vs. 67.1%; $p > 0.05$). Minipercoll® and Minioptiprep® groups had lower cleavage rate than Conventional Percoll® (65.8%, 57.9% vs. 70.4%; $p < 0.05$). At Day 8 blastocyst rate for Minipercoll, Minioptiprep and Conventional Percoll were 16.1%, 16.9%, and 18.4%, respectively ($p > 0.05$). Among the minigradientes, the Miniisolate had higher blastocyst yield than Minipercoll and Minioptiprep (21.1% vs. 16.1% and 16.9%, respectively; $p < 0.05$). In conclusion, the minigradients, in the concentration and volume used in our experiment, reached similar results to the Percoll Conventional group. Therefore, they can be use in the routine of IVP bovine embryos as alternatives with same viability and lower time consuming and production cost.

Key Words: Sperm preparation method, Percoll, MiniPercoll, MiniOptiprep, MiniIsolate

1801

Atp content and gene expression profile in cultured bovine morula resulting from high glucose exposure before compaction

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To characterise the energy-related stress of embryos caused by *in vitro* culture (IVC), bovine embryos were exposed to sub-lethal condition of high glucose [(HG) = 5 mM, (control glucose) = 0.2 mM] during the pre-compaction period and assessed for developmental, ATP content and transcriptomic outcomes. IVC was done in drops of Synthetic Oviduct Fluid medium with 0.4% BSA and amino acids at 5% O_2. Results in embryonic development rates indicated that HG treatment decreased the rate of morula-blastocyst transition (control = 76%, HG = 55%), and was associated with a significant ATP/ADP drop in expanded blastocysts. Using the EmbryoGENE DNA chip (Agilent) and following a 2-colors microarray design with 4 replicates of 10 embryos each, transcriptome comparison (Quantile and Loess for extra and intra-array normalisation respectively, Limma as statistical test) between control and HG treated morulae revealed a large number of differentially expressed genes (DEGs) which were functionally associated with mitochondrial dysfunction and TP53 signalling. This transcriptomic profile was likely to represent a mixed population including a fraction of dying morulae that are about to stop development and a fraction of surviving morulae that are adapting to stress and would reach the blastocyst stage. Further analysis of HG-related DEGs in morula compared to blastocyst suggested that HNF4a under-expression would be an early adaptive response to HG in the establishment of a Warburg-like effect. Taking together, these results shed new light on how *in vitro* culture may drive early bovine embryos to progressive and sometimes lethal modifications of their transcriptomic profile.

Key Words: Morula, energetic metabolism, *in vitro* culture, diabetes, Warburg

1802

Pregnancies after transfer of bovine embryos cultured in a novel microfluidic device applied with a trap and release system

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Recently microfluidic culture systems have been developed for embryos in several species. We have developed a microfluidic device for culture of individual bovine embryos which integrates an automatic trap and release system for embryos. Embryos are arrayed and tracked in the flow channel of microfluidic system and their development is recorded by time-lapse cinematography. Selective retrieval of individual embryos can be achieved using electrolytically-generated bubbles. In the first experiment, cumulus oocyte complexes (COCs) aspirated from ovaries of slaughtered cows were *in vitro* matured for 22 h, and fertilized with frozen-thawed semen for 6 h. After 24 h of *in vitro* culture, 2-cell embryos (n = 263) were loaded in the device without selective retrieval system and cultured in CR1aa medium supplemented with 5% calf serum at 38.5 C, 5% CO_2, 5% O_2 and 90% N_2 with 1ï l/min flow rate for additional 6 days. In the control group, 2-cell stage embryos (n = 257) of the same source were cultured in a conventional micro-droplet system. In the second experiment, COCs collected by ovum pick-up were matured, fertilized and cultured for 24 h as in experiment 1, and then 2-cell embryos were loaded in a device applied with the selective retrieval system for 6 days. To demonstrate their *in vivo* developmental competence, blastocysts derived from a microfluidic device were transferred into the uterine horn on the ipsilateral side of corpus luteum of recipient cows. In the control, blastocysts derived

from the microdroplet system (WOW system, Sugimura et al., Biol Reprod 83(6): 970–8, 2010) were transferred as for the microfluidic device group. Rates and morphological classification of blastocysts on Day 7 (Day 0 = the day of insemination) of *in vitro* culture were recorded and data were analyzed by ANOVA. In experiment 1, the rates of blastocyst formation in the microfluidic device (49.1 ± 4.7%) tended to be higher ($p = 0.056$) than those in the micro-droplet (32.9 ± 6.3%). The percentage of high quality (Code 1; IETS manual ver. IV) blastocysts in the microfluidic device (79.2 ± 3.6%) was significantly ($p < 0.05$) higher than that in the micro-droplet (51.7 ± 8.0%). In experiment 2, in total 88 embryos were cultured in the device, and 48 (54.5%) embryos developed to the morula stage or beyond on Day 7. Then, 19 blastocysts (code 1) were transferred into recipient cows, and two pregnancies (10.5%) were detected. The pregnancy rate in microfluidic was significantly ($p < 0.05$) lower than that in the control (47.1%, $n = 17$). In conclusion, the microfluidic device increased the percentage of high quality blastocyst as compared to the micro-droplet, but embryos obtained by microfluidic device applied with a novel selective retrieval system gave a reduced pregnancy rate. This work was supported by the Research and Development Program for New Bio-industry Initiatives.

Key Words: Microfluidic, embryo culture, *in vitro* fertilization, pregnancy, bovine

1803

In vitro production of Mediterranean buffalo embryos using sexed semen results in low fertilization and development rates

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The use of embryo-based biotechnologies could significantly advance buffalo genetic improvement especially in view of the completion of buffalo genome sequencing. *In vitro* embryo production (IVP) and sexed semen can play an important role in exploiting superior female genetics. At present, very few reports are available on the use of sexed semen for the production of buffalo embryos (Liang et al., *Theriogenology* 69 (7), 822–6). Therefore the scope of this work was to compare the efficiency of IVP using for *in vitro* fertilization (IVF) sexed and unsexed (control) semen from eight different buffalo bulls. This was achieved by: (i) screening the *in vitro* fertilization ability of sexed buffalo semen using bovine oocytes, and (ii) using the best buffalo bull for IVF to investigate the developmental competence of buffalo oocytes fertilized with sexed semen vs. unsexed semen. Holstein Friesian and Mediterranean buffalo oocytes were recovered at the slaughterhouse, matured *in vitro* for 22–24 h (bovine) and 18–20 h (buffalo) in medium TCM199 supplemented with 10% FCS and 0.5 IU of FSH and LH in 5% CO_2 at 38.5°C. Frozen semen of 8 buffalo bulls was used for IVF. After thawing the semen was separated on a Redigrad gradient then diluted into TALP-IVF medium supplemented with 1 μg/ml of heparin and penicillamine, hypotaurine and epinephrine (PHE). Unsexed semen was used at a concentration of 2 million sperm/ml and sexed semen at a 3–4 million/ml. Presumptive hybrid bovine/buffalo zygotes were fixed at 18–20 h post IVF and stained with lacmoid to assess pronuclei formation, while buffalo zygotes were transferred in mSOF supplemented with MEM aminoacids and cultured for 7 days. Embryos were graded for freezing on day 6 and 7. The average fertilization and polyspermy rates of bovine oocytes were 94% (range 62–100) and 21% for control semen, 34% (range 28–50) and 2% for sexed semen respectively. Buffalo zygotes sired by the best performing bull were assessed for cleavage and blastocyst rate. Cleavage, development to freezable blastocyst/oocytes and blastocyst/cleaved was 34% (254/748), 3.9% (29/748) and 11.4 (29/254) for sexed semen and 59.3% (1433/2418), 9.3% (224/2418) and 15.6% (224/1433) for control semen respectively. In conclusion our data indicate that buffalo IVF with sexed semen results in a very significant reduction in the efficiency of embryo production (from 9.3% to 3.9%; $p < 0.01$, Chi Square). This is due to a lower fertilization and cleavage rate rather than to a reduced developmental competence because we found no significant difference in the ability of oocytes fertilized with sexed semen to form freezable blastocysts as compared to oocytes fertilized with unsexed semen (11.4% vs. 15.6%). More work is required to reduce the damage inflicted to sperm during sexing procedures and by adapting the IVF conditions in order to achieve higher fertilization rates comparable to control semen. This work was supported by Regione Lombardia, Por Fers 2007-2013, n°13827741, InnovaB.

Key Words: IVF, embryo development, sexed semen, buffalo, oocyte maturation

1804

Heparin and cumulus cells affect *in vitro* fertilization and developmental competence of goat oocytes

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Considerable research has been focused on *in vitro* production (IVP) of goat embryos to improve its efficiency. However, simplified methods providing high and reproducible results would allow wider use of this promising technology. The aim of this study was to observe the effects of FSH in maturation medium, the use of heparin in fertilization medium and the time of cumulus cell removal on embryo development in goats. During six replicates, slaughterhouse-derived oocytes were randomly allocated to two IVM groups: TCM 199 supplemented with 10 ng/ml EGF and 100 μM/ml cysteamine with or without 50 ng/ml ovine FSH (Ovagen). Matured oocytes were incubated with frozen-thawed semen in synthetic oviduct fluid (SOF), containing 10% heat-inactivated sheep serum, 40 μg/ml gentamicin and the presence of 5 μg/ml heparin or not. The cumulus oophorus was removed by vortex before (denuded oocyte group; DO) or after (cumulus oocyte complex group; COC) fertilization (total of 8 different treatments). After fertilization, presumptive zygotes were cultured for 8 days in groups of 25 in oil overlaid droplets of 25 μl of SOF medium supplemented with 10% FCS at 48 hpi. The proportions of oocytes that cleaved and developed to the blastocyst stage were assessed at Days 2 and 8 post-insemination, respectively. Cleavage rate and blastocyst yield were compared using chi-square analysis with $p < 0.05$ considered significant. No differences were observed in cleavage rate among all conditions. Similarly, FSH did not influence embryo development (not shown). In COC, the presence of heparin significantly increased the yield of blastocysts from oocytes entering into IVM ($p < 0.05$; Table 1). However, this effect was not significant when cleavage rate, or development of cleaved embryos to the blastocyst stage, were considered separately. This resulted in a significantly increased yield of blastocysts from oocytes entering into IVM ($p < 0.05$; Table 1). This effect was not observed in DO. In conclusion, these data indicate that when EGF and cysteamine are added to maturation media, FSH offers no further benefit to developmental competence. However, embryo development can significantly be affected by the moment oocytes are denuded and the presence or absence of heparin during fertilization. These results suggest that cumulus cells have an important role in the regulation of *in vitro* fertilization of goat oocytes and heparin enhances these interactions.

Key Words: IVF, caprine, blastocyst

Table 1. Effect of heparin on intact cumulus–oocyte complexes (COC) or denuded oocytes (DO) during IVF on *in vitro* production of goat embryos (six replicates)

Conditions	Heparin	Oocytes (n)	Cleavage (%)*	Blastocyst (%) from cleaved**	Blastocyst (%) from oocytes**
COC	-	365	66[a]	63[a]	42[a]
COC	+	364	72[a]	75[a]	54[b]
DO	-	360	64[a]	59[a]	38[a]
DO	+	358	68[a]	61[a]	41[a]

Within a column, values with different superscripts differ significantly ($p < 0.05$). *Day 2 pi; **Day 8 pi; pi: post-insemination.

1805

The effect of phytoemagglutinin on *in vitro* embryo development of ovine prepubertal oocytes

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The *in vitro* embryo production from oocytes of prepubertal animals is still suboptimal due to poor yield and quality of blastocyts which result in low survival to term after embryo transfer. Improvements of *in vitro* culture could enhance the efficiency of *in vitro* embryo production from prepubertal oocytes. Phytoemagglutinin (PHA), a sugar-specific lectin with a wide variety of biological activities, including agglutination and mitogenesis, increases the percentage of porcine embryo development and blastocyst quality and improves the efficiency of somatic nuclear transfer in cattle. The objective of this study was to investigate the influence of PHA on *in vitro* embryo development of prepubertal ovine oocytes after *in vitro* maturation and fertilization. Oocytes recovered from prepubertal ovine ovaries (30–40 days of age) were *in vitro* matured and fertilized with frozen-thawed ram semen. Zygotes were cultured in SOF +0.4% BSA for 7 days. PHA (15 μg/ml) was added to the culture system at different interval times: (A) from 1 to 7 day of IVC; (B) from 4 to 7 day of IVC; (C) from 4 to 5 day of IVC; (D) IVC without PHA (control). At the end of culture development up to the blastocyst stage was recorded and the number of nuclei was counted by Hoechst 33342 staining. *In vitro* embryo development and blastocyst cell number were analyzed with chi-square test and analysis of Variance (ANOVA), respectively. Results showed that PHA did not significantly increased the percentage of blastocysts in systems A (37.8%) and B (39.5%) compared to those of control group (39.1%). Treatment with PHA from 4 to 5 day of culture (C) significantly, increased embryo development to the blastocyst stage (52.4% vs. 39.1%, $p < 0.05$) and total nuclei number compared to control group (122, 69 ± 6.95 vs. 81.37 ± 4.52, $p < 0.01$).Our study demonstrates that PHA improves the blastocyst yield and quality when added from 4 to 5 day of embryo IVC. Molecular mechanisms by which PHA affect embryo development are not clear at present. Higher blastocyst cell number could be ascribed to mitogenic activity of PHA. Moreover, PHA could positively affect morula compaction and transition to blastocyst stage.

Key Words: Ovine oocytes, phytoemagglutinin, *in vitro* embryo development

1806

The effect of culture medium changes on *in vitro* production of sheep embryos

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Medium change during *in vitro* cell culture provides fresh nutrients and is useful for the withdrawal of toxic metabolites. Although this practice is essential for *in vitro* cell culture, it is not well defined for *in vitro* embryo culture in sheep, and is variable among laboratories. The aim of this experiment was to determine the effect of embryo culture medium change on *in vitro* culture of sheep embryos. A total of 712 cumulus oocyte complexes (COCs) surrounded by at least two layers of granulosa cells and homogeneous cytoplasm were selected after follicle aspiration from ovaries obtained at a slaughterhouse (four replicates). For *in vitro* maturation (IVM) the selected COCs were incubated in TCM199 drops supplemented with estrous ovine serum (EOS), FSH, LH, cysteamine and antibiotics; covered with embryo tested mineral oil for 24 h, at 39°C and 5% CO_2 in air. For *in vitro* fertilization (IVF), matured oocytes were incubated for 22 h in synthetic oviductal fluid (SOF) drops supplemented with EOS, heparin and hypotaurine; using 1×10^6 frozen-thawed spermatozoa selected by swim-up method. The beginning of IVF was considered Day 0. After IVF, cumulus cell removal was performed by gentle pipetting and *in vitro* culture of presumptive zygotes was performed in 100 μl drops of SOF supplemented with amino acids and bovine serum albumin, with 25–30 embryos/drop covered with mineral oil at 39°C and 5% O_2, 5%CO_2, 90% N_2. At the end of IVF, presumptive zygotes were divided in three experimental groups where the culture medium was replaced on Day 6 (n = 232), as a control group; Days 3 and 6 (n = 243); or Days 2, 4, and 6 (n = 237). The medium changes consisted of replacing 80 μl of old medium (i.e. 80%) with fresh medium previously equilibrated in the incubator, using a micropipette. Cleavage rate (two cell embryos/oocytes), morula and blastocyst rate (morulae and blastocysts/oocytes), and blastocyst rate (blastocysts/oocytes) were recorded on Day 2, Day 6 and Day 7–8 after fertilization, respectively. Statistical analysis was performed by logistic regression. Results are shown in Table 1. An improvement on the number of blastocysts/cleaved embryos was found when culture medium was changed on Day 3 and 6, but not when medium was changed every 48 h. These results highlight the importance not only of the medium change, but also of the optimal moment to do it. Probably, these results are explained by the maintenance of the equilibrium between providing fresh nutrients, taking out toxic metabolites, and preserving the microenvironment provided by embryo secretions.

Table 1. Effect of medium changes during IVC on the cleavage and development rates of sheep embryos

	Cleavage rate (Day 2)	Morulae and blastocysts/ oocytes (Day 6)	Blastocysts/ oocytes (Day 7–8)	Blastocysts / cleaved (Day 7–8)
Day 6	82.8%[a] 192/232	45.7%[a] 106/232	33.6%[ab] 78/232	40.6%[a] 78/192
Day 3 and 6	80.2%[a] 195/243	51.9%[a] 126/243	42.0%[b] 102/243	52.3%[b] (102/195)
Day 2, 4 and 6	83.1%[a] 197/237	48.9%[a] 116/237	30.8%[a] 73/237	37.1%[a] 73/197

Values within column with different letters differ ($p < 0.05$).

1807

Preliminary comparison between different ovum pick-up intervals and different maturation methods

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Commercial ICSI is hampered by low oocyte yield and embryo production. Multiple ovum pick-ups (OPU/cycle) could be an alternative method to increase cumulus-oocyte complex yield (COC/cycle). This study had a double objective: (i) to compare efficiency of different OPU intervals in providing developmentally competent COCs, and (ii) to compare the ability of two different maturation methods to provide *in vitro* matured COCs recovered by OPU. Six mares were initially punctured at D0 (>21 days pause after last OPU) and received the 1st dose of the Progesterone treatment (kindly provided by BET laboratory, Lexington, KY), (300 mg/cc, 7cc IM every 7 days). Following, each mare was punctured at 10, 14 and 21 days intervals (D10; D14 and D21) in different orders to compensate season effect. Number of follicles punctured (foll/session) and recovered COCs (COCs/session) were recorded and COCs were stained with Brilliant Cresyl Blue for qualitative assessment of developmental stage at recovery (BCB+ and BCB−). On 2nd part of the study, different intervals were not taken into account, but BCB+ and BCB− were matured separately *in vitro* either immediately (imm-IVM= IVM in maturation medium: DMEM-F12 + 50 ng/ml FSH and 10 ng/ml LH+ 50 ng/ml EGF + 10%FBS + 1%antibiotic antimycotic Gibco; 38.2°C, 5% CO_2) or after a pre incubation of 18 h in follicular fluid collected from dominant, non pre-ovulatory follicles followed by IVM in the same maturation medium, 38.2°C, 5% CO_2 (del-IVM). Following maturation, oocytes were evaluated for polar body extrusion (MII). MII oocytes were manipulated by conventional ICSI and cultivated for 48 h at 38.2°C in 4 wells dishes containing 20 μl DMEM-F12 + 10%FBS + 1%antibiotic antimycotic Gibco in plastic pockets inflated with 5% O_2, 5% CO_2, 90% N_2. Cleavage was observed under microscope and confirmed by Hoechst staining. Foll/session; COC/session; BCB + COC/session results are expressed as mean and SEM. We performed ANOVA for comparison of means and chi-squre for percentages. D0 presented the highest foll/session (20.2 ± 2.9) and

COCs/session (10.0 ± 1.9) ($p < 0.05$). D10 was the less efficient ($p < 0.05$) for COCs/session (4 ± 0.82), BCB + COCs/session (2.8 ± 0.7) and recovery rate (29%). D14 and D21 were intermediary for foll/session (14 ± 2.5 and 15.2 ± 2.9) and COCs/session (6.3 ± 2.4 and 7.2 ± 1.2) respectively. Recovery rate, 50%; 45%; 47%, and BCB + COCs/session (5.8 ± 1.49; 4.8 ± 1.08 and 5.5 ± 1.67) were similar for D0, D14 and D21 respectively. Del-IVM improved the maturation rate of BCB-COCs (63%, n = 30 del-IVM vs. 25%, n = 12 imm-IVM; $p < 0.05$) and did not influence maturation rate of BCB + COCs (60%, n = 70 deI-IVM vs. 59%, n = 44 imm-IVM; $p > 0.05$). We observed a non significant ($p > 0.05$) improvement of cleavage rate in del-IVM COCs compared to imm-IVM COCs (BCB+: del-IVM = 27%, n = 41 vs. imm-IVM = 15%, n = 21); (BCB-: del-IVM = 13%, n = 20 vs. 0%, n = 3). Summarizing, interval of more than 21 days (D0) is the most work efficient, followed by D14 and D21. The low efficiency of 10 days OPU interval prevents its clinical application. Delayed maturation can improve maturation rates of BCB-COCs.

Key Words: Equine, ICSI, *in vitro* maturation, oocytes, ovum pick-up

1808

Assessment of cytoplasmic markers after simulated stress in *in vitro* maturation of porcine oocytes

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Stress can affect reproduction by altering the female endocrine environment. An altered milieu where the oocyte is developing may influence oocyte competence. The relocation of mitochondria throughout oocyte maturation and the polymerization of actin into microfilaments are important for the progression of meiosis; hence, both parameters are useful markers of cytoplasmic maturation. Cytoplasmic maturation was assessed after exposure to blood plasma from sows which had experienced simulated stress through repeated injections of adrenocorticotropic hormone (ACTH; 5 μg/kg every 4 h for 48 h from the standing estrous) before ovulation. Pig oocytes were exposed to 10% plasma from ACTH-treated sows (ACTH group), NaCl-treated control sows (no-ACTH group) or media with bovine serum albumin (BSA group) for 46 h of *in vitro* maturation. The plasma used was collected at 36 and 12 h (±2 h) before ovulation (for the first 24 h + last 22 h of maturation, respectively). Cortisol and progesterone levels were higher in the ACTH-group than in the control-group. Actin microfilaments (MF) and mitochondrial patterns were evaluated by laser scanning confocal microscopy using Alexa Fluor-488 Phalloidin and MitoTracker Orange CMTMRos, respectively. To assess nuclear maturation, oocytes were stained with Hoechst 33342. A group of non-matured oocytes was included in the analysis as a negative control of meiosis progression. Data were analyzed using ANOVA, Cramér's V coefficient and Pearson chi-squared test. Nuclear maturation did not differ between treatments. A complete cortex MF pattern was present in 60% of oocytes in all groups, but only in 20% of non-matured oocytes ($p < 0.001$). Lower number of oocytes had an even pattern of cytoplasmic MF in the plasma groups (19%, n = 36; 45%, n = 29; 11%, n = 35; ACTH, BSA and no-ACTH groups, respectively; $p < 0.01$). In most of the non-matured oocytes, cytoplasmic MF were not observed (84%, n = 63, $p < 0.001$). No differences were detected between treatments in the presence of transzonal cumulus cell projections after maturation (ranging from 41 to 64%). In comparison, 98% of non-matured oocytes presented transzonal projections ($p < 0.001$). There was an effect of treatment and maturation status ($p < 0.001$) on the distribution of active mitochondria. The peripheral pattern was the most abundant in non-matured oocytes (60%, n = 62), whereas the diffused pattern was predominant in ACTH (44%, n = 32) and BSA (54%, n = 28) groups. However, in the no-ACTH group, a higher proportion of oocytes had a polarized mitochondrial pattern (37%; n = 38) and lower number of oocytes displayed a diffused pattern (24%). In summary, the mitochondrial pattern and actin MF differed according to the maturation stage of the oocyte. After simulated stress, subtle differences were observed in actin MF in matured oocytes and the mitochondrial pattern was associated with the treatment. Funded by Formas and Edvard Nonnens stipendiefond.

Key Words: Oocyte, *in vitro* maturation, actin microfilaments, mitochondria, laser scanning confocal microscopy

1809

Effects of dibutyryl cAMP on ROS and GSH of porcine oocytes, apoptosis of cumulus cells, and embryonic development

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Dibutyryl cyclic adenosine monophosphate (dbcAMP) can be used to block nuclear maturation and allow the oocytes to improve their developmental competence. Glutathione (GSH) content is one of the possible marker of oocyte competence and might protect oocytes against reactive oxygen species (ROS). Cumulus cells (CC) support oocyte competence and CC apoptosis affects oocyte competence. Therefore, the current study was conducted to investigate the effects of dbcAMP supplemented into porcine maturation medium on the levels of ROS and GSH of oocytes, and CC apoptosis. In addition, embryonic development following *in vitro* fertilization (IVF), chemical activation (CA), or electrical activation (EA) were determined. In the first experiment, cumulus-oocyte-complexes (COCs) were cultured at 39°C in a 5% CO_2 atmosphere in North Carolina State University 23 (NCSU-23) supplemented with 0.5, 1, 5, or 10 mM dbcAMP for 22 h, and for another 22 h after removal of dbcAMP. COCs cultured for 44 h without dbcAMP were regarded as control. GSH and ROS levels of oocytes, and CC apoptosis were assessed at 44 h of culture. In the second experiment, COCs were cultured in 0.5 or 1 mM dbcAMP for 22 h and an additional 22 h without dbcAMP and then fertilized *in vitro* or activated parthenogenetically. Embryonic development and cell number of blastocyst were investigated. The ROS levels of oocytes and embryos were examined according to the dichlorohydrofluorescein diacetate (DCHFDA) method. GSH levels of oocytes were detected by Cell Tracker Blue 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CMF2HC). CC apoptosis of COCs cultured *in vitro* for 44 h were assessed by terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling (TUNEL) assay kit. All data were analyzed by Duncan's multiple range test using the Statistical Analysis System ver. 8x (SAS, Cary, NC, USA). As the results, GSH levels of 0.5 mM dbcAMP and the control groups were higher than those of 1, 5, and 10 mM dbcAMP groups (74.2 ± 2.7 and 76.1 ± 0.7, respectively vs. 63.9 ± 1.6, 54.1 ± 1.8, and 34.6 ± 4.1, respectively; $p < 0.05$). ROS levels of oocytes in 0.5 mM, 1 mM dbcAMP and the control groups were lower than those of 5 mM and 10 mM dbcAMP groups (104.0 ± 4.5, 116.7 ± 8.7, and 102.6 ± 4.4, respectively vs. 136.8 ± 4.8 and 144.9 ± 11.3, respectively; $p < 0.05$). Apoptosis levels of CC in 0.5 mM, 1 mM dbcAMP and the control groups were lower than those of 5 mM and 10 mM dbcAMP groups (1.4 ± 0.5%, 1.1 ± 0.3%, and 2.2 ± 0.5%, respectively vs. 18.1 ± 4.9% and 22.7 ± 4.4%, respectively; $p < 0.05$). Cleavage rates and blastocyst formation rates, cell number of blastocyst were not significantly different among 0.5 mM dbc AMP, 1 mM dbcAMP and the control groups. Within the same experiment and among groups, no differences were observed between CA and EA method. In conclusion, there is no difference between the 0.5 mM dbcAMP and the control groups for the studied parameters. The increase of GSH is accompanied by a decrease of ROS in 0.5 mM dbcAMP treatment.

Key Words: Porcine oocytes, dbcAMP, ROS, GSH, apoptosis

1810

Effects of the zona pellucida on *in vitro* sperm penetration into porcine oocytes

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The zona pellucida (ZP) is considered to play an important role in prevention of polyspermy in mammalian oocytes. In porcine oocytes, however, polyspermy occurs with high frequency and is considered to be an obstacle to efficient production of normal embryos. The function of the ZP is still not well understood in pigs, even during *in vitro* fertilization (IVF). In the present study, we investigated the effects of

the ZP on sperm penetration *in vitro*. We collected *in vitro*-matured oocytes with a first polar body after denudation of cumulus cells (ZP+ oocytes). We treated some of them with 0.5% pronase and freed them from the ZP by pipetting (ZP− oocytes). Firstly, to evaluate the function of the ZP, we performed IVF and compared the sperm penetration statuses of ZP+ and ZP− oocytes using frozen-thawed epididymal spermatozoa from four boars. About 10 oocytes were co-incubated with sperm in a 100-μl drop of Pig-FM (Suzuki et al. 2001) for 3 h at a concentration of 1×10^4/ml. More than three replications were carried out in each experiment thereafter. At 10 h after initiation of IVF, the proportions of ZP+ oocytes penetrated by sperm were significantly higher than those of ZP− oocytes for two boars ($p < 0.05$; 93.2% vs. 51.2% and 97.4% vs. 71.1%, respectively). The average numbers of penetrated sperm per oocyte were also higher in ZP+ oocytes for three boars (3.5 vs. 2.3, 4.5 vs. 1.6 and 3.9 vs. 2.0 sperm/oocyte, respectively) (a total of 74–149 oocytes were used for each group). Secondly, to elucidate whether polyspermy was prevented by the ZP and/or oolemma, we evaluated the penetration statuses of ZP+ and ZP− oocytes for a single sperm lot at 1–10 h after IVF. The proportions of oocytes penetrated by sperm in both groups increased significantly with time. The number of penetrated sperm per oocyte increased significantly in ZP+ oocytes, but not in ZP− oocytes. Thirdly, to examine whether or not the oolemma prevented polyspermy, we evaluated the effects of prolonged insemination (5 h) on sperm penetration of ZP− oocytes in comparison with the control (3 h). At 10 h, the proportion of oocytes penetrated by sperm in the 5 h group was significantly higher than that in the control group ($p < 0.05$; 71.1% and 47.2%, respectively). The number of sperm per oocyte in the 5 h group was significantly higher than that in the control (1.8 and 1.5 sperm/oocyte, respectively) (a total of 67–142 oocytes were used for each group). Finally, to evaluate the effects of pronase treatment on sperm penetration, we denuded the ZP mechanically using a micromanipulator without pronase treatment and compared the sperm penetration with that of ZP-oocytes treated with pronase. There was no difference in the sperm penetration statuses between the two groups. These results suggest that the ZP and oolemma are not competent factors for prevention of polyspermy in our present porcine IVF system. However, when the ZP is absent, it appears that sperm penetration is inhibited.

Key Words: Pig, oocyte, IVF, polyspermy, zona pellucida

1811

Hyaluronan and proteoglycan binding link protein 1 and versican enhance cumulus expansion during *in vitro* maturation of porcine cumulus oocyte complexes

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Cumulus expansion, which is characterized by the intercellular deposition of hyaluronan secreted from cumulus cells during oocyte maturation, is important for fertilization and the developmental potential of early embryos. The expression of hyaluronan-related genes, hyaluronan synthetase (HAS) 2, hyaluronan and proteoglycan binding link protein (HAPLN) 1, and versican (VCAN), was measured during *in vitro* maturation (IVM) of porcine cumulus oocyte complexes (COCs). The effect of adding HAPLN1 or VCAN to a chemically defined maturation medium on cumulus expansion, *in vitro* fertilization of COCs and subsequent embryo development was also examined. Porcine COCs were collected from the ovaries of slaughtered prepubertal gilts and matured in porcine oocyte medium (POM) alone and POM containing recombinant human FSH (0.5 IU/ml), TGF-α (10 ng/ml) or both for 44 h. At 0, 20 and 44 h after maturation culture, COCs were harvested and expression of HAS2, HAPLN1 and VCAN transcripts were quantified by real-time RT-PCR. Addition of both FSH and TGF-α enhanced ($p < 0.05$) cumulus expansion, as assessed by the area of COCs, at 20 h (283%) and 44 h (916%) after maturation, compared with no addition. Expression of HAS2 mRNA in COCs after 20 h culture was upregulated by addition of either FSH or TGF-α or both compared with no addition ($p < 0.05$), but downregulated at 44 h after culture ($p < 0.05$). Addition of both FSH and TGF-α upregulated ($p < 0.05$) HAPLN1 mRNA in COCs at 20 h after culture compared with no addition and further increased ($p < 0.05$) at 44 h. Expression of VCAN mRNA was higher ($p < 0.05$) in the presence of FSH at both 20 and 40 h after culture compared with addition of TGF-α alone. When HAPLN1 or VCAN was added at various concentrations to POM supplemented with FSH and TGF-α (POM + FT), addition of 200 ng/ml HAPLN1 (146%) or 4 μg/ml VCAN (137%) enhanced ($p < 0.05$) cumulus expansion compared with no addition. However, when COCs were matured in POM + FT, POM + FT supplemented with 200 ng/ml HAPLN1 or 4 μg/ml VCAN and then fertilized *in vitro*, rates of penetration (73.7–77.3%), normal fertilization (47.7–55.2%), male pronuclear formation (53.6–64.9%) and polyspermy (9.3–18.1%) did not differ among treatments. Moreover, no differences were found among treatments for cleavage (69.5–75.3%) and blastocyst formation (41.6–44.1%) rates of presumptive zygotes and for total cell number in blastocysts (43.3–43.7 cells). Our results indicate that cumulus expansion of porcine COCs during IVM could be stimulated by FSH and TGF-α associated with regulation of the expression of hyaluronan-related genes.

Key Words: *In vitro* maturation, cumulus expansion, hyaluronan-related genes, pig

1850

Effect of the reproductive status of crossbred Zebu cows on *in vitro* embryo production

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In order to improve the oocyte selection for *in vitro* embryo production, the present work evaluated the effect of the reproductive status of postmortem crossbred Zebu cows [pregnant (P) vs. non-pregnant (NP)] on the number of recovered oocytes, the cleavage (number of cleaved embryos/number of recovered oocytes) and the blastocyst production (number of blastocyst/number of cleaved embryos) rates. Type I or II (Le Guienne, 1999) cumulus oocytes complexes (COCs) were selected, counted and separated depending on the donor status: P cows with or without a corpus luteum (CL) and NP cows with (CL) or without (NoCL) a CL. Data for number of recovered oocytes was analyzed using the GENMOD procedure assuming a Poisson distribution, whereas data for cleavage and blastocyst production rates were analyzed using a logistic regression assuming a binomial distribution. The statistical model for all the analyses included the effects of the reproductive status (P vs. NP), presence or not of a CL (CL vs. NoCL) and the interaction of reproductive status x CL. A total of 4658 oocytes obtained from 437 ovaries was evaluated. The number of oocytes collected per ovary from P cows (12.5 ± 2.8) was significantly higher ($p < 0.03$) than from NP cows (9.4 ± 2.5), independently of the presence of a CL; whereas the presence (CL: 10.3 ± 2.6) or not of a CL (NoCL: 11.4 ± 2.7) and the interaction (P-CL: 12.5 ± 2.9; NoP-CL: 8.6 ± 2.5; P-NoCL: 12.6 ± 2.9; NoP-NoCL: 10.2 ± 2.7) did not affect the number of oocytes collected. On the other hand, the cleavage rate was significantly higher ($p < 0.006$) in NoP cows (42.3 ± 0.01%) than in P cows (34.5 ± 0.02%), but the presence or not of a CL did not have any effect (CL: 38.6 ± 0.02 vs. NoCL: 38.0 ± 0.02). Nevertheless, the interaction between reproductive status by CL affected ($p < 0.006$) the cleavage rate: P-CL: 31.1 ± 0.02% (76/244); P-NoCL: 38.0 ± 0.03% (98/258); NoP-CL: 46.7 ± 0.03% (312/738) and NoP-NoCL: 38.0 ± 0.03% (143/376). There were not differences in blastocyst production rate for cleaved embryos from P cows (10.7 ± 0.02%; 17/160) compared with NoP cows (10.0 ± 0.02%; 30/312), independently of the presence or not of a CL [CL: 9.7 ± 0.02% (22/239) vs. 10.6 ± 0.02% (25/233)] and neither the interaction between the reproductive status × CL was different: P-CL: 11.4 ± 0.04% (8/70); P-NoCL: 10.0 ± 0.03% (9/90); NoP-CL: 8.2 ± 0.02 (14/169) and NoP-NoCL: 11.2 ± 0.03 (16/143). These results show that the reproductive status of a cow must be taken into account for oocyte selection, in order to improve embryo production.

Key Words: *In vitro* embryo production, crossbred Zebu cows, reproductive status

1851

Improved bovine embryo production by *in vitro* fertilization with X-sorted sperm using *in vivo* matured oocytes obtained by follicle superstimulation and ovum pick-up

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Since *in vivo* matured oocytes show higher developmental competence compared with *in vitro* matured ones, their use might improve embryo production in *in vitro* fertilization (IVF) systems in which sex-sorted sperm is used. Our aim was to establish an efficient system for the production of female dairy cattle embryos by IVF using sex sorted sperm and *in vivo* matured oocytes collected by ovum pick-up (OPU). Superstimulation of follicles in non-lactating Holstein cows (n = 24) was achieved by CIDR insertion (Day 0), dominant follicle removal on Day 5 and administration of FSH twice a day, starting on the evening of Day 6 until morning of Day 9 in decreasing doses (6, 6, 4, 4, 3, 3, 2, 2 mg; 30 mg in total). On the evening of Day 8, CIDR removal and the administration of 0.225 mg d-croprostenol was performed. For ovulation induction, 200 μg GnRH were injected on the morning of Day 10 (0 h). In experiment 1 (n = 12), ovulation observation was performed in every 3 h from 20 to 41 h after GnRH by an ultrasound scanner to fix the optimal timing for aspiration of *in vivo* matured oocytes and IVF after GnRH on Day 10. Estimated ovulation time was calculated as the median of each two checkpoints. In experiment 2 (n = 18), embryo production from *in vivo* and *in vitro* matured oocytes using commercially available X-sorted frozen-thawed semen was assessed. After dominant follicle ablation (DFA) on Day 5 (Group A, n = 6) or 100 μg GnRH on Day 5 (Group B, n = 6), *in vivo* matured oocyte were collected by OPU at 25–26 h and inseminated at 30 h after GnRH on Day 10. Group A and B were crossover designed. Immature oocytes obtained by OPU of non-stimulated cows (Group C, n = 6) and those by aspiration of slaughterhouse-derived ovaries (Group D, n = 282) were used as control for *in vitro* maturation. Data (means) were compared by Tukey's test after ANOVA. The peak of ovulation occurred at 30.5 h after GnRH administration in 48.1% of total ovulated follicles. Numbers of recovered oocytes in total among Group A, B and C did not differ (19.8, 24.6 and 18.8, respectively). The recovery rate of total oocytes in Group A was higher than Group C ($p < 0.05$, 83.5% vs. 65.8%, respectively), however no difference in Group B (71.3%) compared with Group A or C. There was no difference between Group A and B in numbers of recovered oocytes with expanded cumulus (14.6 and 18.5, respectively). Blastocyst rates among Groups A, B, C and D did not differ (58.0%, 52.8%, 49.9% and 32.1%, respectively). However, rates of high quality blastocysts (Code 1, according to International Embryo Transfer Society) was higher in Group A compared with Groups B and C ($p < 0.05$, 54.9% vs. 21.5% and 36.1%, respectively). Our results demonstrate that high quality blastocysts can be produced by IVF with X-sorted frozen sperm using *in vivo* matured oocytes derived from superstimulated cows combined with DFA. We were supported by the Research and development projects for application in promoting new policy of agriculture, forestry and fisheries (22016).

Key Words: *in vivo* matured oocyte, sexed sperm, superstimulation, OPU, IVF

1852

Effects of brilliant cresyl blue staining on the efficiency of *in vitro* fertilization and parthenogenetic activation of oocytes in buffalo (*Bubalus bubalis*)

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Oocyte quality is critical for oocyte maturation and embryo production *in vitro*. However, at present there are still no effective methods to evaluate oocyte quality at the start of maturation. Therefore, the objective of this study was to evaluate the quality of buffalo oocytes in terms of embryo development *in vitro* using brilliant cresyl blue (BCB) staining. Buffalo oocytes collected from slaughterhouse ovaries were randomly divided into three groups: control group (immediately *in vitro* maturation cultured), holding-control group (kept in D-PBS supplemented with 0.4% BSA for 90 min before cultured), and experimental group (staining-incubated with 26 μM BCB for 90 min before cultured). Treated oocytes (stained with BCB) were then divided into BCB+ (coloured cytoplasm) and BCB− (colourless cytoplasm) according to their ooplasm BCB coloration. After IVM for 22–24 h, oocytes were fertilized *in vitro* and cultured in TCM199 supplemented with 0.33 mM pyruvate and 10% NCS with granulosa cells for 6–8 days, or oocytes were treated with 5 μM Ionomycin for 5 min and 2 mM 6-DMAP for 3–4 h, then cultured for 6–8 days *in vitro*. The maturation rate, embryo development rate of IVF and partenogenetic activation (PA) in each group were evaluated. A total of 2638 oocytes were used in this study. Each experiment was replicated for six times. Data were analyzed by ANOVA procedures with replicates and treatments in the model. The results revealed that: the rate of nuclear maturation in BCB+ group (65.70%) was significantly higher than those of control group (59.86%), holding-control group (58.42%) and BCB− group (48.86%) ($p < 0.05$); there were no significant differences in the rate of blastocyst formation between BCB+ group (27.08%) and control group (25.49%) ($p > 0.05$), but the rate of blastocyst formation in BCB+ group was significantly higher than those of holding-control group (20.50%) and BCB− group (8.45%) ($p < 0.05$); There were no significant differences in blastocyst rate of PA among the BCB+ group (21.44%), control group (20.68%) and holding-control group (19.13%) ($p > 0.05$), but blastocyst rate of PA among the three groups were significantly higher than that of BCB− group (11.63%) ($p < 0.05$). In conclusion, buffalo oocytes selected by BCB staining before IVM were of superior quality in terms of nuclear maturation, however, no effect was found in embryo development. This study was supported by the Guangxi Department of Science and Technology (0815008-2-4, 1123005-3).

Key Words: Brilliant cresyl blue, buffalo, oocyte, *in vitro* maturation, *in vitro* fertilization

1853

Cumulus cells are involved in oocyte maturation and fertilization in *in vitro* produced ovine embryos

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Cells from Cumulus-Oocyte Complexes (COCs) are normally removed soon after *in vitro* maturation (IVM) in most of the laboratories working in embryo production in sheep and goats. However, the possible effect of cumulus cells on *in vitro* fertilization (IVF) of ovine oocytes is not clear enough. The aim of this experiment was to determine the effect of removing cumulus cells during IVM or IVF on embryo production in sheep. A total of 806 COCs surrounded by at least two layers of granulosa cells and homogeneous cytoplasm were selected after follicle aspiration from ovaries obtained at the slaughterhouse (six replicates). The selected COCs were divided in three experimental groups with cumulus cells being removed prior to IVM (n = 277), prior to IVF (n = 302) or after IVF (n = 227). Cell removal was performed by gentle pipetting in 3 ml of TCM199 supplemented with fetal bovine serum, HEPES and antibiotics. For IVM the oocytes were incubated in drops of TCM199 supplemented with estrous ovine serum (EOS), FSH, LH, cysteamine and antibiotics; covered with embryo tested mineral oil for 24 h, at 39°C and 5% CO_2 in air. For IVF (Day 0), matured oocytes were incubated for 22 h in drops of synthetic oviductal fluid (SOF) with EOS, heparin and hypotaurine, using 1×10^6 frozen-thawed spermatozoa selected by swim-up method. *In vitro* culture was performed in drops of SOF supplemented with amino acids and bovine serum albumin covered with mineral oil at 39°C and 5% O_2, 5%CO_2, 90% N_2. Cleavage rate on Day 2 (2 cell embryos/oocytes) and development rate on Day 6 (morulae and blastocysts/oocytes) and Day 8 (total blastocysts/oocytes) were analyzed by logistic regression. Results are showed in Table 1. In conclusion, removal of cumulus cells after IVF improves the cleavage and developmental rates of *in vitro* produced sheep

embryos, suggesting a positive effect of cumulus cells not only on maturation, but also on fertilization of the ovine oocytes.
Key Words: Embryo, ewe, granulosa

Table 1. Effect of time of cumulus cells removal on cleavage and development rates of IVP sheep embryos

	Cleavage rate (Day 2)	Morulae and blastocysts/ oocytes (Day 6)	Total blastocysts/ oocytes (Day 8)
Prior to IVM	54.5% (151/277)[a]	6.9% (19/277)[a]	1.4% (4/277)[a]
Prior to IVF	77.2% (233/302)[b]	28.8% (87/302)[b]	16.6% (50/302)[b]
After IVF	85.0% (193/227)[c]	51.5% (117/227)[c]	37.0% (84/227)[c]

Different letters within columns indicate significant differences ($p < 0.05$).

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In vitro development of feline embryos after intracytoplasmic sperm injection with 7-day refrigerated testicular sperm

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Cold storage of testicular tissue is a valuable tool for short-term preservation of genetic potentials from animals in case that embryo production or tissue cryopreservation cannot be immediately performed (besides the technical aspects it also is interesting to study the resistance of testicular sperm preserved at cold temperatures). However, prolonged cold storage can reduce sperm quality especially sperm plasma membrane contributed to increase the risk of sperm DNA damage which affected the embryo development. This study aimed to assess the *in vitro* embryo development following intracytoplasmic sperm injection (ICSI) with testicular sperm after cold storage for 7 days. Cat testes from adult male cats subjected to routine castration were used in this study. Testes were decapsulated and then mechanically minced with sharp-ended scissors in Hepes-bufferred M199 supplemented with 1.6% BSA and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Spermatozoa were further extracted from minced tissue by gentle pipetting. The sperm suspension was finally filtered through 40 μm cell strainer and cold-stored at 4°C for 7 days. A testicular spermatozoon with morphologically normal head, midpiece and tail was selected for ICSI. Injection with fresh testicular sperm ($n = 186$) and sham injection (injection without sperm; $n = 82$) served as controls. The ICSI-oocytes were then cultured at 38.5°C in a humidified condition of 5% CO_2 in air for 7 days (the day of injection was defined as day 0). The proportions of cleavage, morula and blastocyst (related to the number of injected oocytes) were determined on day 2, 5 and 7, respectively. All day 7 embryos were fixed and stained with DAPI in order to examine the blastocysts and their quality according to the cell numbers. The chi-square test was used to compare the percentages of embryos. The total cell numbers of blastocyst were analyzed by one-way ANOVA. In all cases, differences were considered significant with $p < 0.05$. The proportions of cleavage, morula and blastocyst after ICSI using cold stored testicular sperm ($n = 206$) were similar to fresh control (44.7/37.6, 13.6/16.8 and 7.3/9%, respectively, $p > 0.05$). An average cell number of blastocysts in cold-stored group did not significantly differ ($p > 0.05$) from fresh group (116 ± 16 vs. 128 ± 23 cells, respectively). A small number of oocytes in sham injection group (4.9%) were cleaved. However, they did not develop beyond morula stage (1.2%). In conclusion, 7-day cold stored testicular sperm can fertilize *in vitro* matured cat oocyte via ICSI without artificial activation. The developmental competence of embryos derived from fresh or refrigerated testicular sperm was similar and this technology may be useful for conservation of wild felid species.

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Key Words: Testicular sperm, cold storage, domestic cat, ICSI, embryo

1855

Effect of donor age on ovum pick-up results in buffaloes

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The aim of this study was to determine the effect of donor age on OPU results in buffaloes. In total, 180 donors were used in the experiment and equally allocated to three groups. The first group consisted of heifers aged between 3 and 5 years (nulliparous buffaloes), in the second group the age of cows was between 6 and 10 years (multiparous buffaloes) and cows which were older than 10 years were assigned to the third group. All buffalo donors were subjected to five successive OPU sessions performed at 3 days interval without using any hormonal treatment. Before follicles were punctured the diameters were assessed using the internal ultrasound calliper on the screen and assigned to one of the following diameter classes: 2–5 mm, 5–10 mm, and >10 mm. After OPU and oocyte recovery, the cumulus-oocyte complexes (COCs) were identified using a stereomicroscope. The COCs were classified according to the IETS guidelines and graded A to C. Multiple Comparison Procedures were used (Duncan's Test) to estimate the means of visible follicles, oocytes recovered and oocytes assessed as suitable for IVP. The results of this study show that the number of visible follicles prior to OPU significantly differed among the age groups of donors. The mean numbers of visible follicles per donor were 9.14 ± 0.19, 7.64 ± 0.14, 6.18 ± 0.21 (groups: 3–5 years, 6–10 years, > 10 years; $p < 0.01$), respectively. This difference becomes evident when looking at the number of small follicles (7.91 ± 0.19, 6.06 ± 0.13. 4.78 ± 0.20; $p < 0.01$). In contrast, the total number of recovered oocytes and the number of oocytes per donor assessed as suitable for IVP did not reveal any age dependent effect. Only the number of high quality oocytes (grade A and B) depended on age ($p < 0.0001$). The younger the donors the higher the percentage of high quality oocytes (3–5 years: 66.40%; 6–10 years: 62.13%; > 10 years: 54.40%, see the Table). The present data let us conclude that there is an age dependent effect of the donors on OPU. Younger donors provide better oocytes compared to older ones. This work was supported by the International technical cooperation projects 2008DFA30320 and the Guangxi scientific research Projects (1123005-3).
Key Words: Water buffalo, OPU, donor ages, oocyte

Table 1. Effects of Donors Ages on ovum Pick-up results in Buffaloes

Age of the donor	Puncture session	oocytes suitable for IVP	Oocytes recovered	High quality oocytes(%)
3–5 years	998	5.50 ± 2.13	7.76 ± 2.98	66.40 ± 1.66a
6–10 years	622	7.47 ± 4.72	10.75 ± 6.81	62.13 ± 1.49a
Over 10 years	232	2.04 ± 0.15	3.03 ± 0.22	54.40 ± 2.75b
Average	–	5.73 ± 1.96	8.17 ± 2.79	63.46 ± 8.79
p	–	0.699	0.697	<0.01

Values with different superscripts within columns are very significantly different (a,b: $p < 0.01$).

1856

Season effect on ovum pick-up oocytes of water buffalo with FSH treatment

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The season effect on the recovery and morphology of Ovum Pick up oocytes were determined in the present study. Buffalo donors were treated with FSH in March-May (SG-FSH: spring group, N = 3), and in August-September (AG-FSH, N = 3), or served as controls (SG-CT, N = 3 and AG-CT, N = 4) respectively. The donor buffaloes were injected with 10 mg FSH on the first day, and 5 mg/day in the following 2 days. Ovum pick up (OPU) was conducted on the fourth day. The OPU was repeated five times in SG groups and seven times in the AG-groups with a 3 days interval. The number and size of follicles were recorded before puncture. The recovered oocytes were graded according to their morphological appearance following IETS criteria. Only oocytes of Grade A and B were classified as usable oocytes. The recovered oocytes and usable oocytes were counted and analyzed with SPSS according Duncan Continuous variables. The results showed there was no significant difference in the SG-FSH and SG-CT group in the average recoved oocytes (4.36 ± 0.34 vs. 5.15 ± 0.49) and usable oocytes (3.54 ± 0.31 vs. 3.73 ± 0.38). The recovered oocytes of AG-FSH group was similar to that of AG-CT (5.91 ± 0.31 vs. 5.40 ± 0.63). However, the usable oocytes of AG-FSH group was significant lower than the AG-CT group (1.86 ± 0.30 vs. 4.06 ± 0.52, $p < 0.001$). Comparing SG-FSH and AG-FSH groups, they were not significant different in recovered oocytes (4.36 ± 0.34 vs. 4.06 ± 0.52), but there were significant higher in usable oocytes (3.54 ± 0.31 vs. 1.86 ± 0.30, $p < 0.005$) in the SG-FSH group. Comparing SG-CT and AG-CT groups, there were no significant different in recovered ooctyes and usable oocytes (5.15 ± 0.49 vs. 5.40 ± 0.63 and 3.73 ± 0.38 vs.4.06 ± 0.52 respectively). In conclusion, there were no significant diffences between spring season and autumn season in the number of oocytes recovered however the injection of FSH decreased the number usable oocytes in buffalo OPU. This work was supported by the International technical cooperation projects (2008DFA30320) and the Guangxi scientific research Projects (1123005-3).

Key Words: Water buffalo, FSH, OPU, effect, breeding seasons

1857

The effect of sperm preparation and brief gamete coincubation length on ovine oocytes fertilization, subsequent development and embryo quality

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Over the past 70 years, egg yolk (EY) is frequently used as a cryoprotectant in semen diluents and has been confirmed highly effective for the maintenance of sperm fertility. However, the preparation of uniform diluents containing EY is hard. Since Cheng, (1985) reported the beneficial effect of overnight gamete oocytes coincubation length in IVF, this protocol was used a recommended protocol in several species. However, this protocol leading to exposure of oocytes and embryos to toxic effects associated with excessive generation of reactive oxygen species. The aims of this study were to examine (i) whether brief exposure of mature ewe oocytes to frozen-thawed semen prepared with different semi-defined diluents may affect fertilization, enhanced embryonic development, embryo quality before and/or after freezing vs. long coincubation length, and (ii) explore the accompanying effects of different semi-defined diluents and coincubation length on fertilization and embryo development rates. Matured oocytes were divided randomly into 3 groups and fertilized with frozen-thawed spermatozoa prepared with either 10%, 15% or 20% BSA lacking EY (Ali and Naitana, AETE 2009 pp.132). The oocytes and spermatozoa were coincubated briefly for 1, 2, 3 or 20 h as a regular coincubation length, and then transferred directly into culture wells. The numbers of cleaved and expanded blastocysts/group were evaluated after 46 and 144–192 h post-insemination (hpi), respectively. The quality of embryos was evaluated accordingly to Sifer C et al. (*Hum Reprod* 20(10): 2769–75, 2005). Data were analyzed by Chi-square tests SAS/STAT. Among all treatments, the cleavage rates increased highly with prolonged coincubation length. However, increased coincubation length ≥20 hpi had no effects on cleavage and blastocysts rates vs. brief lengths. Maximum cleavage, blastocysts rates were observed after 3 hpi in 10% and 15% BSA groups (93/110, 84.5%), (83/100, 83.0%) and (54/110, 49.1%), (49/100, 49.0%), respectively. While, cleavage and blastocysts rates were increased higher in 20% BSA group after 2 hpi vs. regular coincubation length (96/109, 88.1%), (53/109, 48.6%) and (92/124, 74.2%), (45/124, 36.6%), respectively. Interestingly, diluents with 20% BSA improved ($p < 0.001$) cleavage rates vs. 10 and 15% BSA after 1–2 hpi (83/107, 77.6%), (42/100, 42.0%), (59/109, 54.1%) and (96/109, 88.1%), (61/101, 60.4%), (91/114, 79.8%), respectively. However, there were no ($p > 0.05$) effects in blastocyst rate among all concentration of BSA, or between brief and long coincubation lengths. In summary: (i) both cleavage and blastocyst rate increased linearly with coincubation length, and (ii) better fertilization results were obtained when gamete-oocytes coincubated > 2 h; and prolonged coincubation > 3 h had no additive effects on fertilization, embryo development efficiency and quality. Finally, reducing coincubation length did not improve early embryonic development and embryo quality vs. regular coincubation length.

Key Words: Semi-defined semen diluents, *in vitro* fertilization, brief coincubation length, embryonic development and quality

1858

In vitro development and DNA fragmentation of domestic cat ICSI embryos subjected to antioxidant conditions and ionomycin activation

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ICSI has gained importance to support feline reproduction and preserve existing genetic biodiversity when poor seminal quality is observed. The objective of this study was to determine the best conditions to generate ICSI embryos in the domestic cat, as a model of wild felids. Ovaries were recovered from cats subjected to ovariectomy. Cumulus-oocyte-Complexes of good quality were *in vitro* matured in standard maturation medium (SMM): TCM 199 containing 1 IU/ml HCG, 10 ng/ml ECG, 2.2 mM calcium lactate, 0.3 mM pyruvate, 0.3% BSA and 3% antibiotic-antimycotic; or SMM supplemented with 1 μl/ml of insulin, transferrin and selenium (ITS, a free radical reducer). In the first experiment, we compared *in vitro* development of ICSI embryos after oocyte maturation in SMM and zygotes cultured in atmospheric oxygen tension (21% O_2), respect to maturation with ITS supplementation and low oxygen tension in culture (5% O_2). Moreover the effect of ionomycin activation after injection (Io) was evaluated. Control SHAM groups were included (Table 1). *In vitro* embryo development was compared by non-parametric Fisher exact test ($p \leq 0.05$). Cleavage rates in ICSI groups increased when Io was employed but blastocyst rates were higher when ITS–5% O_2 was used, regardless Io exposure. Karyotype analyses of cleaved embryos from ICSI–2 and ICSI–4 groups were done to assess if the increment in cleavage, when Io was employed, was a result of parthenogenetic activation instead of sperm fertilization. Nevertheless, we confirmed that nearly 70% of cleaved embryos were diploid in both groups. In the second experiment we determined total cell number and DNA fragmentation by TUNEL assay in ICSI blastocysts from all treatments, after 7 (Bd7) and 8 (Bd8) days of *in vitro* culture. Differences in total cell number were analyzed using one-way ANOVA and the proportion of fragmented nuclei over total cell number was analyzed by the Difference of proportions test. The ICSI–4 group showed the least amount of cells after 7 and 8 days, respect to the other groups which did not differ among them. Moreover, proportion of TUNEL+ cells increased in ICSI–2 and ICSI–4 Bd8, respect to Bd7 (55.5% and 37.4% for Bd7 vs. 89.9% and 67.6% for Bd8; $p \leq 0.05$), in contrast to the other two groups that remained the same proportion of

fragmented cells. We conclude that chemical activation enhances cleavage but not blastocyst formation, and that antioxidant conditions improve embryo development *in vitro* but increase DNA fragmentation of Bd8. Therefore, we consider that the best conditions to generate ICSI embryos in the cat are ITS–5% O_2, but embryo transfers and pregnancy rates are needed to assess this assumption.

Key Words: Cat, ICSI, DNA fragmentation, oxygen tension, embryos

Table 1. Development of cat embryos after ICSI and SHAM

Groups	Io	ITS-5% O_2	n	% Cleaved	% Blastocysts
ICSI-1	-	-	138	52.17^{a}	$8.7^{a/\alpha}$
ICSI-2	-	+	206	$56.8^{a/\alpha}$	$20.87^{b/\alpha}$
ICSI-3	+	-	152	71.1^{b}	$11.12^{ac/\alpha}$
ICSI-4	+	+	109	69.7^{b}	$17.43^{bc/\alpha}$
SHAM-1	-	-	60	38.3^{a}	$0^{a/\beta}$
SHAM-2	-	+	67	$28.4^{a/\beta}$	$0^{a/\beta}$
SHAM-3	+	-	68	64.7^{b}	$0^{a/\beta}$
SHAM-4	+	+	72	65.3^{b}	$6.9^{b/\beta}$

(a,b,c) Values for the same method with different superscripts are significantly different. (α,β) Values for the same conditions of ICSI and the control SHAM with different superscripts are significantly different.

19. Male reproductive tract:

1900

Epididymosomes transfer epididymal sperm binding protein 1 to dead spermatozoa during epididymal maturation

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Previously, we showed that the epididymal sperm binding protein 1 (ELSPBP1) characterises spermatozoa already dead before ejaculation in bovine. In this study, we investigated the presence of ELSPBP1 in bull genital tract as well as its acquisition by spermatozoa during epididymal maturation. As assessed by Real-Time PCR, ELSPBP1 was highly expressed in the caput and the corpus epididymis but presented basal expression levels in the testis and the cauda epididymis. Immunohistochemistry revealed the same expression pattern. However, western blot on tissue homogenates showed some discrepancies as ELSPBP1 was found in comparable concentration all along the epididymis. This difference was due to the presence of ELSPBP1 in the epididymal fluid. In both caput and cauda epididymal fluid, ELSPBP1 was associated with epididymosomes, small membranous vesicles secreted by epithelial cells of the epididymis and implicated in the transfer of proteins to spermatozoa. As assessed by immunocytometry, ELSPBP1 was found on a subset of dead spermatozoa in caput epididymis but was found on all dead spermatozoa in cauda epididymis. To assess ELSPBP1 acquisition by spermatozoa, caput epididymal spermatozoa were incubated with cauda epididymosomes under various conditions. ELSPBP1 detection by immunocytometry assay revealed that only spermatozoa already dead before incubation were receptive to ELSPBP1 transfer by epididymosomes. This receptivity was enhanced by the presence of zinc in the incubation medium. This specificity for a sperm subpopulation suggests that an underlying mechanism is involved and that ELSPBP1 could be a tag for the recognition of dead spermatozoa during epididymal maturation.

Key Words: Epididymosomes, sperm maturation, ELSPBP1

1901

Incorporation of various dietary omega-3 fatty acids into bull sperm and the effects on physiological parameters of fresh and frozen-thawed sperm

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The objectives of this study were to determine the rate of incorporation of various dietary omega-3 (n-3) fatty acids (FA) into bull sperm and the effects on physiological parameters of fresh and frozen-thawed sperm. Fifteen bulls were assigned to three groups and supplemented for 13 weeks with encapsulated fat: (i) SFA – 360 g/day per bull saturated FA, (ii) FLX – 450 g/day per bull providing 84.2 g/day C18:3n-3 (ALA) from flaxseed oil, and (iii) FO – 450 g/day per bull providing 8.7 g/day C20:5n-3 (EPA) and 6.5 g/day C22:6n-3 (DHA) from fish oil. Blood samples were taken every 2 weeks and semen was collected weekly. Fatty acids profiles were determined in blood plasma and sperm, and physiological parameters were measured in fresh and frozen-thawed sperm by Sperm Quality Analyzer-Vb. The data were analyzed as repeated measurements using the PROC MIXED procedure of SAS and the model included the effects of treatment, bull and week. The proportion of ALA in blood plasma was 2.6 and 2.1-fold higher in FLX than in SFA and FO bulls (9.27, 3.61 and 4.33%; $p < 0.01$), respectively. The proportion of C22:5n-3 in blood plasma was 2-fold greater ($p < 0.001$) and that of DHA was 70 and 20-fold higher in FO than in FLX and SFA bulls, (0.28, 0.004 and 0.014%; $p < 0.001$), respectively. The incorporation of n-3 FA into sperm was first expressed on the 6th week of supplementation, and analysis of FA from week 7 to 12 showed dramatic changes in FA profile. The ALA proportion in sperm was relatively low and in FLX was eight fold higher than in SFA and FO bulls (0.08, 0.01 and 0.01; $p < 0.001$), respectively. The DHA proportion in sperm was 36 and 10% greater in FO than in SFA and FLX (29.3, 21.5 and 26.4%; $p < 0.001$), respectively. Simultaneously, the C22:5n-6 FA (DPAn-6) proportion in sperm decreased and was 16.7, 11.8 and 8.8% in SFA, FLX and FO bulls, respectively ($p < 0.001$). No effects were observed in physiological parameters of fresh semen; however, treatments improved the sperm survival following freeze-thaw procedure, expressed by reduced fading rate calculated as progressive motility after thawing (without incubation) compared to fresh sperm; 53.6, 43.0 and 48.6% for SFA, FLX and FO, respectively ($p < 0.01$). Moreover, motility and progressive motility in frozen-thawed sperm was higher in FLX than in SFA and FO bulls (55.5, 47.7, 50.9; SEM = 0.88 and 43.4, 37.0, 38.8%; SEM = 1.07; $p < 0.01$), respectively. The velocity of sperm also was higher in frozen-thawed sperm of the FLX than in SFA (38.8 and 34.1 μm/s, respectively; SEM = 0.1.06; $p < 0.001$). In summary, the percentage of DHA in sperm was elevated on the expense of DPAn-6 FA in FO, but also in FLX bulls, presumably due elongation and desaturation capacity in testicles. The incorporation of DHA into sperm improved the survival, motility, progressive motility and velocity of sperm in frozen-thawed sperm. While a moderate exchange of DHA and DPAn-6 was demonstrated in FLX sperm, beneficial effects were noted in their physiological parameters, suggesting that the ratio between these two FA plays an important role in sperm function.

Key Words: Omega-3, sperm, bull

1902

Recommended minimum scrotal circumference for tropically-adapted beef bulls in northern Australia

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Scrotal circumference has long been regarded as a readily-measured indicator of beef bull fertility and is therefore routinely included in bull breeding soundness examinations. However, concerns have been raised that current minimum thresholds for tropically adapted genotypes do not account for the large variations in northern Australia bull nutrition. The primary objectives of this study were to examine the interaction and effects of weight and age on scrotal circumference and to develop minimum thresholds indicative of suboptimal testicular development. Scrotal circumference measurements (n = 9300) from 1958 bulls ≤3 years of age, were analysed. Breeds included Brahman (n = 559), Droughtmaster (n = 133), Santa Gertrudis (n = 460) and other tropical composites (n = 806). The bulls were located on multiple sites across Queensland, and most continuously grazed native or improved pastures of varying quality, with some receiving commonly-used supplements. Some bulls were lot fed for a period to achieve growth rates of approximately 1 kg/day. Both weight (r = 85.8%) and age (r = 78.5%) had significant positive correlations with scrotal circumference. The Gompertz function, a relatively common non-linear model used to describe growth, best described testicular development in relation to weight in all breeds (R^2 = 80.4). Breed differences in testicular development were small, although tropical composite bulls matured at a slightly earlier age/weight than the other breeds studied. The minimum recommended scrotal circumference within weight ranges were derived for field use as less than the lower 5th percentile (Table 1).

Key Words: Testis, scrotal circumference, bovine, nutrition, bull

Table 1. Average and recommended minimum scrotal circumference (SC) by bodyweight range for tropical bulls

Weight range (kg)	Average SC (cm)	Minimum recommended SC (cm)
393–418	32	28
419–446	33	29
447–479	34	30
480–518	35	31
519–567	36	32
568–631	37	33
632–728	38	34
729–943	39	35
943–1000	39	36

1903

Ultrasonography of the scrotal contents for breeding zebu bulls extensively reared in Costa Rica

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Ultrasonography is a valuable aid to assess the soundness of reproductive organs, since many abnormalities cannot be diagnosed by clinical inspection. Ultrasound assessment of the scrotal contents (US) was performed on 12 Brahman sires extensively reared aged 34.0 ± 1.3 months. Bulls were sound at clinical examination, mainly regarding scrotal circumference (34.9 ± 2.5 cm) and testicular and epididymal consistency and symmetry. They fitted normal sperm morphology and motility (%) for breeding bulls (87.9 ± 11.0 and 78.3 ± 15.4 respectively). Scrotal wall (SW), testicular capsule (TC), vaginal cavity (VC) and mediastinum testis (MT) were assessed at 5 MHz on lateral (L), anterior (A), medial (M) and posterior (P) longitudinal surface of left (LT) and right (RT) testicle. Every plane was divided on top (1/3), middle (2/3) and bottom third (3/3). SW diameter (mm) at the scrotal neck ranged from 6.0 ± 0.6 to 6.6 ± 1.8 for LT and RT respectively ($p > 0.05$). It was thicker ($p < 0.0001$) compared to SW at any other surface (i.e. overall SW for LT and RT at (P) was 3.6 ± 0.5 and 3.5 ± 0.4 mm respectively). VC was visible at the scrotal neck in seven sires as a thin continuous anechoic space dorsal to TC. Conversely, VC could be seen hardly ever at other surfaces and when visible, it was thinner ($p < 0.0001$) than at the neck (0.6 ± 0.2 vs. 1.2 ± 0.8 mm width respectively). TC was visible on all surfaces as a thin continuous hyper-echoic line surrounding the parenchyma. Its diameter was similar ($p > 0.05$) in LT compared to RT at any surface assessed (overall 0.8 ± 0.08 mm; range 0.3–1.8 mm). In three bulls small superficial blood vessels could be seen enclosed in TC. The MT was easily observed as a hyper-echoic straight structure centrally located extending for about 3/4 along the long axis of the testicle. It was visible in all bulls by placing the probe on 2/3 at (P). On the contrary, it could be seen sporadically in 1/3 in LT and RT too (two and five out of 12 bulls respectively). In most sires the initial portion of MT was evident at 3/3 position. MT diameter (mm) in (P) on 2/3 ranged from 4.3 ± 1.5 to 5.1 ± 1.6 for LT and RT respectively. Epididymis head (EH), body (EB) and tail (ET) were hypoechoic compared to parenchyma and showed a triangular, lineal and ovoid shape respectively. EH length and width (mm) were 23.2 ± 4.0 and 12.2 ± 4.9 for LT vs. 22.7 ± 3.6 and 10.9 ± 3.0 for RT respectively. EB diameter was 2.9 ± 0.8 and 2.5 ± 0.7 mm for LT and RT respectively. The whole ET was not easy to visualise at a time given its shape and location at the bottom of the scrotum. Testicular parenchyma showed a fine homogeneous echotexture with moderated echogenicity except one sire that showed diffused hyper-echoic foci in right testicle. Knowing the normal echographic picture of the scrotal contents in bulls extensively reared is of outmost importance to interpret the findings during the US.

Key Words: Zebu bulls, ultrasonography, breeding soundness evaluation, scrotal contents

1904

Thermographic monitoring of scrotal surface during GnRH test in young bulls with low semen quality

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The regulation of the testis functionality is based on general and local information through hypophyseal LH and particularly GnRH that causes a rapid increment of interstitial testosterone level due to a local action. High interstitial testosterone level after stimulation might activate a mechanism of secretion based on the neural local regulation of the blood flow and muscular contraction, therefore the thermographic monitoring may be a suitable method to identify the testicular functionality considering the relationship between scrotal surface temperature (SST) testosteronemia. Aim of this work was to identify relationship between SST and testostenonemia (T) in young bulls with low semen quality. 120 young bulls productive performances were considered to select the subjects with low semen quality (18 bulls). A thermocamera was used to evaluate the SST (five times every 15 min for 1 h) and blood samples were taken to evaluate the testosteronemia (two times 0 and 60 min) during the GnRH test. The selected bulls were divided in two groups depending on the thermic response to the GnRH administration (busereline) only at 60 min. Group 1 (eight bulls) shown a drop of SST at 60 min (mean ± SEM of the delta temperature 0–60 min (SST) = −0.875 ± 0.187 °C) and significantly different ($p < 0.001$) compared with Group 2 (10 bulls), which had an increased SST value (SST = 1.055 ± 0.285°C). Group 1 had a lower basal T level than Group 2 (516 ± 120.04 and 665.3 ± 109.16 ng/dl

for group 1 and 2 respectively) while had a higher T at the end of test (1392.5 ± 50.02 and 1032.9 ± 109.93 ng/dl for group 1 and 2 respectively) showing a significant difference ($p < 0.05$) in term of T (876.5 ± 177.6 vs. 367.6 ± 104.3 ng/dl). Thermic responses at 15, 30 and 45 min were variables on individual basis and have not significant relation with T. Quality parameters of semen were evaluated after GnRH test. Group 1 had improved reproductive performances faster than Group 2 in terms of motility and progressivity post freezing-thawing. The Pearson correlation index between SST and T in young bulls with low quality semen was −0.554 ($p < 0.05$). This relationship may be related to the vascular contraction and to the blood flow decrease expressing a better testicular function.

Key Words: Bull, GnRH test, termography, testicular functionality

1905

Echographic assessment of scrotal contents in extensively reared bulls

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Ultrasound assessment of the scrotal contents (US) is not yet a routine step of breeding soundness evaluation (BSE) in extensively reared bulls. However, this technique is an invaluable aid since many abnormal changes cannot be diagnosed during the clinical inspection of the scrotal sac. US was performed during routine BSE achieved under field conditions in 301 bulls extensively reared in Costa Rica. Sires belonged to *Bos indicus* (BI n = 178), *Bos taurus* (BT n = 48) and crossbreeds (BIxBT n = 75). After the BSE, bulls were classified as sound, deferred and unsound for breeding. The US was performed using a 5 MHz probe placed on posterior longitudinal surface of left (LT) and right testicle (RT). Bulls with normal echographic appearance (NEA) (38.2% n = 115) were characterized by having a testicular parenchyma with a fine homogeneous echotexture and moderated echogenicity, a hyper-echoic mediastinum (MT) centrally located extending about 3/4 along the long testicular axis and a thin hyper-echoic testicular capsule (TC) surrounding the testicle below the scrotal wall. Vaginal cavity was not visible or it was < 1 mm diameter when present in normal bulls. The hypo-echoic homogeneous appearance typical of a normal epididymis was evident along this organ and was accentuated towards epididymis cauda. Sires with abnormal echographic appearance (AEA) accounted for 61.8% (n = 186), being diffused hyper-echoic foci in testicular parenchyma the most common finding (96.7% n = 180). This abnormality was found alone (64.5% n = 120) or combined with other pathologies (32.2% n = 60) such as large fibrotic masses in parenchyma (17.7% n = 33), enlarged superficial TC vessels (9.7% n = 18), hydrocele (2.7% n = 5), anechoic cyst-like dilatations in MT (1.0% n = 2) and spermiostasis with cavity-like formations in caput and cauda epididymis (1.0% n = 2). The overall % of bulls with AEA was higher ($p < 0.01$) either in LT and RT for BT (64.5 and 60.4) than in BI (44.9 and 42.8) and crossbreeds (28.0 and 32.0) respectively. Likewise, bulls > 4 ≤ 7 years-old showed higher levels (%) ($p < 0.0001$) of lesions than younger sires (64.9 vs. 32.5 for LT and 59.7 vs. 32.5 for RT). Bulls classified as sound, deferred and unsound for breeding accounted for 58.8% (n = 177), 6.7% (n = 20) and 34.5% (n = 104) respectively. In those categories the AEA was 58.8% n = 104; 65.0% n = 13 and 59.6% n = 62 respectively ($p > 0.05$). The lack of association between BSE classification and the echographic appearance would be due to the fact that many sires suffering specially of accute testicular degeneration may still showing NEA. Besides, the presence of mid cases of hyper-echoic foci in testicular parenchyma was frequent in sound bulls without causing detrimental changes in spermiogramme or clinical examination, at least at the time BSE was performed. The higher prevalence of lesions found in BT compared to other genotypes studied may be consequence of its greater sensibility to environmental tropical conditions. The presence of hyper-echoic foci in testicular parenchyma seems to be the most common finding during US in bulls extensively reared.

Key Words: Ultrasound, bulls, reproductive pathology, testis

1906

Immunohistochemical localization of aromatase in goat testis and accessory glands

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Cytochrome P450 aromatase is a terminal enzyme in the conversion of androgen into estrogen. Presence of aromatase in male reproductive tract of some species is already demonstrated whereas its presence in male goat is not reported. In this study, immunolocalization of aromatase in the testis, proximal and distal ductus epididymis, ampulla, prostate and seminal vesicle tissues of five sexually mature male goats were investigated by immunoperoxidase technique. Mouse anti-aromatase polyclonal antibody and rabbit anti-mouse IgG antibody were applied in the assay as primary and secondary antibodies respectively. Two additional tissue sections were prepared from each tissue sample and served as negative controls. The first negative control was processed in the absence of primary antibody. In the second negative control section, the primary antibody was replaced by normal mouse serum. No positive staining was observed in the negative control sections whereas strong immunoperoxidase reaction were recorded in the tissue sections from sexually mature ram testis, considered as positive control in the assays. Cytochrome P450 aromatase antigen was detected in the Leydig cells, seminiferous tubule cells in different stages of spermatogenesis, epithelial cell cytoplasm of proximal ductus epididymis, seminal vesicle and prostate tissues of all animals tested. Meanwhile, goat distal ductus epididymis and ampulla were negative in the conducted immunoperoxidase assay. The highest aromatase antigen intensity was observed in the prostate samples, as shown by dark-brown positive reaction. This is the first evidence of aromatase presence in male goat reproductive tract including leydig cells, seminiferous tubules, epithelial cells of proximal ductus epididymis, seminal vesicle and prostate tissues. Considering the presence of estrogen receptors in male reproductive tract of most species including male goat, it seems that estrogen and its receptor are important for male reproductive tract function and fertility.

Key Words: p450 Aromatase, male goat, reproductive tract, immunoperoxidase

1907

Major proteins of the ram seminal plasma: Attributes and interaction with the sperm membrane

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Binder of sperm proteins (BSPs) are major seminal plasma proteins in ruminants and we recently showed that ovine BSPs, known as Ram Seminal Vesicle Proteins (RSVP) 14 and 22 kDa, are the most abundant proteins of the seminal plasma as well. Thus, the purpose of this work was to study certain attributes of RSVPs as well as their ability to interact with the sperm. Semen samples were collected from five adult and reproductively sound Morada Nova rams and centrifuged to separate sperm from seminal plasma. Sperm membrane proteins were extracted and separated using 2-D electrophoresis. One gel was transferred to a PVDF membrane and blotted with anti-BSP antibodies. Spots that reacted with anti-BSP were identified by tandem mass spectrometry (MS/MS). Seminal plasma proteins were also separated using heparin- and gelatin-affinity chromatography. Our results showed that RSVP14 was not detected

in membrane extracts from epididymal sperm but present as the major protein in extracts from ejaculated sperm. Two spots reacted with anti-BSP antibodies (13.7 and 14.5 kDa, pIs 5.0 and 5.2) and were identified by MS/MS. Peptides identified from such spots matched to fibronectin type II conserved domains. Based on immunocytochemistry associated with confocal microscopy, using the same antibodies, we show that RSVP14 binds to the acrosome and equatorial region of ejaculated sperm and with less intensity to the midpiece. This binding pattern changed with capacitation, with RSVPs relocating from the acrosomal to the equatorial and post-equatorial regions. Despite a high homology in amino acid sequence (81%), biochemical differences exist between bovine and ovine BSPs. RSVPs from the seminal plasma do not bind to heparin and only show very weak affinity for gelatin, while bovine BSPs have strong affinity for those components. As evaluated by qPCR, RSVP14 gene is mainly expressed in the vesicular glands and, based on our previous results, RSVP14 is also the most abundant protein in the vesicular gland fluid. Taken together, our findings show that RSVPs are expressed and secreted by the vesicular glands and bind to ovine sperm at ejaculation, suggesting its participation in the modulation of sperm function. In the bovine, where most studies have been conducted, BSPs bind to sperm at ejaculation and mediate sperm capacitation. This event involves BSP binding to heparin and HDL and selective removal of cholesterol and phospholipids from membranes. Given some similarities between ovine and bovine BSPs in sperm, it is possible that the RSVPs retain some of those functions. However, considering that affinity for heparin is an important step towards BSPs participation in capacitation-associated events, specific roles must also exist as a result of some of the RSVP unique attributes.

Key Words: Ram, seminal plasma, proteomics, sperm

1908

Colour-coded and pulsed Doppler measurements of testicular arterial blood flow in male donkey (*Equus asinus*)

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The use of ultrasonography in equine medicine is helpful for the diagnosis of various conditions, especially those affecting the cardiovascular, musculoskeletal and reproductive system. The aims of the present study were to (i) evaluate the potential use of two-dimensional ultrasound in combination with colour-coded (Duplex) and pulsed Doppler (Triplex) sonography, (ii) characterize arterial blood flow of the donkey testis, and (iii) establish reference values for Doppler measurement Two-dimensional ultrasound in combination with colour-coded and pulsed Doppler sonography was used to study the blood flow of the testis in 4 healthy male donkey aged 3–5 years, (100–120 kg body weight). Ultrasound examinations were performed with a 6.5–7.5 MHz microconvex transducer (Sonoace 8800 Kretztechnik AG Tiefenbach, Austria). The donkey were placed in standing position restrained without tranquillization. In each subject the testicles were evaluated. The blood flow of the testicular artery was measured in the spermatic cord and in the marginal aspect of the artery on caudal pole of the testis. The detection of the vessels was made by colour-coded Doppler sonography and the blood flow measures were determined by pulsed Doppler sonography. The systolic peak velocity (SPV), the end-diastolic velocity (EDV), the pulsatility index (PI) and the resistance index (RI) were measured. The value obtained on the three sweeps were averaged to obtain a single mean value for each measure at each location. All measurements were obtained with an angle $< 60°$ and angle correction was applied. The values were calculated as mean ± standard deviation (SD). The physiological testicular artery flows patterns were biphasic with resistive waveform in the spermatic cord and biphasic non-resistive waveform in the marginal aspect. The value of flow parameters were reported in Table 1. To our knowledge, this is the first report regarding the physiological testicular flows patterns in male donkey and the present results give basal informations on the blood perfusion in healthy donkey testis. In conclusion the Doppler ultrasonography could be a non invasive diagnostic method to evaluate physiological and pathological condition also in male Donkey, as it is routinely used in Human Medicine.

Key Words: Donkey, testicular blood flow, Doppler sonography

Table 1. Blood flow parameters of testicular artery (n = 8)

Parameters	Spermatic cord	Marginal
SPV (cm/s)	24.5 ± 3.87	18.22 ± 3.96
EDV (cm/s)	3.55 ± 2.62	6.09 ± 2.35
PI	1.83 ± 0.56	1.11 ± 0.47
RI	0.84 ± 0.13	0.66 ± 0.11

Values are means ± SD.

1909

Testicular impact of intratesticular injection of propofol in inducing general anaesthesia in adult male dog

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Rich vascular structure and easy accessibility of testes render intratesticular injection as a potential route of drug administration. It has been used in different species for different purposes. Intravenous propofol has been tried to induce general anaesthesia in adult male dogs before. The aim of this study was to introduce a new and safer method of induction of general anaesthesia in small sized animals while trying to measure its impact on testicular tissue and function. Eighteen adult male dogs destined for selective castration were divided into three groups. In each group (on day 0), four dogs received intratesticular administration of 4 mg/kg propofol while two dogs received intratesticular normal saline (0.9%) of the same volume. All dogs later on went on to undergo an open prescorotal routine castration (with an IV induced normal general anaesthesia) in groups I, II and III on days one, three and fourteen, respectively. Plasma testosterone was measured in all groups before intratesticular administration of propofol and saline, as just prior to castration. Furthermore, histopathology evaluations of the testes were carried out in all cases following the castration. Testosterone levels showed a dramatic decrease subsequent to intratesticular administration of propofol with a strong positive correlation with the time ($p < 0.05$). Histopathologic examination revealed changes such as congestion, haemorrhage, oedema, fibrosis and tubular atrophy in the testes of treated animals which were more acute in delayed castration ($p < 0.05$). In conclusion, it seems that intratesticular administration of propofol for inducing general anaesthesia severely compromises the integrity of testicular tissue jeopardising the reproductive ability of a male dog in the future.

Key Words: Dog, testes, propofol, testosterone, anaesthesia

1910

Identification of matrix metalloproteinase (MMP-2, MMP-9) and their cognitive inhibitors (TIMP-1, TIMP-2) in the male canine reproductive system

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Matrix metalloproteinases (MMPs) together with their tissue inhibitors (TIMPs), are involved in many physio-pathological processes. This study was aimed to investigate the presence of MMPs and TIMPs in canine seminal plasma (SP), sperm, testis and epididymis, and to discover their association with semen quality traits (i.e., motility, morphology, vitality and concentration). Study of gel zymography revealed that both latent and active forms of MMP-2 and MMP-9 were present in SP (n = 46), and the latent forms appeared to be the predominant proteins. The gelatinase activities of proMMP-9 in poor quality semen samples (n = 31) was significantly higher than that of normal semen while proMMP-2 activity was significantly higher in

normal semen samples (n = 15, p < 0.01). Interestingly, relatively higher proMMP-2 activity and lower proMMP-9 activity were detected in the vigorously motile sperm samples (>70%, n = 21 vs. <70%, n = 25, p < 0.01). proMMP-2 and proMMP-9 showed their enzymatic activities that were significant higher in the epididymal sperm rich fraction than the water-like prostatic fraction. Immunofluoresecent studies demonstrated expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 on fresh sperm membrane of both head and flagella whereas all MMPs and TIMPs were barely observed on abnormal morphological sperm. In addition, the immunohistochemistrical study showed that MMP-2 and MMP-9 were located within seminiferous tubule where significant staining was observed in spermatocytes and spermatids. On the other hand, both MMPs and TIMPs showed intensive localization in the pseudostratified epithelium of epididymis. Conclusively, it is suggested that MMPs is involved in the process of spermatogenesis, sperm transit during epididymis and possibly subsequent sperm functionality. Potential clinical applications of MMPs remain to be elucidated.

Key Words: Canine, matrix metalloproteinase, tissue inhibitor of metalloproteinase, seminal plasma, sperm

1950

Intratesticular injection of zinc gluconate as a permanent contraceptive for cats

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Recently, we reported that a commercial zinc gluconate (ZG) preparation (Testoblock®) disrupted spermatogenesis and caused apparently permanent sterilization in male dogs. However, there is a paucity of reports regarding similar approaches in the male cat. Therefore, the objective was to evaluate the efficacy of intratesticular injection of ZG (Testoblock®) as a permanent contraceptive for domestic male cats. Sixteen sexually mature mongrel cats were assigned to two groups, Control (n = 5) and Treated (n = 11), with a single injection of saline or Testoblock®, respectively, into each testis on Day 0. The volume injected was based on 1 ml per 27 mm testis width, approximately twice the dose (per gram of testis) injected in dogs. Physical examination, testis width, semen characteristics, hematology, clinical chemistry (hepatic and renal function), and libido were assessed on Days 0 (before injection) 60, and 120. Testis width did not change significantly in Control cats. However, in Treated cats, there was evidence of testicular atrophy, based on reductions in testis width on Days 60 and 120 relative to Day 0 (30% and 37%, respectively, p < 0.05), with reductions (relative to Control cats) on those two moments of evaluation of approximately 17% (p = 0.06) and 23% (p = 0.118). Regarding sperm parameters, on Day 60, the ejaculates of 10 Treated cats were azoospermic, whereas the remaining cat had lower sperm counts and reduced motility, whereas on Day 120, the ejaculates of 8 (72%) cats were azoospermic, one had necrospermia and two still had viable sperm. In contrast, Control cats had excellent semen quality throughout the study. Values for hematology and clinical chemistry consistently remained within reference ranges for all cats, with no significant difference between groups. In Treated cats, there was substantially reduced libido, less mounting, aggression and urine marking (spraying) from Day 60 to the end of the study. Furthermore, penile spines (which are testosterone-dependent) were either decreased (six of 11) or absent (four of 11) in Treated cats, except for one cat which still had well-developed penile spines on Days 60 and 120. In contrast, penile spines remained well-developed in control cats. To our knowledge, the present study was the first report of a careful clinical/spermatological investigation of the effects of sterilization of cats by one-time bilateral intratesticular injection of ZG. We concluded that intratesticular injection of the zinc gluconate-based chemical sterilant Testoblock® has great potential as a permanent contraceptive for male cats.

Key Words: Contraception, zinc gluconate, testis, semen, cat

20. Nutrition and reproduction:

2000

Concentrations of enterolactone in plasma and pre-ovulatory follicles of dairy cows supplemented with flaxseed and the correlation with intra-follicular estradiol concentrations

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Flaxseed is the richest source of the plant lignan secoisolariciresinol diglycoside, a precursor to the mammalian lignan enterolactone (EL). Lignans have weakly estrogenic and anti-estrogenic properties, and they inhibit enzymes involved in the metabolism of sex hormones. The objectives of this study were to examine the concentrations of EL in plasma and follicular fluid aspirated from pre-ovulatory follicles of dairy cows fed extruded flaxseed, and the association between intra-follicular EL and estradiol concentrations. Twenty multiparous, 256-day-pregnant Israeli-Holstein cows were fed either a standard diet both pre- and postpartum (control; n = 9) or supplemented with extruded flaxseed (EX-FLAX; n = 11, prepartum 1 kg/day/cow and postpartum 9.2% of dry matter). The pre- and postpartum diets contained 1.49 and 1.76 Mcal/kg dry matter net energy for lactation, and 13.3 and 17.0% crude protein, respectively. Follicular aspirations began at 60 days in lactation; 7–8 days following behavioral estrus the cows received prostaglandin F2-alpha treatment and 48 h later all follicles >7 mm were aspirated. Follicles were regarded as estradiol-active when estradiol:progesterone ratio was >1 and these were chosen for further analysis. In total, nine control and 11 E-FLAX estradiol-active follicles were analyzed. No differences in follicular progesterone or estradiol concentrations were observed between dietary treatments. Concentrations of EL were determined in follicular fluids (FF) and in plasma sampled at day of follicular aspiration. The concentrations of EL in plasma were numerically, but not significantly, higher in EX-FLAX than in control cows (0.33 and 0.25 μM for EX-FLAX and control, respectively). However, the concentrations of EL in FF obtained from estradiol-active follicles were higher in the EX-FLAX than in the control group (0.19 and 0.11 μM EL for EX-FLAX and control, respectively; p < 0.05). Data was logarithmically transformed for correlation analysis, and across treatments analysis revealed a positive correlation between EL concentration in plasma and in FF (r = 0.61, p < 0.05). In addition, EL concentrations in FF were positively correlated with estradiol concentrations (r = 0.50, p < 0.04) and estradiol:progesterone ratio in FF (r = 0.65, p < 0.003). In conclusion, feeding dairy cows with extruded flaxseed increased EL concentrations in pre-ovulatory follicles. The positive correlation between plasma and intra-follicular EL concentrations implies that EL is transferred into the pre-ovulatory follicle. Furthermore, the positive correlation between follicular EL and estradiol suggests that EL may affect follicular steroidogenesis; however, this assumption requires further research.

Key Words: Enterolactone, flaxseed, follicle, estradiol

2001

The impact of maternal nutrition during pregnancy on fetal calf growth and muscle gene expression in beef cattle

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Influences of nutrition during pregnancy on offspring performance have been established in many mammalian species, and could represent a window of opportunity to ensure/enhance genetic potential via dietary means in beef cattle. To investigate the impact of maternal nutrition on fetal growth and development in the latter half of pregnancy, 22 mature (3–6 years-old) multiparous pregnant cross-bred cows were divided into two groups (n = 11 cows/group) and fed two levels of nutrition; ad-libitum intake (˜140% above maintenance; HIGH) and restricted to 85% maintenance intake (LOW). The diet consisted of a haylage-based total mixed ration containing 20% wheat straw. All cows started the feeding trial simultaneously at 147 ± 15 days of gestation and were slaughtered over 6 weeks (blocks), in groups of four (two cows from each group/week) at 247 ± 10 days of gestation. Total weight gain and average daily weight gain were less ($p < 0.005$) in cows in the LOW group when compared to cows in the HIGH group (53 vs. 104 kg and 0.53 vs. 1.07 kg/day, respectively), indicating that nutritional restriction impacted the dam. No significant main effects were observed on fetal phenotypes determined after slaughter with the exception of lung weight, for which a significant treatment-by-block interaction was observed. Liver IGF1 gene expression was decreased ($p < 0.05$) and IGF1R gene expression was increased ($p < 0.05$) in fetuses from cows in the LOW group when compared to those from cows in the HIGH group [0.96 vs. 1.42 relative units (ru) and 0.96 vs. 0.78 ru, respectively]. Moreover, muscle (longissimus dorsi) expression of IGF and myogenesis family genes were also increased ($p \leq 0.05$) in fetuses from cows in the LOW group when compared to those from cows in the HIGH group (IGF1: 1.76 vs. 1.13 ru, IGF1R: 1.08 vs. 0.76 ru, IGF2R: 2.38 vs. 1.63 ru, INSR: 1.35 vs. 1.0 ru, MYOD1: 1.46 vs. 1.01 ru, and MYOG: 1.39 vs. 0.93 ru, respectively). There were negative correlations ($p \leq 0.05$) between fetal weight and muscle IGF1 ($r = -0.55$), IGF1R ($r = -0.48$), IGF2 ($r = -0.65$), IGF2R ($r = -0.65$), MYOG ($r = -0.48$) and SRF ($r = -0.61$) mRNA abundance. This analysis is noteworthy since it displays the variation in response that individual animals can experience to nutritional challenges, and emphasizes the importance of identifying nutritionally susceptible animals in commercial herds to avoid detrimental consequences of sub-optimal pre-natal nutrition. Our results clearly illustrate that restricted feed intake during pregnancy can alter the expression of growth and myogenic genes in muscle and could be detrimental to the fetus even if no obvious phenotypes are observed. Future experiments are required to determine if such changes affect economically important traits such as longevity or meat production and quality.

Key Words: Fetal programming, gene expression, nutrition, beef cattle

2002

The effects of high and low protein diet intake on the mRNA abundance of mid-luteal phase endometrial candidate genes and ovarian dynamics in high producing lactating dairy cows

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Pregnancy rates (PR) in high producing lactating dairy cows have declined drastically over the past several decades, but those of heifers have remained constant. Reduced PR could be due to multiple causes and the underlying physiological mechanisms are still unclear. High dietary protein intake is one of the reasons for decreased PR observed in dairy cows. The objective of this study was to examine the effects of high and low protein diet intake on mRNA expression of mid-luteal phase endometrial fertility candidate genes (IGF1, IGFBP1, IGFBP3, FGF2, BAX, BCL2, HSPA1A, IL1A, TNF, SERPINA14), and ovarian follicular and corpus luteum (CL) dynamics. Holstein cows (n = 24), blocked by parity (2nd or 3rd), previous milk production, and body condition score (3.25–4.25), were assigned to a high (17.3% of dry matter) or low (14.8%) protein diet group at calving. The diet was composed of corn silage, grass silage, alfalfa hay and concentrate. The protein content in the diet was manipulated by altering the levels of ingredients in the concentrate. In addition, the diets were balanced for fermentable carbohydrate. All cows were housed in a single experimental pen with 24 free stalls fitted with rubber mattresses covered with sand. The pen was equipped with an automated feed and water intake system. Cows received 'Ovsynch' treatment approxiamtely 35–40 days after calving and endometrial biopsies were obtained 11–12 days after synchronized ovulation and mRNA expression was determined by qRT-PCR. Trans-rectal ultrasonography was performed weekly, starting 2 weeks post calving, to evaluate ovarian follicular (number and diameter) and CL (fist appearance and diameter) dynamics. Milk production of cows was recorded daily and samples were collected weekly for evaluation of milk urea nitrogen (MUN) levels. IL1A expression was lower in cows fed with high protein diet ($p < 0.05$), but expression of all other genes were not different between the two groups. Milk analysis showed lower ($p < 0.05$) MUN levels in the low protein group relative to that of high protein group (6.9 ± 0.2 vs. 9.5 ± 0.2 mg/dl). Mean number of small (2.5 ± 0.3 vs. 1.2 ± 0.2) and large (1.3 ± 0.1 vs. 1.1 ± 0.1) follicles, mean size of the largest follicles (16.1 ± 0.6 vs. 14.4 ± 0.4 mm), and the mean CL size (27.1 ± 1.4 vs. 23.9 ± 0.7 mm) were greater ($p < 0.05$), and the number of days to first ovulation was shorter (20.8 ± 1.3 vs. 25.7 ± 2.1 d; $p < 0.05$) in cows fed with low protein to those fed high protein. Milk yield and body condition score were not affected by low protein diet intake. In conclusion, lower dietary protein level increased endometrial IL1A gene expression and had beneficial effects on ovarian function in postpartum lactating dairy cows.

Key Words: Pregnancy rate, protein intake, dairy cows, genes, ovarian parameters

2003

Effect of undernutrition on embryo production and quality in superovulated ewes

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The objective of this study was to determine the effect of undernutrition on embryo production and quality in mature Rasa Aragonesa ewes. Ewes were allocated into two groups to be fed diets that provided either 1.5 (control; n = 20) or 0.5 (low nutrition; n = 25) times the daily requirements for maintenance (AFRC, 1998). Diets consisted of 0.8 or 0.5 kg of barley straw and 0.55 or 0.1 kg of pellets (79% barley, 15% soy bean, 6% mineral supplement) per ewe per day for control and low nutrition groups, respectively. Different diets were offered from the day that estrus synchronization treatment was initiated until the day that embryos were collected. Estrus was synchronized using intravaginal fluorogestone acetate sponges (30 mg) for 14 days. Superovulation was induced with 210 IU pFSH and 500 IU eCG, i.m., administered 48 h before sponge withdrawn. Rams of known fertility were placed with the ewes 24 h after sponge withdrawal and ewes were examined for signs of estrus every 8 h. Embryos were collected through mid-ventral laparotomy 7 days after the onset of estrus. Bodyweight (BW) was determined at time of sponge insertion, sponge withdrawal, and embryo collection. In addition, blood samples were collected at the day of embryo collection to determine plasma progesterone concentrations. Low nutrition resulted in lower BW at the time of sponge withdrawal ($p = 0.06$) and embryo collection ($p < 0.05$) (53 ± 1 and 53 ± 2 kg, respectively) when compared to controls (56 ± 1 and 57 ± 2 kg, respectively). Although the number of corpora lutea and plasma progesterone concentration were greater ($p < 0.05$) in the low nutrition group (27.6 ± 2.6 CL and 62.7 ± 7.0 ng/ml, respectively) when compared to controls

(17.4 ± 1.6 CL and 44.7 ± 4.1 ng/ml, respectively), the total number of recovered structures (oocytes and embryos) did not differ between the groups (15.4 ± 1.8 and 12.4 ± 1.3 structures in the low and control groups, respectively); therefore, structure recovery rate was lower ($p < 0.05$) in the low nutrition (60%) than in control group (73%). In addition, low nutrition also resulted in lower ($p < 0.1$) total number of embryos and number of viable-transferable embryos (5.0 ± 1.3 and 3.4 ± 1.1 embryos, respectively) when compared to controls (8.4 ± 1.4 and 6.2 ± 1.3 embryos, respectively). In conclusion, undernutrition during the period of superovulation and early embryonic development in ewes resulted in increased number of ovulations, but reduced total and viable number of embryos. These effects might be mediated by disruption of endocrine homeostasis, oviduct environment, and/or oocyte quality. Project AGL2010-15004

Key Words: sheep, undernutrition, superovulation, embryo

2004

Maternal undernutrition of recipient ewes reduces pregnancy rate regardless level of nutrition of donor ewes

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This study was designed to determine the effects of maternal undernutrition on pregnancy rate of recipient ewes after transfer of embryos collected from control or undernourished donor ewes. Forty-five donors and 52 recipients Rasa Aragonesa ewes were synchronized in estrus (Day 0) using intravaginal sponges (30 mg fluorogestone acetate) for 14 d. At the time of sponge insertion, ewes were allocated to one of two groups to be fed diets that provided either 1.5 [control group (C); donor n = 20; recipient n = 29] or 0.5 [low group (L); donor n = 25; recipient n = 23] times the daily requirements for maintenance (AFRC, 1998). Diets consisted of 0.8 or 0.5 kg of barley straw and 0.55 or 0.1 kg of pellets (79% barley, 15% soy bean, 6% mineral supplement) per ewe per day for control and low nutrition groups, respectively. These diets were offered up to the day of embryo collection and transfer. To induce superovulation, donor ewes received 210 IU pFSH and 500 IU eCG (i.m.) 48 h before the intravaginal sponge was removed. Embryos were collected 7 days after the onset of estrus, and after subjective classification, 102 embryos (compacted morulae and early/expanded blastocysts) were transferred into recipient ewes (two embryos per ewe). Recipient ewes were assigned to four groups on the basis of experimental diets: CC (embryos collected from a C donor and transferred to a C recipient; n = 14); LC (donor L-recipient C; n = 11); CL (donor C-recipient L; n = 15) and LL (donor L-recipient L; n = 12). Blood samples were collected from recipient ewes at time of sponge insertion (d-14), withdrawal (d-1) and at embryo transfer (d7) to determine plasma insulin and NEFA concentrations. Pregnancy was determined based on plasma progesterone concentrations obtained on 18 and confirmed by ultrasonography 40 days after embryo transfer. Undernutrition resulted in lower ($p < 0.01$) body weight (52 ± 1 kg) and greater ($p < 0.05$) NEFA concentrations on d7 (0.21 ± 0.03 mM) when compared to controls (59 ± 1 kg and 0.10 ± 0.01 mM, respectively). Control ewes also tended ($p = 0.06$) to have greater plasma insulin concentrations on d-1 and d7. Pregnancy rates determined based on progesterone concentrations on day 18 were similar among groups (CC: 79%, LC: 91%, CL: 80%, LL: 92%), but differed when determined by ultrasonography on day 40 (CC: 71%, LC: 73%, CL: 53%, LL: 58%); overall, pregnancy rates tended ($p = 0.1$) to be lower in undernourished recipients (55%) when compared to controls (72%). In conclusion, short-term undernutrition up to the day of embryo transfer in recipient ewes appeared to have resulted in late embryonic death and this effect was independent of the nutritional status of the embryo donor. Project AGL2010-15004

Key Words: Sheep, undernutrition, embryo

2005

Effect of short-term energy supplementation during estrus synchronization on circulating insulin and glucose concentrations and ovulation in Toggenburg goats

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Nutrition might affect a wide range of reproductive events, from gametogenesis to puberty, and the importance of endocrine signals in the regulation of follicular development has long been recognized. The objective of the present study was to evaluate the effects of short-term energy supplementation during estrus synchronization on circulating insulin and glucose concentrations and ovulation in Toggenburg goats during the non-breeding season. The study was conducted between late February and early March in Minas Gerais, Brazil. Thirty-two does were allocated into three groups to receive different levels of energy supplementation. One group received a maintenance diet (Group M; n = 11), one group received 1.5 times the amount of energy offered to the maintenance group (Group 1.5M; n = 10), and another group received two times the amount of energy (Group 2M; n = 11). Rations were formulated with corn meal, soybean meal, sorghum meal, and unsaturated fatty acid (Megalac-E®); corn meal and unsaturated fatty acid amounts were manipulated in order to obtain the different levels of dietary energy. Estrus was induced and synchronized with intravaginal sponges impregnated with 40 mg of medroxyprogesterone acetate for 6 days (Day 0 = sponge insertion). Energy supplementation was offered during the period in which intravaginal sponges were being used. Does were treated with intramuscular eCG (200 UI) and d-cloprostenol (0.4 ml) on Day 4. Blood samples were obtained in the morning from the jugular vein on Days 0, 2, 4, 6, and 16 and plasma insulin and glucose concentrations were determined. In addition, ultrasound exams were performed to detect the number of ovulatory follicles. Energy supplementation did not affect insulin concentrations (overall 8.25 ± 4.41, 8.61 ± 3.76, and 7.17 ± 4.8 μUI/ml in Groups M, 1.5 M, and 2 M, respectively), glucose concentrations (overall 57.72 ± 7.47, 56.48 ± 6.90, 58.35 ± 8.91 mg/dl in Groups M, 1.5M, and 2M, respectively), or mean number of ovulatory follicles (1.27 ± 0.47, 1.11 ± 0.33, 1.18 ± 0.40 follicles in Groups M, 1.5M, and 2M, respectively). In conclusion, short-term supplementation with 1.5 or 2 times the amount of maintenance energy during estrus synchronization did not affect circulating insulin and glucose concentrations and ovulation in Toggenburg goats during the non-breeding season.

Key Words: Goats, ovulatory follicles, energy supplementation, insulin, glucose

2006

Foetal life protein restriction of mink dams affects the birth weight and pre-weaning growth of their offspring

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Low dietary protein (14% of metabolisable energy, ME) offered to mink dams during gestation affects reproductive performance, birth weight, and gene expression of some key hepatic enzymes in both F1 and F2 generations. The objective was to investigate if less severe protein restriction during gestation affected F2 offspring birth weight and pre-weaning growth without affecting female F1 reproductive performance and quantitative metabolism traits. The animals were housed under Danish mink farm conditions. Dietary protein provision was low (L; 19% of ME) or control (C; 31% of ME) during the last

21 ± 3 (F0 dams) and 17 ± 1 (F1 dams) days of gestation. Dams and offspring were fed a standard diet from parturition onwards. One-year-old F1 dams formed four groups according to their protein provision during foetal life and during their own gestation in a 2 × 2 design: low-low (LL; n = 18), low-control (LC; n = 19), control-low (CL; n = 20), and control-control (CC; n = 20). Six dams from each treatment were housed in metabolism cages for evaluation of ME intake, heat production (HE), and retained energy (RE) during late gestation and twice during lactation (week 2 and 4); results of these measurements were averaged for analysis. Data were analyzed using proc GLM in SAS. The ME intake was not different between treatment groups (range 920–970 kJ*kg$^{-0.75}$) but HE was affected by gestation diet ($p < 0.001$) and interactions between foetal life protein provision and gestation diet ($p = 0.01$) resulting in a greater HE in LC, intermediate in CC and CL, and lower in LL dams (728, 662, 635, 602 kJ*kg$^{-0.75}$, respectively), whereas RE was affected by interactions between the foetal life protein provision and gestation diet ($p = 0.04$) resulting in a greater RE in LL, intermediate in CL and CC, and lowest in LC dams (368, 283, 255, and 202 kJ*kg$^{-0.75}$, respectively). Treatment did not affect the percentage of barren dams (range 0–15%) or litter size (range 8.3–10 kits). There were foetal life protein provision ($p = 0.01$) and gestation diet effects ($p < 0.001$) on F2 birth body weight (BW) (n = 513); BW being highest in offspring from LC and CC dams, intermediate in offspring from LL and lowest in offspring from CL dams (10, 9.8, 8.9, and 8.3 g, respectively). However, gestation diet effects ($p < 0.01$) and interactions ($p < 0.001$) between foetal life protein provision, gestation diet, sex and age were observed in F2 offspring pre-weaning BW from 1 to 8 weeks of age; this resulted in female kits from the CC group having the highest, LC and LL intermediate and CL the lowest BW (708, 699, 681 and 666 g, respectively) at 8 weeks of age. In male kits LC and CL had the highest, LL intermediate and CC the lowest BW at 8 weeks of age (818, 816, 811 and 797 g, respectively). In conclusion, foetal life protein restriction affected birth weight of the subsequent generation, and pre-weaning offspring growth.

Key Words: Foetal programming, protein provision, gestation diet

2050

Short-term dietary propylene glycol supplementation affects circulating metabolic hormones and progesterone concentrations and follicular growth in dairy heifers

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Over-feeding and under-feeding are know to have negative effects on follicular growth in cattle. The objective of this study was to determine the effects of dietary propylene glycol (PG; Propypact®, Difagri Picot SA, France) on blood metabolites, metabolic and reproductive hormones, and follicular growth in 10 dairy heifers, 14 ± 1 months-old, weighing 332 ± 26 kg. Treatments consisted of (i) 1.1 kg of beet pulp (control), (ii) 150 g PG (PG150), and (iii) 300 g PG (PG300). Each heifer received all the treatments in different orders (1-3-2, n = 3; 1-2-3, n = 3; and 3-2-1, n = 4). A standard hay/concentrate diet formulated to produce a gain of 850 g/day was supplied at 8:00 and treatments were supplied at 16:00 from Days 1 to 13 of the estrous cycle following induced estrus (Day 0). Estrus induction treatment consisted of a subcutaneous 3 mg norgestomet implant inserted for 9 days combined with GnRH treatment (i.m.) at implant insertion. Two days before implant removal, 500 μg cloprostenol was administered. Blood samples were collected by jugular venipuncture every 2 h for 24 h on Days 0 and 13 for evaluation of insulin, glucose, and β-hydroxybutyrate (BHB) concentrations. Blood samples were also collected once on Days 2, 6, 9, 12 for evaluation of insulin-like growth factor-1 (IGF-1), estradiol, and progesterone concentrations. On the same days, ovarian follicular growth was evaluated; the total number of follicles and their diameters were recorded and classified (2–3 mm, 4–7 mm, and >8 mm). Results were analyzed by Repeated-measures ANOVA. There were no treatment, day, or interaction effects on overall mean insulin, glucose, and BHB concentrations measured on Days 0 and 13. There were treatment, time, and interaction effects on insulin, glucose and BHB concentrations obtained over 24 h on Day 13; glucose concentrations were greater ($p < 0.05$) at 4:00, 8:00, 12:00, 16:00 and 20:00 h, whereas BHB concentrations were lower ($p < 0.05$) at 20:00 and 22:00 h in the PG300 group when compared to control and PG150 groups. There were treatment, day, and interaction effects on IGF-I and progesterone concentrations, and number of small follicles; PG150 treatment resulted in greater ($p < 0.05$) IGF-1 concentration on Day 6, progesterone concentrations on Days 9 and 12, and number of small follicles on Day 2 when compared to controls. These results indicate that short-term dietary PG supplementation affects circulating concentrations of metabolites and metabolic hormones, and increases progesterone concentrations and number of small follicles. Propylene glycol supplementation might be especially useful when combined with treatments to stimulate follicular growth for superovulation or ovum-pick up.

Key Words: Propylene glycol, metabolites, follicular growth, dairy heifers, insulin

2051

Correlations of zinc and selenium concentrations in seminal plasma and serum with sperm count and morphology in domestic cats

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Zinc and selenium are known to play an important role in the proper function of female and male reproductive systems. The objective of this study was to correlate seminal plasma and serum zinc and selenium concentrations to sperm count and sperm morphology in the domestic cat. A total of six adult mixed-breed male cats, 4–7 years old, were used in this trial. All animals were housed at the Veterinary School research cattery and fed dry commercial cat food. Semen collections were performed during the breeding season (October–December) with at least 2-day interval and using an artificial vagina. All cats were submitted to semen collection on the same day (n = 16 total ejaculates/cat). After collection, semen samples were centrifuged during 5 min at 700× *g* and seminal plasma was separated from sperm, re-centrifuged at 1500× *g* for 15 min, and stored at −80°C. Before analysis, seminal plasma samples from the same tom were thawed, pooled, and centrifuged at 3000× *g* for 10 min. In addition, selenium and zinc blood concentration were determined on two different occasions, November and December. Seminal plasma and serum zinc and selenium concentrations were measured by atomic absorption spectroscopy. The pellet containing sperm was resuspended and total sperm number determined. In addition, sperm morphology was evaluated in 200 cells per sample using Rose Bengal and Fast Green FCF staining. Seminal plasma and serum zinc concentrations were 3.9 ± 1.0 and 3.0 ± 0.7 mg/l, respectively, whereas selenium concentrations were 52.2 ± 5.1 and 65.9 ± 9.4 μg/l, respectively. Mean total sperm count among cats varied from 18.7 to 55.0 million spermatozoa, whereas percentage of morphologically abnormal sperm varied from 30.7 to 87.8%. Correlations were made using mean values of sperm count, sperm morphology, and zinc and selenium blood concentrations obtained for each cat. Seminal plasma zinc concentration had a strong positive correlation ($r = 0.71$, $p < 0.05$) with total sperm count, whereas serum zinc concentration had a strong negative correlation ($r = -0.77$, $p < 0.05$) with the percentage of morphologically abnormal sperm. Based on these findings, we can suggest that zinc might be an essential trace element for cat sperm quality by exerting a positive effect upon the process of spermatogenesis. Thus, appropriate zinc supplementation should be provided in the diet of felids used in reproduction programs.

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Key Words: Sperm quality, zinc, selenium, seminal plasma, serum

2052

Effect of dietary energy on sperm membrane integrity and seminal plasma protein concentrations in mature rams

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Nutrition affects testicular mass and daily sperm production in mature rams, but the effects of nutrition on semen quality are unknown. Since sperm function has been associated with seminal plasma proteins (SPP), this study was conducted with the objective of determining the effects of nutrition on SPP concentration and semen quality in adult Merino rams. The experiment was conducted during 85 days and was initiated in July (winter). Although the experiment was conducted after the end of the breeding season, Merinos are not considered strongly seasonal sheep. Two groups of rams (n = 5/group) were fed either a high-energy (HE; 16MJ ME/days) or a low–energy, maintenance diet (LE; 8MJ ME/days. By the end of the experimental period, HE diet had led to a 21% increase in body weight and 11% increase in scrotal circumference (SC), whereas LE diet had led to a 3% decrease in body weight and 17% decrease in SC. Semen was collected using artificial vaginas every 2 weeks for evaluation of sperm production and semen quality. Ejaculate volume, sperm concentration, total sperm number in the ejaculate, percentage of motile sperm, and sperm kinetics (determined by CASA) did not differ significantly between groups. The proportion of membrane-intact sperm (determined with eosin/nigrosin stain) was greater ($p < 0.001$) in the HE (80 ± 1%; mean ± SEM) than in the LE diet group (66 ± 2%). For evaluation of SPP, seminal plasma was separated immediately after collection by centrifugation for 12 min at 12 000 rpm. Seminal plasma was storage at −20°C until concentrations were determined with the Bradford Coomassie Blue Protein Assay using bovine serum albumin as the standard. Seminal plasma protein concentration increased from 20 ± 3.2 mg/ml on the first day of the experiment to 33 ± 2.0 mg/ml by the end of the experiment in the HE diet group, but concentrations did not change significantly in the LE diet group. Overall, SPP concentrations were greater ($p < 0.05$) in the HE (29 ± 3.6 mg/ml) than in the LE diet group (21 ± 2.1 mg/ml). There was a strong negative correlation between SPP concentration and sperm viability in the HE diet group ($r = -0.66$; $p < 0.001$), but not in the LE diet group. In conclusion, nutrition–driven changes in SC in mature rams were associated with changes in sperm membrane integrity and SPP concentrations. Further work is needed to determine whether these observations reflect cause–effect relationships and how SPP might affect sperm membrane integrity.

Key Words: Nutrition, seminal plasma protein concentration, sperm viability, ram

2053

Oral L-arginine supplementation increases vascular perfusion of the ovulatory follicle in post-partum mares

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Increased ovarian blood flow has been correlated to increased pregnancy rates in mares. L-Arginine (L-Arg) is the precursor for nitric oxide, a smooth muscle relaxant and potent vasodilator. The objectives of this study were to determine if oral L-arg supplementation would alter ovarian follicular dynamics and increase ovarian perfusion during the first post-partum estrous cycle in mares. Sixteen mares were blocked by age, parity and expected foaling date then randomly assigned to either a control (n = 8) or L-arg supplemented (n = 8) group. L-Arg mares were fed 100 g L-Arg once daily in addition to the basal diet. L-Arg supplementation began 21 days prior to the expected foaling date and continued until the end of the study. Mares underwent daily transrectal exams starting the day after foaling until ovulation using a Micromaxx Sonosite Doppler ultrasound with a 5–10 MHz broadband linear array transducer (Bothell, WA, USA). Follicles were categorized by diameter (6–10, 11–15, 16–20, >20 mm). The retrospectively identified dominant (F1) and largest subordinate (F2) were mapped via a non-tracking method. Follicular perfusion was defined as the blood flow surrounding the follicle using color Doppler ultrasonography. Perfusion was estimated using a radial grid divided into 32 even slices. The number of slices containing perfusion were counted and compared to the total number of slices. Data was analyzed using the Mixed procedure of SAS (version 9.2; SAS Institute, Cary, NC, USA). No differences were found between groups in the number of follicles by category or F1 and F2. Mean time from deviation to ovulation were 8.0 ± 0.6 days in L-arg mares and 6.6 ± 0.6 days in control mares ($p = 0.10$). Mean deviation diameter of the F1 in L-arg mares was 22.9 ± 1.2 mm and in control mares was 23.0 ± 1.4 mm (NS). Mean perfusion in the F1 was increased in the L-Arg (37.3 ± 2.6%) compared to control (25.4 ± 2.7%; $p \leq 0.05$) 5 days before ovulation (Day 0). L-Arg mares had increased perfusion on Days −3 and −2 ($p \leq 0.05$) and a trend ($p = 0.07$) towards increased perfusion on Day −1. In conclusion, L-Arg supplementation had no effect on follicular dynamics but increased vascular perfusion in the wall of the ovulatory follicle in post-partum mares.

Key Words: Mare, Doppler ultrasound, L-arginine, blood flow, ovary

21. Oocyte development:

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Standardization of RNA extraction from bovine oocytes

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In the last decade, studies of gene expression on a large scale have characterized the transcripts present in oocytes and some of these transcripts have been associated with the competence to develop into an embryo and finally a healthy offspring. The results support the hypothesis that specific mRNA or proteins produced during oogenesis are responsible for the development of oocyte competence. With the increasing understanding of the processes of pre-maturation and final maturation of the oocyte, it may be possible to improve the procedures of *in vitro* embryo production. The investigation of oocytes matured *in vitro*, using global analysis of mRNA technology, can detail the genes involved in meiosis, cytoplasmatic maturation and embryonic development. For this, the mRNA extracted must be of good quality to provide a reliable result. Thus, the aim of this study was to standardize the procedures for RNA extraction from bovine oocytes. Ovaries were collected from slaughtered cows and transported to the laboratory. The oocytes were aspirated, selected and matured for 24 h at 5% carbonic gas and maximum humidity. Subsequently, they were denudated with hyaluronidase and RNA extraction was performed by one of two methods: RNeasy MicroKit (Qiagen) following the manufacturer's instructions and the Trizol® method. The concentration and quality of RNA was determined by Nanodrop ND-1000 and optical density 260/280 ηm. Seeking to achieve a higher concentration of RNA, as well as better quality, the number of extracted cells (7, 15, 20, 30, 50, 100) and speed of centrifugation (12000 rpm or 14000 rpm) were changed. According to the Nanodrop ND-1000 and optical density results, it was observed that regarding the number of oocytes used in each extraction, it wasn't possible to make any association with the RNA concentration and quality, in both the RNeasy MicroKit and Trizol® methods. On the other hand, when the speed of centrifugation was changed to 14000 rpm in the RNeasy MicroKit method, a slight improvement was noted in the ratio of 260/280 ηm. The best concentrations of RNA were obtained using the Trizol® method, ranging between 20 and 60 ηg/µl. The RNeasy MicroKit resulted in lower concentrations between 0.7 and 15 ηg/µl, associated with the observation that a larger sample size does not guarantee a higher concentration of RNA. In respect to the quality of RNA (260/280 ηm reason), it is expected that this ratio is about 2.0. Despite not reaching values as close to 2.0, the Trizol® method exhibited better values (between 1.66 and 1.79) than extraction using the RNeasy MicroKit (between 1.06 and 1.62). In conclusion, the Trizol® method was more efficient for the extraction of RNA from bovine oocytes. However, new changes in the methodology should be proposed seeking a better quality of RNA.

Key Words: Oocyte, RNA extraction, bovine

2101

Transcript and protein stockpiles, lipid content and ultrastructure of bovine oocytes enclosed or not within cumulus cells are differently affected by *in vitro* maturation: implications for developmental competence

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The oocyte is surrounded by cumulus cells (CC), which support its growth through bidirectional intracellular communications and help to metabolize external energy substrates. During final meiotic maturation, CC play an important role in oocyte acquisition of developmental potential: indeed, bovine oocytes matured *in vitro* without CC have a lower capacity to support embryo development. We aimed to compare the effects of optimized *in vitro* maturation (IVM) procedures on the transcriptome, proteome and morphology of cumulus denuded oocytes (CDO) and cumulus enclosed oocytes (CEO), and on subsequent embryo development. Cumulus-oocyte complexes were aspirated from cow ovaries; half of the oocytes were denuded of CC, and then CDO and CEO were cultured separately for 22 h in metabolically-equilibrated serum-free TCM199-enriched medium. After IVM, metaphase-II rate was similar (89.3 ± 3.3% vs. 88.2 ± 3.0%); however after *in vitro* fertilization, blastocyst development was significantly lower in CDO compared to CEO (20.3 ± 4.3 vs. 43.5 ± 5.4%, $p = 0.015$). In contrast, when CDO and CEO were subjected to ionomycin/6-DAB parthenogenesis, cleavage and blastocyst rate of parthenotes did not differ, only the proportion of hatched blastocysts from cleaved oocytes from CEO tended to be higher than from CDO (16.0 ± 3.5% vs. 6.9 ± 0.7%; $p = 0.066$). Global transcriptomic analysis using a microarray detected 13 609 transcripts in oocytes with polar bodies after IVM; among them only 45 (0.33%) were differentially expressed between CDO and CEO ($p < 0.05$, fold change range: 0.6–2.1). They corresponded to 41 genes regulating transcription, translation and metabolism. At the protein level, using profiling of intact cells MALDI-TOF mass spectrometry of single metaphase-II oocytes ($n = 12$ per group), we found that 3.05% of detected m/z peaks, corresponding to low weight protein species, significantly varied between CDO and CEO ($p < 0.05$). Transmission electron microscopy of CDO and CEO at metaphase-II stage revealed differences in quantity and distribution of lipid droplets, nutritive vacuoles and mitochondria between the two groups. Moreover, significantly diminished total lipid content was evidenced in CDO compared with CEO by quantifying Nile Red fluorescence emission ($p < 0.01$). In conclusion, during IVM in optimized enriched medium, absence of CC only slightly affected oocyte transcriptome and had no impact on nuclear maturation. Differences between the development of IVF embryos and parthenotes originating from oocytes matured without CC suggests a possible alteration of the factors regulating paternal genome involvement. Differences of ultrastructure, proteome and lipid content between these two groups indicated that metabolism in denuded oocytes was more affected by IVM, as compared with CEO, and this led to suboptimal cytoplasmic maturation and consequently to lower embryo rate. Financial support: programs Ovogenae2 (ANR-07-GANI-004/ApisGene), OSCILE (ANR-08-GENM-033).

Key Words: Oocyte, cumulus, transcriptome, proteome, ultrastructure

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Follicle size of origin affects glucose 6 phosphate dehydrogenase activity and developmental competence of oocytes from Boran cows

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In vitro embryo production (IVEP) is rapidly gaining popularity as one of the tools of choice for the improvement of cattle genotypes in certain countries. In sub Saharan Africa, IVEP is yet to be fully optimized especially with the *Bos indicus* breeds. However, IVEP is still faced with challenges that render it incapable of fully recapitulating *in vivo* embryo production conditions. Oocyte competence prior to *in vitro* maturation has been identified as one of the key areas affecting this process and it has been associated with the glucose 6 phosphate dehydrogenase (G6PDH) activity as well as follicle size. Nevertheless, some features of ovarian follicular dynamics are different between *Bos indicus* and *Bos taurus*, which may influence oocyte competence. The aim of this study was to evaluate the effect of follicle size on G6PDH activity and oocyte developmental competence in Boran (African Zebu breed) cattle using brilliant cresyl blue (BCB) staining and *in vitro* embryo production. Oocytes were collected from Boran cows at slaughter, their follicles were measured and classified into 3 groups; 1–3 mm, >3–6 mm and >6 mm in diameter. These follicles were aspirated and cumulus oocytes complexes (COC) with a homogenous nucleus and over two layers of compact cumulus cells were selected. These COC were exposed to BCB stain for 1 h and observed for cytoplasmic coloration. Those that retained the blue coloration (BCB positive) were deemed competent (low activity of G6PDH) while those that did not have any cytoplasmic coloration (BCB negative) were deemed incompetent (high activity of G6PDH). A higher proportion ($p < 0.01$) of BCB positive oocytes was found in >6 mm follicles (81.1%) than in 1–3 mm (73.1%) and >3–6 mm (76.5%) follicles. BCB positive oocytes from 1 to 3 mm follicles had higher ($p < 0.05$) blastocyst rate (18.94%) than BCB negative oocytes (9.7%); however, no significant difference was found in their cleavage rate ($p > 0.05$). No difference in cleavage and blastocyst rates was found between BCB positive and BCB negative oocytes from follicles >3 to 6 mm in diameter. The cleavage and blastocysts rates with BCB positive oocytes increased as follicle diameter increased, with oocytes from follicles larger than 6 mm diameter producing the highest blastocyst rate (29.03%). In conclusion, as follicle size increases the activity of G6PDH decreases in Boran oocytes whereas the developmental competence increases. Boran follicles as small as 1–3 mm in diameter are able to produce oocytes with low G6PDH activity and competence to develop to the blastocyst stage.

Key Words: IVEP, Boran, brilliant cresyl blue, follicle size

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Organelle modulation in bovine oocytes during dominant follicle growth and maturation

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Our objective was to understand the changes in number, size and distribution of organelles in the ooplasm during the growing, static, regressing and preovulatory phases of dominant follicle development. We hypothesized that ooplasmic organelles undergo changes in number and spatial distribution in a phase-specific manner. Hereford heifers were monitored daily by transrectal ultrasonography and cumulus-oocyte-complexes were collected by transvaginal ultrasound-

guided follicular aspiration on Days 3–4 of wave 1 (growing follicles, n = 5; Day 0 = ovulation), Days 6–7 (static follicles, n = 5), Days 10–12 (regressing follicles, n = 7) and Days ≥17 (preovulatory follicles, n = 5). Oocytes were processed and transmission electron micrographs were obtained representing peripheral (ooplasm within 10 μm of plasma membrane) and central regions. Standard stereological countings (points and line-intersections) were performed by randomly placing a transparent test-grid over the electron micrograph (×10 000). The surface area density ($\mu m^2/\mu m^3$), volume density ($\mu m^3/\mu m^3$), and numerical density (number/1000 μm^3) of organelles were analyzed by 2-way ANOVA (region and day). The mitochondrial numerical density was higher ($p \leq 0.04$) in oocytes from regressing follicles (193 ± 10.4) compared to those from growing, static and preovulatory stages (118.7 ± 14.4, 125.6 ± 20.8 and 150.5 ± 28.7, respectively). More than 70% of mitochondria in oocytes from growing, static and preovulatory follicles were located in the peripheral regions, whereas oocytes from regressing follicles had an even distribution (region*day interaction $p < 0.001$). The volume occupied by lipid droplets was higher ($p < 0.01$) in oocytes from regressing follicles (3.5 ± 0.7%) than those from growing and preovulatory follicles (1.1 ± 0.3% and 1.6 ± 0.2%) while oocytes from static follicles were intermediate (2.3 ± 0.5%). Oocytes from growing follicles had a greater proportion of lipid droplets in the peripheral region than in the central region (86.9% vs. 13.1%), whereas all other stages had an even distribution (region*day interaction $p \leq 0.01$). The percent surface area of mitochondria in contact with lipid droplets was lower ($p \leq 0.04$) in the oocytes from growing follicles (2.3 ± 0.9%) than static, regressing and preovulatory follicles (8.9 ± 0.2%, 6.1 ± 1.2% and 6.2 ± 1.4%, respectively). The number, size and distribution of smooth endoplasmic reticulum, vesicles, cortical granules, and Golgi did not differ among phases. Our hypothesis was partially supported in that mitochondrial number increased and translocation occurred from a peripheral to an even distribution as follicles entered the regression phase. In addition, lipid droplets underwent spatial reorganization from a peripheral to an even distribution during the growing phase and mitochondria-lipid contact area increased with development. Supported by the Natural Sciences and Engineering Research Council of Canada.

Key Words: Oocyte, bovine, ultrastructure

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Comparison between different maturation systems acting through the manipulation of cAMP dependent pathways in bovine oocytes

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Recent studies reported that delaying meiotic resumption of bovine oocytes through the modulation of cAMP mediated pathways can increase *in vitro* developmental competence (Albuz et al., *Hum Reprod* 2010; 25:2999). The aim of this study was to compare different maturation systems in which oocytes were treated with modulators of intracellular cAMP levels for different times and concentrations. In the first group (G1), cumulus-oocyte complexes (COCs) were collected, treated with an adenylate cyclase activator (forskolin) and a non selective phosphodiesterase (PDE) inhibitor (IBMX) for 2 h, then cultured in maturation medium (MM: TCM199 + 0.4%BSA, 0.2 mM Na-Pyruvate, 2 mM Glutamine, 0.1 mM Cysteamine, 0.1 IU/ml rhFSH), supplemented with 20 μM cilostamide, a type 3-specific PDE inhibitor (Albuz et al., *Hum Reprod* 2010; 25:2999). In the second group (G2), COCs were collected in IBMX supplemented medium, cultured for 6 h in MM with 10 μM cilostamide followed by 20 h culture in inhibitor-free MM (Luciano et al., *Biol Reprod* 2011; 85:1252; Lodde et al., *Biol Reprod* 2009; 81:281). In the control group (G3) COCs were collected and cultured in MM without any inhibitor. Oocytes were fertilized with the same bull at 26 h post IVM for G1 and G2 and at 20 h for G3 group. Presumptive zygotes were cultured in mSOF + BSA + aa for 7 days in 5% CO_2 5% O_2 at 38.5°C. Maturation rate after 18 and 30 h of IVM was similar while at 24 h it was significantly lower in G1 vs. G2 and G3 (17 vs. 48 vs. 64%) ($p < 0.05$ Student's *t*-test), while no differences were observed between G2 and G3. Fertilization rates were not different but a high polyspermy rate was observed in G1 compared to G2 and G3 (38 vs. 15 vs. 7%). The percentages of cleavage and blastocyst development to D + 7 were lower in G1 (47% and 13%) vs. G2 and G3 ($p < 0.05$) respectively, while no differences were observed between G2 (61% and 19%) and G3 (68 and 25). Embryo quality and cell number was significantly lower in G1 (G1: 130, G2: 159, G3: 150). Our results indicate that treatment of bovine oocytes with high concentrations of cilostamide during the whole IVM phase together with a pre-IVM with IBMX and forskolin is detrimental for fertilization and embryo development. On the other hand, short-term treatment with cilostamide slightly increases embryo quality but not blastocyst rate. In conclusion, the definition of an optimal maturation system needs further studies in order to modulate more accurately the intracellular cAMP levels especially during the first hours of culture. Work supported by Grant n 26096200 (project Ex Ovo Omnia) from Regione Sardegna & Lombardia.

Key Words: Oocyte maturation, cilostamide, cAMP, IBMX, bovine

2105

Synchronization of follicular development does not improve ovum pick up yield in *Bos taurus indicus* cattle

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The aim of this work was to evaluate the efficiency of a follicular development synchronization protocol using estrogen, progesterone, and prostaglandin $F2_\alpha$ to improve ovum pick up (OPU) yield in Nelore (*Bos taurus indicus*) cattle. Embryo donors cows (n = 15) were raised in pasture (*Cynodon* spp.), supplemented with mineral salt ad libitum, maintained in a farm located in Brasilia, DF, Brazil (15°46′47″S, 47°55′47″W), during 2009–2010. The OPU procedures (n = 84) were performed in the same animal prior to and after treatments with the synchronization protocol, which was comprised of 2 mg of estradiol benzoate I.M. (Estrogin®, Farmavet, Brazil), 3 mg of norgestomet ear implant (Crestar®, Akzo Nobel Ltda – Divisao Intervet, Brazil), and 30 μg of cloprostenol I.M. (Prolise®, ARSA SRL, Argentina). Hormones were administered on Day 0, the ear implant was removed on Day 5, and the OPU procedures were also done on Day 5. Cumulus oocyte complexes (COC) were recovered from follicles using an Aloka SSD-500 ultrasound (7.5 MHz intravaginal transducer). Prior to oocyte collection, all cows received an epidural anaesthesia of 4 ml of 2% lidocaine (sp) with 2% epinephrine (Pearson®; Eurofarma Laboratory, SP, Brazil). All accessible follicles (2.0 mm in diameter) were aspirated using an 18-G needle attached to a 50 ml Falcon tube. A vacuum of 50 mmHg was applied during aspiration, equivalent to a flow rate of 15 ml/min. Oocytes were collected in sterile Petri dishes containing phosphate-buffered saline and classified according to morphology as viable and non viable (without cumulus cells and degenerated). The variables studied were viable oocyte production (VOP), non viable oocytes production (NOP), and total oocyte production (TOP) which were the sum of the two others. For statistical analysis, the normality of the data and the homogeneity of the variances were assessed, and then, all variables were analyzed by ANOVA with paired *t*-test at a significance level at 5%, using R statistical software. Oocyte recovery from animals before the use of the protocol, i.e., without synchronization, was 11.36 (VOP), 4.01 (NOP), and 15.37 (TOP), while after treatment was 12.36 (VOP), 4.31 (NOP) and 16.68 (TOP). There was no difference considering the administration of hormones: VOP ($p = 0.591$), NOP ($p = 0.586$), and TOP ($p = 0.563$). *Bos taurus* indicus cattle exhibit a greater number of follicular waves and also a greater number of follicles per wave, compared to Holstein; thus the control of ovarian function by hormonal means should be slightly different from that of *Bos taurus* cows. Probably this is one explanation for the data observed in this work.

Key Words: Oocytes, ovum pick up (OPU), *Bos taurus indicus*, follicular development synchronization

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Recombinant bovine somatotropin administration improves ovum pick up yield in Nelore (*Bos taurus indicus*) cattle

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The aim of this work was to evaluate whether recombinant bovine somatotropin (rbST) improves ovum pick up (OPU) yield in Nelore (*Bos taurus indicus*) cattle. Nelore embryo donors cows (n = 20) were raised in pasture (*Cynodon* spp.), being supplemented with mineral salt ad libitum, maintained in a farm located in Brasilia, DF, Brazil (15°46′47″S, 47°55′47″W), during 2009–2010. The OPU procedures (n = 92) were performed in the same animal prior to and after treatment with 500 mg S.C. of rbST (Boostin® Coopers, Brazil) up to the 20th day after administration. Cumulus oocyte complexes (COC) were recovered from follicles using an Aloka SSD-500 ultrasound (7.5 MHz intravaginal transducer). Prior to oocyte collection, all cows received an epidural anaesthesia of 4 ml of 2% lidocaine (sp) with 2% epinephrine (Pearson®; Eurofarma Laboratory, SP, Brazil). All accessible follicles (>2.0 mm in diameter) were aspirated using an 18-G needle attached to a 50 ml Falcon tube. A vacuum of 50 mmHg was applied during aspiration, equivalent to a flow rate of 10–15 ml/min. Oocytes were transferred to sterile Petri dishes containing saline or phosphate-buffered saline (PBS) and classified according to morphology as viable (degrees I, II, and III) and non viable (without cumulus cells (denuded) and degenerated). The oocytes, then, were carried at 37°C to the laboratory in order to be matured and fertilized. The variables studied were viable oocyte production (VOP), non viable oocyte production (NOP), and total oocyte production (TOP) which were the sum of the two others. For statistical analysis, the normality of the data and the homogeneity of the variances were assessed. Data with a non normal distribution (VOP and TOP) were submitted to logarithmic transformation, and then, all variables were analyzed by ANOVA with paired *t*-test at a significance level at 5%, using the R statistical software. Oocyte recovery (mean) from animals before the use of somatotropin was 10.38 (VOP), 3.85 (UOP), 14.22 (TOP), while after treatment was 13.20 (VOP), 5.49 (UOP) and 18.56 (TOP). The rbST promoted an enhancement in all variables: VOP ($p = 0.0287$), UOP ($p = 0.0037$), and TOP ($p = 0.0067$). Probably the increase of oocyte production observed in this work was due to IGF-1 secretion after rbST administration, which has a synergic effect in the follicle gonadotropin receptors, augmenting the recruited follicle numbers in the follicular wave, and also by a direct action of somatotropin, diminishing granulosa cells apoptosis.

Key Words: Recombinant bovine somatotropin, oocytes, ovum pick up, *Bos taurus indicus*

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Mitochondria differ between adult and prepubertal sheep oocytes during *in vitro* maturation

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Oocytes from prepubertal animals are known to have a reduced ability to undergo normal embryo development and produce viable offspring (Armstrong, 2001. Theriogenology 55:1303–1322). The correct number, distribution, and function of mitochondria in oocytes is essential for successful embryo development (Van Blerkom, 2009. Semin. Cell Develop. Biol. 20:354–364). The aims of this project were (i) to determine if the volume, distribution or morphology of mitochondria in ooyctes from adult ewes change during *in vitro* maturation (IVM), and (ii) to determine if similar changes are observed in oocytes from prepubertal lambs. As determined by electron microscopy and stereology, the average volume of mitochondria in adult ewe oocytes (n = 12) increased during *in vitro* maturation (IVM) from 58.5 mm^3 to 85.7 mm^3 ($p < 0.01$). However, no such increase was observed in prepubertal lamb oocytes (n = 12) with the mitrochondrial volume being similar to adult ewes before maturation (59.2 mm^3) and less than in adults following maturation (64.6 mm^3; $p < 0.05$). In adult oocytes prior to IVM, the density of mitochondria was higher ($p = 0.001$) in the periphery than in the centre of the cytoplasm while in the prepubertal oocytes the density was not significantly different between the periphery and centre. Following IVM, the mitochondria were evenly dispersed across the diameter of the cytoplasm in both the adult and lamb oocytes. As we previously reported, the percentage of hooded mitochondria increased during IVM in the adult oocytes and decreased in the lamb ($p < 0.01$). In conclusion, this study has identified that mitochondria change during IVM and furthermore, these changes are different between oocytes from adult and prepubertal ewes which may contribute to the reduced developmental competence of prepubertal oocytes. Future work will determine if blastocyst rate can be improved by treating sheep oocytes with factors that increase mitochondrial biogenesis during IVM.

Key Words: Sheep, oocyte, mitochondria, prepubertal

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CD9 expression by ovine oocytes and its role in sperm-oocyte interactions

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Oocyte CD9 is needed for gamete fusion; measuring the expression of CD9 in mammals could provide a useful marker for predicting conventional IVF fertilization success in mammals with normal sperm parameters. The present study was conducted to examine whether CD9 is present in ovine oocytes and whether it functions during the sperm-oocyte interactions. First, the present of CD9 in ovarian tissue, oocytes and spermatozoa was examined by immunohistochemistry, immunofluorescence and immunoblotting. The results showed that CD9 was present on the membrane of many kinds of ovarian cells. The immunostaining was stronger on granulosa cell membranes than that on the oocyte plasma membrane in preantral follicles, but the staining on the oocyte plasma membrane was almost the same as that on granulosa cell membrane in fully grown follicles. The CD9 staining was observed on the membrane of oocytes at various stages including germinal vesicle (GV), metaphase I (MI) and metaphase II (MII). The staining was stronger as the oocyte nuclear stage proceeded to MII than at earlier stages. There was no immunostaining of CD9 on the membrane of the sperm when the sperm were stained by two different immunofluorescent procedures. By immunoblotting, a 25 kDa protein was found in the oocytes at GV, MI and MII stages, and the density was increased significantly during oocyte maturation. Sperm binding and fusion with oocytes was then examined by *in vitro* fertilization. When the zona pellucida-free matured oocytes were fertilized, both sperm binding to ooplasma and sperm penetrating into oocytes were significantly ($p < 0.01$) reduced in CD9 antibody treated oocytes (1.2 ± 0.4 per oocyte and, 20.1% respectively) compared with untreated control oocytes (2.5 ± 0.3 per oocyte and, 75% respectively), indicating that the CD9 antibody potentially inhibits sperm-oocyte binding and fusion. These results demonstrated that the CD9 present on ovine oocytes is involved in sperm-oocyte binding and fusion during fertilization. This work was supported by the National Natural Science Foundation of China (No 30860189).

Key Words: CD9, expression, sheep, oocyte

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Oocyte mitochondrial bioenergy potential and oxidative stress in adult superovulated ewes: Within-/between-subject, *in vivo* vs. *in vitro* maturation and age-related variations

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The aim of this study was to analyze within-/between-subject, maturation procedure (*in vivo* vs. *in vitro*), and age-related variations of mt bioenergy potential and oxidative status of metaphase II (MII) oocytes recovered from hormonally stimulated sheep. Ovulated MII oocytes collected by oviductal flushing (*in vivo* MIIs) and MII oocytes obtained after *in vitro* maturation (IVM) of fully grown immature oocytes (IVM MIIs) retrieved from the ovaries of the same syncronized (Fluorogestone acetate + D-cloprostenol) superovulated (pFSH/pLH and eCG) subjects (n = 10), were analyzed. Ovariohysterectomy was performed 54 h after vaginal sponge removal. Oocytes underwent nuclear chromatin, mt and ROS staining with Hoechst 33258, MitoTracker Orange CMTM Ros and 2′,7′-dichloro-dihydro-fluorescein diacetate (DCDHFDA) and evaluation by confocal microscopy (Ambruosi et al., *PLoS One* 2011;6:e27452). Within-subject variation coefficient (CVw), between-subject variation coefficient (CVb), and interclass correlation coefficient (ICC) for oocyte mitochondrial activity (MitoTracker fluorescence intensity), intracellular ROS (DCDHFDA fluorescence intensity) and mt-ROS colocalization (Pearson′s correlation coefficient) for *in vivo* vs. IVM MIIs in young vs. aged subjects were compared (Table 1). Oocyte bioenergy potential was shown to be affected by high between-subject variation (high CVb). This finding is much more interesting in subjects sharing breed, dietary regimen and superovulation protocol. It was also influenced by the maturation process (*in vivo* vs. *in vitro*), with higher variation found in IVM MIIs, indicating that a certain degree of variability characterizes currently used IVM conditions; and by donor age, with higher variation in young subjects yielding both *in vivo* and IVM oocytes. Intracellular ROS level was more stable (lower CVb) than mt activity. Even in this parameter, higher variation was observed in IVM oocytes and in oocytes derived from young donors. Colocalization of intracellular ROS and actively respiring mitochondria showed the lowest CVb and was a reliable marker of *in vivo* MII oocytes. In conclusion, oocyte/oxidative status is affected by within-/between-subject, *in vivo* vs. IVM and age-related variations. Mt/ROS colocalization could be considered as a predictive biomarker of oocyte quality.

Key Words: Sheep oocyte, donor age, mt distribution pattern and activity, intracellular ROS levels, mt/ROS colocalization

Table 1. CVw, CVb and ICC of bioenergy/redox parameters in ovine oocytes

Parameter	MII oocytes				
	in vivo (n = 69)	IVM (n = 28)	Young (n = 71)	Aged (n = 26)	Total (n = 97)
Mitochondrial activity					
CVw (%)	18.56	24.45	28.28	16.50	26.13
CVb (%)	68.47	94.33	90.94	58.44	73.66
ICC	0.93	0.94	0.91	0.93	0.89
Intracellular ROS levels					
CVw (%)	17.26	30.87	24.18	15.93	22.04
CVb (%)	51.56	67.59	78.05	44.69	66.67
ICC	0.90	0.83	0.91	0.89	0.90
Mitochondrial ROS Colocalization					
CVw (%)	18.28	27.45	25.89	18.07	23.87
CVb (%)	25.24	56.25	48.92	35.85	43.53
ICC	0.66	0.81	0.78	0.80	0.77

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Factors affecting *in vitro* maturation of alpaca oocytes

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We investigated factors that might affect *in vitro* maturation of alpaca oocytes. A 2 × 2 × 2 factorial design having age (old vs. young), oocyte collection method (aspiration vs. slicing) and media with or without fetal bovine serum (FBS) were utilized to determine optimum maturation rates. We hypothesized that oocytes aspirated from young alpacas and placed in maturation media supplemented with FBS would have greater maturation rates than those incubated in any other factorial combination. Oocytes were collected from the ovaries of six young alpacas (5, 5, 6, 7, and 9 years old) and five old alpacas (13, 16, 18, 18 and 18 years old). Ovaries were aspirated using 10 ml syringes and 20-ga 1-inch needles. Once all follicles ≥2 mm were aspirated, a scalpel blade was used to recover oocytes ≤1 mm in diameter by sequential slicing of the ovarian cortex. Oocytes collected were classified as morphologically normal oocytes (MNO) and deemed suitable for incubation if ≥3 compact layers of cumulus cells and a homogenous evenly granulated cytoplasm were observed. They were then kept separate and halves of each group were randomly divided and matured 24 h in chemically defined maturation media with or without 10% FBS (FBS; Invitrogen). Maturation was defined by the visualization of a polar body at the end of the incubation period. The proportion of matured oocytes in each group was analyzed by chi-square or Fisher Exact Test, whenever appropriate. Significance was set at $p \leq 0.05$ and probabilities between $p > 0.05$ and ≤0.1 indicated that the difference approached significance. A total of 276 oocytes were collected and 118 (42.7%) were classified as MNO. Overall aspiration yielded a greater percent of MNO than those obtained following slicing, 61.6% vs. 36.8% respectively ($p < 0.001$). There were no significant differences in the percent of MNO recovered following aspiration or slicing of the ovaries between young and old alpacas. However, within age group more MNO were collected by aspiration than slicing: 60.4% vs. 40.3%, respectively, in the young group, and 63.1% vs. 30.3%, respectively, in the old group. The overall oocyte maturation rate was 17.8% (21/118) and no differences were observed between age groups regardless of collection method or media used. More MNO matured when collected by aspiration (15/51; 29.4%) compared with slicing (6/67; 8.9%), regardless of age group or media used ($p = 0.008$). Within the old group, there was a trend ($p = 0.056$) for greater maturation rates when oocytes were incubated in media supplemented with FBS (5/10; 50%) than without it (1/12; 8.3%). In conclusion, more MNO can be collected via aspiration of antral follicles >2 mm and are more likely to reach maturation than those obtained from follicles ≤1 mm via ovary slicing. Addition of FBS to the maturation media may improve *in vitro* maturation rates of oocytes obtained from older alpacas.

Key Words: Alpaca, oocyte, *in vitro* maturation, fetal bovine serum, camelid

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Developmental competence of equine oocytes after chemical or mechanical activation and assisted fertilization (ICSI)

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A study of parthenogenesis is an important approach to understanding the fundamental aspects of early embryonic development. Recently, it has been possible to produce equine embryos through somatic cloning (Hinrichs, 2005). Understanding artificial activation is relevant to cloning research since artificial activation of oocytes is an essential component of current nuclear transfer protocols (Kim et al., 1996). The aim of this study was to compare the developmental competence of equine oocytes after artificial activation by chemical or mechanical methods and assisted fertilization (ICSI). Equine oocytes were collected by scraping ovarian follicles of slaughtered mares; only

compact cumulus-oocyte complexes were used for the activation treatment. The oocytes were matured for 30 h in TCM 199 with Earle's salts supplemented with 20% FBS, 5 mg/ml FSH, 1 mg/ml oestradiol and cultured at 38.5°C in air containing 5% CO_2. After maturation the oocytes were denunded of cumulus cells by pipetting them in hyaluronidase (300 i.u./ml PBS) and those showing the first polar body were used for the artificial activation treatments and ICSI procedure.Treatment 1–5 μM ionomycin in TCM 199 medium (5 min) + 10 μg/ml cycloheximide in TCM medium + 10%FBS (24 h). Treatment 2–50 μM calcium ionophore in TCM 199 medium (5 min) + 10 μg/ml cycloheximide in TCM medium + 10%FBS (24 h). Treatment 3–5 μM ionomycin in TCM 199 medium (5 min) + 2 mM 6-DMAP in TCM medium + 10%FBS (3.5 h). Treatment 4–50 μM calcium ionophore in TCM 199 medium (5 min) + 2 mM 6-DMAP in TCM medium + 10%FBS (3.5 h). ICSI and 'scheme ICSI' was performed as described previously by Palermo et al. (1992). After artificial activation and ICSI oocytes were transferred into DMEM F 12 medium supplemented with 10% FBS and cultured at 38.5°C in 5% CO_2 in air. The results of this experiment are summarized in Table 1. In the case of chemical activation the embryonic divisions stopped at the 2-cell stage. Therefore, attempts were made to improve efficiency by modifying treatment 3. Concentration of ionomycin was increased to 10 μM and the time of incubation in 6-DMAP was extended to 4 h. Oocyte activation protocols used successfully in other animal species are ineffective in the case of horses. Equine oocytes require stronger stimuli to induce partenogenetic embryos divisions.

Key Words: Equine oocytes, parthenogenesis, artificial activation

Table 1. Efficiency of activation of mare oocytes

Method of activation	No. oocytes	No. 2-blastomers (%)	No. 4-blastomers (%)	Total number of cleaved oocytes (%)
Treatment 1. I.O. + CHX	104	0	0	0
Treatment 2. C.I + CHX	90	0	0	0
Treatment 3. I.O + 6-DMAP	97	17 (17.5)	0	17 (17.5)
Treatment 4. C.I. + 6-DMAP	107	10 (9.2)	0	10 (9.2)
Modified Treatment 3	53	11 (20.7)	8 (15.1)	19 (35.8)
Schame ICSI	76	0	0	0
ICSI	44	9 (20.4)	8 (18.25)	17 (38.65)

I.O., ionomycin; CHX, cycloheximide; 6-DMAP, 6-dimethylaminopurine.

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WTAP gene expression in *in vivo* and *in vitro* porcine oocytes

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The Wilms' tumor 1 suppressor gene WT1 appears to play an important role in both transcriptional and posttranscriptional regulation of cellular genes. WT1 and WT1-associating protein (WTAP) are localized throughout the nucleoplasm as well as in speckles and partially colocalized with splicing factors. WTAP is a factor essential for cyclin A2 mRNA stabilization and regulation of G2/M cell-cycle transition in somatic cells. For cell-cycle regulation of WTAT, WTAP might be involved in regulation of oocyte maturation. The mechanism of WTAP in somatic cells has been previously characterized, however its biological function in oocytes still remains to be elucidated. To investigate the role of WTAP in oocytes, the abundance of its mRNA was examined in *in vivo* and *in vitro* matured porcine oocytes by using deep sequencing. More reads for WTAP mRNA were detected in *in vivo* than in *in vitro* matured oocytes (12.5 vs. 0). The expression of cyclin A2 in *in vivo* was higher than *in vitro* (0.5 vs. 8.4). However, the expression of cell cycle related genes (cyclin B1, cyclin B2, CDC20) in *in vivo* oocyte was lower compared to *in vitro*. WTAP gene expression was confirmed by quantitative real-time PCR. The expression level of WTAP variant 1, WTAP variant 2, and cyclin A2 was compared between oocytes matured *in vivo* and *in vitro*; GAPDH was served as internal control. The mRNA abundance of WTAP variant 2 and cyclin A2 in *in vivo* matured oocytes were significantly higher than *in vitro* ($p < 0.05$). To further characterize porcine WTAP, the entire coding sequence of WTAP1 was amplified by PCR and sequenced; WTAP variant 2 is a shorter version of WTAP variant 1 due to a premature stop codon. The sequencing result indicated that porcine WTAP variant 1 is consist of 395 amino acids and showed high identities against human and mouse WTAP sequence; both 94% each respectively. This is the first report of the presence of WTAP in oocytes. This study demonstrates that WTAP is expressed higher in *in vivo* oocytes compared to *in vitro*. This suggests *in vitro* matured oocytes may show lower developmental competence because of lower level of WTAP thereby unstable expression of cyclin A2 in porcine oocytes. This research would be helpful to improve current *in vitro* maturation system and to characterize oocyte maturation.

Key Words: Pig oocytes, WTAP gene, *in vitro* matuation, cell cycle

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Different media used for selection of competent swine oocytes with Brilliant Cresyl Blue staining

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Selection of more competent oocytes increases *in vitro* embryo production rates. Brilliant Cresyl Blue (BCB) is a noninvasive method for oocyte selection, which estimates cellular viability by measuring the activity of glucose-6-dehydrogenase. Oocytes stained by BCB are considered more suitable for *in vitro* maturation (IVM). PBS is the most common medium used for BCB staining, associated with 90 min incubation. The aim of this study was to test the selection of swine oocytes in a richer medium, with two BCB concentrations and shorter incubation period. The maintenance medium tested was the modified porcine zygote medium (PZM4-m). Prior to IVM, 666 oocytes were distributed in the following groups: (i) porcine fluid follicular (PFF), (ii) PBS, (iii) PZM4-m, (iv) PBS with 13 μM of BCB, (v) PBS with 26 μM of BCB, (vi) PZM4-m with 13 μM, and (vii) PZM4-m with 26 μM of BCB. After incubation for 60 min at 39°C in the different media, oocytes were classified as positive (stained) or negative (no staining), except for group 1 which was washed in PFF and were not incubated, being considered the general control. IVM was performed in NCSU-23 m with eCG, hCG, hypotaurine, β-mercaptoethanol, cysteine, EGF, AMP-c and PFF, in the first 24 h, followed by NCSU-23 without eCG, hCG and AMP-c, for additional 24 h. Nuclear maturation was assessed with Hoechst (Sigma H33342) using epifluorescence microscopy. The results were analyzed by Chi-Square using the Software Statistix 9.0. The rate of metaphase II in group 1 was 72% (n = 50); 30% in group 2 (n = 55); 55.6% in group 3 (n = 72); 67.3% in positive group 4 (n = 110); 43.7% in negative group 4 (n = 48); 50% in positive group 5 (n = 44); 45.5% in negative group 5 (n = 33); 48% in positive group 6 (n = 166); 35.7% in negative group 6 (n = 29); 27.5% in positive group 7 (n = 51) and 12.5% in negative group 7 (n = 8). These results showed that maturation rates in PBS in both BCB concentrations 13 μM (67.3%) and 26 μM (50%) was statistically superior to PZM4-m, which had 48% in 13 μM and in 27.5% in 26 μM of BCB. Maturation rates in PBS with 13 μM (67.3) of BCB were superior ($p < 0.05$) than in 26 μM (50%). However, maturation rates in PBS with 13 μM of BCB (67.3%) were lower ($p < 0.05$) than in the general control group (72%). This research showed that is possible to decrease the BCB incubation period from 90 to 60 min in order to obtain effective oocyte coloration. Regarding the tested media PBS was superior to PZM4-m and a lower BCB concentration 13 μM was better than 26 μM. Therefore, both PBS and PZM4-m with BCB showed lower MII rates than control group. Further studies are needed to more precisely define the effectiveness of PZM-4m as adilution media for BCB staining.

Key Words: Oocyte, *in vitro* maturation, Brilliant Cresyl Blue, PZM4-m

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Effects of oxygen concentration on *in vitro* maturation of canine oocytes

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Canine oocytes require an extended period of culture up to 72 h *in vitro* for nuclear maturation to the metaphase II stage which also results in high degeneration rates when cultured in high oxygen gas atmosphere. High levels of fat deposit in these oocytes and incompetent repair mechanisms are expected to predispose these oocytes to oxidative stress and may contribute to high degeneration and reduced nuclear maturation rates. Canine cumulus oocyte complexes (COCs) were isolated by mechanical separation from ovaries collected after routine ovariohysterectomy and cultured in serum free SOF media (synthetic oviductal fluid) incubated at low (5% O_2 + 5% CO_2 + 90% N_2) or high (5% CO_2 in air) oxygen concentrations. Changes in oocyte nuclear maturation rates, H_2O_2 levels within the oocytes and expression of reactive oxygen species inhibitory genes; superoxide dismutase 1 &2 (SOD1&2), glutathione reductase (GSR), glutathione peroxidase (PGX1) and catalase (CAT) were analyzed. Higher meiotic resumption from germinal vesicle break down up to metaphase II was observed in low O_2 (41.75 ± 13.1%) compared to high O_2 (15.76 ± 8.155) ($p = 0.014$) after 52 h of culture ($n = 112$). Extension of the culture period up to 84 h at Low O2 produced the highest meiotic resumption at 72 h (64.10 ± 5.98%; $p = 0.008$), ($n = 457$) compared to 52 or 84 h. Oocytes ($n = 110$) cultured in High O_2 contained higher levels of peroxidase measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) fluorescent assay after 72 h of culture ($p = 0.004$). High O_2 cultured oocytes ($n = 300$) also showed higher fold induction of SOD1, SOD2, GSR, PGX1 and CAT at the mRNA level using relative qPCR analysis. These results for the first time show that Low oxygen gas composition improves nuclear maturation rates and alleviates oxidative stress for canine oocytes during *in vitro* maturation.

Key Words: Canine oocytes, hydrogen peroxide, *in vitro* maturation, oxygen, SOF

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Thermoprotective effects of IGF-I on bovine germinal vesicle oocytes

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Bovine oocytes are succeptible to elevated temperature during oocyte maturation and germinal vesicle (GV) stage. The objective of the first study was to determine the effect of different insulin-like growth factor-I (IGF-I) concentrations on heat-induced apoptosis on bovine GV oocytes. Cumulus-oocyte complexes (COCs) collected from slaughterhouse ovaries were cultured in medium 199 supplemented with 50 μg/ml gentamycin, 0.2 mM sodium pyruvate, 100 μM cysteamine and 12.5 μM butyrolactone (MAM: meiotic arrest medium) containing 0, 12.5, 25, 50 or 100 ng/ml IGF–I for 14 h at control (38.5°C) or heat shock (41°C) temperatures. Then, COCs were transferred to *in vitro* maturation medium at 38.5°C for 10 h. After this period oocytes were mechanically denuded by repeated pipetting and fixed in 4% paraformaldehyde for TUNEL (Terminal deoxynucleotidyltransferase nick-end labeling) analysis. This experiment was replicated six times using 81–111 COCs/treatment. Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS. In the absence of IGF-I the proportion of TUNEL-positive oocytes increased from 1.0 ± 2.8% to 9.7 ± 2.8% in the control vs. heat shock group, respectively ($p \leq 0.05$). In heat-shocked oocytes, addition of IGF-I decreased the proportion of TUNEL-positive oocytes from 9.7 ± 2.8% to 1.4 ± 3.1% in 0 vs. 12.5 ng/ml IGF-I ($p \leq 0.05$). Such thermoprotective effect of IGF-I was not observed at higher concentrations. In the second experiment, the objective was to determine the effect of different IGF-I concentrations on developmental competence of bovine GV oocytes exposed to heat shock. COCs were exposed to control (38.5°C) or heat shock (41°C) temperatures in MAM containing 0, 12.5 or 100 ng/ml IGF-I for 14 h. Then, oocytes were subjected to *in vitro* maturation, fertilization and culture at 38.5°C. The proportion of oocytes that cleaved and developed to the blastocyst stage was evaluated on days 3 and 8 after fertilization, respectively. This experiment was replicated 5 times using 78–109 COCs/treatment. In the absence of IGF-I there was no effect of temperature on cleavage rate, however heat shock reduced blastocyst rates from 26.4% ± 3.6% to 15.5% ± 3.6% in the control vs. heat shock group, respectively ($p \leq 0.05$). In heat-shocked oocytes, addition of IGF-I increased the proportion of oocytes that reached the blastocyst stage from 15.5% ± 3.6% to 30.0% ± 3.6% in 0 vs. 12.5 ng/ml IGF-I ($p \leq 0.05$). Such thermoprotective effect was not observed at 100 ng/ml IGF-I. In conclusion, IGF-I at low doses exerted a thermoprotective effect reducing heat-induced apoptosis and improving developmental competence in bovine GV oocytes.

Key Words: IGF-I, heat shock, germinal vesicle oocyte, apoptosis, bovine

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Identification of deadenylated RNAs in bovine immature oocytes

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In bovine, oocyte maturation and early embryo development (until the 8- to 16-cell stage) depends on maternal storage of specific RNAs with a short poly(A) tail to their 3′ extremity. To date, no large scale analysis has been performed in order to characterize such deadenylated RNAs in mammals. Thus, the aim of our study was to identify deadenylated RNAs in bovine immature oocytes by transcriptomic analysis. First, using magnetic beads coupled to oligodT, we developed a method to isolate deadenylated from polyadenylated RNAs. As a control, we added RNA with known poly(A) tail (synthesized *in vitro* from tomato isolated mRNAs) during immature oocyte total RNA extraction. Three RNA fractions were isolated: (i) RNAs that do not bind to the beads, named for clarity reasons A-, (ii) RNAs that were eluted from the beads at 42°C, and (iii) RNAs that were eluted at 75°C, named A+. The methodology was validated by real-time quantitative PCR. Eighty percent of the control non-adenylated RNA was identified in the A− fraction, while 18 and 2 percent were obtained in A42 and A+ fractions, respectively. Eighty three and twelve percent of the control polyadenylated RNA were obtained in A42 and A+ fractions, while only 5% was obtained in A− fractions. In order to identified RNAs with very short or with very long poly(A) tail length, we amplified RNAs from the fraction A− and A+ and performed hybridization on the EmbryoGENE Agilent chip (44 K). In the A− fraction, 4 181 probes were detected above background, while 5586 probes were identified in the A+ fraction. Among them, 2869 probes were common between both conditions. Using LInear Models for Microarray (LIMMA) analysis with a p-value < 0.05 and at least a fold-change of 2 between both conditions, 610 and 788 probes appeared over-represented in A− and A+ fraction, respectively. Validating our analysis, several genes coding for histones, 18S and 28S RNA were identified in the A− fraction. Moreover, we also identified 230 novel transcribed regions corresponding to embryonic EST (Expressed Sequence Tag) in the A− fraction. We are currently carrying out the analysis of these candidates. Functional annotation in DAVID (Database for Annotation, Visualization and Integrated Discovery) revealed that in A+ fraction, key molecular mechanisms involving ribonucleoprotein as translation or spliceosome are enriched. To conclude, results from this study will pave the way for a better understanding of the maternal RNA storage during oogenesis in bovine. This work was supported by NSERC.

Key Words: Oocyte, adenylation, maternal RNA, oocyte maturation, early embryo development

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Effects of FGF10 on oocyte maturation, quality and capacity to become an embryo

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Fibroblast growth factor 10 (FGF10) acts as a paracrine factor on cumulus-oocyte complexes, increases expression of genes related to oocyte maturation and enhances oocyte competence to become an embryo. Our objectives were to test whether FGF10 improves oocyte maturation, decreases percentage of oocyte apoptosis and influences embryo production rates *in vitro*. In all experiments, oocytes, obtained from an abattoir, were collected from adult cows and matured for 22 h in TCM 199 supplemented with: (i) 2.5 μg/ml FGF10, (ii) 10 μg/ml FGF10, (iii) 50 μg/ml FGF10, or (iv) no FGF10 (control group). In experiment 1, after maturation, oocytes were fixed and stained with Hoechst- 33 342 to evaluate different stages of meiosis (metaphase 1; metaphase 2; or metaphase 2 with extrusion of the first polar body). In experiment 2, after maturation, oocytes were stained with the terminal deoxynucleotidyl transferase enzyme (TUNEL) to determine the percentage of apoptotic nuclei. In Experiment 3, oocytes were fertilized and cultured *in vitro* until the early blastocyst stage. Results showed that 2.5 μg/ml FGF10 increased the percentage of oocytes that extruded their first polar body (36%) when compared with 10 μg/ml FGF10 (13%); 50 μg/ml, FGF10 (12%) or the control group (19%; $p \leq 0.05$). The percentage of apoptosis was lower ($p \leq 0.05$) in groups treated with higher doses of FGF10 (10 μg/ml and 50 μg/ml FGF10, 5% and 6%, respectively) when compared with 2.5 μg/ml FGF10 (15%) or control group (33%). The oocytes undergoing apoptosis were mostly ($p \leq 0.05$) at the metaphase II stage (61%) followed by metaphase II (31%) and extruded polar body (6%). The percentage of oocytes that were in the metaphase II stage and were TUNEL negative did not differ between groups (64%; 64%; 59%; 60% for 2.5 μg/ml; 10 μg/ml; 50 μg/ml and control group, respectively). In experiments 1 and 2, 75–81 oocytes were used per treatment. The results of *in vitro* embryo production did not differ between groups (17%; 10%; 13%; 13% for 2.5 μg/ml; 10 μg/ml; 50 μg/ml and control group, respectively). There were used 135–170 embryos per treatment. In conclusion, addition of 2.5 μg/ml FGF10 to the oocyte maturation media increases the percentage of oocytes which extrude the first polar body; higher concentrations of FGF10 (10 and 50 μg/ml) reduces the percentage of oocytes undergoing apoptosis but none of the different concentrations are able to improve blastocyst development *in vitro*.

Key Words: FGF10, oocyte, embryo, bovine, apoptosis

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FSH/LH, PKC and EGF involvement in bovine cumulus cell expansion during oocyte maturation

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FSH and LH are important regulators of granulose cell proliferation, differentiation, and steroidogenesis. Recent lines of evidence suggest that protein kinase C (PKC) as well as epidermal growth factor (EGF) are involved on FSH/LH induced events. However, in large animals the signal pathway connecting FSH and EGF/EGFR is not clear. Therefore, this study was carried out to examine the participation of PKC in cumulus cell expansion during FSH/LH-induced maturation of cumulus oocyte complexes (COC). COC were collected by slicing of ovaries recovered after slaughter and were matured *in vitro* (TCM 199 + 10% FCS, 22 μg/ml pyruvate and 50 μg/ml gentamicine for 24 h at 38.5°C and 5% CO_2). In experiment 1, COCs were randomly divided into four groups: Control (IVM media); FSH/LH (IVM media + 7 UI/ml LH + 0.5 μg/ml FSH); EGF (IVM media + 0.01 mg/ml EGF) and FSH/LH/EGF (IVM media + EGF, LH and FSH as described previously). To evaluate the role of PKC in bovine cumulus cell expansion, in experiment 2, 10 mM of the PKC inhibitor Bisindolylmaleimide I (BIM) was added to each experimental group as described in experiment 1 (Control-BIM; FSH/LH-BIM; EGF-BIM and FSH/LH/EGF-BIM). After maturation, cumulus expansion was assessed by measuring COC diameter by inverted microscopy using the Image Pro Plus 5.1 software and data are present in pixels. Five replicates were carried out; statistical analyses was performed by Tukey Test and a probability of < 0.05 was considered to be statistically significant. In experiment 1, oocytes matured in the control group had no cumulus expansion (498.96a). The hormonal stimulation (FSH/LH) group efficiently induced cumulus expansion (663.27b) compared to control group. The addition of EGF during maturation was more efficient to induce cumulus expansion (916.08c) than the FSH/LH group. Maximum cumulus expansion was observed in the FSH/LH/EGF group (1055.68d). In experiment 2, oocytes matured with the PKC inhibitor (Control-BIM group) had no cumulus expansion after 24 h maturation (592.4a). PKC inhibition by BIM completely blocked FSH/LH-induced cumulus expansion (FSH/LH-BIM group; 481.28b). On the other hand, PKC inhibition had no effect on EGF-induced cumulus expansion (EGF-BIM group; 929.17c), and cumulus cell expansion was significantly higher than FSH/LH-BIM and control-BIM group. Although cumulus expansion after maturation in the FSH/LH/EGF-BIM group (715.44d) was higher than Control-BIM and FSH/LH group, it was lower than in EGF-BIM group. We show that inhibition of PKC by BIM in FSH/LH-stimulated bovine CCOs strongly inhibits cumulus cell expansion and that BIM effect on cumulus cell expansion was overcome by EGF treatment. It is known that FSH/LH induces EGF production in cumulus cell; the present work provides evidence that PKC is involved and, for the first time, that an FSH/LH-PKC-EGF connection is involved in bovine cumulus cell expansion.

Key Words: Oocyte maturation, PKC, EGF, FSH, LH

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Effect of inhibin A on the expression of brain-derived neurophic factor in porcine granulosa cells and the expansion of cumulus oocyte complexes *in vitro*

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Studies on the autocrine/paracrine actions of inhibins in different cell types have come to the spotlight. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family. In addition to its well-known roles in the neuron, BDNF is also important for the development of nonneuronal tissues (Kawamura et al., 2007. *Dev. Biol.* 311:147–158). Previous studies conducted by our laboratory have shown that BDNF influenced the growth of granulosa cells (GCs) and promotes bovine early embryo development (Yi et al., 2011. *Animal* 2:1786–1794). The objective of this study was to confirm the relationship between inhibin A and BDNF in porcine cumulus cells, and to investigate the effect inhibin A on expansion of cumulus oocyte complexes (COCs) *in vitro*. The COCs were cultured in TCM199 (30 COCs in 100 μl) with 0, 4, 40 and 100 ng/ml inhibin A for 44 h. Total RNA and protein from 30 COCs were extracted using a RNAprep Pure Micro Kit (Tiangen Co., Beijing, China) and a total protein extraction kit (Applygen, China), according to the manufacturer's instructions. The expression levels of BDNF mRNA and protein were evaluated by quantitative real-time PCR and western blotting, respectively. Furthermore, the expansion rate of COCs cultured with 0, 4, 40 and 100 ng/ml of inhibin A was analyzed. The RT-PCR, Western blot, inhibin A treatment experiment were repeated three times. Statistical analysis was performed using one-way ANOVA (as implemented in SPSS 13.0. software) followed by Dunnett's multiple range test. The result shows that expression of BDNF mRNA in porcine cumulus cells very significantly increased with various concentrations of inhibin A ($p < 0.01$), while the expression of BDNF protein was significantly increased only when the concentration of inhibin A were 40 and 100 ng/ml ($p < 0.05$).Addition of 100 ng/ml inhibin A in culture media can significantly increase the expansion rate of COCs ($p < 0.01$). In conclusion, this study provided evidence of a role of inhibin A as a mediator of BDNF action in cumulus cells and highlights the need for greater understanding the local autocrine/paracrine roles of inhibins in GCs. This work was supported by the

State Key Development Program of Basic Research ('973' Program) of China (No. 2011CB944203).
Key Words: Porcine, inhibin A, granulosa cells, BDNF, COCs

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Effects of Bcl-2 inhibitor (ABT-737) treatment on developmental competence, apoptosis and ER-stress in pigs

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The key regulators of apoptosis are the interacting proteins of the Bcl-2 family. Bcl-2, an important member of this family, blocks cytochrome C release by sequestering pro-apoptotic BH3-only proteins such as Bid, Bad, Bax and Bim. The pro-survival family members (Bcl-2, Bcl-XL, Bcl-W) are critical for cell survival, since loss of any of them causes cell death in certain cell type. In previous study, inhibitory effect of Bcl-2 by inducing ER-stress and apoptosis in the various cancer cells was reported. However, its role during early porcine embryonic development is not sufficient. In this study, we traced the effects of Bcl-2 inhibitor, ABT-737, on early porcine embryonic development. We also investigated several indicators of developmental potential, including gene expression (apoptosis-related genes) and apoptosis, which are affected by ABT-737. Porcine embryos were cultured in the PZM-3 medium with or without ABT-737 for 6 days. Also, thirty blastocysts per sample were used for gene expression analysis. Data were analyzed by ANOVA using the general linear model procedure in statistical analysis systems (SAS). Significant differences in developmental potential were detected between the embryos that were cultured with or without ABT-737 (30.3 ± 4.8 vs. 14.7 ± 3.0%). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) showed that the number of cells containing fragmented DNA at the blastocyst stage increased in the ABT-737 treated group compared with control (4.7 vs. 3.7, respectively). Blastocysts that developed in the ABT-737 treated group had lower quality and higher apoptotic nuclei than those of the untreated control, resulting in decreased quality of preimplantation porcine blastocysts. The mRNA expression of the pro-apoptotic gene Bax increased in ABT-737 treated group, whereas expressions of the anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL, Bcl-W) decreased. Also, expression of the ER stress indicator genes (GRP78, XBP-1 and sXBP-1) increased in ABT-737 treated group. These results suggest that inhibition of Bcl-2 in development of pig embryos induces ER stress mediated apoptosis. And these results also indicate that inhibition of Bcl-2 decreased porcine developmental competence *in vitro*. In conclusion, Bcl-2 is closely associated with expression of apoptosis- and ER stress-related genes and developmental potential in pig embryos.
Key Words: Bcl-2 inhibitor, developmental ability, apoptosis, ER-stress, pig

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The beneficial effect of t10,c12–conjugated linoleic acid on *in vitro* maturation of porcine oocyte

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Conjugated linoleic acid (CLA), a group of 18-carbon polyunsaturated fatty acids, is a collective term used to describe positional and geometric isomers of linoleic acid that have been shown to have many positive health benefits. Specifically, trans −10, cis −12 –CLA (t10, c12–CLA) is solely responsible for fat reduction, and it has also been found to have several beneficial bioactive properties related to reproduction. The objective of this study was to shed light on the potential mechanisms mediating the beneficial effects of t10, c12–CLA on *in vitro* maturation of porcine oocytes. In experiment 1, various concentrations (0, 10, 25, 50, 100 μM) of t10, c12–CLA were added to the defined IVM medium. Based on the maturation rates and subsequent developmental competence of parthenogenetic embryos, the appropriate concentration was found and used in all following experiments. In Experiment 2, the redox potential was investigated to evaluate the effect of t10, c12–CLA on cytoplasmic maturation. In experiment 3, U −0126 (a specific selective inhibitor of ERK kinase) was added to the IVM media in the presence or absence of t10, c12–CLA, and the nuclear status and cumulus cell expansion after 44 h culture were estimated. In addition, the involvement of ERK/MAPK pathway mediating the positive effect of t10, c12–CLA on oocyte nuclear maturation was tested. Finally, in experiment 4, prostaglandin-endoperoxide synthase 1 (PTGS1) and PTGS2 expression was focused on to examine the participation of t10, c12 –CLA in prostaglandin synthesize. Compared to the control, IVM medium supplemented with 50 μM t10, c12–CLA significantly increased the proportion of oocytes reaching the metaphase II (MII) stage (60.0 ± 2.7% vs. 48.9 ± 4.4%), and also had greater rates of blastocyst formation (51.9 ± 1.5% vs. 39.6 ± 1.5%). We observed 50 μM t10, c12–CLA also significantly reduced intracellular reactive oxygen species (ROS), and increased glutathione (GSH) concentrations. Furthermore, 50 μM t10, c12–CLA treatment during IVM increased the percentage of phosphorylated/total ERK1/2 in COCs after 22 h maturation and in matured oocytes, and it also promoted the percentage of MII oocytes and cumulus cell expansion in the presence of U −0126. Moreover, t10, c12–CLA affected the expression levels of prostaglandin synthesize related genes in cumulus cells; the level of PTGS1 and PTGS2 transcripts were significantly increased in cumulus cells in the culture containing 50 μM t10, c12–CLA. In conclusion, the results indicate that t10, c12–CLA can exert its beneficial effect on oocyte maturation in pigs via multidimensional pathways.
Key Words: t10,c12-CLA, porcine oocyte, ROS, GSH, ERK

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Exposure of *in vitro*-matured porcine oocytes to Hoechst 33 342 in combination with ultraviolet irradiation induces mitochondrial dysfunction

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This study was designed to investigate the effects of Hoechst 33 342 staining (H342) and ultraviolet irradiation (UV) on developmental ability and mitochondrial activity of porcine oocytes. *in vitro*-matured oocytes were divided into 2 experimental groups: (i) oocytes stained with 5 μg/ml H342 for 12 min and exposed to UV for 30 s (H342/UV), (ii) untreated oocytes (Control). Thereafter, oocytes were incubated for 5 h with 1000 frozen-thawed spermatozoa per oocyte and cultured for 7 days to evaluate the embryo development. Mitochondrial distribution and number of mitochondrial DNA (mtDNA) copies were estimated, using confocal laser scanning microscopy with MitoTracker Deep Red and the real-time polymerase chain reaction procedure, respectively, in different stages of development: *in vitro*-matured after 1 h treatment, zygotes, 2- to 4- cells and blastocyst. The oocytes exposed to H342/UV showed lower ($p < 0.001$) cleavage (40.9 ± 12.2%) and blastocyst (10.6 ± 1.6%) rates than untreated oocytes (62.4 ± 12.2% and 24.4 ± 1.6%, respectively). In most of control oocytes, active mitochondria were dispersed within the cytoplasm being more abundant in the peripheral cytoplasm. In control zygotes, 2- to 4-cells embryos and blastocysts, active mitochondrial aggregations were associated with the pronuclei, the nuclear region of each blastomere or the inner cell mass and trophectoderm, respectively. A similar distribution was observed in only 68.4%, 58.3%, and 43.3% of H342/UV oocyte, zygote and 2- to 4-cell groups, respectively. Moreover, a high percentage (36.6%) of H342/UV 2- to 4-cell embryos showed blastomeres with poor or no staining for mitochondria. However, embryos achieving the blastocysts stage from H342/UV treated oocytes shown a similar mitochondrial distribution that control blastocysts. The mean mtDNA copy number was similar for Control and H342/UV oocyte (347 023 ± 46 334 and 370 441 ± 77 126, respectively), zygote (100 575 ± 20 895 and 85 909 ± 19 503, respectively) and blastocysts (320 056 ± 52 590 and 557 115 ± 176 717, respectively) groups. However, H342/UV treated 2- to 4-cell embryos showed lower mtDNA copy numbers than those from the Control group. In conclusion, the results of the present study demonstrate that the combination of H342 staining and UV irradiation has a clear deleterious effect on the developmental ability of porcine oocytes

which is associated with mitochondrial dysfunction. Supported by SENECA (04543/GERM/07).
Key Words: Mitochondria, oocyte, porcine, Hoechst, UV irradiation

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Immunolocalization of GDF9 and BMP15 during *in vitro* maturation of canine oocytes

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Communication between mammalian oocytes and their companion cumulus cells is essential for development of both types of cells. Growth-differentiation factor 9 (GDF9) and Bone morphogenetic protein 15 (BMP15) have been shown to be critical regulators of many granulosa cell processes that are fundamental to oocyte development; however, these proteins have not been described in dogs. Therefore, we proposed to identify these two growth factors in the oocyte and cumulus cells, and the temporal relationships between the presence of GDF9 and BMP15 with canine oocyte maturation. Cumulus-oocyte-complexes were collected from adult bitches following ovarihysterectomy; a total of 1200 oocytes with and without cumulus cells were randomly allocated in four groups: non-matured and *in vitro* matured for 48, 72 and 96 h (TCM-199 with Earle's salt supplemented with 25 mM Hepes, 10% FCS, 0.25 mM pyruvate, 10 IU/ml of hCG, 300 IU/ml penicillin, and 20 mg/ml streptomycin, at 38.5°C and 5% CO_2). In every time point, the oocytes with and without cumulus cells were incubated with first antiboby (Human GDF9 or BMP-15 Polyclonal Antibodies) and then with a second antibody (FITC-conjugated goat anti-rabbit IgG) for indirect immunofluorescence. Samples were evaluated with an inverted epifluorescence microscope. The data were analyzed by longitudinal model (dependent on time) using logistic regression. The preliminary evidence indicated the presence of both proteins in canine oocytes and cumulus cells before and during culture. The intensity of fluorescence of both proteins was higher in non-matured GV oocytes and decreased with time of culture ($p < 0.05$) in MI-MII oocytes with and without cumulus cells and also in cumulus cells. The immunodetection was lower in cumulus cells in comparison to oocytes in all maturation periods. These results might indicate that throughout *in vitro* development, canine oocyte restricted the secretion of these proteins, which represent the first approach of GDF9-BMP15 dynamic in dog oocyte maturation. Supported by Grant FONDECYT 1110265
Key Words: Oocyte, canine, maturation, GDF9, BMP15

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Juxtacrine and paracrine mutual oocytes-granulosa cells interaction: a proposed model

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The bidirectional communication between oocytes and granulosa cells may be mediated by several factors via a local feed back loop(s). This experiment was carried out to study the mutual interaction of direct (juxtacrine) and indirect (paracrine) co-culture of porcine denuded oocytes and granulosa cells *in vitro*. Denuded immature oocytes were divided into two groups; (i) direct contact, and (ii) indirect co-culture with granulosa cells. Transwell 0.4 μm polyester membrane inserts (Corning Inc.) were used to permit oocyte-granulosa cells communication with a distance of 2 mm between them in co-culture. Oocytes were cultured with granulosa cells (initial count was 1×10^5 cell/ml) in a defined basic maturation medium, TCM-199 supplemented with 1 mg/ml FSH, 10 ng/ml epidermal growth factor (EGF), 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 mg/ml insulin and 1% (v/v) Pen-Strep at 39°C in a humidified atmosphere of 5% CO_2 for 44 h. The temporal expression (0, 22 and 44 h of culture) of oocyte-secreted factors (OSFs), growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) was studied using RT-PCR. Oocytes were subjected to parthenogenetic electrical activation to assess their developmental competence. Steroid hormones (progesterone, P4 and estradiol, E2) in the culture medium were measured using RIA and granulosa cells' steroidogenesis enzymes gene expression were quantitatively measured using real-time PCR after 44 h of culture. Granulosa gap junction connexin-43 (CX43) expression was studied by immunoflurescence. Data were analyzed with one-way ANOVA and the significance was determined when $p < 0.05$. GDF9 and BMP15 expression progressively declined by the end of culture in the case of direct contact with granulosa cells while it increased progressively in the indirect co-culture group; however, oocytes that were cultured in direct contact showed a significant increase in blastocyst development after parthenogenetic activation compared to those in the indirect co-culture group (20% vs. 11.5%, respectively). The granulosa cell count in the direct contact group showed a significant decrease compared to the indirect co-culture group (1.2×10^5 cell/ml vs. 2.1×10^5 cell/ml, respectively). Progesterone concentration (13.6 ± 1.4 ng/ml), B-HSD expression, estradiol concentration (78.2 ± 2.5 ng/ml) and CYB19A expression were all significantly increased in indirect co-culture compared to direct contact (P4 was 4.01 ± 0.3 ng/ml and E2 was 26.7 ± 3.1 ng/ml). CX43 was more highly expressed in the granulosa of the direct contact group than the indirect co-culture group. These results indicate that difference in mutual communication between oocytes and granulosa cells exist depending on whether they are in direct contact (juxtacrine) or a short distance apart (paracrine) and represent a new approach to study different ovarian follicular cells interaction. Research supported by MKE (#10033839-2011-13), IPET (#311011-05-1-SB010/ #311062-04-1-SB010) and research institute for veterinary science. IMS and AE contributed equally.
Key Words: Oocyte, steroidogenesis, CX43, paracrine communication, granulosa cells

22. Parturition & post-partum physiology:

2200

Effects of two different hormonal treatments (progesterone and GnRH + PGF2a) on the dairy cows with postpartum anoestrus

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The objective of present study was to compare the efficacy of two hormonal programs for recycling of postpartum anestrous cows. Two hundred Holstein cows from a dairy herd (with 2560 cows) located on the suburb of Tabriz (North-west of Iran) were examined during a year (January–December 2011). These cows showed no visible oestrous signs until at least 60 days postpartum. In this herd, routine oestrus detection is carried out visually by two expert technicians four times/day starting on day 45 (VWP) postpartum. Clinical examinations were performed twice, with a 10 day interval, and blood samples for progesterone analysis were collected simultaneously with every rectal palpation. Progesterone was measured by ELISA kit (Diaplus Inc., USA). Based on progesterone values and clinical examination only cows with lower progesterone levels (P4 < 2 ng/ml) at both samplings and nonfunctional ovaries (at two consecutive examinations) were randomly allocated into two groups: A&B (n = 100). Cows with laminitis, mastitis or functional structures on the ovaries (like cysts or corpus luteum) were removed from the study and replaced by the other anoestrus cows. In group A, cows received 0.021 mg buserelin acetate (5 ml Vetocept, manufactured by Abu-Reihan Pharmaceutical Company, Tehran, Iran) in day 1st followed by 25 mg dinoprost tromethamine (5 ml Vetalyse, manufactured by Abu-Reihan Pharmaceutical Company, Tehran, Iran) injection on the 8th day. In group B, all of cows received 125 mg progesterone (5 ml Vetagesteron, manufactured by Abu-Reihan Pharmaceutical Company, Tehran, Iran) intramuscularly for 8 days. Then estrus detection was continued by two expert technicians four times/day (8 am, 12, 6 pm and 2 am) for the next 5 days. In groups A and B the rate of observed oestrus were 13% and 86% respectively. Significant difference was observed between two groups ($p \leq 0.01$). It was concluded that 8 days of progesterone treatment is superior to a single injection of buserelin followed by

prostaglandin for inducing cyclicity in cows with postpartum anoestrus.
Key Words: Progesterone, postpartum, anoestrus, dairy cows, GnRH

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Endocrine profiles during the peripartum of primiparous rangeland beef cows of different body condition score (BCS) at calving

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The objective of this study was to evaluate temporal changes on endocrine profiles during the peripartum and early lactation, and its association with milk energy (NEL) output and resumption of luteal activity, of rangeland beef cows of different BCS at calving. Twenty primiparous crossbred (Hereford × Angus; 32 months) spring-calving cows with a BCS of 6 (scale 1–8) at −112 days from parturition (days; Day 0 = parturition) were selected from a contemporary group (n = 60) according with their BCS at −35 ± 6 days. Cows were classified into low (BCS < 4.5) or moderate (BCS ≥ 4.5) BCS groups and blocked by expected calving date (calving dates within a 28 day period). Changes in BCS from −112 to −35 days were not due to dietary treatments as all cows grazed together on a native pasture paddock (60 ha), with an average forage mass available of 503 ± 60 kg dry matter/ha (13.8% crude protein and 26.5% acid detergent fiber) from −49 to +49 days. Cow BCS was recorded every 2 weeks from −112 to +49 days and serum samples were collected weekly from −49 to +120 days. Milk yield was obtained at +15 and +35 days by machine milking, samples were collected for milk composition, and NEL output was calculated according to NRC. Resumption of luteal activity was determined by progesterone analysis and considered as the interval from calving to first luteal sample (P4 ≥ 1 ng/ml). Data were analyzed as repeated measures with a mixed model that included BCS group, days (−49 to +49 days), and their interaction as fixed effects. Cow BCS was greater ($p < 0.05$) for moderate than low throughout the period evaluated. Leptin concentrations tended to be greater in moderate than low cows (4.8 vs. 4.3 ± 0.3 ng/ml; $p = 0.10$) and did not vary during the period evaluated. Adiponectin concentrations were less in moderate than low cows (152 vs. 106 ± 18 ng/ml; $p < 0.05$) and were affected by days ($p < 0.05$). Serum adiponectin increased from −49 to −21 days, decreased from −21 to +21 days, and remained stable through +49 days. Concentrations of IGF-I were only affected by days ($p < 0.05$) as serum IGF-I did not change from −49 to −7 days, decreased at +7 days, increased at +21 days, and remained stable thereafter. However, when only the prepartum period was considered, serum IGF-I was greater in moderate than low cows. Although there was no effect of days on insulin concentrations, serum insulin was less in moderate than low cows (1.28 vs. 1.88 ± 0.38 μUI/ml; $p < 0.05$), due to increased ($p < 0.05$) serum insulin after +15 days in low cows. NEL output during the first 35 days was greater in moderate than low cows (16.4 vs. 12.1 ± 1.3 MJ/days). Resumption of luteal activity was earlier in cows with moderate BCS (94.5 vs. 113.3 ± 2.3 days). Primiparous beef cows of moderate BCS tended to have greater serum concentrations of leptin and reduced concentrations of adiponectin and insulin during the periparturient period and had greater prepartum concentrations of IGF-I. The endocrine profiles of beef cows with moderate BCS were associated with greater NEL output and earlier resumption of luteal activity.
Key Words: Beef cattle, postpartum anestrus, grazing

2202

Gene expression profiles and differentiation in new developing bovine trophoblastic cell lines

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To characterize bovine trophoblastic cells, we have been developing cell lines. We raised 12 new cell lines (BT-A to -L) from blastocysts that were produced by *in vitro* production using abattoir oocytes. The aims of this study were to analyze global gene expression profiles in 12 cell lines and some differentiation induced cell lines with Matrigel (BD Matrigel™ Basement Membrane Matrix; BD Biosciences, USA). We used a custom-made oligo-microarray and a quantitative real-time PCR (qPCR) for analyzing gene expression. Three cell lines, which were selected by the intensity of CSH1 expression (BT-1: less but was developed previously, -C: less and -K: higher), were cultured on thick Matrigel prepared using the manufacturer's instructions. Totally about 7000 genes were selected for expression analysis. Gene ontology analysis showed no difference among the cell lines except BT-J that expressed a much smaller number of significant genes compared to that of *in vivo* embryos. New cell lines were divided into three major sub groups with a hierarchical clustering analysis and a principal component analysis. Group 1 involved cells that expressed trophoblastic binucleate cell (BNC) specific genes like CSH1, PRP1, and PAGs. Group 2 contained cells with less intensity of BNC specific genes. Group 3 was similar to Group 1 but was mainly characterized by some other trophoblastic genes. Some undifferentiated-marker genes like Oct3/4, Sox2, c-Myc, were found in most cell lines. We only applied BT-C for microarray analysis in Matrigel culture, and the analysis showed differences in expression profiles: the intensities of 120 genes were increased more than 2-times after on-Matrigel culture. They contained various trophoblast specific genes. The differentiation from trophoblastic mononucleate (MNC) to BNC on-Matrigel culture was found in BT-1 and C but not in BT-K: the intensities of CSH1, PRP1 and PAG1 genes expression were confirmed more than 10-times by qPCR. IFNT was significantly increased 2-times in BT-C on-Matrigel culture. These increments were confirmed with immunochemical analysis. These results suggest that BNC derives from MNC, and for this purpose BT-1 and -C cells are an excellent model. CSH1, PRP-1 and PAG1 are good indicators for the differentiation from MNC to BNC, and IFNT is an indicator as MNC. New developing cell lines maintain the undifferentiated status and contain trophoblastic stem cells similar to the trophoblast cell lineage in early embryos. They are a useful tool for analyzing bovine trophoblast cell lineage. This study was supported by a Grant-in-Aid from JSPS (Kiban-Kenkyu B 23380162).
Key Words: Trophoblast, gene, differentiation, cell, bovine

2203

Relationship between acute phase proteins and subsequent fertility of dairy cows after postpartum uterine inflammation

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The potential relation of serum Hp, SAA, and Fb with subsequent fertility in cows suffering from acute puerperal metritis (APM), clinical metritis (CM) or clinical endometritis (CE), treated with different methods were studied. Late pregnant cows (n = 138) from two farms were used. Diagnosis of APM and CM was made on the 3rd and 5th day postpartum (PP) in farm 1 and 2 respectively. Clinical metritis was

defined if an atonic uterus with a purulent or mucopurulent vaginal discharge was found, without changes in the general health condition. Presence of APM was confirmed when increased body T°, anorexia, an enlarged and atonic uterus and odoriferous discharge were found. In both farms animals were randomly matched, by blood fibrinogen, into three groups to ensure minimal variation of physiological response to inflammation within the groups. The treatments started on the 3rd and 5th day PP in farm 1 and 2 respectively. In farm 1 group A was treated by i.m. Ceftiofur for 5 days in combination with Flunixin for 3 days. Group B was treated by i.m. Ceftiofur for 5 days followed by two injections of PGF2α with an interval of 8 h on the 8th day PP. In farm two diagnosis of CM was done on 5th day PP using the same methodology as in farm 1. Group A was treated by intramuscular Ceftiofur from the 5th day PP for 5 days followed by two injections of PGF2α with an interval of 8 h on the 10th day PP. Animals from group B were re-examined on day 28 PP and (if examination showed existence of CE) treated using the same scheme as group A starting on the 30th day PP. Negative and positive control groups were formed in both farms. Blood samples were taken once before parturition and during 6–7 weeks PP. Date of first insemination up to 90 days PP (days to first service 90, DFS 90), first service conception rate (FSCR), date of successful insemination up to 150 days PP (DO 150) and the number of services per pregnancy (NSP) were recorded for all cows. Animals with higher Hp levels in the first week PP had a lower probability of a first insemination during the first 90 days PP ($p = 0.037$). A similar association of Fb and DFS 90 was found in the second week PP ($p = 0.042$). Serum amyloid A showed no association with DFS 90. Cows with lower Fb levels in the 7th week PP had a negative association with FSCR in the 7th week PP ($p = 0.009$). There was no association found between SAA and Hp with FSCR. The association between SAA concentration in the 7th week and DO 150 was statistically significant ($p = 0.029$). Animals with higher concentrations of Hp in the 6th week PP had a tendency to have a smaller likelihood of successful insemination until 150 days PP ($p = 0.027$). Animals with higher concentrations of Hp in the 6th week, and cows with higher levels of SAA and Fb in the 7th week PP, needed a higher number of services per pregnancy than the cows with normal concentrations ($p = 0.05$, 0.047 and 0.04 for HP, SAA and Fb, respectively). The experimental groups, animals with APM or CM, showed no significant effect for the association of SAA, Hp, and Fb with fertility parameters.

Key Words: Cows, fertility, fibrinogen, haptoglobin, serum amyloid A

2204

Use of lectin histochemistry to study the development of the bovine placentome during gestation

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Lectins bind to glycoproteins and can be used as molecular markers to identify the types of glycoproteins present in tissues. A study was undertaken to characterise changes in glycoproteins at the feto-maternal interface of the bovine placenta throughout gestation, by evaluating lectin binding patterns. Three lectins, Dolichos buflorus (DBA), Glycine max (SBA) and Phaseolus vulgaris leuko agglutinin (PHA-L), were used as markers, and, with the assistance of computer-based software, the intensity of lectin binding to placental tissue was quantified. Uteri ($n = 25$) from early to near term pregnant cows were collected from the abattoir and categorized into five stages of gestation, based on fetal crown rump length (100–125 days $n = 5$; 126–150 days $n = 5$; 151–175 days $n = 5$; 176–200 days $n = 5$; ≥200 days $n = 5$). Dissected placentomes were embedded in paraffin wax and cut into 5 μm sections and mounted on slides for lectin histochemistry. The binding of the lectins were observed and recorded with a fluorescent microscope. The images captured were subjected to computer-assisted software to measure the intensity of fluorescence. The intensity of binding of PHA L was significantly higher ($p < 0.01$) than SBA and DBA at all stages of gestation. PHA-L, which binds to bi, tri and tetra-antennary branched Galβ1, 4GlcNac1, 2Manα1,6, shows intensive staining by Day 100 of gestation, decreases to Day 125, after which more cells were intensively stained, until it reaches a peak by Day 175. Thereafter, staining was reduced from Day 200 of gestation onwards. PHA L seems to bind with a wide range of glycoprotein produced by the binucleate cells as well as binding to the maternal epithelial cells in some cases. This also implies that there are sub-types of glycan being produced during gestation. DBA and SBA showed similar binding patterns to each other at all stages of gestation, with DBA slightly lower by Day 200 of gestation. This indicates the presence of α linked GalNAc (DBA) and α/β linked GalNAc or galactose (SBA) produced by the BNC granules. These lectins showed low binding patterns until Day 175 and increases afterwards. To our knowledge, this study is the first to quantify lectin binding patterns with a computer-assisted method, and demonstrate changes in the type of glycoproteins and glycosylation patterns exhibited within the feto-maternal junction during gestation. These results could be used to identify the functional role of glycoprotein in the development of the bovine placenta during pregnancy.

Key Words: Cattle, pregnancy, placenta, lectin staining, glycoprotein

2205

Induction of parturition in cattle using dexamethasone profoundly suppresses maternal corticosteroid secretion and alters maternal plasma electrolytes

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Steroid hormone profiles in cattle change dramatically in the last 2 weeks before parturition in response to maturation of the fetal pituitary-adrenal axis and fetal corticosteroid production. Synthetic glucocorticoids have long been used to induce calving in dairy cattle. In this study, we investigated the change in the maternal steroid hormone profiles in response to a two stage dexamethasone calving induction regimen. Seven pregnant, near term heifers were each given a single injection of 25 mg of long-acting dexamethasone (DEX) trimethylacetate (Dexavet AP, Bomac Ltd., New Zealand) 10 days before scheduled delivery. Aweek later, a second injection of 25 mg of short-acting DEX sodium phosphate (Dexol 5 Bomac, Ltd., New Zealand) was given. Blood samples were collected, starting weekly 4 weeks prior to the scheduled calving date and then daily before and after the DEX injections until 2 days post-partum. All calves were delivered unassisted and were healthy. Plasma samples were extracted with ethyl acetate and a panel of 14 steroids was measured from the same sample using HPLC and mass spectrometry. The first DEX injection initiated a decrease ($p < 0.05$) in plasma progestagens, with a concomitant increase in circulating estrogens. Estrone and estradiol levels peaked 2 days before parturition. Complete progesterone withdrawal, necessary for parturition, occurred only after the second DEX injection, and this was accompanied by a rapid decline in estrone and estradiol, and an increase pregnenolone, the precursor substrate of all steroids. Progesterone and estrogen levels were at basal levels 2 days after calving. After the first DEX injection, all maternal corticosteroids (cortisol, corticosterone, cortisone and aldosterone) were profoundly suppressed ($p < 0.001$) until 2 days post-partum. Analysis of plasma sodium (Na), potassium (K) and total calcium (Ca) showed that after the first DEX injection, plasma Na dropped and then rose again, whereas plasma K rose sharply from, 4.1 to 6.5 mM, to above the normal range (3.9–5.8 mM) before dropping back to normal a week after the DEX injection. There was no appreciable effect on Ca levels. These results show that: (i) peri-partum progesterone and estrogen profiles in this induced calving regimen resembled that reported for natural calving, (ii) the rise in precursor substrates (pregnenolone, etc) that accompanied the final decline in progesterone and estrogen suggests a down-regulation of 3β-hydroxysteroid dehydrogenase activity in the placenta/corpus luteum, and (iii) the suppression of all maternal corticosteroid secretion is likely due to quelling of maternal adrenocorticotrophin release and this in turn, led to perturbations in plasma Na and K. Other maternal physiological responses may be affected in ways that are not fully understood.

Key Words: Parturition, dexamethasone, corticosteroids, steroid hormone profiles

2206

Oxidative stress in Holstein calves born under distinct obstetrical conditions

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Dystocia or prolonged parturition is one of the main causes of neonatal mortality, primarily due to asphyxia during calving. Hypoxia leads to placental hypoperfusion giving rise to fetal and neonatal reactive oxygen species. Oxidative stress reactions may trigger adverse neonatal outcome. The time in which a calving dam should be assisted may vary considerable, depending on the level of parturition difficulty. The decision to obstetrically intervene is doubtful, mainly regarding to when medical assistance should be undertaken. The aims of the present study were to compare the oxidative stress of calves born under distinct obstetrical conditions and to establish a relationship with neonatal vitality. Holstein calves were grouped according to the duration of calving and obstetrical assistance: 0–2 h of spontaneous labor (Group 0-2SPO; n = 11), 0–2 h of assisted labor (Group 0-2ASS; n = 10), 2–4 h of spontaneous labor (Group 2-4SPO; n = 9), 2–4 h of assisted labor (Group 2-4ASS; n = 9), 4–6 h of spontaneous labor (Group 4-6SPO; n = 6) and 4–6 h of assisted labor (Group 4-6ASS; n = 8). Cows of the ASS Groups were submitted to extraction of the calf within the same time passing as the SPO Groups but still presenting normal parturition. Calves were subjected to blood glutathione peroxidase (GPX) analysis, an antioxidant enzyme, and blood thiobarbituric acid reactive substances assay (TBARS) at birth, 2 and 4 h after calving. Neonatal oxidative status was further correlated with the clinical assessment through the Apgar score (a method to evaluate neonatal vitality adapted from human neonatology and previously standardized for calves), arterial blood gas analysis and blood glucose concentration. Statistical analysis was performed by repeated measures with $p < 0.05$. No statistical differences were verified among groups regarding oxidative stress. On the other hand, TBARS concentration was statically superior after 4 h (369.2 ± 19.7; p = 0.01) comparing to birth (295.1 ± 14.9), whereas GPX levels decreased after 2 h of calving. Moreover, TBARS correlated positively with Apgar score ($r = 0.22$; p = 0.005) and negatively with body temperature ($r = -0.21$; p = 0.008) and pCO_2 ($r = -0.19$; p = 0.02). A negative correlation among GPX and Apgar score ($r = -0.22$; p = 0.006), blood pH ($r = -0.23$; p = 0.004), base-excess ($r = -0.20$; p = 0.01), SO_2 ($r = -0.20$; p = 0.01) and bicarbonate ($r = -0.17$; p = 0.03) was verified; as well as a positive correlation between GPX and glycemia ($r = 0.36$; $p < 0.0001$) and body temperature ($r = 0.15$; p = 0.05). In conclusion, calves suffer oxidative stress during the initial 4 h of life, regardless of the calving period or obstetrical intervention. However, the conversely profile of TBARS and GPX suggests a physiological function of the reactive oxygen species during neonatal adaptation period. As pulmonary gas exchange improves, neonatal oxidative stress diminishes leading to clinical recovery and metabolic balance. Furthermore, Apgar score and body temperature assessment can be employed as clinical predictors of oxidative stress in bovine neonates. FAPESP grant 06/50485-7.

Key Words: Reactive oxygen species, vitality, calving, neonate, bovine

2207

The effect of lactation on post partum uterine involution in Holstein dairy cows

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Following parturition, complete uterine involution is a prerequisite to any subsequent pregnancy. The objective of this experiment was to examine the effect of lactation on the rate of uterine involution in postpartum dairy cows. Holstein primiparous autumn-calving cows were used (n = 19, mean age 3.9 ± 0.1 years). All cows were pregnant to a synchronized estrus following insemination with semen from the same bull. At calving, cows were randomly assigned to one of two treatment groups, lactating (n = 11) or non-lactating (i.e. dried off at calving, never milked, n = 8). Examination of the reproductive tract was carried out by ultrasonography twice weekly until week 7 postpartum. Blood samples were collected twice weekly for analysis of progesterone to indicate resumption of cyclicity and metabolites indicative of energy status (insulin, IGF-1, glucose, NEFA, BHBA). Uterine involution was assessed in terms of size of the uterine horns, uterine body diameter and uterine fluid volume. Vaginal mucous score was carried out on week 4 postpartum for assessment of uterine infection. Data were analysed using the MIXED procedure of SAS. Progesterone profiles were similar for both groups throughout the 7 week period ($p > 0.05$). Resumption of cyclicity (serum progesterone > 1 ng/ml) had occurred in both groups by week 3 postpartum. Concentrations of NEFA and BHBA were higher while concentrations of glucose, insulin and IGF-1 were lower ($p < 0.05$) in lactating compared to non-lactating cows. Lactating cows had a smaller uterine diameter ($p < 0.05$) than non-lactating cows from week 4 to 6 postpartum (week 4: 20.2 ± 1.3 v 24.9 ± 1.5 mm, respectively), and had a lower uterine fluid volume up to week 7 ($p < 0.05$). By week 7, there was no difference in uterine diameter (15.2 ± 1.8 v 15.2 ± 1.6 mm) or uterine fluid volume (0.11 ± 0.38 vs. 0.18 ± 0.46) between lactating and non-lactating cows, respectively. Vaginal mucous score revealed no evidence of uterine infection in either group. In conclusion, while lactation induced significant alterations in metabolic status, it did not have a major effect on the rate of uterine involution as defined by the parameters measured in this current study.

Key Words: Infertility, dairy cattle

2208

Relationships between serum adiponectin concentrations and postpartum luteal activity in High-producing dairy cows

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There is accumulating evidence showing the role of adiponectin, a newly discovered adipocytokine secreted from adipose tissue, in the regulation of the ovarian function in dairy cows. Adiponectin plays an important role in the control of lipid metabolism, glucose homeostasis and energy balance in different species. No information is available regarding the relationship between the serum adiponectin and the luteal activity in dairy cows postpartum. Therefore, the present study was performed to specifically characterize the relationship between serum adiponectin profiles with the pattern of luteal activity in the postpartum period of clinically healthy high producing dairy cows. Coccygeal vein blood samples were collected from 71 high producing dairy cows twice weekly (3 days apart) from the 1st to 7th week postpartum. Only cows with a history of being clinically healthy during the peri- and postpartum period were used in the present study to enable us to exclude the possible adverse effects of clinical diseases on the resumption of postpartum luteal activity. Serum progesterone (P4) concentrations were determined twice weekly using a validated commercial radioimmunoassay kit (Immunotech kit, France). Based on the progesterone profile, the pattern of the postpartum luteal activity was determined in the cows as normal luteal activity (NLA), prolonged luteal phase (PLP), delayed first ovulation (DOV), and anovulation (AOV). Serum adiponectin was determined in a randomly selected subset of cows from the NLA (n = 5), PLP (n = 6), DOV (n = 6), and AOV (n = 5) luteal groups every 2 weeks using a validated commercial ELISA kit (Bovine Adiponectin, Cusabio Biotech Co., Ltd., China). The analytical sensitivity of the test was typically < 3.12 μg/ml. Biweekly changes in mean serum adiponectin concentrations in groups of different luteal activity from the 1st to the 7th week postpartum were analyzed using repeated measure ANOVA. Differences between the mean serum adiponectin concentrations of luteal activity groups were compared using a one-way ANOVA (LSD test was used to locate differences). Mean serum adiponectin concentrations decreased gradually until the third week postpartum in NLA group and then increased whereas in the AOV and DOV groups, the serum adiponectin continued to decrease after the third week postpartum ($p < 0.05$). Moreover, the mean (±SE) serum adiponectin concentrations in cows in the NLA (3.53 ± 0.21 μg/ml) and PLP

(3.50 ± 0.39 μg/ml) groups were higher than cows in the AOV (2.34 ± 0.40 μg/ml) group at 7 weeks postpartum (p = 0.02). The data shows for the first time the presence of a relationship between serum adiponectin concentrations and luteal activity in postpartum period in High-producing dairy cows.
Key Words: Adiponectin, luteal activity, postpartum, dairy cows

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Application of polymerase chain reaction for fetal gender determination using cervical mucous secretions and maternal serum in the cattle

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Determination of bovine fetal sex is potentially of economic and practical importance. Ultrasonography and karyotyping have been used for sexing bovine fetuses. Our aim was to use a novel approach using PCR to amplify DNA from bovine (*Bos taurus*) fetal cells recovered through post-slaughter sampling of cervical secretions and maternal serum to assess the effectiveness of this method in gender determination. The PCR target sequence was intron five of the bovine amelogenin gene located on both X and Y-chromosomes. Fetal-derived Y-chromosome was not detected in any samples from cows carrying female fetuses (n = 8). We were able to demonstrate the presence of fetal-derived Y-chromosome in seven cervical mucus samples out of 13, which were collected from cows with male fetuses. None of positive cervical samples were below 70 days of gestation, but negative samples were distributed below or over this age. No fetal DNA material was amplified in maternal serum samples collected from cows with female or male fetuses. These results indicate that amplification of Y-chromosome segments from cervical mucus of pregnant cows after 70 days of pregnancy is highly specific for presence of a male fetus, but that this approach currently lacks sensitivity. Inability to distinguish maternal and fetal DNA makes it impossible to confirm presence of a female fetus. This is the first report on validating the presence of fetal DNA material in bovine cervical mucus. Further investigations are needed to maximize the accuracy and evaluate the practicality of this approach in real field situations to sex bovine fetuses.
Key Words: Fetal sexing, cervical mucus, PCR, cattle

2210

Concentrations of copper and its main antagonists in colostrum and serum of Bergamacia sheep

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In addition to its role in neonatal immunity colostrum is an important source of protein, carbohydrates, lipids, vitamins and minerals, elements that participate in nutrition and thermoregulation of the newborn. (Simões et al., 2005. Pesq. Vet. Bras., 25(4):219–224).The aim of this study was to assess the colostral copper and its main antagonists (Zn, S, Fe and Mo) by analyzing of colostrum and serum in Bergamacia sheep. We used 20 Bergamacia sheep aged 3–5 years old kept confined from 15 days before lambing, fed sorghum silage, concentrate, hay, mineral and water ad libitum. Colostrum was collected from dams (30 ml) at 0, 24, 48 and 72 h after lambing. Blood samples were collected only after lambing (0 h). Analysis of levels of molybdenum, zinc, iron, sulfur and copper in the sorghum silage, hay, mineral salt and colostrum were performed by atomic absorption spectrophotometer. The obtained values of minerals from the concentrate diet, sorghum silage, mineral and hay are in agreement with those found in the literature for a proper diet for pregnant ewes. The values obtained from analysis of hay, feed, silage and mineral salt were in mg/kg for copper 9.00, 16.71, 5.14 and 412.69; for zinc 39.59, 103.76, 29.86 and 5426.81; for sulfur 701.40, 1268.18, 531.36 and 7899.55; for iron 94.74, 220.04, 140.58 and 4859.14 and finally to the molybdenum 1.91, 1.40, 2.35 and 0.02 respectively. The reference values of colostrum and the milk at 72 h were in mg/kg for copper 13.99 and 6.35; for zinc 8.83 and 5.20; for iron 12.18 and 6.12; and finally to the molybdenum 6.71 and 6.71 respectively. The results are presented as concentrations minerals in colostrum 0, 24, 48 and 72 and serum (0 h) of Bergamacia sheep (Table 1). The values of the minerals in colostrum in the present experiment were below those found by Kracmar et al. (2005. *Small Rumin. Res.*, 56:183–188), but had the same descendent trend lines until the time of 72 h. The concentrations of immunoglobulins, peptides, amino acids and minerals are high in colostrum of all mammals. These concentrations of virtually all components are reduced gradually and stabilize within 5 days after parturition when the characteristics of normal milk are attained (Kracmar et al., 2005. *Small Rumin. Res.*, 56:183–188). The values of serum Cu and Zn are presented adequate, seconds Pugh (Sheep and goat medicine. 1st ed. Roca, 2005. 347 pp), who suggests that Cu should be between 0.7 and 2.0 ppm, and Zn between 0.8 and 2.0 ppm. We conclude that all minerals have higher concentrations in colostrum than serum at parturition and achieve values similar to normal milk at 72 h.
Key Words: Bergamacia sheep, colostrum, copper, antagonists

Table 1. Minerals concentration in colostrum of Bergamacia sheep 0, 24, 48 and 72 h after lambing and the serum on 0 h

Mineral (mg/kg)	Time (h)	Time (h)	Time (h)	Time (h)	Time (h)
	0	24	48	72	0 (serum)
Cu	2.3 ± 1.3[a]	2.1 ± 0.9[a]	1.9 ± 0.9[a]	1.9 ± 1.1[a]	1.6 ± 0.3
Zn	7.8 ± 1.8[a]	6.2 ± 2.5[b]	4.3 ± 1.2[c]	3.8 ± 1.0[c]	1.2 ± 0.4
Fe	7.0 ± 4.3[a]	6.6 ± 2.9[a]	5.3 ± 1.7[a]	6.2 ± 2.6[a]	6.0 ± 1.5
S	1997.7 ± 422.0[a]	1022.3 ± 327.3[b]	653.5 ± 167.3[c]	602.6 ± 175.4[c]	578.4 ± 93.1
Mo	3.0 ± 2.4[a]	2.9 ± 2.6[a]	3.6 ± 2.7[a]	3.2 ± 2.6[a]	0.9 ± 0.4

Tukey's test: different letters in the same row are significantly different (p < 0.05).

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Transcervical administration of PGF2α analog to interrupt gestation in mares

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Interruption of gestation of the undesired sex is becoming a common practice in Argentinean embryo transfer programs after fetal sexing around day 60 or later. Polo breeders are interested mainly in female offspring, therefore some programs decide to interrupt undesired gestations. This study analyses retrospectively the procedure to interrupt pregnancies in a large scale embryo transfer program. During the 2010/11 reproductive season, pregnancies were sexed between days 58–65 by ultrasonography according to the genital tubercle location. One hundred four undesired gestations (mostly male fetuses) were interrupted by transcervical administration of 500 μg of cloprostenol in 10 ml of sterile saline solution with an insemination pipette after perineal cleaning. The exact location to discharge the cloprostenol solution was the internal cervical os avoiding tearing the chorioallantoic membrane. Serum eCG concentrations were not measured and only a descriptive analysis is presented here. Ninety eight mares (94.2%) aborted and expelled completely the conceptus within 24 h with no retained placenta or other complications observed. Twenty mares were scanned 1 and 3 h after the treatment. At 1 h the fetuses showed hypermotility, increased heart beat and dispersed hyperecoic dots within the amniotic and alantoic fluids. By 3 h, all fetuses were dead or highly depressed with very low heart rates. Only six mares (5.8%) were found with a dead fetus in the uterus or in the

vagina at 24 hs after the treatment; in these six mares the chorioallatois had been torn accidentally at the time of treatment. Fourty-nine (47.1%) of the aborted mares were used as recipients again in the same season; with a pregnancy rate of 79.6% (39/49) and embryo loss rate at 60 days of gestation of 7.7% (3/39). The same treatment was applied to three mares between 120 and 140 days of gestation. In these three cases the fetuses were found dead within the uterus with obvious signs of decomposition by 48 h. These mares were treated with uterine lavages and antibiotic infusion and responded successfully. In conclusion, although it is not known if some of the abortions occurred by the transcervical manipulation itself (no control group), transcervical administration of cloprostenol in mares can effectively interrupt gestation around 60 days without major complications and with a favorable prognosis for fertility. In contrast, in more advanced gestations (120–140 days) is not recommended in the absence of additional treatment.

Key Words: Interruption, gestation, mare

2212

Activin A during Thoroughbred pregnancy and immunohistochemical localization of its receptors and intracellular mediators in equine fetal gonads

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Immunolocalization of Activin has been evidenced in equine fetal gonads and placenta, but the Activin action on these tissues and the plasma Activin concentrations in the mare are still unknown. Thus, our objectives were to characterize the plasma Activin A concentrations and to immunostain Activin signaling molecules in fetal gonads and the utero-placental unit. Activin A was measured by sandwich ELISA (Quantikine, R&D systems, MN, USA) in weekly plasma samples from six pregnant Thoroughbred mares. The intra- and inter-assay CVs were 2.3% and 7.1% respectively. Differences in Activin A levels at different stages of pregnancy were analyzed by Duncan's multiple range test at $p < 0.05$. Fetal testes (day 120, 180, 225 and 314), ovaries (day 110, 140, 180 and 270) and the respective utero-placental units from a different set of pregnant thoroughbred mares were immunohistochemically analyzed for Activin receptors (IIA/B and IA/B) and its intracellular mediators (smad 2, 3, and 4). Activin A concentration remained low until around day 50 (43 ± 30 pg/ml) which then significantly increased at day 96 (91 ± 31 pg/ml) and maintained this level until the second significant peak (398 ± 94 pg/ml) on the day of parturition. Activin receptors type IIA/B and IA/B immunostained the interstitial and germ cells of fetal testes and ovaries along with the utero-placental units throughout all stages of pregnancy. Intracellular mediators of the Activin signal, Smad proteins 2, 3, and 4 were also immunolocalized in all of these tissues. Thus it can be concluded that Activin action is possible in these tissues as they express all of the signaling molecules for Activin. For the first time, present study characterized the plasma Activin A status during equine pregnancy where the first significant increment coincides with the time when the fetal gonadal size of horse start increasing and the second increment coincided with the parturition.

Key Words: Activin, horse, pregnancy, fetal gonad

2213

Cloprostenol-induced changes in endometrial proteins and gene expression during the third week of pregnancy in mares

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A major objective of this study is the identification of proteins that are potential biomarkers or functional mediators of early pregnancy failure in mares. This report details some changes in selected uterine proteins after early pregnancy is compromised by administration of IM cloprostenol, a prostaglandin F2α analogue, 1–4 days before sample analysis on Days 16–20 after confirmed ovulation and pregnancy. Samples from control and cloprostenol-treated mares included uterine flush fluid and endometrial biopsies. From conceptuses recovered in the saline flush, yolk-sac fluid, yolk-sac wall (trophoblast) and capsule were obtained. Proteomic profiles of flush and yolk-sac fluids were analysed by LC MS/MS with Scaffold 2 interpretation, or by SDS-PAGE and immunoblots. Transcriptomes (by Agilent 44K expression microarrays) were determined for endometrium and trilaminar trophoblasts collected on Days 19 or 20 from mares given saline or cloprostenol on Day 18 (5–6 per treatment for each day). Endometrial samples from 5 non-pregnant mares were also analysed. Secretoglobin 1A1 (SCGB), also termed uteroglobin or CCSP, was expressed in large amounts in normal pregnant endometrium and the protein was variably abundant in one or two forms in uterine flush fluids. After cloprostenol, SCGB increased in uterine flush fluid and was immunohistochemically most abundant in secretory cells and secretions of larger nested endometrial glands. However, there were few SCGB transcripts in trophoblasts and the protein was not detected in normal or cloprostenol-treated yolk-sac fluids. The forms of SCGB that increase in compromised pregnancy are still being characterized. By comparison, secretory phospholipase A2 (sPLA2), a protein previously shown to increase markedly in the embryonic capsule after cloprostenol, also increased in the uterine flush fluids. SPLA2 was present in yolk-sac fluids of conceptuses from treated but not from control mares. Corresponding increases in sPLA2 gene transcripts were found in the endometrium 1 or 2 days after cloprostenol, but not in the trophoblasts in which sPLA2 expression was very low. Endometrial expression of SCGB and sPLA2 was consistently much higher in mares that were not pregnant. These results indicate that endometrial production of SCGB is reduced in pregnancy, consistent with its proposed role in innate immunity in mucosal surfaces, but it increases when pregnancy is compromised in this model. The results also suggest that endometrium-derived sPLA2 increases rapidly and markedly after cloprostenol and enters the conceptus before the pregnancy fails. These findings further support the hypothesis that sPLA2 is an endometrial product detrimental to the survival of the early equine conceptus. Supported by NSERC, Grayson Jockey Club Research Foundation, Equine Guelph and OMAFRA.

Key Words: Uteroglobin, phospholipase, transcriptome, trophoblast, conceptus

2214

Uterine fluids and fertility during the first postpartum ovulatory period in Thoroughbred mares under tropical conditions

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The practical importance of the post-partum period is aimed to decrease the inter-foaling interval to produce a foal per mare per year. During the postpartum period one of the major events related to the uterine involution process is the elimination of lochia. In addition, it has been shown that the presence of uterine fluids reduces the mare's

fertility. Therefore, in order to describe the dynamics of the postpartum uterine secretions and discharge and its relation to fertility of Thoroughbred mares located near the equator (09°55′N, 67°20′W), seven postpartum periods of mares were studied. Mares were, 6–10 years of age, without history of assisted foaling, retained fetal membranes or endometritis and were bred during the first postpartum ovulatory cycle. Each mare was monitored by daily uterine ultrasound, looking for the presence or not of fluid in the uterine lumen. Daily ultrasound examination started on day 1 after foaling (day of foaling was assigned as day 0) and continued until the second postpartum ovulation. Physiological events were assigned to the day in which they began. Data were analyzed using descriptive statistics and Kruskal–Wallis test. Eighty five percent of the mares had uterine fluid during the first 5 days of the postpartum period, whereas 29% of the mares had uterine fluid between days 7 and 10, and only 14% of the mares had uterine fluid after day 10 postpartum. By day 14 postpartum no mares showed intrauterine fluid collection. Of the mares evaluated (n = 7), five mares had totally eliminated the intrauterine fluid before day 5 postpartum (Group 1) and two out of seven mares eliminated the uterine fluid after day 7 postpartum (Group 2: group with a delay in intrauterine clearance). After breeding during the foal heat, 57.2% of the mares (four out of seven) were pregnant. However, the pregnancy rate of G1 (80%; 4 out of 5 mares) whereas no mares that retained fluid after D 5 (G2) became pregnant (0%; zero out of two mares). The delay in the clearance of intrauterine fluid appears to affect the chances of pregnancy in foal heat breeding.

Key Words: Uterine fluids, fertility, Thoroughbred mares, postpartum, tropical

2215

Foaling characteristics and retained foetal membranes (RFM) in mares conceiving under natural day-length and foaling at pasture

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A retrospective study was made of foaling records including the incidence of RFM (membranes expelled >3 h after foaling) from 1118 Standardbred mares. The mares conceived under natural day-length by artificial insemination and foaled at pasture, with daily veterinary care, over 4 breeding seasons in New Zealand. Data had been collected by staff on one stud farm, with mares observed every 20 min or more often. Gestation length was 348.8 ± 0.4 days (mean ± SEM; range 312–393 days); 2.4% of foals were born dead; 49.9% were fillies, 50.1% were colts. Most foalings (88%) occurred between 1 October and 31 December and 79% took place between 2100 and 0759 h. For 90% of mares, foaling duration (from mare down and straining or membranes visible) was <20 min (median 12 min). Within 15 min of foaling; 89% of mares stood again. 7% of mares foaled standing. After birth, 72% of foals stood within 60 min and 63% suckled within 120 min. RFM occurred in 42% of mares. The % of mares with RFM was 37.5, 41.4, 48.8 and 61.2 for foaling duration of 0–10, 11–20, 21–30 and >30 min, respectively. Mares that foaled standing had 67% RFM. If foals first suckled within 120 min of birth, 32% of mares had RFM compared to 52% when first suckle was after 120 min. Foaling characteristics of individual mares did not appear to be repeated from year to year. There was no apparent effect of month of foaling, timing of foaling, gestation length, mare parity or age, and time until foal stood on the incidence of RFM in this pool of mares. We suggest that foaling duration, mare's foaling position, and time until foal's first suckling may affect placenta delivery time.

Key Words: Mare, foaling, retained placenta

2216

Concentration of lactate and cortisol in feline amniotic and allantoic fluids throughout pregnancy

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In the present study, lactate and cortisol concentration of amniotic (AM) and allantoic (AL) fluids and maternal serum in pregnant queens was compared after performing an ovariohysterectomy. Eleven pregnant queens were included and distributed in four different groups, day 30 (D30), day 40 (D40), day 50 (D50) and day 60 (D60), according to their gestational age (Knospe, 2002. Anat. Histol. Embryol. 31:37–51). Once measured, the embryo vesicles were dissected and AM and AL fluids were aspirated. Additionally, a blood sample from every queen was obtained from the jugular vein. Lactate was analysed with an Olympus AU400 Clinical Chemistry Analyzer (Olympus Diagnostic Systems, Olympus, Germany) and OSR reagents (Olympus System Reagent®, Olympus, Ireland). Cortisol was analyzed with the Salivary Cortisol ELISA kit (DRG Instruments, Marburg, Germany) and the lecture was performed with the EMS Reader MF V.2.9-0 (Labsystems, Helsinki, Finland). Results were analyzed using the SAS statistical package. A General Lineal Model procedure (PROC GLM) was used to evaluate significant differences ($p < 0.05$) among samples, whereas the LSMEANS procedure was used to list these mean differences. Data were first analyzed comparing the three groups of samples; MS, AM and AL fluids, at different pregnancy times. Also, data from every group of samples were analyzed among the different phases of pregnancy for each group to detect variations along the pregnancy. Lactate showed similar concentrations in AL, AM and MS when compared among them. Concentrations were also constant throughout the pregnancy with the exception of AL on D50 (8.91 ± 0.61 mM) and AM on D60 (12.05 ± 0.63 mM) that showed significant ($p < 0.05$) higher values of lactate than in other pregnancy periods. Cortisol level in AL and AM was significantly ($p < 0.05$) lower than in MS from D30 to D50 of pregnancy. On D60, cortisol concentration in AL showed a significant ($p < 0.05$) increase when compared with the other gestational phases (55.8 ± 7.2 ng/ml on D60 vs. 0.6 ± 5.9 ng/ml on D30, 1.9 ± 5.9 ng/ml on D40, and 6.1 ± 4.6 ng/ml on D50). AL and AM showed higher values of lactate concentration on D50 and D60 respectively. It is known that the accumulation of lactate is an indicator of tissue hypoxia. Hypoxia development during the time period between foetal removal from the mother during surgery and sample collection could be a contributing factor for lactate production. Additionally, at the end of pregnancy foetuses reach the inherent space limitations of the uterus. The lack of space induces foetal stress, which can be translated into tissue hypoxia. Stress induces the release of foetal adrenal corticotrophin (ACTH) which in turn will induce foetal corticoids. This hypothesis is sustained by the fact that cortisol in AL and AM also increased at the end of pregnancy.

Key Words: Lactate, cortisol, amniotic fluid, allantoic fluid, pregnancy

2250

Uterine blood flow at various stages of pregnancy in dairy buffaloes

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Throughout pregnancy, functional and structural changes occur in the uterine vascular system depending upon fetal nutritional requirements. Middle uterine artery, the major blood vessel, adapts to these hemodynamic changes to ensure adequate blood supply to the developing placenta/fetus. This study was conducted on 54 Murrah buffaloes, six in each month of gestation beginning from 2 to 10 months. The middle uterine artery ipsilateral and contralateral to

the fetus were scanned using a Doppler ultrasound device (Exago, France) in pulsed-wave mode using a 7.5 MHz linear probe with a filter of 100 Hz, power of 50%, pulse repetition frequency (PRF) of 4000 Hz and doppler angle varying between 20 and 60 degrees. Blood flow was reflected by parameters such as resistance index (RI), pulsatility index (PI), time-averaged maximum velocity (TAMV), diameter of the vessel (D), volume (Vol) and rate (R) of blood flow (Table 1). RI of the ipsilateral artery was positively correlated with PI ($r = -0.94$, $p < 0.05$) and negatively correlated with all other parameters (TAMV: -0.65 $p < 0.0001$, D: -0.63 $p < 0.0001$, Vol: -0.64 $p < 0.01$ and R: -0.45 $p < 0.0001$), whereas positive correlation occurred between TAMV: D ($r = -0.78$, $p < 0.0001$), TAMV:-Vol ($r = -0.98$, $p < 0.01$) and Vol:D ($r = -0.89$, $p < 0.01$). RI and PI decreased continuously during the first 8 months of gestation and remained fairly constant thereafter. The difference in the volume of blood flowing through the ipsilateral and contralateral artery was low at 2 months (1563.8 ml/min), after which it started increasing until the 5th month(3543.01 ml/min) and thereafter remained fairly constant till the 8th month. In the last month, difference rose to 6192.34 ml/min between the ipsilateral and contralateral blood vessels which may be due to the requirements of the growing fetus. Volume of blood recorded in ipsilateral and contralateral uterine arteries of buffaloes in the present study was higher compared to dairy cows as revealed from available literature. Diameter of the ipsilateral artey rose from 12.5 to 15.4 mm and that of the contralateral artery from 11 to 14.2 mm from 2 to 10 months of gestation. The rate of blood flow in the ipsilateral artery was lower compared to the contralateral artery which may be due to the proportionate increase in the diameter of the blood vessel. The notch signal disappeared by 20–24 weeks which denotes completion of development of placenta. Transrectal Doppler ultrasongraphy proved to be a useful non-invasive method which can be used to assess the uterine blood flow during pregnancy in buffaloes.

Key Words: Buffalo, gestation, blood supply, middle uterine artery

Table 1. Parameters used to assess blood flow

Gestation in months	2	3	4	5	6	7	8	9	10
RI i	0.75	0.64	0.62	0.55	0.45	0.48	0.27	0.42	0.33
TAMV–m/s i	59.06	57.14	68	70.13	74.65	67.31	73.38	96.77	128.57
PI i	1.92	1.26	1.07	1.04	0.71	0.75	0.48	0.57	0.43
R–bpm i	67.6	61.7	67.5	70.5	79.88	71.4	65.43	79.7	77.9
R–bpm c	58.5	64.5	58.2	75.8	124	80	83.1	79.3	85.5
Vol–L/min i	4.35	4.21	3.6	5.16	5.85	5.87	5.93	9.72	14.37
Vol–L/min c	2.78	1.91	0.54	1.62	3.99	3.8	3.62	6.72	8.18
D–mm i	12.5	12.5	10.6	12.5	12.9	13.6	13.1	14.6	15.4
D–mm c	11	11	6.88	9.38	12.1	12.7	10.4	13.3	14.2

I, ipsilateral; c, contralateral.

2251

Lung morphometry of preterm puppies after maternal betamethasone treatment

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In human perinatology the use of prenatal corticosteroids in order to improve lung function in premature infants is an important intervention. The use of antenatal betamethasone induces changes in fetal lung structure in different species. Hence, our hypothesis is that the use of betamethasone in a pregnant bitch has an effect on the structural maturation of the canine lung tissue of premature neonates, thus promoting the improvement in respiratory condition. We used 25 neonates delivered by cesarean section, allocated into two groups: a control group (CONT) – maternal administration of saline solution (0.9% NaCl) at 55 days of gestation (n = 15) and Betamethasone Group (BETA) – administration of a single dose of 0.5 mg/kg maternal body weight of betamethasone (Celestone® Chronodose Injection) at 55 days of gestation (n = 10). The day of ovulation was determined as day 0. In the CONT group, neonates were assessed at 55 (CONT55), 57 (CONT57) and 63 (CONT63) days of gestation, while in the BETA group, at 57 (BETA57) and 58 (BETA58) days of gestation. Lung morphometry was performed to assess the volume density (%) of respiratory bronchioles, saccules, terminal saccules, alveoli and septae. Moreover, lung immunoistochemistry (performed on two animals per group) was carried out to identify cell proliferation (Proliferating Cell Nuclear Antigen – PCNA) and tagging of cytokeratin to distinguish respiratory epithelia from lung parenchyma (saccules and alveoli). The data was analyzed by ANOVA and LSD at $p \leq 0.05$. We observed a significantly higher percentage of septation in the CONT63, BETA58 and BETA57 groups. The neonates from the CONT63 group had subsaccular development significantly greater than other groups. The treated groups showed significantly greater subsaccular development than the CONT55 and CONT57 groups. The saccular formation was more evident in the BETA57 and CONT57 groups, followed by the BETA58 and CONT55 groups. We observed a low alveolar percentage in the CONT55, CONT57 and BETA 57 groups, with significantly higher values in the CONT63 and BETA58 groups. The neonates from the CONT55 group showed a significantly higher percentage of respiratory bronchioles tissue than the other groups. The BETA57, BETA58 and CONT63 groups presented a remarkable delimitation between respiratory epithelia and lung parenchyma. Moreover, the neonates from the CONT57 and CONT55 groups showed more diffuse tagging for cytokeratin in the lung tissue. We identified a greater cell proliferation in the CONT57 and CONT55 groups in relation to BETA57, BETA58 and CONT63 groups. These results allow us to confirm that the administration of betamethasone to pregnant bitches induces structural changes of neonatal lung parenchyma, with increased formation of gas exchange structures and differentiation of lung tissue, issues that can promote an improvement in neonatal respiratory condition. FAPESP grant no. 2009/52269-8 and 2009/06752-9.

Key Words: Morphometry, lung, canine, betamethasone, premature

2252

Epithelial cell differentiation regulated by microRNA-200a in mammary gland

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Mammary gland epithelial cells undergo periodic cycles of proliferation, differentiation and involution. Many works have been reported that miRNAs known as small non-coding RNAs and post-transcriptional regulator that influence a variety of biological processes. Here we found that one miRNA, miR-200a, had relatively high expression in epithelial cells-rich organ, such as mammary gland, lung and kidney. The objective of this work was to assess possible role of miR-200a in mammary epithelial cells. In mice, the miR-200a expression in mammary gland was increased from mid pregnancy to lactation and its expression in mammary epithelial EpH4 cells was stimulated by treatment of lactogenic hormone, insulin, dexamethasone and prolactin. The lactogenic hormone also induced the expression of milk protein β-casein mRNA (marker of cell differentiation) and E-cadherin mRNA (marker of epithelial cell). However, the knockdown of miR-200a prevented the increase of β-casein and E-cadherin mRNA expression. Immunofluorescence analysis revealed that E-cadherin and ZO-1 (another marker of epithelial cells) signals were decreased by the knockdown of miR-200a. Finally, in a three-dimensional culture system that models lumen-containing mammary duct, the knockdown of miR-200a decreased rate of cavity formation, indicating a reduction of epithelial cell polarity. These observations suggest that miR-200a is important for the maintenance of epithelial cell phenotype, which contributes to lactogenic hormone inducing cell differentiation in mammary gland.

Key Words: Mammary gland, miRNA, cell polarity, epithelial cell, lactation

2253

Effect of passive immunization against inhibin-α subunit and non-steroid bovine follicular fluid (NSBFF) on mammary gland growth and development in primiparous female Wistar rats

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The role of immunoneutralization against inhibin-α subunit and NSBFF on mammary gland growth and development in primiparous female rats was investigated. Eight mature male rabbits were injected with NSBFF (1 ml, sc, per each for five times; 1 week interval). One month later, blood was collected, centrifuged and antiserum was obtained. Twenty four pregnant rats were assigned into three groups, injected, on 5th, 10th, and 15th days of gestation, with saline (control), NSBFF antiserum (NSFF-Ant.), and inhibin-α antiserum (Inh. Ant.). After parturition, females were anesthetized, blood samples were obtained for assessment of LH, FSH, estrogen, prolactin, and inhibin-B, and mammary tissues were obtained for histophysiological study. Gestational periods were significantly increased in Inh. Ant. and NSBFF-Ant. groups. FSH and prolactin concentrations were significantly increased, whereas estrogen and inhibin-B concentrations were significantly decreased in the two treated groups. NSBFF-Ant. treated females revealed marked decline in mammary ductal elongation and aleveolar morphogenesis, whereas Inh. Ant. treated groups showed moderate decline in mammary ductal elongation but have marked development in aleveolar morphogenesis. Fat pad was retarded in the two treated groups. Females of NSBFF-Ant. group showed abnormal mammary ductal elongation and alveolar morphogenesis, whereas those of Inh.Ant group showed normal morphogenesis and differentiation, but their secretory cells showed mild retardation. In conclusion, inhibins can perform important role in parturition, whereas activins play positive role in mammary gland ductal elongation and alveolar morphogenesis.

Key Words: Inhibin, activin, mammary gland, non-steroid bovine follicular fluid, passive immunization

2254

Abortion induced by cloprostenol does not affect the fertility of dairy heifers

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The aim of this study was to compare the fertility of dairy heifers after induced abortion at first trimester of gestation with cloprostenol. The effect of pregnancy interruption was evaluated in 52 Holstein heifers (18–24 months age) inseminated by conventional semen, carrying male fetuses ranging from 55 to 68 post conception diagnosed by ultrasound (Esaote Aquila™, the Netherlands). Abortion was induced by one administration IM of 0.530 mg of sodium cloprostenol (Ciosin™ MSD, Brazil) after fetal gender diagnosis. A second ultrasound evaluation was made 15 days after to confirm abortion. In heifers that had no fetuses and with a corpus luteum, a new application of the same product was given IM at this time. Vaginoscopic evaluation was made 15–20 days after the second cloprostenol treatment. All heifers without alterations at this evaluation were inseminated in the next normal estrus with the same semen used before abortion. The efficiency of abortion induction was 86.53% (45/52). The occurrence of clinic uterine infections diagnosed by vaginoscopy was low (4.4% −2/45). The periods from first cloprostenol to first insemination and first cloprostenol to conception were 38.94 + 4.96 and 47.32 + 15.89 days, respectively. There was no difference on the number of inseminations per conception before or after pregnancy interruption (1.32 + 0.79 vs. 1.44 + 0.98; $p > 0.05$). We conclude that cloprostenol is an efficient method for interrupting gestation in the first trimester and that the induced abortion does not affect fertility in heifers. Supported by Fapemig.

Key Words: Bovine, gestation, cloprostenol, abortion, reproductive performance

23. Pregnancy & placenta physiology:

2300

Environmental regulation of pregnancy-specific protein B concentrations during late pregnancy in dairy cattle

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Environmental factors, such as photoperiod and heat stress, can be manipulated during the dry period to influence health, productivity and reproductive performance of dairy cows in their subsequent lactation. Photoperiod and heat stress effects on subsequent lactation are related to alterations in prolactin (PRL) signaling. Additionally, exposure of cows to heat stress during the dry period decreases gestation length; however, the mechanism involved in this process is unknown. The objective of these experiments was to evaluate the influence of environmental factors (i.e., heat stress and photoperiod) during late gestation (i.e., dry period) on pregnancy specific protein B (PSPB) concentrations in plasma of dairy cows. In Experiment 1, cows were dried off approximately 46 days before expected calving and randomly assigned to heat stress (HT; n = 30) or cooling (CL; n = 30) treatment. CL cows were housed with sprinklers, fans and shade, whereas HT cows were provided only shade. In Experiment 2, cows were dried off at approximately 60 days before expected calving and randomly assigned to three treatments: long day photoperiod (LDPP: 16L:8D; n = 15), short day photoperiod (SDPP: 8L:16D; n = 14) and SDPP + PRL implant (12 mg/days of PRL at 28 days or16 mg/d of PRL at 39 days relative to calving; n = 11). In both experiments, plasma samples were collected at dry off and at −32, −18, −7, −3 and 0 days relative to calving. In Experiment 1, greater concentrations of PSPB were detected in plasma of CL vs. HT cows (388.3 ± 24.7 vs. 287.4 ± 23.8 ng/ml; $p < 0.01$). Concentrations of PSPB did not differ between −46 and −18 days (66.0 ng/ml). However, PSPB concentrations (ng/ml) were greater ($p < 0.01$) for CL cows at days −7 (534.7 > 357.2), −3 (807.2 > 572.2) and 0 (800.8 > 563.5). Additionally, HT cows had increased PRL plasma concentrations compared to CL cows (21.01 ± 1.6 vs. 13.78 ± 1.6 ng/ml). In Experiment 2, no differences were detected in plasma concentrations of PSPB (ng/ml) among LDPP, SDPP or SDPP + PRL groups on days −60 (41.5) −32 (51.7), −18 (58.5), −7 (532.9), −3 (838.2) and 0 (729.4) relative to parturition. Photoperiodic PRL concentrations were 10.81, 7.84 and 4.22 ng/ml for LDPP, SDPP + PRL and SDPP respectively. Results indicate that HT alters PSPB concentrations in late pregnancy, suggesting that placental activity is compromised in cows exposed to excessive elevated temperatures around the time of calving. However, the mechanism involved is not associated with photoperiod or PRL secretion.

Key Words: Environmental factors, late pregnancy, prolactin, pregnancy-specific protein B

2301

The use of 3D ultrasonography for determination of fetal gender in the bovine fetus during early pregnancy

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The utilization of 2D real time ultrasonography for the determination of fetal viability, biometry and fetal gender was described shortly after transrectal ultrasonography became a technology available and utilized in bovine reproduction. Here we report the utilization of 3D imaging technology to determine the ultrasonographic morphology of the bovine genital tubercle in female and male fetuses. We hypothesized that the determination of the bovine fetal gender would be achievable using evaluations of the 3D imaging information obtained by examining *ex vivo* pregnant uteri and live pregnant cattle. Ultrasonography examinations were carried out using a portable ultrasound equipment (Voluson i BT09; GE Healthcare Systems, Austria) equipped with a 3D/4D real time multi-frequency (5–9 MHz) micro-convex transducer (RNA 5–9RS). The transducer had a foot print of 32.2 × 29.1 mm. Eight *ex vivo* pregnant uteri and two live

animals were examined. The two live animals were restrained in stocks and examined via palpation per rectum transrectal ultrasonography without sedation. The *ex vivo* uteri were opened to reveal the actual fetal gender once the ultrasonography examinations were completed and recorded. Macroscopically, the genital tubercles appeared to have a single lobular structure. The two live cows calved one live fetus each and the gender was recorded. Among the eight *ex vivo* uteri, five were male and three were female fetuses; the gestational ages were estimated to be between 58 and 120 days based on fetal biometry measurements. The live pregnant cows had one female and one male fetus aging 63 and 120 days, respectively, based on known breeding dates. The male and female genital tubercles were readily visualized once the acquisition of the 3D scanning was completed (2–4 s). For fetuses older than 90 days, the scrotum and mammary papillae were visualized in addition to the visualization of the genital tubercles. All 10 determinations of fetal gender using 3D ultrasonography were correct. Contrary to the bilobular ultrasonographic appearance of the genital tubercles (male and female) as determined by 2D ultrasonography, the rendered 3D imaging revealed the genital tubercles to be characterized by a unilobular appearance. The unilobular appearance seen in the 3D images and macroscopic examinations are in agreement with the findings of Tainturier et al. (2004) who conducted a histological study using H&E sections of male and female genital tubercles of bovine fetuses and concluded they were unilobular. They further argued that the bilobular appearance observed with 2D ultrasonography was the result of specular reflection artifacts owing to cell density differences within the tubercles. In summary, 3D ultrasonography of the early bovine fetus presents as a valuable tool to study fetal bovine anatomy during the first trimester *in utero*.

Key Words: 3D Ultrasonography, bovine, fetus, fetal sexing, gender

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Digital rectal palpation: a technique for gynaecological examination and pregnancy diagnosis in red Sokoto goat

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Digital Rectal Palpation (DRP) was used to palpate the internal genitalia and carry out comparative pregnancy diagnosis with ultrasonography (UL), progesterone assay (PA), ballotment (BL) and non-return to oestrus rate (NOR) in red Sokoto goat. Fifty two does were randomly divided into three groups of 18, 18 and 16 as prostaglandin F2-alpha ($PGF_2\alpha$), progesterone (P_4) pessaries and control groups. A double injection protocol of $PGF_2\alpha$, 12-days apart, and progesterone pessaries, inserted for 12-days were used to synchronize oestrus, while no treatment was administered to the control group. Thirteen bucks were used in the experiment, seven as breeder bucks while six were apronized and used for heat detection, with two bucks in each group. Breeding was by hand-mating (Natural service) method. Does were palpated with the first (index) finger weekly from commencement of the experiment until foetal parts were distinctively detected on three consecutive palpations. First (index) finger and pubic lengths were measured with a measuring tape and ruler. The vagina, cervix, uterine body, uterine horns and ovaries were palpated (100% in all the does) as normal reproductive organs. Pregnancy diagnosis for PGF_2?, P_4 and control were 88.9% – DRP, 87.5% – UL, 83.3% – PA, 75.0% – BL, and 77.8% – NOR; 100% – DRP, 100% – UL, 50.0% – PA, 33.3% – BL, 50.0% -NOR and 100% – DRP, 83.3% – UL, 75.0% – PA, 83.3% – BL and 83.3% – NOR, respectively. There was no significant differences ($p > 0.05$). Amniotic vesicle was detected (100% in all pregnant does with DRP and UL) from day 22 to 49 and foetus (100% in all pregnant animals with DRP and UL) between days 50 and 56, and with BL between days 74 and 105 of gestation as positive signs of pregnancy and their ages. Length of the first (index) finger (10.0 cm) was directly proportional to pubic length (10.0 cm) externally (from vulva to the point of pubic symphysis). It was concluded that DRP technique can be used to examine the normal internal genitalia and positively diagnose and age pregnancy as early as 22–28 days of gestation in Red Sokoto goat.

Key Words: Digital, rectal, palpation, pregnancy, goat

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Activation of the fibroblast growth factor-system during pre-implantation equine pregnancy

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Approximately 10–20% of equine pregnancies confirmed at day 15 fail to reach term, with the majority of losses occurring before definitive placenta formation (day 40 after ovulation), i.e. in the pre-implantation period. Fibroblast growth factors (FGFs) 2, 7 and 10 play important roles in maternal-conceptus signaling, implantation and embryo organogenesis in various species. This study examined whether gene expression patterns for FGF2, its receptors (FGFRs 1, 2, 2IIIc, 3 and 4) and binding protein (FGFBP), and for FGFs 7 and 10 and their common receptor (FGFR2IIIb), in endometrium and trophoblast were consistent with a role in the establishment of equine pregnancy. Endometrial biopsies were recovered from 4 mares at each of late estrus, days 7 and 14 of diestrus, and days 7, 14, 21 and 28 of gestation. Conceptuses were recovered from the pregnant mares by uterine lavage (day 7) or using a video-endoscope guided net (days 14–28). For day 21 and 28 conceptuses, bilaminar trophoblast alone was used in gene expression studies. Expression of mRNA for the genes of interest was analysed by real-time RT-PCR and normalized with respect to 3 stable housekeeping genes using GeNorm. FGF2 mRNA was up-regulated in endometrium during estrus ($p < 0.05$). Of its potential receptors, endometrial expression of FGFR1 was elevated on day 14 of the estrous cycle and day 7 of pregnancy ($p < 0.05$), while endometrial FGFR2 was up-regulated during estrus and on day 28 of pregnancy ($p < 0.05$). The endometrial expression of FGFBP and other potential FGF2 receptors (FGFRs 2IIIc, 3 and 4) did not vary between the stages examined. Endometrial FGF10 expression was low and unaltered across the stages of cycle and early pregnancy examined. However, while endometrial FGF7 expression was also stable during the estrous cycle and the first 21 days of pregnancy, it increased nine fold on day 28 ($p < 0.05$), at which time-point the receptor for FGF7 (FGFR2IIIb) was also up-regulated ($p < 0.05$). In the conceptus, mRNA expression for all of the genes examined increased progressively during early pregnancy ($p < 0.05$). The temporal pattern of endometrial FGF2 expression suggests estrogen-dependency, as reported in women. By contrast, the marked increase in endometrial FGF7 gene expression between days 21 and 28 suggests a function in uterine preparation for placentation. We propose that dissolution of the blastocyst capsule between days 18 and 22 improves trophectoderm-endometrium contact, thereby enabling the trophectoderm to trigger the increase in endometrial FGF7 expression and/or allowing endometrial FGF7 to stimulate trophectoderm proliferation and adhesion to the endometrium.

Key Words: FGF, endometrium, pre-implantation, trophectoderm, horse

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Identification of the gp130 receptor family genes and their expression in the endometrium and conceptus during implantation period in mares

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Leukemia inhibitory factor (LIF), interleukin-6 (IL6) and IL11 are included in gp130 receptor cytokine family which plays crucial roles to establish pregnancy in some mammalian species. However, in horses, the open reading frame (ORF) of the LIF and IL11 genes are not annotated in GenBank database. Therefore, in this study, we identified ORF sequence of these genes first. Then gene expression of equine

LIF, IL6, IL11, their specific receptors (LIFR, IL6R and IL11RA) and gp130, a common signal transducer, in the endometrium and conceptus during pre-implantation period in mares were examined by real-time RT-PCR and represented individually. Endometrial samples from 10 mares were collected from caudal part of uterine horns, where fixation of conceptus occurs, on cyclic day 13-, and on pregnant days 13, 19, 25 and 30 (n = 2). Conceptus samples were collected on pregnant day 30 and separated to four parts. The length of sequenced LIF and IL11 ORFs were 651 and 597 bp, respectively. Sequence analysis of equine LIF and IL11 ORF showed high homologies with the other animals (89% and 88% of humans, 80% and 85% of mice, and 89% and 82% of cows). The similar high homology of corresponding amino acid sequences for equine LIF and IL11 were also observed that indicated a conserved function of these cytokines. The expression of LIF mRNA increased in the endometrium on days 19 and 25 regardless of the existence of a conceptus. On the other hand, the expression of IL6 mRNA increased in the endometrium on day 25 and 30, especially in gravid horn. The expression of IL11 mRNA didn't change throughout the experimental period. Among the specific receptors, the expression of LIFR and IL11RA mRNA kept relatively in higher level in the endometrium until day 25, and then decreased at day 30. In contrast, IL6R and gp130 mRNA were constantly expressed in both endometrium and conceptus throughout the experimental period. Additionally, endometrial expression of these mRNAs on cyclic day 13 was similar to those on pregnant day 13. Temporal expression of LIF with its receptor before implantation indicates that LIF possibly play a critical role on implantation process like in mouse. Although IL11RA mRNA also expressed before implantation, specific expression of IL11 mRNA was not recognized. Thus, biological role of IL11 on equine implantation is unclear. Moreover, time and space-specific expression of IL6 mRNA in the endometrium with developed conceptus suggests the presence of direct interaction between conceptus and endometrium. These results indicate time- and space-specific action of gp130 receptor cytokine family during pre-implantation period in mares.

Key Words: Horse, implantation, cytokine, gp130

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MicroRNA biogenesis-associated genes expression and miRNA expression profile in porcine embryos

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In the last years microRNAs (miRNA) have emerged as a new group of molecules with a great regulatory potential for posttranscriptional controlling of gene expression. miRNAs are believed to play an important role in the female reproductive tract and to regulate implantation and embryo development. Our previous studies revealed altered expression of the microprocessor complex genes (Drosha, Dicer, Ago1-4) in porcine embryos/trophoblasts. In the present study three other genes encoding components crucial for miRNA synthesis and transport were analyzed, DiGeorge syndrome critical region gene 8 (DGCR8; cofactor for Drosha), TAR RNA binding protein 2 (TARBP2; cofactor for Dicer1) and Exportin-5 (XPO5), which mediates nuclear export of pre-miRNAs. Quantitative real-time RT-PCR was applied to analyze expression of these genes in early porcine embryos from day (D) 10, 11 (elongated), 12 (filamentous), D16 and D20 trophoblasts. In addition, microarray experiments were performed to explore miRNA expression profiles in the same samples. DGCR8 mRNA expression did not differ between analyzed groups. XPO5 showed highest expression on D11 (vs. D12, $p < 0.01$ and days 16–20, $p < 0.001$). Likewise, increased mRNA level of TARBP2 on D11 (vs. D10; $p < 0.05$) was observed, which gradually decreased on D16 and 20 (vs. D10, $p < 0.01$ and $p < 0.001$, respectively). Analysis of microarray data was performed with BioConductor R packages, 'vsn' and 'limma'. Statistical analysis revealed a sharply distinct miRNA expression pattern for D20, when compared to embryos from D10 to 12. The comparison of D20 trophoblasts with D10 and D11 blastocysts revealed higher expression of 31 and 37 miRNAs and lower expression of 39 and 45, respectively (fold change ≥ 2, false discovery rate 5%). All results obtained for compared days are presented in Table 1. Number of differentially expressed miRNAs indicate weather there was up- or down-regulation in later vs. earlier embryo stage. In summary, together with our previous findings this data revealed co-expression of the major components of the miRNA processing machinery, such as Drosha-DGCR8 and Dicer-TRBP2. Moreover, we demonstrate temporal regulation of miRNA expression in the porcine conceptuses and trophoblasts, which may contribute to embryo development/transition and establishment of efficient embryo-maternal interactions during early pregnancy.

Key Words: Pregnancy, microRNA, embryos, pig

Table 1. Number of differentially expressed miRNAs in porcine embryos

Embryo stage	D10	D11	D12	D16	D20
D10		0^a; 0^b	5^a; 2^b	17^a; 14^b	31^a; 39^b
D11	0^a; 0^b		1^a; 0^b	14^a; 11^a	37^a; 45^b
D12	5^a; 2^b	1^a; 0^b		12^a; 15^b	40^a; 49^b
D16	17^a; 14^b	14^a; 11^b	12^a; 15^b		36^a; 42^b
D20	31^a; 39^b	37^a; 45^b	40^a; 49^b	36^a; 42^b	

a – up regulated; b – down regulated.

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Use of an ELISA for serum PSPB in dairy cows with twin or single fetuses

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Since 2003, approximately 95 000 blood samples have been routinely assayed between 30 and 36 days post AI, by BioPRYN® ELISA (detects pregnancy-specific protein B, PSPB) for early pregnancy detection in 22 Hungarian dairy herds. Assay data [the optical density (OD) values for sample and plate cutoff] were recorded in our Bopella data base. Comparison of data at different times and in different plates was feasible by using the ratio (OCR) of sample OD/plate cutoff OD. A greater OCR means there was a greater serum concentration of PSPB. All calving data (n = 8456) of three dairy farms (Farm A, N and O) were collected from a farm registry. There were 7875 single (S) and 581 twin (T, 6.9%) deliveries. T rate varied among farms (6.7%, 5.7% and 8.6%; $p < 0.01$) and months (Range, 4.5% (May) to 8.8% (November); $p < 0.01$). Daily milk production did not differ between T and S cows (33.6 kg vs. 32.8 kg). The OCR for T vs. S was different (2.3 vs. 1.92; $p < 0.01$) suggesting that it may be possible to predict T. However, variation of OCR was high within S and T groups (min–max: 1.07–6.05 vs. 1.01–14.7, respectively) resulting in less value of the prediction. T calving was less for primiparous vs. multiparous cows (2.1% vs. 7.4%; $p < 0.001$) but a strong farm effect was observed within Farm A (1.4% vs. 7.7%) compared to within Farm N (3.3% vs. 5.4%) and Farm O (6.1% vs. 9.0%). Hormone treatments (Ovsynch or PGF2α) before AI did not significantly affect T over non-hormone treated cows (6.7% vs. 7.6%; $p > 0.1$). However, more T were recorded for PGF2α treated than for Ovsynch treated cows (9.4% vs. 5.6%, $p < 0.01$). Reports were published on the prediction of T pregnancy in cows (Vasques et al., 1995. *Anim. Reprod. Sci.* 38:279–289), sheep (Willard et al., 1995. *J. Anim. Sci.* 73:960–966) and moose (Huang et al., 2000. *J. Wildlife Mgmt.* 64:492–499), using PSPB radioimmunoassays. Also, during routine dairy farm pregnancy checks with BioPRYN (BioTracking LLC, unpublished), higher OD values were observed for T but it was not possible to examine differences until the Bopella data base for BioPRYN ELISA for PSPB was accumulated. The data suggest that T can be detected using BioPRYN. High individual variation in PSPB may require testing later in pregnancy (perhaps 60 days) rather than at the 30–36 days of this study. Additionally, loss of one of the twin fetuses early in pregnancy while providing a single birth can contribute to some of the variation.

Key Words: Cattle, pregnancy, twin, PSPB, ELISA

	Total calving	*T* calving	*T* calving rate (%)
Farm A	5139	344	6.7
Farm N	1645	94	5.7
Farm O	1672	143	8.6
Primiparous cows	4082	86	2.1
Multiparous cows	7672	565	7.4
Non treated cows	6904	463	6.7
Hormone treated cows	1552	118	7.6
Ovsynch treated cows	682	38	5.6
PGF2α treated cows	800	75	9.4

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Conceptus development and global gene expression at preimplantation stages in lactating dairy cows of distinct genetic groups and estrous cyclic statuses

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Anovular cows have impaired reproduction because of reduced establishment and maintenance of pregnancy compared with estrous cyclic cows. In addition, the genetic background of dairy cows can influence reproductive performance, and crossbreeding Holsteins cows with other dairy breeds has been shown to improve pregnancy per insemination (p/AI) compared with purebred cows. The objectives were to compare conceptus development and global gene expression at preimplantation stages in anovular Holstein (AH), estrous cyclic Holstein (CH), and estrous cyclic Jersey/Holstein crossbred (CC) lactating dairy cows subjected to a synchronized ovulation. On postpartum d 29, cows of both breeds were randomly selected within a grazing herd and received an injection of prostaglandin. Ovaries were scanned by ultrasonography on postpartum d 29 and 39 to determine estrous cyclic status. Based on the presence or absence of corpus luteum (CL), cows were then grouped in AH (n = 10), CH (n = 25) and CC (n = 25) and subjected to the Ovsynch protocol. The day of AI was considered study d 0. Blood was sampled and analyzed for concentrations of progesterone and estradiol on study days −10, −3, −1, 7, 15. On study d 15, uteri were flushed and interferon-tau (IFN-τ) concentrations in fluid measured. Recovered conceptuses were subjected to global analysis of gene expression using Affymetrix Gene Chip® Bovine Array. Size and morphology of the conceptuses were recorded. Microarray data was log2 transformed and analyzed by *t*-test. Gene expression was considered significantly different when $p \leq 0.05$ and fold change ≥2.0. Estrous cyclic status within the same genetic background had a greater impact on conceptus gene expression than genetics within the same estrous cyclic status. Considering CH as the reference, 417 and 65 genes were differentially expressed compared with AH and CC, respectively. Similarly, 284 and 29 genes were differentially expressed when only elongated embryos were considered. Although CC ovulated a smaller follicle, they had greater concentrations of steroid hormones during the Ovsynch protocol and after AI, tended ($p = 0.11$) to have greater P/AI and presented advantages in conceptus morphology/gene expression compared with CH. Conversely, although P/AI was similar between the two Holstein groups, AH had more advanced conceptuses than CH, likely because of a faster rise in progesterone concentrations after AI. In conclusion, expressive differences on gene expression of important biological processes were identified and might help explain the differences in fertility observed between estrous cyclic and anovular cows of distinct genetic background.

Item	AH	CH	CC
Follicle size d-1, mm	23.5 ± 0.9[a]	20.7 ± 0.6[b]	19.1 ± 0.6[c]
Estradiol d-1, pg/ml	4.8 ± 0.9[b]	5.4 ± 0.6[b]	7.1 ± 0.6[a]
Progesterone, ng/ml			
d-10	0.1 ± 0.7[b]	4.0 ± 0.4[a]	3.6 ± 0.4[a]
d-3	2.2 ± 0.8[c]	8.1 ± 0.5[b]	9.4 ± 0.5[a]
d7	4.1 ± 0.3[a]	3.0 ± 0.2[b]	4.0 ± 0.2[a]
d15	7.6 ± 0.6[ab]	6.7 ± 0.4[b]	7.7 ± 0.4[a]
Pregnant d15, % (n)	70.0 (10)	64.0 (25)	84.0 (25)
IFN-τ, ng/ml	7.27 ± 1.5[a]	0.57 ± 1.1[b]	0.85 ± 0.9[b]
Conceptus lenght, mm	47.8 ± 8.8[a]	9.4 ± 5.8[b]	25.3 ± 8.7[a]
Ovoid embryos, % (n)	0.0 (7)[b]	37.5 (16)[a]	0.0 (21)[b]

Values on the same row with different superscripts differ ($p < 0.05$).

Key Words: Anovular, crossbred, conceptus, gene expression, pregnancy

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Analysis of perinatal uterine artery and umbilical cord Doppler velocimetry in sheep

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Monitoring uteroplacental blood flow is an important predictor of fetal well-being and adverse perinatal outcome, as well as a tool to diagnose uterine pathological conditions during pregnancy. Pulsed-wave Doppler indices such as resistance index (RI), systolic-diastolic ratio (S/D) and pulsatility index (PI) are indicative parameters of fetal and placental perfusion and can be provided by a non-invasive technique. The aim of the present study was to investigate uterine artery (UA) and umbilical artery (UMA) blood flow during pregnancy, parturition and postpartum in sheep. For this purpose, 15 pregnant sheep were examined at 60, 90 and 120 days of pregnancy. The right and left UA were evaluated by rectal sonography, while UMA was analyzed through transabdominal ultrasound. Induction of labor was performed at 135 days of gestation with aglepristone, an antiprogestin, administered at 0.33 ml/kg, i.m., daily, for 2 days. Before (M1) and after 12 h of the initial injection (M2), as well as between each injection (M3), all ewes were evaluated by pulsed-wave Doppler ultrasound. During the postpartum period, analysis was performed on Days 1, 3, 5, 7, 15 and 30. No differences were observed comparing the right and left uterine artery for RI (0.68 ± 0.01 and 0.68 ± 0.01, respectively), PI (1.36 ± 0.56 and 1.51 ± 0.10) and S/D (5.01 ± 0.77 and 5.96 ± 0.98) throughout the perinatal period. During the induction of labor, all UA vascular parameters remained constant. However, UMA hemodynamics parameters statistically decreased after the onset of treatment for RI (M1: 0.61 ± 0.02; M2: 0.58 ± 0.01 and M3: 0.55 ± 0.02), PI (M1: 0.99 ± 0.06; M2: 0.90 ± 0.04 and M3: 0.85 ± 0.04) and S/D (M1: 2.64 ± 0.14; M2: 2.44 ± 0.08 and M3: 2.29 ± 0.09). In addition, uterine artery RI, PI and S/D increased during the postpartum period. The therapy used here to induce labor, with an antiprogestin, did not promote adverse effects on uteroplacental perfusion, as UA indices remained unchanged during treatment. Simultaneously, an increase in fetal blood flow was evidenced during the onset of labor. Doppler indices (RI, PI and S/D) are known to have a negative correlation with progesterone levels in women, as shown previously. On the other hand, the present results suggest that progesterone has little or no significant effect on uterine vascular relaxation in sheep. We can infer that the increase in hemodynamics indices may be due to the postpartum uterine involution and increased UA perfusion is unnecessary during this period. Further studies are necessary to better understand the physiological mechanisms responsible for the underlying uterine vasodilator effect in ovine.

Key Words: Uterine artery, umbilical cord, doppler, pregnancy, sheep

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Metalloproteinase 2 (MMP-2), osteopontin (OPN) and leukemia inhibitory factor (LIF) genes expression in the oviduct and uterus of pregnant and nonpregnant bitches

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The aim of the present study was to analyze the expression of the genes MMP-2, OPN and LIF, which are presumably involved in the signaling of embryo implantation on canine pregnancy. For that, 19 pregnant bitches were ovariohysterectomized (OSH) after being divided into four groups according to gestational age (Group A: 05 females with 8 days of gestation, Group B: 05 females with 12 days of gestation, Group C: 05 females with 21 of gestation, Group D: 04 females with 60 days of gestation). Others five non-pregnant bitches were ovariectomized 12 days after the onset of the LH preovulatory surge (Group E) and were used as control group. After OSH, the oviduct and uterine tissue were removed and submitted to real-time PCR aiming investigate the MMP-2, OPN and LIF genes. Data were statistically analyzed and differences were considered significant with $p < 0.05$. It was observed a significant increase in the expression of mRNA LIF in the oviduct (0.1271 ± 0.07670, $p = 0.0234$) and uterus (1.268 ± 0.05728, $p < 0.0001$) in group D in relation to the others groups. The presence of this gene in oviduct throughout the entire gestation suggest a significant role of LIF in maintain and regulate the embryonic development, nevertheless the higher expression observed in uterine tissue during late pregnancy could emphasize the production of this cytokine by elements of the placenta, similar as occur in humans (Hsen-Fu Chen, 2004).The mRNA OPN expression was significantly higher in the oviduct in relation to the uterine tissue ($p = 0.0004$) in the early stages of cyclic diestrus (Group E: 0.1618 ± 0.02097, vs. 0.02992 ± 0.007471) and pregnancy ($p = 0.0026$) (Group B: 0.3642 ± 0.02702 vs. 0.06547 ± 0.03533), presumably due to the synthesis of this molecule in the oviduct, as occur in humans and cattle (Gabler et al., 2003). Osteopotin may perform a beneficial role in the physiology of the oviduct and in canine embryonic fertilization. The expression of mRNA MMP-2 was significantly higher in the oviduct ($p = 0.0011$) in initial stages of pregnancy (Group A: 5.750 ± 0.5851 vs. Group E: 1.203 ± 0.5147) and also higher in the uterus ($p = 0.0361$) in late pregnancy (Group D: 9.105 ± 0.8856 vs. Group B: 4.336 ± 0.8091). MMPs are secreted by the placenta and human embryo and contribute to the development of fetal tissue (Bischof and Campana, 2000). This is necessary to ensure the embryonic tissue invasion on the maternal uterine in a controlled manner. Based on these results, the role for MMP-2 would be preparing the uterine wall to the placentation process.The expression of MMP2, OPN and LIF in oviduct and uterus in all studied groups and the presence of significant variations on its expression during the pregnancy phases and non-pregnat bitches suggest that these factors have a great importance in canine pregnancy development as well as occur in another species. FAPESP for financial support.

Key Words: Metalloproteinase 2, osteopontin, leukemia inhibitory factor, pregnant, bitches

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Visualisation of pregnancy in the Tammar wallaby *Macropus eugenii*

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Tammar wallabies give birth to tiny, altricial young that undergo most of their development in the pouch. Mothers mate within hours post-partum, and the resulting conceptus normally enters diapause, under lactationally mediated inhibition, as a 0.25 mm blastocyst of 100 cells. Removing the pouch young (RPY) leads to reactivation of gestation with birth occurring about 25–28 days later. To correlate histologic and macroscopic observations to clinical findings and to determine factors influencing gestation length, we have monitored pregnancy following RPY ($n = 20$) using transabdominal ultrasound via the pouch opening (Voluson i, GE, Austria; 5–9 MHz array). From Day 4 RPY onward, exams were performed daily in at least two randomly chosen females. The blastocyst was first detected ultrasonographically, varying between individuals from Day 11 to 14 RPY, as an anechoic round vesicle in the uterus with a diameter of 1 mm. Embryonic development from this stage until birth is a chronologically fixed sequence with nearly no time variation between individuals. Thus, variation between individuals in gestation length after RPY is due to variability in the timing of the reactivation of the blastocyst, which is dependent on the progesterone level. This variation of about 3 days is precisely reflected in the variation of gestation length. Using ultrasound, we could predict the sequence of the births of the females, and we confirmed this by observing the birthing order. Subsequently, we defined ultrasonographic milestones as a function of the days prior to parturition (p.P.), including the first detection of the live embryo with heartbeat (Day 11 p.P.) and the detection of the allantois (Day 9 p.P.). From Day 2 p.P. onward we observed embryonic movements mimicking those required to climb to the pouch demonstrating that complex behaviour in the wallaby begins in utero at a highly altricial stage of mammalian development.

Key Words: Pregnancy, marsupials, embryogenesis, ultrasonography, prenatal development

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Early detection of dystocia in Holstein cows using plasma pregnancy- specific protein B (PSPB), progesterone and some hematological and blood biochemical characteristics

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Dystocia is reported the primary cause of calf loss in beef and dairy cattle. This figure may be slightly higher in dairy cattle due to the metabolic pressures placed on calcium stores. The early identification of dystocia may have an economic impact. This study investigated the early detection of dystocia in Holstein cows using plasma pregnancy-specific protein B (PSPB), progesterone and some hematological and blood biochemical characteristics. Thirty eight cows were divided at calving into two groups. The first group ($n = 9$) involved cows with dystocia, while the second group ($n = 29$) calved normally (Intact). Blood samples were collected from each cow at the 8th (days 230–232 of gestation) and 9th (2–4 days pre- expected calving) months of gestation. Plasma progesterone concentrations were significantly greater ($p < 0.01$) in cows with dystocia compared with intact cows. There was a tendency for PSPB to be higher (22.4%) in dystocia (416.5 ± 80.32 ng/ml) compared to intact (323.3 ± 28.55 ng/ml). The overall mean of plasma alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were significantly ($p < 0.05$) lower in dystocia (21.78 ± 1.53 and 50.18 ± 7.68 Unit/liter respectively) as compared with intact (30.00 ± 2.10 and 74.23 ± 6.28 U/l respectively) during the 8th month of pregnancy. On the other hand, at the 9th month of gestation, plasma ALP ($p < 0.05$) and globulin percentage ($p < 0.01$) were lower while, plasma albumin ($p < 0.01$) and albumin to globulin ratio ($p < 0.05$) were higher in dystocia compared with intact. Excluding data of PSPB, these blood variables concentrations may prove useful for predicting dystocia in Holstein cows during 8th and 9th months of pregnancy.

Key Words: Progesterone, PSPB, blood, dystocia, Holstein cows

2356

Altering rumen degradable to undegradable protein ratio: influence on nutrient consumption, blood urea nitrogen, involution of uterus, ovarian cyclicity and pregnancy rate in Nili-Ravi buffaloes

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Biological and physiological mechanisms associated with reproductive performance of dairy animals may be influenced by concentration and type of dietary protein. Adequate concentration of rumen degradable protein (RDP) may be used to modulate nutrient consumption and utilization, which has direct link with not only milk yield but also influences ovarian and metabolic profiles during early lactation to influence efficiency and profitability of production. Therefore, this study was designed evaluate the responses of altering RDP concentrations on nutrient consumption, blood metabolite profiles, involution of uterus, uterus tonicity, ovarian cyclicity and pregnancy rate in early lactating Nili Ravi buffaloes. Twenty four early lactating Nili-Ravi buffaloes, eight in each group, were used in a Randomized Complete Block Design. Three experimental diets were formulated such as to contain 50%, 66% and 82% RDP of dietary crude protein and were denoted as T1, T2 and T3, respectively. The findings revealed that dry matter intake increased ($p < 0.05$, $p = 0.001$) in buffaloes fed T1 (14.85 kg/days) diet than those fed T2 (12.3 kg/days) and T3 (11.12 kg/days) diets. Organic matter intake also followed the same trend. Blood urea nitrogen was higher ($p < 0.05$, $p = 0.001$) in buffaloes fed T3 (34.18 mg/100 ml) diet than those fed T2 (29.20 mg/100 ml) and T1 (25.25 mg/100 ml) diets. At 45 days postpartum, more number of buffaloes (6/8) fed on T1 diet indicated complete uterus involution process compared to those fed on T2 (4/8) and T3 (4/8) diets. At 75 day postpartum, buffaloes (4/8) fed on T1 diet had better uterus tonicity than those fed on T2 (2/8) and T3 (2/8) diets. More number (6/8) of buffaloes on T1 diet showed better ovarian activity compared to those received T2 (4/8) and T3 (4/8) diets, at 90 day postpartum. Pregnancy per AI in buffaloes fed T1 (75%) diet was higher than those fed T2 (50%) and T3 (50%) diets. The outcome of the experiment suggested feeding 50% RDP of the total protein content in early lactating buffaloes improved nutrient consumption, ovarian cyclicity and pregnancy per AI.

Key Words: RUP:RDP, nutrient consumption, ovarian cyclicity, buffalo

2357

Assessment of plasma profile of pregnancy-specific protein B and progesterone in Iraqi riverine buffaloes (*Bubalus bubalis*) throughout gestation and post-partum

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During the last decades, a polymorphic family of placental proteins has been discovered in ruminants and used for pregnancy detection. The pregnancy specific protein B (PSPB) is one of these proteins that can be detected readily in the maternal peripheral circulation from the time of initial placentome formation. This study was conducted to demonstrate plasma PSPB and progesterone profile in Iraqi riverine buffaloes (Bubalus bubalis) during gestation and 60 days post-partum periods. Nine female riverine buffalo of 7 years old were used in this study. Animals were naturally mated following estrus detected by monitoring both day and night. Pregnancy was checked for these buffaloes using BioPRYN enzyme linked immunosorbent assay (ELISA) test on 22–24 days post mating (PM) and assured by rectal palpation on Day 61 pm. Blood samples (10 ml) were collected biweekly via jugular venipuncture from each buffaloes from mating (Day 0) until 60 Days post-partum (PP), to measure plasma PSPB and progesterone concentrations. Mean PSPB concentrations increased progressively ($p < 0.01$) from week 4 to 36 of gestation (from 2.085 to 8.448 ng/ml) and reached peak at week 38 (9.755 ng/ml). Post delivery, PSPB concentrations were significantly ($p < 0.01$) and steadily declined to reach its lowest levels at Days 45 (0.928 ng/ml) and 60 (0.321 ng/ml) PP. Furthermore, plasma progesterone concentrations was low at Day 0 (0.271 ng/ml) and increased progressively ($p < 0.01$) to 4.053 ng/ml at week 4 pm. Peak concentration (5.771 ng/ml) of progesterone was noticed at week 12. Progesterone concentrations were declined slightly during the last month of gestation and sharply at delivery and PP periods. This study indicated for the first time the plasma profile of PSPB and progesterone concentrations in riverine buffaloes throughout gestation and PP periods.

Key Words: PSPB, progesterone, profile, water buffalo

2358

Serum biomarkers of health in pregnant buffaloes during last trimester

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The aim of the study was to investigate and determine the health biomarkers particularly when the animal is near to parturition. Animals were divided into pregnant and non pregnant buffaloes. Blood samples were taken from clinically healthy pregnant ($n = 25$ during last trimester) and non pregnant ($n = 35$ dry not pregnant) buffaloes for collection of serum. Total oxidant status (TOS), total antioxidant status (TAS), homocysteine, paraoxonase and ceruloplasmin in serum was analyzed. Data was analyzed statistically with SPSS computer software. It was revealed from the results that TOS, homocysteine and ceruloplasmin concentration were significantly high ($p < 0.05$) in pregnant buffaloes as compared to non pregnant. Paraoxonase activity was significantly low ($p < 0.05$) in pregnant buffaloes. The Oxidant status of pregnant buffaloes was 0.68 ± 0.09 as compared to non pregnant 0.56 ± 0.06 μmol/H_2O_2 Equvi/l. Like wise the Total antioxidant status of pregnant buffaloes was determined to be 0.28 ± 0.04 while it was 0.35 ± 0.09 mmol trolox/l in non-pregnant animals. These differences were non significant statistically. It was concluded from the study that internal homeostasis, in term of these biomarkers, alter in late pregnancy in buffalo. Increased productions of oxidants during late pregnancy have affected the concentration of TAS, homocysteine, paraoxonase and ceruloplasmin. Moreover, these measurements gave complementary information about the metabolic status of pregnant buffaloes.

Key Words: Buffaloes, health biomarkers, pregnancy, oxidants, antioxidants

24. Puberty & neuroendocrine regulation of reproduction:

2400

Puberty and sexual maturity in Senepol bulls raised semi-intensively in central Brazil

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The Senepol breed has been strategic in crossbreeding with zebu cattle, by its constitution and genotypic adaptation to tropical climate, with few studies about the reproductive aspects especially in puberty and sexual maturity. The objective of this study was to determine the andrologic profile, CAP (Andrological Classification by Points), similar to the Breeding Soundness Evaluation, but with specific scores for Zebu and European bulls, testosterone plasma concentrations, seminal total proteins and libido (assessed in corral for 5 min/bull with six heifers in heat). In Step 1, PO 13 bulls, sired from five different bulls (IVF), with an average age of 12.22 ± 0.16 months, supplemented with commercial ration (1 kg/animal/day). Every 30 days, for 3 months, it was evaluated: body weight (BW), scrotal circumference (SC), physical and morphological semen characteristics, total plasma testosterone and seminal proteins analyzed by 1D system (Bradford-mod) by spectrometry (isoelectric focusing-SDS-PAGE). The variables were subjected to tests of normality Shapiro-Wilk, Kolmogorov-

Smirnov and asymmetry and kurtosis (SAS, 2002). In Step 2, performed at 16.19 ± 0.16 months old, it was also assessed CAP and libido (1 = vulva smelling and licking; 2 = flehmen reflect; 3 = female persecution; 4 = aborted mounting 5 = complete mounting). Comparisons of the different variables throughout the reproductive development and between groups (immature and mature within the time of evaluation) were performed using the MIXED procedure of SAS statistical package (SAS, 2002). In Step 1, to pubertal animals (12.22 ± 0.16 months) and Stage 2 (16.19 Â ± 0.16 months) for pubertal but immature animals (immature) and those with complete sexual maturity (mature), it was registered, respectively, PC (kg) of 304.07 ± 12.98a; 353.57 ± 11.83a and 374.00 ± 18.53b ($p < 0.05$), SC (cm) 28.15 ± 0.55a, 31.00 ± 0.57a and 32.05 ± 0.06b ($p < 0.05$); sperm motility (%) of 23.84 ± 5.06a, 61.42 ± 3.78b and 66 b 66 ± 5.16b ($p < 0.05$); sperm concentration ($\times 10^6$) of 88.84 ± 7.23a, and 788.29 ± 159.73b and 801.93 ± 136.52b ($p < 0.05$); major sperm defects (%) of 69.84 ± 6.85a, 48.28 ± 5.18a and 13.16 ± 0.98b ($p < 0.05$); total sperm defects (%) of 82.30 ± 9.00a, 63.14 ± 8.64a and 23.16 ± 4.26b ($p < 0.05$); plasma testosterone (ng/ml) to 8.85 ± 1.95a, 5.63 ± 1.70a and 6.51 ± 1.38a ($p > 0.05$) and seminal total protein (mg/ml) of 18.60 ± 5.89a, 14.08 ± 0.86a and 18.37 ± 5.66a ($p > 0.05$). Within mature 57.15% compared to 42.85% of immature animals expressed high libido (actions 4 and 5); 33.34% of mature and 66.66% of immature animals showed medium (actions 2 and 3) libido ($p > 0.05$). None of the animals expressed low libido. The Senepol breed, selected for tropical adaptation showed andrologic variability evaluated at 12 and 16 months of age, even though not differences in plasma testosterone and total seminal proteins levels were registered among the studied animals in both steps.

Key Words: Andrological classification by points (BSE), andrologic evaluation at puberty, Senepol, semen and sexual behavior, sexual maturity

2401

FSH polymorphism as a molecular marker of sexual precocity in Nellore bulls

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The animal breeding programs objective animals with some favorable reproductive characteristics like fertility and sexual precocity. Thus, genes responsible for these kinds of features could be important molecular makers and assist in choosing of better breeders. Among these genes, the FSH (follicle stimulating hormone), wich one controls the proliferation and differentiation of Sertoli and Leydig cells, besides being one of those responsible for sexual maturation in heifers; could be a potential molecular marker of sexual precocity in bovine. This gene, located in chromosome 11, has two SNPs (single nucleotide polymorphisms) those results in GG, CG or CC genotype. So, the aim of this study was to determine whether these SNPs may be correlated with sexual precocity in bulls. For this, 220 bulls belonging to the Genetic Improvement Program of the Nellore Breed (Faculty of Medicine of Ribeirão Preto, Brazil) were divided in two groups, according to their Expected Progeny Difference (EPD) for scrotal circumference at 365 days since this EPD is directly correlated to the sexual precocity in bulls. In the first group, 110 animals have EPDs for SC at 365 days > 1 cm (short cycle animals group) and the second one having 110 animals with EPD < 1 cm (long cycle animals group). Then, a 306 bp fragment was obtained from genomic DNA with polymerase chain reaction (PCR), using specific primers, to all the animals. Later, the polymorphisms were analyzed by Restriction Fragment Length Polymorphism (RFLP) with the *Alu*I enzyme restriction. As results, every long cycle animals presented heterozygosis (CG). In relation to the short cycle animals, it was verified the heterozygosis in 105 bulls; and GG genotype was present in the five animals remaining. CC genotype wasn′t found. Therefore, among the long cycle animals, the allelic frequency of G and C were 0.5 and their genotypic frequency were 100% CG. On the other hand, in the short cycle animals group, the G and C allelic frequency were 0.52 and 0.48, respectively. In respect to its genotypic frequency, 4.5% was GG and 95.5% was CG. The results were comproved by chi-square, showing statistically significant difference ($p = 0.021$). With these data, we can infer that the GG genotype in this SNP is a potential molecular marker of sexual precocity in Nellore bulls, may, after more studies, be used as a selection tool.

Key Words: Animal breeding, sexual precocity, SNP, gene FSH, molecular marker

2402

Decrease in progesterone concentration was not followed by an increase in plasma luteinizing hormone in Nellore calves (*Bos taurus indicus*) after birth

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During sexual maturation in Bos indicus heifers, it is important an increase in luteinizing hormone (LH) secretion, consequence from the increase of steroid positive feedback resulting in the LH pre-ovulatory peak and first ovulation. The aim of this study was to evaluate LH and progesterone (P4) concentration from eight Nellore heifers (Bos taurus indicus), born within short intervals (maximum 45 days apart). Blood samples were collected, via jugular veni-punctured, at birth and once a day for 7 days. Concentration of progesterone were determined using a Coat-a-Count RIA kit, the inter-assay coefficient of variation (CV) were 12.59% for high control (14.77 ng/ml), 0.35% for low control (0.43 ng/ml) and the assay sensitivity was 0.004 ng/ml. LH concentration was evaluated by in house RIA, average intra-assay CV was 4.09% for high control (1.99 ng/ml) and 3.44% for low control (0.42 ng/ml); the inter-assay CV was 9.35% for high and 18.14% for low control, the assay sensitivity average was 0.037 ng/ml. Progesterone concentration decreased from birth to the seventh day of life ($p = 0.007$, repeated measures ANOVA) 0.148 ± 0.03 ng/ml; 0.129 ± 0.31 ng/ml; 0.027 ± 0.02 ng/ml; 0.020 ± 0.00 ng/ml; 0.018 ± 0.01 ng/ml; 0.035 ± 0.003 ng/ml; 0.010 ± 0.00 ng/ml; 0.005 ± 0.00 ng/ml respectively but the LH concentration was constant ($p = 0.28$) from birth until the seventh day of life: 0.224 ± 0.20 ng/ml; 0.328 ± 0.01 ng/ml; 0.226 ± 0.05 ng/ml; 0.263 ± 0.07 ng/ml; 0.258 ± 0.05 ng/ml; 0.263 ± 0.07 ng/ml; 0.273 ± 0.19 ng/ml; 0.270 ± 0.14 ng/ml respectively. Although progesterone secretion (from placental origin) decreased LH concentration did no increased. Apparently around birth calve hypothalamic-pituitary axis did not respond to progesterone concentration decrease.

Key Words: Sexual maturation, negative feedback, puberty, gonadal contention, partum

2403

The effects of a soybean and canola diet on sexual maturation in dairy heifers

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Soybeans are rich in isoflavones, phytoestrogen compounds, which when fed at high levels are capable of exerting estrogenic effects in mammals. While several studies suggest an important role of dietary phytoestrogens on the rate of sexual maturation in mammals, there is minimal literature on sexual maturation of dairy cattle fed soybeans. This study examined the effects of feeding a soybean and canola diet during pre-pubertal development (during the period of early gonadotropin rise) on the sexual maturation and growth of dairy heifers. At 8 weeks of age Holstein heifer calves (n = 24) were randomly assigned to receive canola or soybean meal calf starter diets until 24 weeks of age. Treatments were balanced for age, heights, weights, and calf sire. Calves were fed approximately 1.5 kg of feed per calf per day and ad libitum hay and water. Diets were formulated to be isocaloric, isonitrogenous, and isolipidic. From 8 weeks of age to puberty, bi-weekly transrectal ovarian ultrasonography was performed and

heights and weights were taken. Ovarian ultrasonography records were used to evaluate follicular and luteal development. Puberty was defined as the time a corpus luteum (CL) was first observed. A minimum of two separate CL structures were observed for verification. Overall, heifers fed the canola diet tended ($p = 0.09$) to be heavier between 8 weeks of age and puberty, but there were no differences in their heights ($p = 0.13$). At 24 weeks of age, heifers fed the canola diet tended to be heavier than heifers in the soy group (183.4 ± 5.9 kg vs. 164.9 ± 5.8 kg; $p = 0.07$), however there were no differences in their heights (109.4 ± 1.2 cm vs. 106.8 ± 1.2 cm; $p = 0.20$). The maximum follicle diameter observed at 24 weeks of age was 11.6 ± 0.7 mm vs. 10.7 ± 0.6 mm ($p = 0.39$) for canola and soy treatments, respectively. Heifers fed the soy diet were older (42.4 ± 1.2 weeks vs. 35.7 ± 1.2 weeks; $p = 0.0004$) and tended to be heavier (285.8 ± 7.6 kg vs. 259.5 ± 7.9 kg; $p = 0.06$) at puberty compared to the canola fed heifers. The precise effect of phytoestrogens on reproductive function has not been fully elucidated and is dependent on the level of inclusion, basal diet, and stage of physiological maturity of the female being supplemented. It is critical to understand how a diet high in phytoestrogens may affect the attainment of puberty in dairy heifers as this can influence age at first breeding and subsequent age at calving. In summary, heifers fed a soybean calf starter diet achieved puberty later and tended to gain weight slower than heifers fed a canola calf starter diet.

Key Words: Sexual maturation, heifers, phytoestrogens, soybean, canola

2404

Expression of kisspeptin neurons in the arcuate nucleus of the goat during the follicular and luteal phases – A preliminary study

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Hypothalamic kisspeptin (KP) is regarded as a key factor regulating GnRH release from the hypothalamus. Using immunohistochemial staining techniques, neurons expressing KP have been identified in the hypothalamus of many species. However, there seems to be no report on the change of expression of KP in the female goat hypothalamus over physiological stages. A preliminary trial was therefore carried out to study the pattern of KP expression in the arcuate nucleus of sexually mature native goats (Abadeh goats: age: 3–5 year, mean BW: 40 kg) during the follicular ($n = 2$) and luteal ($n = 2$) phases of the ovarian cycle, as determined by plasma progesterone analysis. The diencephalons were removed following fixation of the brain using 10% formalin solution. Sections (30 μm) were prepared from the cranial (rostral), middle and caudal regions of the arcuate nucleus, and stained immunohistochemically using the AC#566 kisspeptin antibody. The data, subjected to the test of normality and homogeneity of variance, were subsequently analyzed by Proc Genmod of the SAS, using a model in which the effects of the stage of cycle, region of the arcuate nucleus and their interaction were included. No significant interaction was found between the phase of the cycle and the region of the arcuate nucleus ($p = 0.420$). A higher number of KP-containing neurons was identified in the arcuate nucleus in the follicular (185 ± 90) compared to the luteal (117 ± 71) phase of the cycle ($p < 0.03$). The distribution of KP neurons was different between the regions of arcuate nucleus ($p < 0.0001$). A higher number (mean ± SD) of KP neurons was recorded in the caudal region (229 ± 46) than in the middle region (143 ± 46), which was higher than in the cranial region (43 ± 13). In a subsequent trial, using a larger number of animals, we intend to study the distribution of KP neurons, in cyclic and anestrous goats.

Key Words: Goat, kisspeptin, follicular phase, luteal phase, arcuate nucleus

2405

Transrectal ultrasonography in measurement of uterine diameter in ewe lambs after progestagens stimulus

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The beginning of sexual activity for both males and females is very important when it comes to animal rearing, especially due to the profit gains that only begins when the animals return to the productive phase (Monteiro et al., 2010). At puberty progesterone cyclical changes occur followed by estrogen stimulation and it is likely that previous exposure to progesterone change the mechanisms by which tissues respond to estrogen in the female prepubertal causing the final development of the reproductive tract (Lewis & Berardinelli, 2001).The aim of this study was to evaluate the response of prepubertal Santa Ines ewe lambs to exogenous administration of norgestomet (CRESTAR®). Eighteen Santa Ines prepubertal ewe lambs with average of 160 days-old and 29.6 ± 0.32 Kg of weight and 91.6 ± 2.5 of corporal mass index (Monteiro et al., 2010) were used. The females were randomly assigned to three different groups. In the first group (G1) the ewe lambs were isolated from the other two groups and were not subjected to any treatment. In the second group (G2) the females were submitted to the insertion of ear implants of 1.5 mg norgestomet (CRESTAR®) for 12 days. In the last group (G3), the females were submitted to the insertion of ear implants of 1.5 mg norgestomet (CRESTAR®) for 24 days (two consecutive insertions). After treatments (first removal (M1) and second removal (M2) of the implants), during 5 days, every 24 h all females were submitted to transretal ultrasound exams to measurement the diameter of uterus. For rectal examinations the Aloka Prosound®) two was used with a 7.5 MHz prostatic probe. Data was analyzed by ANOVA followed by SNK test (Student Newman Keuls $p < 0.05$). The means and standard deviations of measurements of diameter of the uterus in the right and left in M1 were 0.93 ± 0.10 and 0.95 ± 0.06 for G1, 1.16 ± 0.12 and 1.10 ± 0.17 for G2 and 1.11 ± 0.11 and1.2 ± 0.10 for G3, respectively. In M2 were 1.02 ± 0.04 and 0.98 ± 0.05 for G1, 1.11 ± 0.01 and 1.10 ± 0.08 for G2 and 1.17 ± 0.07 and 1.2 ± 0.12 for G3, respectively. In the first and second moments measures of right and left uterine diameter of G1 were statistically different from G2 and G3 ($p \leq 0.05$) but G2 and G3 did not differ between them. It can be deduced that the Santa Ines lambs subjected to administration of a norgestomet promoted final development of the reproductive tract but no difference between one treatment (12 days of exposure) or two treatments (24 days of exposure) was observed. Financial support of FAPESP (2009/15270-8 and 2009/18419-2).

Key Words: Ewe lambs, progestagens, puberty, ultrasound, uterus

2406

Effect of melatonin treatment on plasma IGF-I level, and gene expression of β-lactalbumin, BCL2, BAX, and BCLX in the mammary epithelium in sheep

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Long-term melatonin treatment used for cycle induction affects negatively milk production in lactating dams. One of the proposed way of action is that persistent melatonin signal has inhibitory effect on the growth hormone (GH) – insulin-like growth factor-I (IGF-I) – mammary gland axis. On the other hand significant data from literature showed the favorable, anti tumor-growth impact of melatonin treatment when used for estrogen-sensitive breast cancer, both *in*

vitro and *in vivo*. Melatonin enhanced apoptosis in tumor tissues via direct action on its receptors. This brought on the idea that melatonin may act directly on mammary epithelium also in sheep and thus enhancing apoptosis leads to early involution and decreased milk production.
Fifteen healthy, high producing, 4 years old, spring-lambing Awassi ewes, were involved in the trial. On day 0 (40–60 days of lactation) animals were allocated into three groups (n = 5 in each) according to their previous milk yield. Control group received no treatment. Simple group was treated with 18 mg (one piece), Double group with 36 mg (two pieces) of slow release melatonin implant (Melovine®, CEVA, Liburne, France). Basal plasma non-esterified fatty acid (NEFA), β-OH butyrate (BHB), thyroxin (T4), triiodothyronin (T3), IGF-I and insulin levels were assayed on D0, D25, D46. On the same days we performed TRH-challenge (600 μg TRH iv.) and evaluated the T3, T4 and IGF-I response. On D0 and D46 mammary gland biopsies were collected from all animals to examine MT1 receptor and β-lactalbumin mRNA gene expression as well as genes involved in apoptosis. Basal T3 and T4 did not differ between groups, although IGF-I level was lower in melatonin treated groups compared to control on D46 ($p = 0.06$ Simple; $p = 0.18$ Double). Concerning TRH-challenge, marked elevation of thyroid hormones was observed (138–155% T4; 156–201% T3), however IGF-I response remained under our expectations (maximum elevation was 108–132%). BHB and NEFA did not differ between groups. According to data available from mammary gland biopsies expression of β-lactalbumin mRNA decreased in all groups by D46 compared to the pool of samples of D0. This decrease was statistically significant in melatonin-treated groups ($p = 0.066$ Control; $p = 0.007$ Simple; $p = 0.013$ Double). Expression of BCL2 and BAX increased compared to D0 in Simple group ($p = 0.03$ and $p = 0.05$ respectively), contrarily BCLX expression decreased (NS). We found no significant differences in the other two groups. MT1 receptor gene expression was not detectable in the mammary epithelium. Long-term melatonin treatment had negative effect on basal plasma IGF-I levels. Although the applied TRH treatment evoked the expected effect on the adenohypophysis – thyroid axis, it was apparently not suitable to stimulate the GH - IGF-I axis. The observed changes of gene expression by D46 may indicate enhancement of apoptosis of mammary epithelium in melatonin treated animals. Supported by NKB 15706.
Key Words: Melatonin, milk production, apoptosis, mammary gland, insulin-like growth factor I

2407

Glutamatergic receptors are expressed at pronounced higher rate in the ovine fetal hypothalamus than pituitary during the late gestation period

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The neuroendocrine activation between hypothalamus and pituitary in the fetal brain is critical for the parturition process. The objective of this study was to identify those genes that are transcribed at high rate in the ovine fetal hypothalamus compared to pituitary during the last stage of gestation. The products of these genes could be essential components of the signal cascade that triggers parturition. Global gene expression in the ovine fetal hypothalamus and pituitary was measured at 120, 130, 145 days of gestational life (the gestation term is around 154 days) and 1 day of extra-uterine life using the microarray technique. The data was analyzed employing the Empirical Bayes principle to rank the genes according the difference in temporal profiles among hypothalamus and pituitary. Top ranked genes were grouped by similar expression using hierarchical clustering. The clusters of genes with highest difference in expression between both regions were selected. The cluster of highly transcribed genes in hypothalamus compared to pituitary contained 228 genes. Expected genes in this cluster were those codifying for hypothalamic hormones, like corticotropin releasing hormone, gonadotropin releasing hormone, growth hormone releasing hormone and for neuropeptides like oxytocin, neuropeptide Y and agouti related protein. The list of 228 genes was subjected to network analysis using the Cytoscape software, submitting the list to the GeneMania plugin for network inference and enrichment analysis. The top overrepresented biological process was glutamate receptor activity ($p < 0.001$). Genes involved with this process and validated by RT-PCR were ionotropic gluatamate receptors: N-methyl D-aspartate (NMDA), AMPA 2, AMPA3 and AMP4 and metabotropic receptors 3 and 8. The significant differences in the gene expression observed by microarray were also detected by RT-PCR, since the fold changes in the mRNA for all the mentioned genes were significantly higher ($p < 0.05$) in the hypothalamus compared to pituitary. The mRNA expression for the NMDA receptor in hypothalamus showed the highest difference in fold change compared to the pituitary, being from 700 times greater at 120 days of gestational life to more than 2000 times greater at 145 days. In conclusion, this study suggests that neurotransmission in the ovine fetal hypothalamus at the end of gestation is overwhelmingly mediated by glutamate receptors, which are important inputs to neurons in the paraventricular nucleus controlling the hypothalamus-pituitary-adrenal axis and, ultimately, parturition in this species.
Key Words: Ovine fetal hypothalamus, parturition, hypothalamus-pituitary-adrenal axis, glutamate receptors, NMDA receptor

2408

Serum levels of free thyroid hormones and cortisol during the mare′s oestrus cycle

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In most mammals, both male and female, the thyroid hormones play an important role in the regulation of the reproductive function. Some studies support the hypothesis that thyroid hormones may influence the ovarian function stimulating the follicular growth by acting directly on the ovary. Most of the thyroid research in horses seems to be focused on the effects of hormones bound to the carrier protein (T3–T4). Nathless, is well know that free fractions (fT3, fT4) have a biologically activity, and their serum levels are more adequate than T3–T4 to a better monitoring of the thyroidal function. An interaction between thyroid axis and adrenocortical function is reported and glucocorticoids have been also frequently indicated as one major factor mediating the suppressive effects of stress on reproductive functions. In an attempt to clarify the thyroid gland involvement in the mare reproductive activity, this study investigated the changes of fT3 and fT4 and cortisol during the oestrus cycle of 25 clinically-healthy mares from February through July in South-Italy (41°2′N; 16°53′E; 85 m), at the Centre for Artificial Insemination at Faculty of Veterinary Medicine of Bari. The mares (7.8 ± 2.7 years old) were monitored throughout two consecutive normal oestrus cycles using transrectal palpation and ultrasonographic examination. Blood samples were collected 7 and 14 days post ovulation (T_0 and T_1) during the first cycle; at the beginning of estrous (T_2) (16.9 ± 0.8 days), at late estrus (T_3) (19.9 ± 0.9 days) and then every 12 h (T_4–T_8) to few hours post ovulation (T_9) (21.5 ± 1.1 days), during the second cycle. Serum cortisol, fT3 and fT4 were evaluated with immunoenzyme assay (EIA) method. Data were analyzed with ANOVA test and p value < 0.05 was considered statistically significant. All the mares spontaneously ovulated without treatment between 36 and 72 h post late oestrus (T_3). The fT3 and fT4 trend curves were similar. A progressive increase of free thyroid hormones was observed in the first period of estrus from T_1 to T_3 (fT3: 1.6 ± 0.3 vs. 2.4 ± 0.6 pg/ml; fT4: 11.1 ± 1.2 vs. 19.7 ± 1.4 pg/ml; $p < 0.05$), followed by a baseline phase from T_3 to T_5/T_6, and then, by a new but less evident increase with a significant peak in the last 24–12 h before ovulation (T_6/T_7; fT3: 1.9 ± 0.4 pg/ml; fT4: 15.4 ± 0.6 pg/ml; $p < 0.05$). The cortisol curve increase from the 14th day after ovulation (T_1) showing two peaks: the first one at late oestrus (T_4; 85.2 ± 13.7) and the second one 12 h before ovulation (T_6/T_8; 72.6 ± 13.4) ($p < 0.05$). The fT3 and fT4 peacks in the early oestrus and those at the time of ovulation suggest that thyroid hormones may have a direct stimulatory effect on ovarian function, especially regulating the follicular maturation and ovulation. Increased cortisol secretion, partly due to the AI management of mares, seems to have no effect on fT3 and fT4 serum levels.
Key Words: Thyroid gland, free thyroid hormones, cortisol, oestrus cycle, horse mare

2409

Preliminary assessment of the relation between luteinizing hormone and progesterone concentrations on day 14 after insemination in sows

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In pigs, progesterone release is not dependent on LH release before day 12 of pregnancy. However, Parvizi et al. (1976) showed that, in late pregnancy, vena cava progesterone levels follow the pulsatile luteinizing hormone (LH) release with a 20–60 min delay and results of Virolainen et al. (2005) suggest that this may also be true at Day 22 of pregnancy. This experiment evaluated possible relations between LH and progesterone release as measured in the caudal vena cava at day 14 of gestation, during the period of placental attachment in pigs. On day 13 after first insemination, seven second parity sows received a catheter in de caudal vena cava as well as an ear vein catheter. One day later, blood samples were taken from the vena cava every 15 min and from the jugular vein every hour, from 800 until 1800 h. The samples were analysed for LH and progesterone concentrations. One of the sows was omitted from the results due to abnormal high progesterone concentrations (>400 ng/ml) in the vena cava samples. Similar to results in literature, LH and progesterone were released in a pulsatile manner. The mean number of LH pulses was 4.0 ± 1.1 per 10 h, with an amplitude of 0.86 ± 0.3 ng/ml. Basal and average concentrations of LH were, respectively, 0.35 ± 0.07 and 0.68 ± 0.1 ng/ml. The pattern of progesterone release to the vena cava was very irregular and pulses were hard to define. The number of surges above 100 ng/ml was 4.7 ± 1.1 per 10 h. Basal and average progesterone concentrations were, respectively, 33.6 ± 13.1 and 65.5 ± 19.8 ng/ml. The average concentration of progesterone measured in the jugular vein was 27.6 ± 1.5 ng/ml. On average 1.5 ± SD (0–3) of the LH pulses was followed by a progesterone surge above 100 ng/ml within 15–60 min after maximum LH. Average jugular vein progesterone concentrations were negatively correlated with LH concentrations (r = -0.95, p = 0.01). In conclusion, at day 14 of pregnancy, progesterone release, as measured in the vena cava, shows a very variable pattern, which only seems partly related to LH release.

Key Words: Pigs, progesterone, LH, gestation

2410

Evaluation of the reproductive potential of 1-year-old female Tasmanian devils and their prospective contribution to the captive breeding program

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In 2006, a captive insurance population of Tasmanian devils (Sarcophilus harrisii) was initiated in response to the wild population decline as a result of the fatal, transmissible Devil Facial Tumour Disease. Despite the successful introduction of males to females in most recommended pairings, the successful production of joeys is suboptimal (range 26.3–53.0%; 2007–2011). As a means of increasing the number of potential pairings, while causes of reproductive failure are investigated, we examined the reproductive potential of 1 year old female devils which are currently considered outside the optimal breeding age range of 2–4 years of age. Temporal fecal progesterone metabolite (FPM) analysis was used to determine the occurrence of the bi-phasic progesterone estrous cycle in yearling females and compare the dynamics of detected cycles with those of pregnant adult female devils. In devil estrous cycles, elevated FPM concentrations are associated with the proestrous phase and estrus (follicular phase) and the short luteal phase, with low concentrations between. Estrous cycles were confirmed in 50% (8/16) of 1 year old female devils examined. The interval between the end of the proestrous elevation in FPM concentrations and the beginning of the luteal phase, assumed to be a period of sperm storage, was 11.3 ± 4.0 days in yearling females which was greater than the interval observed in pregnant females (7.6 ± 2.3 days; $p < 0.05$). The length of the luteal phase in yearling females was similar to that observed in pregnant females (11.6 ± 1.8 vs. 12.5 ± 1.4 days respectively; $p > 0.05$) indicating that these are likely normal estrous cycles. Providing female devils with breeding opportunities during their first estrous cycle, may increase the lifetime reproductive potential of the individual thereby increasing their overall contribution to the captive breeding program.

Key Words: Tasmanian devil, estrous cycle, puberty, progesterone, fecal

2450

A comparison of puberty traits in angus, brahman, hereford, and senepol heifers

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During four years, age and weight at puberty were collected and analyzed on 137 Angus, 126 Brahman, 107 Hereford, and 72 Senepol heifers. The research was conducted at the USDA Subtropical Station at Brooksville, Florida. Also, blood samples were taken to study correlations between selected blood metabolites as indicators of nutritional status for age and weight at puberty. Puberty was determined using Brahman and Angus teaser bulls fitted with chin ball markers and checked three times a week. Palpation of ovaries for corpus luteum, blood sampling for progesterone levels and body weight were taken every 28 days. Blood insulin levels were negatively (r -0.24) correlated to age at puberty in heifers born in 1985 and 1986. Urea nitrogen was not correlated to age at puberty. Urea nitrogen was positively correlated (r -0.034) to weight at puberty. Seasonal anestrous in Brahman was noted by a definite decrease in the percentage of heifers cycling in January that rose to a peak level in March, immediately before the beginning of the breeding season. The two Hereford lines differed significantly (p = 0.0001) in age at puberty, while the two Angus lines differed (p = 0.0012) only in weight. Of the three frame sizes in Brahman, (large, medium, small) the small frame size was significantly younger at puberty than the large. Age at puberty in both Angus and Hereford breeds were affected by sire, but sire differences in weight were not significant in either breed. Sire differences were significant for both age and weight among Brahman sires. Senepol sires had no significant effect on either age or weight at puberty. Statistical analysis was done by least-squares fixed model procedures (SAS version 6, 1965). The average age and weight at puberty for the four breeds were: Angus 503 days, 259 kg; Brahman 602 days, 324 kg; Hereford 590 days, 284 kg; Senepol 583 days, 300 kg. There were obvious breed differences in age and weight at puberty among the four breeds, which could be used as a guide in breed selection in commercial cattle.

Key Words: Puberty, heifers, age, weight, breed

2451

Detection by immunohistochemistry of the *N*-methyl-D-aspartate receptor subunit-1 (NMDAR-1) in the intact sheep hypothalamus

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The issue of whether the gonadotropin releasing hormone (GnRH) neurons express N-methyl-D-aspartate (NMDA) and other glutamate receptors has been quite controversial. An immunohistochemical study was performed to evaluate the presence and localization of the N-methyl-D-aspartate receptor subunit-1 (NMDAR-1) in GnRH neurons in hypothalamus sections (2–3 Âμm) of adult rams. The immunereaction conditions used were: thermal antigenic recovery (citrate pH 4), primary antibodies dilutions of 1/100 for anti-GnRH (rabbit anti goat GnRH; Novus Biologicals Inc, Littleton, CO, USA) and of 1/25 for anti-NMDAR-1 (rabbit anti-human NMDAζ1

subunit; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation times selected were: 1 and 12 h for anti GnRH and anti NMDAR-1 antibodies, respectively. The immune amplification and development system used was peroxidase. The immune-labeling intensity of the studied structures was assessed using a cross scale. It was possible to detect the presence of NMDAR-1 at the plasma membrane level and in the cytoplasm of reactive cells of the hypothalamic preoptic region. These results demonstrated an antigenic stability of the NMDR-1 epitopes against 10% formalin buffer. Results show the likelihood of using the immunohistochemical technique with paraffin-dipped samples in the study of the NMDA receptor in rams.

Key Words: NMDAR-1, hypothalamus, immunohistochemistry, ram

2452

Effects of melatonin on salsolinol-induced prolactin secretion in goats

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Salsolinol (SAL), a dopamine (DA)-derived compound, is a putative endogenous prolactin (PRL)-releasing factor not only in rats but also in ruminants. However, the precise mechanism involved in PRL secretion by SAL or the secretory pattern of PRL induced by SAL in various physiological states in animals remains unknown. We have recently demonstrated that photoperiod modulates PRL-releasing response to SAL and a long photoperiod highly enhances the PRL-releasing response to SAL in goats. The pineal gland acts as a neuroendocrine transducer and converts photoperiod into a daily pattern of melatonin (MLT) secretion. Therefore, the objective of this study was to clarify the effect of MLT on SAL-induced PRL release in goats. Female goats were placed into a room kept at 20°C and 16 h of light (08.00–24.00 h): 8 h of darkness (24.00–08.00 h), and animals were orally administered saline or MLT (4 mg/head) twice a day (16.00 and 20.00 h) for five weeks. A single intravenous (i.v.) injection of SAL (5 mg/kg b.w.), thyrotropin-releasing hormone (TRH) (1 μg/kg b.w.) or a dopamine (DA) receptor antagonist, sulpiride (0.1 mg/kg b.w.) was given to the goats three weeks after the first oral administrations of saline or MLT, and PRL-releasing response to SAL was compared to that of TRH or sulpiride. SAL as well as TRH or sulpiride stimulated the release of PRL promptly after each injection in both saline- and MLT-administration groups. The area under the response curve (AUC) of PRL for the 60-min period after i.v. injections of saline (controls), SAL, TRH and sulpiride in saline-administration group was greater than each corresponding value in MLT-administration group. There were no significant differences in the AUC of PRL among the values produced after the injection of SAL, TRH and sulpiride in saline-administration group. However, the values produced after the injection of SAL and sulpiride were significantly greater than those produced after the injection of TRH in MLT-administration group. To our knowledge, this is the first report regarding the effect of MLT on SAL-induced PRL secretion in ruminants. Our results show that MLT is able to reduce the PRL-releasing response to SAL as well as TRH or sulpiride in goats, and provide further evidence that SAL is involved in the regulatory processes of PRL secretion in ruminants.

Key Words: Salsolinol, prolactin, melatonin, dopamine, goat

2453

The relationship between the hormones involved in growth and reproduction in trichogaster trichopterus male – A model for the suborder of Anabantoidei fish

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The blue gourami (Trichogaster trichopterus) belongs to the Anabantoidei suborder. These fish adapt to water having low oxygen concentrations because of the presence of an air-filled breathing cavity (the labyrinth organ), and they exhibit a specific sexual behavior. The male sexual behavior and pheromones induce final oocyte maturation and spawning in mature females. The complex interaction of the hormones in males and females along the brain-pituitary-gonad axis during growth and sexual behavior has been studied in our laboratory for over 20 years. Key genes encoding to hormones involved in growth and reproduction were cloned, and their relative gene expression together with steroid levels during the reproduction cycle were measured during the reproductive cycle. These data, together with the hypophsiotropic effect of brain peptides, led us to propose a model describing the endocrine regulation of blue gourami males. In mature non-reproductively active males, upregulation of gonadotropin-releasing hormone 1 and 2 (GnRH1 and GnRH2, respectively), as well as pituitary adenylate cyclase activating polypeptide related peptide (PRP), promote spermatogenesis and growth through gonadotopin (GtH)-11 ketotestosterone (11KT) and growth hormone (GH)- insulin-like growth factor-1 (IGF-1) pathways, whereas in mature reproductively active males, the GnRH3-luteinizing hormone (LH)-11KT pathway is upregulated to promote spermiation. Pituitary adenylate cyclase activating polypeptide (PACAP) and GnRH3 promote sexual behavior, however, they cooperate to attenuate early stages of spermatogenesis and weight gain via downregulation of follicle stimulating hormone (FSH) and GH, respectively. These findings support the biological importance of evolutionary events influencing the GnRH and PACAP hormone families.

Key Words: GnRH, PACAP, GH, gonadotropin

2454

Development of specific immunoassay systems for canine pituitary hormones using hetero-antibody fluor-immunoassay (FIA) method

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There are many difficulties to develop a homologous immunoassay for small or rare animal pituitary hormones. Here we present hetero-antibody FIA to prepare easy and convenient immunoassay methods using Europium (Eu) molecule labeling. Porcine pituitary hormones were isolated by successive chromatographic steps, and then were immunized against rabbits (LH, FSH, TSH), guinea pigs (PRL) and a goat (GH). The purified canine LH, FSH (provided from Prof. Wakabayashi) and GH and PRL (purchased from NIDDK, NIH) were labeled with Eu, respectively. Those assays were performed in 96-well microtiter plates coated with second antibodies. The characteristics of each assay systems were shown in Table 1.

The data suggest that (i) the canine pituitary hormones assay systems developed using the antibodies to porcine pituitary hormones are very specific and high sensitivities; (ii) their assay systems can measure each hormone in peripheral blood serum; (iii) their assay methods can make clear the cyclic hormonal changes of canine pituitary hormones as pulsatile, diurnal, estrous cycles, and during pregnancy.

Key Words: Canine, pituitary hormone, fluoro-immunoassay, hetero-antibody

Table 1. Characteristics of FIAs for canine pituitary hormones

Hormone	LH	FSH	PRL	GH
dilution of Ab	20 000	20 000	200 000	800 000
IC50 (ng/ml)	2.5	2.3	3.4	2.0
range (ng/ml)	0.1?100	0.05?100	0.1?100	0.05?100
cross-reactivity	all < 0.1%	all < 0.1%	GH;0.5%	PRL;0.4%
♂ (ng/ml)	11.13 ± 6.17	2.70 ± 1.12	23.87 ± 13.55	9.42 ± 6.86
♀ (ng/ml)	12.43 ± 10.86	3.77 ± 2.54	36.10 ± 17.65	16.88 ± 23.06

25. Reproductive behavior:

2500

Influence of bull exposure on resumption of ovulatory activity of postpartum anoestrous dairy cows

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Exposing postpartum anoestrus suckled beef cows and heifers to bulls, either with close physical contact or by fenceline contact, can accelerate resumption of ovulatory activity. In that regards, the procedure can be a significant approach for dairy breed. Thus, the main objective of this study was to examine the effect of bull exposure, particularly focussing on resumption of ovulatory activity in high-producing Holstein-Friesian dairy cows during early lactation in an intensive farming system. Twenty multiparous anoestrus dairy cows (0–50 DPP) were split into two treatment groups; no bull contact throughout the experiment (NBC; n = 8, average DPP = 25), and fenceline bull contact for at least 30 days, until the onset of cyclicity (BC; n = 12, average DPP = 30). They were housed in free-stall cubicles and fed with a total mixed ration daily. Milk samples were collected twice a week for milk progesterone analysis and the first sustained rise in P4 was taken to indicate that ovarian cyclicity had resumed after calving. Walking activity was monitored by pedometers using a Fullwood Crystal monitoring system. Video cameras were installed to record oestrus behaviour and any interactions with the bull. Differences between treatments groups (NBC and BC) were assessed using repeated measures ANOVA and Chi-square tests. More cows in BC group showed oestrus behaviour during the observation period compared to cows in NBC (BC = 58%, NBC = 33%; X2 = 1.307). However, bull exposure had no effect on hastening oestrus, as determined by milk progesterone (NBC 10–60 DPP, BC 10–90 DPP; X2 = 0.069, p = 0.795). There was no significant difference in days to resumption of ovulatory activity between NBC and BC cows ($p > 0.05$) as measured from milk P4 concentration. Cows in the BC group showed an increase (15–20 min in normal day, 45–50 min during oestrus; $p < 0.05$) in time spent visiting the bull on the day of oestrus. There was no significant difference ($p = 0.079$) in change of walking activity on the day of oestrus in cows from both treatment groups. Exposure to a bull of cows during the early postpartum period has no absolute effect on resumption of cyclicity but may encourage some luteal activity which commit with increased of oestrus behaviour. Further research is required to understand the influence of bull exposure on ovulatory activity in dairy cows.

Key Words: Progesterone, bull contact, no bull contact, days postpartum

2501

Investigations on semen quality and freezability of Cholistani bulls – A preliminary study from Cholistan desert of Pakistan

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The Cholistani breed of cattle is a thermo-tolerant and tick resistant breed being reared under pastoral system by the nomadic herders of Cholistan desert of Pakistan. This indigenous breed has been neglected because with most attention being focused on Sahiwal and Red Sindhi breeds of cattle. This study was designed to generate a preliminary data regarding the quality and freezability of semen from Cholistani breeding bulls (n = 06) maintained at the Semen Production Unit, Karaniwala, Punjab, Pakistan. Semen was collected weekly from October to December, 2011, with an artificial vagina. A total of 101 ejaculates were collected with a mean of 17 collections per bull. The mean ejaculatory volume (ml), mass motility (Scale 1–5) individual motility (%) and sperm concentration (milliom/ml) were 4.45 ± 0.76, 1.94 ± 0.14, 64.38 ± 2.64 and 918.05 ± 65.79, respectively. The fresh semen attributes regarding sperm viability and normal morphology (through supra-vital staining), and acrosome integrity (through formaldehyde-citrate solution) revealed mean values of 84.60 ± 1.27, 83.97 ± 0.61 and 83.85 ± 0.54%, respectively. Semen was diluted by a slow one step method in TRIS egg yolk extender, loaded into 0.5 ml straws, equilibrated at 4°C for 6 h and stored in liquid nitrogen tanks. Post thaw assessment of viability, normal morphology and acrosomal integrity indicated reduced cryoinjury to sperm from Cholistani bulls compared to Sahiwal bulls. Future studies with a larger sample size expanded over all seasons and which considers age and testicular biometry are necessary to establish seasonality (if any) of the Cholistani breed of cattle.

Key Words: Indigenous cattle, Cholistani cattle, semen quality, freezability

2502

Sexual and social behaviour signs during the oestrus cycle in dairy cows

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Fertility in high producing dairy cattle has declined. This may in part be explained by a weak oestrus expression and difficulties to record and interpret oestrous behaviour signs. The aim with this study was to investigate the importance and occurrence of different sexual and social behaviours. Local (e.g. discharge) and behaviour (mating, standing) signs of oestrous were routinely recorded in Swedish Red and Holstein cows at the university herd. Based on this, oestrous intensity was scored in five classes from weak (one, local signs only) to very strong (five, local and behaviour signs). The data set contained 1096 oestrus periods recorded during 1992–2008. Conception rate in cows with oestrous scored 1 was 36.0% and in cows scored 5 it was 44.7% implying expression of behaviour sexual signs as important for fertility. In a recent intensive behaviour study in the same herd the presence of sexual and social behaviour during a sexual cycle were studied during 7 days at 08.00–10.00, 13.00–15.00, 20.00–22.00 and 02.00–04.00, in total 56 h. The number of times a cow performed or received a sexual or social behavior was recorded. Milk progesterone profiles were used to evaluate the stage of the sexual cycle for each cow. Nine cows ovulated during the study. These were matched according to breed, days after calving and production level with nine non-ovulating cows. The most common performed and

received sexual behavior in the group of ovulating cows was mating (eight and 5.9 times per cow) followed by chin resting (3.9 and 3.9) and licking vulva (2.1 and 1.4). Standing and mounting behavior was observed in four and six cows, respectively, of the nine ovulating cows. Mounting was performed from behind in 90% of the registrations and from the head and the side in 6% and 4%. Licking (head, neck, body), head to head butting and head to body pushing were the most common performed and received social behaviors (1.7–2.1 per cow). Sexual behavior signs were more common during night time and when more than one cow was in oestrus at the same time. In the non-ovulating cows, performed sexual behavior signs was about one third as common as in the ovulating cows. The most common behavior was chin resting (1.9 times per cow) followed by licking vulva and mounting. The most common social behavior was head to head butting (2.3 times per cow). We conclude that behavior signs are important for fertility and that it is a large variation among cows in expression of these signs. There is a need for a more thorough definition of 'good oestrous expression' before including the trait in future breeding programs.

Key Words: Dairy cattle, reproduction, social behaviour, sexual behaviour, oestrus signs

2503

Vomeronasal organ blocking and its influence on sexual behavior of Nellore males raised on pasture

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In cattle, reproductive events are associated with aggressive and sexual behavior, which increases handling difficulties. In zebu animals the vomeronasal organ (VNO), located between the oral and nasal cavity plays an important role in detecting pheromones, which modulate the steroid concentrations that influence sexual behavior. Blockage of the vomeronasal organ, a less invasive technique than castration, could be an important management tool, which might result in improved economic performance in the beef cattle business by reducing the aggressiveness of bullâ€™s and increasing carcass fat deposition. The aim of the present study was to evaluate the effect of blocking the incisive duct of the VNO on the sexual behaviours of sniffing and licking the female genitals and on the flehmen reflex. The experiment was conducted from August/2011 to January/2012, using 60 Nellore bulls, approximately one-year-old, divided into three groups: G1 = control (intact), G2 = orchiectomized and G3 = incisive duct of the VNO blocked. The VNO was blocked by the introduction of a nylon wire (4 cm × 2 mm) into the incisive ducts. All treatments were applied in compliance with the legal procedures related to animal comfort and welfare recommended by the ethics committee of the Federal University of Minas Gerais. The average number of the sexual events, sniffing and licking of female genetalia and flehmen reflex were recorded for a period of 5 min in the presence of 10 females, of which three were in heat (Table 1). Observations were made 60 days after treatments were completed and were compared by Kruskal–Wallis test, using the statistical package SAS (2002). Licking of the inguinal region recorded in G1 was the sexual event observed in greatest number ($p > 0.05$) when compared to G2, but was not different from G3. Low frequencies of the sexual events were recorded in this study. This was possibly due to sexual immaturity of the animals, which may explain the lack of effects observed from blocking of the VNO on the variables studied. It was concluded that the effect of the VNO incisive duct obstruction should not be evaluated in sexually immature and inexperienced animals.

Key Words: vomeronasal organ, castration, flehmen reflex, sexual behavior, pheromones

Table 1. Frequency of sexual events observed for 5 min in Nellore males when exposed to females in heat

Sexual events	G1	G2	G3
Sniffing and licking female genital area	2.17 ± 0.55[a]	0.55 ± 0.19[b]	1.43 ± 0.39[ab]
Flehmen reflex	1.13 ± 0.45[a]	0.27 ± 0.19[a]	0.62 ± 0.20[a]
	$p < 0.05$ (in line)		

2504

Sexual preference of ewes for rams medicated with naloxone

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Sexual preference in the ewe depends on several factors; ram attractiveness is important. In this work two rams were treated with 0.5 mg naloxone im for 15 days, and other two control rams were treated with saline injections. To study sexual preference 12 ewes were sinchronized to display estrus at the end of naloxone treatment of the rams. In each test, two rams, one treated and one control, were tied in a 20 × 20 m paddock at equal distances from the point of entrance of the ewes. Maximum mobility for each ram was 3 m. On the following day after sponge withdrawal (first trial); ewes were allowed to enter at the opposite end of the paddock where the chosen rams were tied. When an ewe directed her attention to a ram and was mounted and mated it was withdrawn to allow the ram to mate another approaching ewe; procedure repeated twice a day at 8:00 am and 6:00 pm.Proceptive and receptive behavior were noted in the ewes. Plasma testosterone levels were monitored through the duration of the experiment in both treated and control rams. It was observed that on the first week of treatment, testosterone levels were not significantly different between treated and control rams; but at the beginning of the second week of treatment testosterone plasma levels in naloxone treated rams increased until values reached levels of 15 ng/ml compared with 6 ng/ml in control rams. When ewes were exposed to treated and control rams, 11 ewes showed high proceptivity and receptivity for the naloxone treated ram ($p < 0.01$) and they were mated, for the control ram only one ewe showed complete preference. Behavior data were analyzed by the Wilcoxon signed rank test. Simple linear correlation coefficients were calculated to examine within-ram interrelationships between serum Testosterone concentrations. Probability values of $p < 0.05$ were considered statistically significant.It was concluded that naloxone treatment facilitated the secretion of testosterone in rams and the high concentration of this androgen motivated ewes to prefer mating with the naloxone treated ram.

Key Words: Behavior, naloxone, sexual preference, ewe, rams

2505

Some factors that affect the pregnancy rate in alpacas

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Alpacas is a domestic specie of south American camelids with a great importance to highland populations because of its high quality fiber but the reproductive performance is very deficient with a birth rate of 40%. Two experiments were design to evaluate the effect of some factors on the pregnancy rate at first service in alpacas female lactating from 3–8 years old and without parity problems. A first experiment with a total of 85 animals was carried out to evaluate the effect of age of female (n = 85), time of mating (n = 80) and interval parity to mating (n = 67) and a second experiment with a total of 174 animals to evaluate the effect of month of calving: January (n = 56), February (n = 67) or March (51) on conception rate. Animals were mated with male fertile and after of a receptivity test and confirmation of presence of a dominant follicle ≥7 mm by ultrasonography. Pregnancy was determined by ultrasonography 25 days after mating. Results to first experiment indicate a 57.9, 66.7 and 47.2% of pregnancy rate to females of 3, 4–6 and more of six years age, respectively ($p < 0.05$); 50.0, 54.5 and 59.0% to 15, 16–25 or more 25 min of mating; 48.0 and 59.4% to interval parity – mating of 20 and more 20 days ($p < 0.05$). Second experiment, pregnancy rate was 58.3, 70.7 and 82.1% to January, February and March, respectively ($p < 0.5$). The results suggest that age of female, interval parity-mating and month of calving are factors that affect the pregnancy rate and a reproductive management system would improve the pregnancy rate in alpacas under highland Peruvian conditions.

Key Words: Alpacas, reproduction, management

2506

Effect of lactation and boar exposure during early lactation on expression of oestrus in multiparous sows

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During lactation, sow ovarian follicle growth and ovulation are normally prevented by suckling. However, we have recently demonstrated that provision of boar contact during late lactation results in high incidences of lactation oestrus in sows suckling 3, 5, 7 and 10 piglets (Terry et al., 2011. Manipulating Pig Production XIII, Australasian Pig Science Association, Werribee). The aim of this study was to determine how boar contact and lactation length affect the ability of the sow to express oestrus and ovulate during the first 26 days post-partum. Sows were randomly allocated to one of four treatments; weaning at 26 days and no boar contact (control); weaning at 26 days and boar contact (BC); weaning at 7 days and boar contact (BC + Short Lactation (SL)); and weaning at 7 days and no boar contact (Short Lactation (SL)). Boar contact commenced on day 7 post-partum and consisted of 15 min of daily, physical boar contact in a detection mating area. Timing of oestrus was determined using a back pressure test. Sows were artificially inseminated at first detection of oestrus and daily until last detection of oestrus. Sow live weight and P2 backfat were measured on days 1, 7 and 26 post-parturition. Differences between treatments were determined using a general analysis of variance model (Genstat, 10th Edition). There was no effect of treatment on sow LW and P2 on day 1 (262 ± 3.8 kg, 23 ± 0.7 mm) or day 26 (252 ± 3.4 kg and 22 ± 0.6 mm) of lactation. A significantly higher proportion of BC and BC + SL sows expressed oestrus during lactation compared to control and SL sows (Table 1). However, it was observed that of sows that exhibited oestrus within 26 days of parturition, BC + SL and SL sows expressed oestrus significantly earlier in lactation (13.1 ± 0.6, n = 14; and 13.0 ± 1.6 days, n = 2) than control and BC sows (18.3 ± 1.3, n = 3; and 16.0 ± 0.7 days, n = 10) ($p < 0.05$). Pregnancy rates of sows expressing oestrus within 26 days of parturition were similar for control (33%); BC (60%), BC + SL (64%) and SL (100%) sows. These data support previous work from our group demonstrating that boar contact is an effective stimulant of lactational oestrus.

Key Words: Lactation oestrus

Table 1. The effect of lactation and boar contact on the percentage of sows exhibiting oestrus within 26 days of farrowing; 27–33 days of farrowing; and more than 34 days of farrowing

		Percentage of sows expressing oestrus		
Treatment	n	day 26 of farrowing	day 27–33 of farrowing	day 34 of farrowing
Control	18	17[a]	61[a]	22[a]
BC	15	67[b]	33[a,c]	0[a]
BC + SL	15	93[b]	0[b]	7[a]
SL	15	13[a]	7[b,c]	80[b]

[a,b,c]Different superscripts within a column indicate $p < 0.05$.

2507

Boar contact is an effective stimulant of lactation oestrus

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The ability to stimulate behavioural oestrus and ovulation during lactation allows piglet age at weaning to be increased without reducing sow reproductive output. The first objective of this study was to assess the efficacy of boar contact as a stimulus of ovulation in lactating sows at different stages in early lactation. The second objective was to compare the responsiveness of multiparous (MP) sows and primiparous (PP) sows to boar stimulation during lactation. On day 3 of lactation, the litters of 50 MP (parity 3 ± 0.2; liveweight 263 ± 5.0 kg) and 38 PP (liveweight 215 ± 5.6 kg) sows were standardised to 11 piglets. Within parity group sows were randomly allocated to commence 15 min of daily, physical boar contact (BC) in a detection mating area on day 10 (BC10), 14 (BC14), or 18 (BC18) of lactation, or no BC (NBC) during lactation. Sows were weaned on day 27.5 ± 0.09 of lactation. Prior to day weaning, NBC sows were tested for oestrus behaviours by removing them from the farrowing crate for 5 min daily from day 2 of lactation. Exposing both multiparous and primiparous sows to a mature boar during lactation increased their capacity to express lactation oestrus compared to those not receiving boar contact ($p < 0.05$). Sows in the BC10 treatment exhibited oestrus earlier in lactation than those in the BC18 and control treatment groups ($p < 0.05$). Following the commencement of boar contact MP sows exhibited lactation oestrus more rapidly than PP sows ($p < 0.05$). Overall, these data demonstrate that daily boar contact is an effective stimulant of lactation oestrus, and that commencing boar contact earlier in lactation results in a shorter farrowing to lactation oestrus interval. The longer interval from boar contact to lactation oestrus expression of primiparous sows suggests a reduced responsiveness to boar contact compared to multiparous sows.

Key Words: Lactation oestrus

Table 1: Proportion sows exhibiting lactation oestrus and interval from farrowing or boar contact to lactation oestrus

	Treatment				Parity	
	BC10	BC14	BC18	NBC	MP	PP
N	23	22	23	20	50	38
Proportion sows expressing oestrus in lactation	0.57[a]	0.36[a]	0.61[a]	0.05[b]	0.52	0.26
Farrow to lactation oestrus interval, day	17.5 ± 0.8[a]	19.5 ± 1.0[ab]	22.3 ± 0.8[b]	23.1 ± 3.0[b]	19.3 ± 0.6[a]	21.7 ± 1.0[b]
BC to lactation oestrus interval, d	7.5 ± 0.8[a]	5.5 ± 1.0[a]	4.3 ± 0.8[a]	N/A	5.4 ± 0.6[a]	7.8 ± 1.0[b]

[ab]Within row, and main effect, indicate significant difference; $p < 0.05$.

2550

Lack of a relationship between social rank and reproductive patterns in semicaptive pampas deer (*Ozotoceros bezoarticus*) males

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In some ruminant species male social rank is related with reproductive characteristics. Pampas deer is an endangered species from the southern cone of South America. The aim was to determine if individual hierarchical position is related with body weight, neck perimeter, and reproductive pattern (testicular volume, testosterone concentrations and seminal parameters) in pampas deer males allocated in single male groups. Four groups of 4–6 adult males (total = 19) were allocated in 0.5 ha paddocks, in which they could graze and browse, and received dairy ration in amounts considered as ad libitum (approximately 600 g/animal/day). Spontaneous agonistic encounters were recorded (100, 225, 242, and 387 interactions in each

group) during the breeding season (February–April), and success index was calculated for each animal according to the proportion of individuals in the group dominated (range 0–1). During the breeding season (March–April, autumn), animals were anesthetized (ketamine, xylazine and atropine), body measurements were recorded, blood samples for testosterone measurement by RIA were collected, and, semen was obtained by electroejaculation. Basic seminal parameters were determined (total spermatozoa in the ejaculate, sperm quality, percentage of alive, motile, progressive, and normal spermatozoa). Success index was not significantly related with any measured parameter, and only tended to be positively related to sperm quality ($r = 0.31$; $p = 0.1$). Mean ± SEM values of the other measured characteristics were: body weight: 28.5 ± 0.8 kg; neck perimeter: 39.1 ± 1.0 cm; testicular volume: 10.9 ± 0.7 cm^3; testosterone: 13.6 ± 1.9 nM; total spermatozoa: 271.2 ± 76.7 million spermatozoa, sperm quality: 3.5 ± 0.2; alive spermatozoa: 66.2 ± 6.7%; motile spermatozoa: 61.8 ± 6.2%; spermatozoa with progressive motility: 60.6 ± 6.3%; normal spermatozoa: 41.3 ± 5.5%. This results differ with what has been observed in some ruminant species, but it should be considered that pampas deer seems to be a light hierarchical species, as males may coexist in freedom groups during the whole breeding season. Moreover, in this experiment, animals were allocated in homogeneous groups according to age, and received enough food to avoid competition and have free access to it despite individual success index. Overall, pampas deer male reproductive patterns were unrelated with success index, at least in the conditions described in this experiment.

Key Words: dominance, semen, hierarchy, social behavior, cervid

2551

Reproductive hormone patterns of pregnant, pseudo-pregnant and acyclic captive African wild dogs (*Lycaon pictus*)

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With a population of < 5500, African wild dogs are considered one of the most endangered canid species. They are cooperative breeders with only the dominant pair in each pack breeding once per year. Litters are large but pup survival is poor and is further impacted by disease and human conflict. Research and breeding management are hampered by a poor understanding of wild dog reproductive biology. To improve our basic knowledge we monitored hormone patterns during the oestrous cycle with non-invasive techniques. We collected faecal samples from 15 females held in a variety of captive housing conditions during the breeding season. By comparing longitudinal hormone profiles with behavioural and anatomical changes, we classified females according to three reproductive classes: pregnant (n = 1), pseudo-pregnant (n = 9) and acyclic (n = 4). We also monitored a single female in which contraception was induced with a deslorelin implant. We divided the oestrous cycle into four phases: anoestrus, pro-oestrus, oestrus and luteal and aligned profiles to an oestradiol peak, with ovulation assumed to occur two days later. We used changes in behaviour and anatomy, documented in our own and other studies, to define the length of pro-oestrus (e.g. onset of male interest, vulva oedema; 9 days), oestrus (ovulation to mating; 12 days) and luteal phases (pregnancy 70 days). Oestradiol concentrations during the pro-oestrus phase (12.6 ± 1.4 ng/g) was significantly higher than all other phases across pseudo-pregnant and acyclic classes ($F = 24.02$, $p < 0.01$). These two classes did not differ in overall oestradiol levels but did in progestagen concentrations ($F = 43.41$, $p < 0.01$). Progestagen profiles showed that anoestrus values (582 ± 100 ng/g) were significantly lower than luteal (832 ± 56 ng/g; $F = 11.93$, $p < 0.01$). We had only one pregnant female and her profile was similar to that of the pseudo-pregnant females. The female treated with contraceptive maintained low oestradiol and progestagen concentrations, similar to acyclic females. Interestingly, most females classed as pseudo-pregnant were found in single-sex groups suggesting that, as with other canids, female wild dogs are spontaneous ovulators and that subordinates are unlikely to be physiologically suppressed. These findings have important implications for captive breeding programs and development of reproductive technologies. Non-invasive sampling has significant value for studying reproduction and can play an important role in conserving this enigmatic species.

Key Words: Canid, faecal steroid, oestrous cycle

2552

Reproductive problems in Landhi Buffalo Colony, Karachi, Pakistan

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The present Landhi Buffalo Colony is the largest of its kind on the face of earth, was established in 1958 about 21 km away from Karachi down town. Spread over an area of 750 acres, it carried about 8000 heads of water buffaloes along with some cows. The main objective was to remove animals from thickly populated residential areas (mohallahs) of sprawling city to improve upon cleanliness and reduce environmental pollution. Since then during the half a century or so the colony was progressively developed into thickly populated township inhibited by more than 0.85 million water buffaloes and cows and about 225 000 are human. Unlike rest of the world, this colony faces a very unusual phenomenon that the animals that are bred and raised elsewhere are brought to Landhi Buffalo Colony and its expansions/extensions to cater the milk needs of the Karachi city. These animals are high producers. Because of high feeding costs in Karachi, the average producers do not produce economically. The 95% of the animals are not mated. They consider the animals as disposable or use once commodity. The milk producers at the end of their lactation sell these animals for slaughter causing a shear loss of superior germ plasm. There are two enclosures in the slaughter house, (i) Slaughter animals, 75% percent animals are slaughtered and (ii) the remaining nearly 20% that are prototype, young and sound in appearance are purchased by the villagers at beef prices, (unsold animals still go under knife) who take them in the interior to breed them again. The follow up revealed that 83% of such animals bred normally. The parturitions that take place in this colony, ten percent of them suffer from retention of placenta. Nearly 5% recover while the remaining 5% develop complications like metritis, endmetritis, mastitis, and loss of milk etc. Some other problems like few of the farmers let loose the breeding bulls in the herd. Such bulls would mount even such animals that are not in heat. In some of the cases it leads to bleeding and viginitis. The cows in the colony mostly repeat but water buffaloes do not. When such repeater cows were sold and sent to the remote farms, they bred normally. It is assumed that it could be due to the environmental factors and withdrawal of oxytocin that is being widely used in the colony to cause the milk let down. The dystocia cases are around 1–2% that is successfully corrected by the local veterinarians so the complications are rare and similarly the calves that are dead on arrival cases are also rare in this colony. The predisposing factors regarding the paralysis or prolapsed uterus in fresh calves are because of long distance traveling. Some of the old age farmers resort to some mall practices like putting the tail or by pouring the water into the vagina, which is wide open after the parturition, with view to improve milk production. This practice, quite often leads to infections of various kinds.

Key Words: Water buffalo (Buffalo), reproductive problems, mismanagement, economic loss

26. Reproductive immunology & toxicology:

2600

Relationship between uterine immune function and embryonic mortality in a group of subfertile beef heifers

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The objective was to compare endometrial gene expression profiles in beef heifers yielding viable or degenerate embryos on Day 7 after estrus as a means to explain differences in embryo survival. The focus was on the expression of genes that were associated with either the pro-inflammatory and anti-inflammatory response as well as anti-microbial peptides. Estrus was synchronised in cross-bred beef heifers (n = 157) using a Controlled Intravaginal Drug Releasing device (CIDR) protocol. Heifers detected in standing estrus (within 24–48 h post CIDR removal, n = 102) were inseminated (estrus = Day 0) with frozen-thawed semen from a single ejaculate of a bull of proven fertility. Heifers from which an embryo was recovered (following slaughter on Day 7) were classified as either having a viable (V) embryo (morula/blastocyst stage: n = 32) or a retarded (R) embryo (arrested at the 2- to 16-cell stage: n = 19). The overall recovery rate (V and R combined) was 64%. Endometrial gene expression was determined using quantitative real-time PCR analysis. Of the 31 genes analysed, eleven were differentially expressed in the endometrium of heifers yielding V compared with R embryos ($p < 0.05$). Of these 11 genes, expression of Beta defensin (DEFB), Interferon alpha (IFNα), Interferon gamma (IFNγ), Interleukin 6 (IL6), Interleukin 10 (IL10), Forkhead box P3 (FOXP3) and Natural cytotoxicity triggering receptor 1 (NKp46) was higher in endometria from which a R embryo was recovered compared with heifers that yielded a V embryo. Expression of Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NKFB1), Transforming growth factor beta (TGFβ), Interferon gamma-inducible protein 16 (IFI16) and Interleukin 21 (IL21) was higher in heifers with a V embryo compared with heifers yielding a R embryo. We propose that increased expression of IFNα, IFNγ, IL10, FOXP3, IL6 and NKp46 combined with the decreased expression of NFKB1, TGFβ, IFI16 and IL21 contribute to a uterine environment that supports embryo development to the blastocyst stage. In contrast, the deregulation of the expression pattern of these genes may contribute to retarded embryo development. Given that TGFβ can regulate whether an immune response will be pro- or anti-inflammatory, its reduced expression in endometria from which R embryos are recovered indicates that disturbance of the very fine balance between the two responses at this stage of embryonic development can have detrimental implications for embryonic survival.

Key Words: Embryo mortality, uterine immunity

2601

Early postnatal immunisation with a novel gonadotrophin-releasing hormone (GnRH) construct has longer reproductive effects in male than female calves

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Immunisation against GnRH impairs reproductive function by reducing synthesis and release of pituitary gonadotrophins (LH and FSH) and gonadal steroids (testosterone and oestradiol). Despite being temporary in peri- and post-pubertal cattle (6–8 months), immunisation during the early postnatal stage induced log-term reproductive effects in sheep. Thirty-six cross-bred calves were immunised against a GnRH-bovine herpes virus 1 gD glycoprotein construct (Pfizer Ltd.; heifers (IH), n = 9 and bulls (IB), n = 9) or received saline (heifers (CH), n = 9 and bulls (CB), n = 9) at 2 (V1), 6 (V2) and 13.5 (V3) weeks of age. Antibody titres against GnRH and endocrine responses to immunisation were measured in samples taken twice monthly (FSH, testosterone and progesterone) or during intensive sampling periods (LH pulse characteristics). Puberty was determined based on progesterone profiles in heifer and testicular size (14 cm diameter) in bull calves. Antibody responses against GnRH were similar between IH and IB, but higher ($p > 0.05$) than CH and CB after V2, reaching a peak antibody titre after V3 (37.06 ± 9.8% and 28.13 ± 4.9% binding at 1:1000 dilution, respectively). Immunisation decreased LH (pulse amplitude, mean and basal concentrations; $p < 0.05$) and FSH concentrations ($p < 0.05$) in IH and IB compared to CH and CB during peak antibody titres at 16 weeks of age ($p < 0.05$), with no differences after this age. In IB, these changes provoked lower testosterone concentrations ($p < 0.05$), reduction in morphologically normal sperm ($p = 0.06$) and smaller testicular size ($p < 0.05$) compared to CB, delaying puberty by ~8 weeks. In IH, the number of medium-sized follicles (4–8 mm diameter) decreased in IH compared to CH during this period (0 vs 4.8 ± 1.1 follicles; $p < 0.05$), without effect on age at puberty (55 vs. 53 weeks of age; $p > 0.05$). Additionally, immunisation increased carcass quality in IB but not in IH. Overall, despite having similar effects on hormonal concentrations and reproductive organ dynamics during high antibody titre periods, immunisation caused a longer-term reproductive effect in male than in female calves. Our findings indicate that there may be a developmental window when testicular development is prone to endocrine disturbance impacting on long-term reproductive function. While a similar window during early postnatal development in female calves may exist, this may have passed by the time antibody reached peak titres able to provoke reproductive disturbance.

Key Words: immunocastration, GnRH-immunisation, puberty, cattle

2602

Treatment with seminal plasma (SP) modulates the endometrial cytokine network and improves fertility in dairy cows

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Epidermal growth factor (EGF) profile in the uterine endometrium has been indicated to be a good marker of fertility in dairy cows. Infusion with SP into the vagina normalized the EGF profile in repeat breeder cows with an abnormal EGF profile and improved fertility. The objective of the present study was to examine if the SP action to normalize EGF profile and to restore fertility is mediated by changes in a cytokine network in the endometrium. Multiparous Holstein cows with normal uterine involution and without an abnormal clinical sign were examined the ovaries daily from day 30 after parturition using ultrasonography. Prostaglandin (PG) F2α was given between 35 and 46 days after parturition when a dominant follicle reached 1.3 mm in diameter in the presence of a functional CL (>2.0 in diameter and >3 ng/ml of plasma progesterone). Cows were infused with 0.5 ml of SP diluted to 20 times (10 ml) with PBS (n = 38) or 10 ml of PBS (n = 38) into the vagina at induced estrus or 72 h after PG treatment. Ovulation within 36 h after the SP treatment was confirmed by ultrasonography. Endometrial tissues were obtained by biopsy on Days 0, 3, 7 and 14 after SP treatment. Levels of cytokine expression in the endometrial tissues were determined by quantitative PCR and EGF concentrations determined by EIA. In the SP group, endometrial EGF concentrations on Days 3 and 14 ($p < 0.01$) and conception rate at the first AI (55.3 vs. 36.8%, $p = 0.07$) and pregnancy rate by 120 days after parturition (73.7 vs. 52.6%, $p = 0.03$) were greater than the control group. Granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-6 showed a similar change: their levels were high on Day 3 and low on Day 14 in the SP group ($p < 0.01$) while their expression were at intermediate levels on all days in the control group. Expression of IL-4 and chemochein 8 (CXCL8) in the SP group showed an opposite change: the levels were lower on Day 3 and greater on Days 7 and 14 than the control group ($p < 0.05$). In cows conceived by the first AI regardless of treatment groups, GM-CSF levels on Days 3 and 7 were greater and CXCL8 levels on Day 3

were lower than the control group. There was no correlation between EGF concentrations and cytokine levels in the endometrium. The present results indicate that SP plays a regulatory role in the endometrial cytokine network and enhance fertility, although the relationship between the profiles of EGF and the local cytokine network remained unclear. Further study is necessary to identify seminal factors responsible for the SP action and to understand the significance of observed changes in cytokine expression in the present study to establish pregnancy.

Key Words: Cytokine, endometrium, fertility

2603

Participation of CD4(+)CD25(+)T cell and WC1(+)γδT cell in pregnancy establishment of cows

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In the pregnancy of human and mouse, regulatory T (Treg) cells have been shown to play a crucial role in suppression of an anti-fetal immune response. On the other hand, it is unclear whether Treg cells participate in the pregnancy of the cow. The objective of this study was to examine the possible role of CD4(+)CD25(+)Foxp3(+)T cell and WC1(+)γδT cell in the regulation of maternal immune system for pregnancy establishment in cows. In experiment 1, seventeen multiparous Friesian-Holstein cows (six pregnant cows, seven non-pregnant cows and four non-inseminated cows) were examined. Blood samples were collected at Day 6, 11, 16 and 20 (Day 0 = estrus). Pregnancy diagnosis was performed on 35 days after insemination. Population of CD4, CD25, WC1, WC1.1 and WC1.2 cells in peripheral blood mononuclear cells (PBMC) were measured by flow cytometry. In experiment 2, mRNA expression of Foxp3, WC1, WC1.2, IL10 and transforming growth factor β (TGFβ) in PBMC of seven multiparous cows (three pregnant cows and four non-inseminated cows) were examined at Day 6 and Day 20 by real-time PCR. In experiment 1, ratio of increase in population of WC1.2 cell from Day 6 to Day 20 in pregnancy cows was significantly higher than that in non-inseminated cows. The cow group and day of blood sampling have no effects on the population of other T cells in PBMC. In experiment 2, mRNA expression of Foxp3 in PBMC at Day 20 in pregnant cows was significantly higher than that at Day 6 in pregnant cows and Day 20 in non-inseminated cows. The ratio of increase in mRNA expression of TGFβ in PBMC from Day 6 to Day 20 in pregnant cows was higher than that in non-inseminated cows. Expression of IL10 mRNA in PBMC was higher in pregnant cows than non-inseminated cows regardless days after estrus. It has been reported that Foxp3 mainly express in CD4(+)CD25(+)T cell and TGFβ stimulate Foxp3 expression in cattle. Increase in Foxp3 and TGFβ mRNA expression in PBMC in pregnant cows suggests that TGFβ released from PBMC induce the differentiation from CD4(+)CD25(+)T cell to CD4(+)CD25(+)Foxp3(+)T cell in pregnant cows. It is known that WC1.2(+)γδT cell show regulatory function on immune system by IL10 secretion. We observed higher expression of IL10 mRNA at Day 6 in pregnant cows before increase in population of WC1.2 cell. Therefore, IL10 might be secreted by another T cell, and may regulate maternal immune system in cows. In addition, greater population of WC1.2(+)γδT cell at Day 20 in pregnant cows may participate for establishment of maternal–fetal tolerance and pregnancy by mechanism except the IL10 secretion. Present study suggests the participation of regulatory T cells in pregnancy establishment of cows during early period after insemination.

Key Words: Cow, pregnancy, regulatory T cell, maternal-fetal tolerance

2604

Effects of prostaglandin F2α on subclinical endometritis and fertility of lactating dairy cows subjected to timed AI

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Treatment of dairy cows with prostaglandin (PG) F2α during diestrus results in luteolysis and synchronizes return to estrus, which has been suggested to enhance uterine immunity and induce mechanical cleansing of the reproductive tract. However, improvements in uterine health following PGF2α administration are often confounded with its effect of presynchronizing the estrous cycle prior to a timed AI (TAI) program. Objectives were to investigate the efficacy of PGF2α to reduce the incidence of subclinical endometritis (SCE) and improve pregnancy per AI (p/AI) in lactating dairy cows subjected to TAI. A total of 1342 Holstein cows at 25 ± 3 days in milk (DIM) were allocated randomly to receive either two treatments with PGF2α at 25 ± 3 and 39 ± 3 DIM (2PGF, n = 414), a single PGF2α treatment at 39 ± 3 DIM (1PGF, n = 474), or to remain as untreated (Control, n = 454). All cows were enrolled in the Double Ovsynch program at 48 ± 3 DIM, and received TAI at 75 ± 3 DIM. A subset of cows (n = 357) had uterine cytology samples collected at 25 ± 3, 32 ± 3 and 46 ± 3 DIM to determine the percentage of polymorphonuclear cells (PMN). Subclinical endometritis was defined by the presence of ≥5% of PMN at 46 ± 3 DIM. Vaginal discharge score (VDS) was evaluated at 25 ± 3 DIM and used to define clinical endometritis (CE). Pregnancy was evaluated at 32 and 60 day after AI. Data were analyzed using the GLIMMIX procedure of SAS. Subclinical endometritis did not affect (p = 0.20) P/AI at 32 day after AI (No SCE = 44.6 vs. SCE = 30.0%). However, cows with SCE had reduced (p < 0.001) P/AI at 60 after AI than healthy counterparts (18.0 vs. 33.9%). A greater proportion (p < 0.001) of SCE cows lost their pregnancies between d 32 and 60 (40.0 vs. 11.7%). Treatments with either one or two injections of PGF2α did not improve uterine health and fertility responses (Table 1). Cows that had combined CE and SCE at 46 ± 3 DIM had reduced (p < 0.01) P/AI compared with healthy counterparts (10.5 vs. 41.7%) and with cows that had CE only (10.5 vs. 32.5%). As expected, SCE impaired fertility of lactating dairy cows, particularly when associated with CE. Nonetheless, treatment with PGF2α before initiation of the TAI program was unable to improve uterine health and fertility in lactating dairy cows.

Key Words: Prostaglandin, uterine health, dairy cows, endometritis

Table 1. Effect of treatment with PGF2α on fertility responses of dairy cows

		Treatments		
	Control	1PGF— % (n/n) —	2PGF	p
Incidence of SCE	13.0 (15/125)	10.4 (13/125)	18.8 (22/117)	0.88
		Pregnant		
d32	38.1 (173/454)	40.7 (193/474)	41.1 (170/414)	0.39
d60	33.7 (153/454)	36.7 (174/474)	35.0 (145/414)	0.69
Pregnancy loss	11.6 (20/173)	9.8 (19/193)	14.7 (25/170)	0.65

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Exposure of pregnant ewes to a complex mixture of environmental chemicals disturbs ovarian development in their offspring

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The effects of exposure to endocrine disrupting compounds (EDCs) on reproductive function have been demonstrated in diverse species, but mainly in males. The objectives of this study were to investigate the effects of 'real life' exposure of pregnant ewes (F0: n = 8–11/group) to EDCs through grazing pastures fertilised with sewage sludge fertilizer (T) vs. inorganic fertilizer (C), during critical windows of fetal development. The effects on the fetal ovarian phenotype were analyzed in both F1 and F2 female fetuses using transcriptomic (with a custom 15K sheep oligo Agilent array), proteomic (2-DE) and histological analyses. Fetal tissues (F1 & F2) were collected on day 140 of gestation, following continuous EDCs-exposure (0–140T), or during specific developmental windows: early (0–80T), middle (30–110T) or late (60–140T) and were compared with unexposed fetuses (0–140C). Transcriptome analysis showed that in F1 fetuses, EDC exposure during the second-half of gestation induced a greater disturbance of ovarian gene expression than early exposure. Most of differentially expressed transcripts were down-regulated in a window-specific manner. Disturbed expression of a set of genes was validated by using qRT-PCR. In ovaries of animals exposed in the second-half of pregnancy, there were significantly increased numbers of type 1a follicles and type 2 follicles with signs of atresia. This gestational period corresponds to two major stages of ovarian differentiation: prophase I meiosis of germ cells and follicle formation. Analysis of the F2 ovaries (NB direct EDC exposure was of the F0 animals pregnant with the F1 generation) revealed that the number of germ cells and follicles, and gene expression patterns, were not significantly modified in fetuses whose pregnant grandmothers were exposed to sewage sludge fertilizer. However, the expression of several proteins, mostly with metabolic functions, was reduced, suggesting alterations in post-transcriptional regulatory processes. This study allows novel insights into consequences of maternal exposure to a complex mixture of low dose EDCs on ovarian development in a large mammal (non rodent) animal model. Supported by EU (FP7; REEF #212885).

Key Words: Ovary, environmental chemicals, maternal exposure, foetal development, sheep

2606

The locally cervical administrated of misoprostrol or follicle stimulating hormone (FSH) increase the mRNA expression of beta defensin 2 in the does (*Capra hircus*) cervical tissue during the estrous cycle

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The cervical relaxation is induced by the administration of Misoprostol or FSH leading to the possibility of intrauterine artificial insemination by the trans-cervically insemination technique. However, the locally cervical administration of Misoprostol or FSH may interrupt the local immune system of the cervix. Beta-defensin two is an antibiotic peptide which is locally regulated by inflammation. This study was to investigate the effect of Misoprostol or FSH on the mRNA expression of beta defensin two in goat cervix. Twenty goats were equally assigned to four groups. They were synchronised using progestagen pessaries and 250 IU PMSG at pessary removal. Intra-cervical hormone was applied at 24 or 48 h after the pessary removal: Group 1: control; Group 2: FSH 2 mg at 24 h; Group 3: FSH 2 mg at 24 h and Misoprostol 1 mg at 48 h; Group 4: Misoprostol 1 mg at 48 h. Cervices were collected 54 h after sponge removal and divided transversely into six sections then the alternate sections were stored at -20°C. The expression of beta defensin two mRNA was determined by the quantitative Reverse Transcription-Polymerase Chain Reaction technique using the beta-actin as the reference gene. Cervical tissue was analysed in three regions (vaginal, mid and uterine region). The relative expression levels of beta defensin two mRNA were analysed by ANOVA. We found that the expression of beta defensin two was greater in the cervices treated with FSH than those treated with Misoprostol ($p < 0.05$). The cervices treated with FSH combined with Misoprostol decreased the mRNA expression of beta defensin two ($p < 0.05$). We also found that the mRNA expression of beta defensin two was greatest in the uterine end of cervix and lowest in the vaginal end of cervix ($p < 0.05$). The results show the beta defensin two has the role in the local immune system in the cervical tissue of goat. The locally cervical administration of the cervical relaxant such as FSH or Misoprostol affected the local defensive mechanism in the cervical tissue. The administration of Misoprostol decreases the expression of beta defensin two which makes the cervical tissue prone to bacterial infection following the administration of induction of cervical relaxation by Misoprostol.

Key Words: Beta-defensin 2, goat, cervix, misoprostol, FSH

2607

The effect of immune modulators on endometrial cytokine expression in mares susceptible to persistent breeding induced endometritis

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Previous research has shown that 6 h after breeding is a critical time in the establishment of persistent breeding induced endometritis (PBIE), a leading reproductive health concern in equine veterinary practice. Multiple treatment strategies aimed at modulating the immune response are available; however, more information is needed to understand the mechanism of action for immunomodulatory treatment of PBIE. The specific aim of this experiment was to evaluate the effects of immune modulation with dexamethasone and mycobacterial cell wall extract (MCWE) on the inflammatory cytokines IL1B, IL6, IL10, IFNG, IL1RN, iNOS, TLR2, and TLR4, and intrauterine nitric oxide (NO) production in mares susceptible to PBIE 6 h after insemination. We hypothesized that these treatments will alter NO production and cytokine expression when compared to non-treated susceptible mares. Six mares susceptible to PBIE were inseminated during three consecutive estrus cycles with 1×10^9 killed spermatozoa (i) alone (control), or in combination with (ii) dexamethasone (50 mg iv) at the time of insemination, or (iii) with MCWE (Settle1.5 mg iv) administered 24 h prior to insemination. All mares received one treatment per cycle in randomized order, and each mare served as her own control. Uterine secretions were collected 6 h after insemination using a sterile tampon inserted into the uterus, and a 200 ml lavage was used to collect remaining secretions. An endometrial biopsy was obtained immediately after uterine fluid collection and stored in RNAlater® until further processing. NO concentrations in the uterine secretions was measured using a commercial NO assay, and total intrauterine NO was calculated using the C1V1 = C2V2 equation. Uterine biopsies were used for qPCR analysis of the inflammatory cytokines IL1, IL6, IL10, IFNG, IL1RN, iNOS, TLR2, and TLR4. Data were log10 transformed and analyzed with an ANOVA. Expression of IL1B mRNA was lower after treatment with dexamethasone ($p < 0.001$) and MCWE ($p = 0.046$) when compared to control. IFNG mRNA expression tended to decrease after treatment with dexamethasone ($p = 0.079$). No differences were detected in the mRNA expression of TLR2, TLR4, IL1RN, IL6, and iNOS after any of the treatments. Total intrauterine NO was decreased after treatment with MCWE ($p = 0.047$), and dexamethasone had no effect on intrauterine NO. In conclusion, treatment with immune modulators alters immune parameters in the uterus after insemination, which may explain how they alter uterine inflammation. A better understanding of the mechanisms of action of immune modulators will allow for continued improvements in the development of treatments targeted at reducing uterine inflammation.

Key Words: Endometritis, equine, immune modulation, uterine, cytokine

2608

Low levels of the herbicide Atrazine contaminated sow feed and water affect litter size, sex ratio of litter and increase mummified fetuses in pigs

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Herbicide contaminated feed of sow may affect the pig embryo and increase the number of mummified fetuses. The objective of this present study is investigate the relationship between the levels of the herbicide Atrazine contaminated sow feed and water in each week during gestation period and the sex ratio of litre and the number of mummified fetuses. The data from 826 l during May 2010 to August 2011 were used in this study. The sampling sow feed and water were collected every week during gestation period to monitor the level of Atrazine. Litter size, sex ratio of litter (female: male) and number of mummified fetuses were recorded. The tissue sample from mummified fetuses were collected and stored at -80°c to check the sex by polymerase chain reaction (PCR). In this present study, we found that the level of Atrazine contaminated in feed and/or water >0.3 ppb during first week to fifth week of gestation increase the sex ratio (2.3 ± 0.9 vs. 1.1 ± 0.7; $p \leq 0.05$) and decrease the litter size (9.8 ± 2.6 vs. 11.3 ± 3.1; $p \leq 0.05$). In the last period of gestation (week 12–16), the level of Atrazine contaminated feed and/or water >0.7 ppb can increase the number of mummified fetuses (3.3 ± 1.2 vs. 1.5 ± 1.4; $p \leq 0.05$) and also increase the sex ratio of total born alive piglets. Additionally, we randomly checked the sex of 100 mummified fetuses and found that the most of them are male (84 vs. 16). These results may help to improve the piglet production and use as the standard level of Atrazine contaminated feed and water in the future.

Key Words: Atrazine, litter size, sex ratio of litter

2609

Squamous metaplasia of the prostate and diffuse alopecia in a 13-year-old castrated dog due to chronic ingestion of exogenous estradiol

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A 13-year-old Italian Greyhound with a generalized progressive alopecia since 2 years was referred to our clinic to perform a low-dose dexamethasone stimulation test and abdominal ultrasound to exclude or confirm Cushing disease. The dog had previously been treated with trilostane for 4 months without having any positive effect. Earlier blood analysis showed no significant abnormalities and ACTH stimulation test was negative. Having bilateral cryptorchidism, the dog was castrated at young age. Abdominal ultrasound revealed a slightly enlarged right adrenal gland (diameter of 1 cm at the cranial part). The prostate was enlarged and heterogeneous with multiple anechogenic cavities, indicating hormonal stimulation. Craniad the prostate and ventrally of the bladder, a hypoechogenic, oval structure (3 cm by 1.7 cm) could be seen, and was suspected to be a remaining cryptorchide testicle. Fine needle aspiration however showed that the structure contained a thick purulent liquid, with large amounts of polymorhonuclear white blood cells. Blood analysis revealed a leukocytosis (29370/mm3) with neutrophilia (23202/mm3). Low-dose dexamethasone test definitely excluded Cushing disease (basal cortisol levels 2.17 μg/dl; cortisol at 4 and 8 h <1 μg/dl). A prostatic wash was performed and revealed a severe prostatitis and presence of keratinized prostatic cells, indicating a squamous metaplasia of the prostate. Measurement of seric LH levels (LH Witness®, Synbiotics) showed an LH concentration lower than 1 ng/ml, indicating a hormonal negative feedback on the hypothalamo-hypophyso-gonadal axis. After a more thorough anamnesis, it became clear that the dog was licking and ingesting a transdermal estradiol containing cream (Oestrogel®, 0.06%, Besins Int., Belgium) from his owner, since 2 years, causing his symptoms. The dog was put on antibiotic treatment for the prostatitis (enrofloxacine 10 mg/kg SID) and underwent surgery to excise the abscess cranial of the prostate. The two ductuli deferentes were attached to this structure. Histopathological analysis revealed this tissue to be embryological remanents or a morphological anomaly with a urogenital origin. Control after 4 weeks showed that the prostate slightly decreased in volume, however, anechogenic cavities where still present of which one increased in volume. The alopecia was still present as well, both probably due to a prolonged action of the estrogens. Even though injectable estrogen preparations are no longer available in veterinary medicine in many countries, a thorough anamnesis towards other exogenous estrogen sources is still necessary and can reduce the number of complementary exams performed.

Key Words: Prostate squamous metaplasia, alopecia, exogenous estradiol

2610

Post-testicular adverse effects after short-term exposure to methylmercury in rats

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Mercury, a highly harmful metal due to its cumulativeness and persistence in the environment, presents methylmercury (MeHg) as its most toxic form. Nourishment based on fish and other foods originating from MeHg-contaminated waters is the main pathway of human exposure to this organic compound. It is known that exposure to MeHg impairs the reproductive system, including decreased fertility and libido, impotence and alterations on sperm morphology and motility. The epididymis is an organ of the male reproductive tract involved in sperm transport, storage, protection, concentration and maturation. The aim of this work was to investigate whether exposure to 1, 5 and 10 mg/Kg MeHg, during the sperm transit time from the caput to the proximal cauda of the epididymis, can alter sperm quality in adult rats. The results were compared using ANOVA or Kruskall–Wallis test. Differences were considered significant when $p < 0.05$. At the end of the treatment ICP-MS technique (Inductively Coupled Plasma Mass Spectrometer) showed dose-dependent bioaccumulation of methylmercury in the epididymis. Body and sexual organ weights, plasma hormone levels (testosterone, LH and FSH), daily sperm production, sperm number and transit time in the epididymis were not affected. However, sperm motility was significantly reduced ($p < 0.05$) and signs of decreased fertility were seen in the highest dose after in utero artificial insemination [median (Q1–Q3): C = 83.33 (80–90.42) n = 10; 10 mg/Kg = 64.29 (32.42–90) n = 9; $p > 0.05$]. It was concluded that the epididymis, in this experimental model, was a sensitive organ for MeHg.

Key Words: Methylmercury, epididymis, sperm motility, reproductive toxicity, fertility

2611

Effects of vitamin C on oxidative stress induced in rat testis by BTS Antenna

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This study, was conducted to evaluate the effect of radio frequency wave (RFW)-induced oxidative stress in the testis and the prophylactic effect of vitamin C on the tissue by measuring the antioxidant enzymes

activity including: glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and malondialdehyde (MDA). Thirty-two adult male Sprague-Dawley rats were randomly divided into four equal groups. The first group as the control group and the second group was control-vitamin C group that received L-ascorbic acid (200 mg/kg of BW/day by gavage) for 45 days. The third group as the test group was exposed to RFW (900 MHz) and the fourth group as the treated group received vitamin C for 45 days in addition to exposure to RFW. At the end of the experiment all groups were killed and testis of all rats were removed and stored at -70°C for measurement of antioxidant enzymes activity and MDA. The results indicate that exposure to RFW in the test group decreased antioxidant enzymes activity and increased MDA compared with the control groups ($p < 0.05$). In the treated group vitamin C improved antioxidant enzymes activity and reduced MDA compared to the test group (0.396 ± 0.01 vs. 0.532 ± 0.03) ($p < 0.05$). It can be concluded that RFW causes oxidative stress in testis and vitamin C improves the antioxidant enzymes activity and decreases MDA.

Key Words: Vitamin C, oxidative stress, radio frequency wave, testis

2612

The effect of probiotic inovo injection in broiler breeding eggs on immune response of their broiler

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Of 360 fertilized eggs from Cobb 500 strain were grouped to three treatments and six repetitions. Of 107CFU of Protexin was injected in each egg on 18th day of incubation, the second group was injected by 0.9% of soluble sodium chloride and the third group, was the group had no injection. Immediately after hatching chicks were randomly divided in a randomized completely block design in 18 cages (three treatments × six replications). At the end of experimental period one bird was slaughtered for evaluation of lymphoid organs. At ages of 7 and 21 days two birds were injected for measuring of antibody titer against sheep red blood cells (SRBC) and 7 and 14 days after each injection, blood samples were taken to the lab. At 17th, 27th and 42th days of age, blood samples were prepared from two birds in each repetition and they were evaluated for antibody against Newcastle virus. At the end of the period to assess the number and percentage of types of white blood cells, blood sample was taken and transferred to the laboratory. There was not significant difference between treatments for antibody against SRBC on day 28, but the results related to the same headline on day 35 showed that the group Protexin had significantly higher than other two groups. The results of statistical analysis for traits such as monocytes, eosinophils and total white blood cells had not significant differences between experimental groups, but the percentage of lymphocytes in experimental sodium chloride group had significant difference with control group .For number of hetrophyls also there was significant difference between control group and sodium chloride group, but protexin and control had not significant difference.

Key Words: Probiotic (protexin), immune response, broiler

2650

A bovine serocolostrum to transfer the passive immunity in foals and calves

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Foals and calves are born agammaglobulinaemic; their survival depends on the passive transfer of maternal immunoglobulins (IgG_1) by colostrum ingestion. Currently, there is still 10–12% of neonatal mortality, half is the consequence of the failure in IgG_1 absorption through the immature gut. During the 24 first hours the gut is able to transfer a wide proportion of native IgG_1 by micropinocytosis. This transport should be enhanced by molecules present in the aqueous phase of the colostrum (serocolostrum). Hence, four serocolostrums were produced by membrane fractionation with modification in soluble molecules composition except for IgG_1 concentration. They were expected to contain factors enhancer of the IgG_1 intestinal absorption in order to be used as a colostrum replacer. Two serocolostrums were freeze-dried to be used in addition to whole and skimmed milk (foals and calves) or to IgG_1 deficient equine colostrum (DEC, foals). Animals were fed in the seven first hours after birth with 4.1 g of IgG_1/kg body weight. Control groups received equine (foals) or bovine (calves) colostrum by tube feeding. Serum samples were taken vs. time and serum concentration in equine and bovine IgG_1 was measured by single radial immunodiffusion. The half life and the absorption coefficient (AC) of the IgG_1 were calculated. The maximal IgG_1 concentration in serum was observed at 18 h after feeding. Regardless the composition of the serocolostrums, when they were given as a substitute, exactly the same failure in IgG_1 absorption was observed. The IgG_1 serum concentration was 2.4 times (˜8 g/kg, $AC = 16 \pm 5\%$) and 3.5 times (˜5 g/kg, $AC = 25 \pm 7\%$) lower in calves and foals respectively, in comparison to the control groups. When serocolostrum was mixed up with whole or skimmed bovine milk, IgG_1 serum concentration was only increased by ˜ ×1.5–1.8 for both species. When it was mixed up with the DEC, the bovine IgG_1 concentration in foal serum was increased by three ([IgG_1] ˜15 g/kg ($AC = 45 \pm 5\%$). Hence, it seems that molecular fractions (caseins and/or fat) present in colostrum but lacking in serocolostrum are involved in IgG absorption either by transfer activation or by avoiding a transfer limitation. Additional experiments are ongoing to test these assumptions. The identification of these factors should permit to better understand the mechanism of absorption in immature enterocytes. Our results also showed that bovine serocolostrum was efficient enough to compensate the failure in passive immunization in calves and foals even if the half time of bovine IgG_1 was reduced in foals.

Key Words: Bovine serocolostrum, passive immunization, calves, foals, molecules enhancer, immunoglobulin gut transfer

2651

Expression of the cytokine IL-23 in gilt endometrium after insemination with seminal plasma, spermatozoa or semen extender

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For a successful pregnancy to occur, signaling between seminal fluid and the female reproductive tract is required. Weak IL-23 p19 immunoreactivity has been detected in human endometrium but has not been described in pig endometrium. In this study, potential function for IL-23 in the inflammatory response to different insemination treatments were examined. Insemination was performed in gilts with seminal plasma (SP, n = 4), spermatozoa in the extender Beltsville thawing solution (BTS) (SPZ, n = 4) or BTS alone (n = 4). In control gilts (n = 4) the insemination catheter only was inserted. Endometrial tissue samples were collected 35–40 h after insemination. The IL-23 mRNA expression was analyzed using quantitative real-time PCR with primer and probe specific for porcine. With immunohistochemistry (IHC) the labelling of IL-23 in tissue was examined. The analyses showed that IL-23 mRNA was expressed in the endometrium. There was a significantly lower IL-23 mRNA expression in samples from gilts in the SPZ, SP and BTS treatment groups, compared with the controls which only had the insemination catheter inserted. IL-23 immunolabelling was detected in a small number of separate cells and in the subepithelial connective tissue of the endometrium. In conclusion, the results show that all fluids used for insemination decreased the expression of IL-23 mRNA in uterus compared to when only a catheter was inserted, indicating a possible role for IL-23 in the inflammatory response after insemination in gilts.
Key Words: Gilt, endometrium, IL-23 mRNA, insemination

2652

The effect of black seed (*Nigella sativa*) extract on gonadotropin, estrogen, progesterone and folliculogenesis in mice

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The black seed a member of the family of Ranunculaceae, have been employed as a spice and curative remedy for numerous disorder. This research with regard to the role of the treatment plant infertility study has been designed. Five groups of mice were selected. group 1 is control, group 2 is placebo, and experimental groups was 3,4 and 5 received IP injection of 50 100 200mg/kg per 2 day extract for 20 days respectively. All of mice received Cloprostenol, after three days plus Progesterone, for synchronization. The control group was kept in natural condition and Placebo group received normal saline.After 10 injection,blood samples were taken from all groups, hormonal measurement, including LH, FSH, estrogen, progesterone were performed by RIA technique. The results is analyzed in signification level about %95 by SPSS software. The important parameter in this research included: The ovarian weight changes, probably histological changes in ovaries, change in number of corpus luteum and follicle, density of level of FSH, LH, estrogen and progesterone. Results showed significance decrease in the level of FSH, LH also significance increase the level of estrogen in all groups, while the level of progesterone in the experimental group 2. Experimental group 3 had a significant increase weight of ovaries. Number of follicles significance increase in the experimental group 3. Also significance increase the number of corpus luteum in experimental group 2. The hydro-alcoholic extract of Nigella sativa may enhance the number of follicle and corpus luteum, that indicated it increase fertility in female mice.
Key Words: *Nigella sativa*, gonadotropin, progesterone, folliculogenesis, mice

27. Reproductive management - cattle:

2700

Reproductive performance of crossbred dairy cows under smallholder condition in Ethiopia

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A study was carried out to assess the reproductive performance of crossbred dairy cows under smallholder conditions in Asella town, Oromia regional state, Ethiopia. A total of 60 crossbred dairy cow farmers that owned 3–5 cows were interviewed using a structured questionnaire to collect information on age at first service, age at first calving, days open and calving interval. Simultaneously, reproductive performances of 250 crossbred dairy cows of the selected farmers were studied. The overall estimated mean values for age at first service, age at first calving, calving interval, days open, and number of service per conception were 24.9 ± 3.8 months, 34.8 ± 4 months, 372.8 ± 5.9 days, 85.6 ± 5.6 days and 1.52 ± 0.9, respectively. In this study, 35% of the respondents come across return rate after the first service in their crossbred dairy cows. In general, the overall observed reproductive performance of crossbred dairy cows was found to be promising considering the management situation and limited supplemental feed utilized in the area. Thus, a sustainable extension service should be established in order to improve animal feed resources management, efficient artificial insemination service and animal health care to bridge the existing gaps.
Key Words: Cattle, Ethiopia, production, reproduction

2701

Transvaginal single needle aspiration: An alternative treatment of ovarian follicular cysts in dairy cows?

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Worldwide discussions arise about the extended use of drugs (i.e. antibiotics and hormones) in veterinary medicine. The One Health concept makes that we have to be critical on the use of drugs and search for alternative treatments in veterinary medicine. In dairy cattle, the transvaginal single needle aspiration technique is used for the collection of oocytes for *in vitro* production of embryos (IVP). Alternatively, in the ambulatory clinic of the department of Farm Animal Health, the previous technique has been validated for the treatment of follicular cystic ovaries. For evaluation, in this study the transvaginal single needle aspiration technique has been compared with the conventional treatment with GnRH. Follicular cysts in cows were detected using rectal examination and ultrasound (5 MHz linear rectal probe, Esaote Pie Medical, Maastricht, the Netherlands) of the ovaries. For the aspiration of the fluid of the cyst a device consisting of two stainless steel tubes (46 cm) with outer tube diameter of 12 mm which protects the inner tube with needle, was used. The inner tube is connected inside to a silicon tubing with 20 ml syringe and at the front to a 18 gauge needle. The device is inserted into the vagina and placed in the fornix vaginae and rectally the cystic ovary is placed against the vaginal wall. The needle is inserted through the vaginal wall into COF and the fluid aspirated into the syringe.After treatment of the follicular cyst with either aspiration (n = 14) or GnRH (5 ml buserelin acetaat 0 0042 mg/ml, Receptal®, Intervet, Boxmeer, The Netherlands; n = 14) cows were evaluated for the day of 1st heat and pregnancy rates after artificial insemination (AI) at the 1st heat following treatment. Cows aspirated showed oestrus significantly earlier than GnRH treated cows, 14.5 ± 5.7 and 29.2 ± 14.4 days (mean ± SD; $p < 0.05$), respectively. Pregnancy rates of 1st AI after treatment were significantly higher in the aspirated group compared to GnRH treated cows, 78.6% and 21.4%, respectively. In conclusion, transvaginal single needle aspiration of a follicular cyst is an effective and reliable method of treatment of follicular cysts under practical conditions.

Moreover, the use of the aspiration technology may decrease the use of hormones in the cattle industry.
Key Words: Ovarian follicular cyst, COF, transvaginal single needle aspiration, GnRH, pregnancy

2702

Effects of bovine somatotropin injection on serum progesterone concentrations in nonlactating dry cows

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Research from our group demonstrated that administration of exogenous glucose and insulin increased serum progesterone (P4) concentrations in cows, likely by reducing hepatic P4 degradation. However, glucose and insulin supplementation may also increase circulating concentrations of other hormones associated with reproductive and hepatic function, including insulin-like growth factor (IGF)-I. Administration of bovine somatotropin is an alternative to increase circulating concentrations of IGF-I in cattle, independently of insulin and glucose. Therefore, the objective of this experiment was to evaluate the effects of bovine somatotropin administration on serum concentrations of glucose, insulin, IGF-I, and P4 in ovariectomized dry cows receiving exogenous P4 (controlled internal drug releasing device containing 1.9 g of P4; CIDR). Ten Gir × Holstein cows were randomly assigned to receive, on day 0 of the study, subcutaneous injections containing 500 mg sometribove zinc every 9 days (BST; n = 5), or subcutaneous saline injections every 9 days (SAL; n = 5). Treatments were applied on days 0, 9, and 18 of the experimental period (day 0 to 27). Cows were maintained on pasture and individually received 4 kg of a corn-based supplement daily at 0800 h. Cows received a CIDR prior to the beginning of the study (day-2), which remained throughout the experimental period. Blood samples were collected daily from day 0 to 27, immediately prior to (0 h), and 1 and 2 h relative to supplement feeding, for determination of serum glucose, insulin, IGF-I, and P4 concentrations. Data were analyzed as repeated measured with the MIXED procedure of SAS using cow as the experimental unit. As expected, BST cows had greater ($p \leq 0.01$) IGF-I concentrations compared to SAL cows beginning on day 2 of the experimental period (treatment × day interaction; $p < 0.01$). Hence, mean IGF-I concentrations were greater ($p < 0.01$) for BST cows compared to SAL cows (351 vs. 109 ng/ml; SEM = 26). Mean glucose concentrations were greater ($p < 0.01$) for BST cows compared to SAL cows (69.4 vs. 65.1 mg/dl; SEM = 0.8), whereas BST cows had greater ($p < 0.05$) insulin concentrations compared to SAL cows on days 8, 9, 10, 11, 16, 17, 19, 20, and 21 of the experimental period (treatment × day interaction, $p < 0.01$). However, no treatment effects were detected ($p = 0.72$) for serum P4 concentrations (1.13 vs. 1.17 ng/ml for SAL and BST cows, respectively; SEM = 0.08). Therefore, bovine somatotropin administration increased serum concentrations of IGF-I, glucose, and insulin, but did not alter serum P4 concentrations in ovariectomized dry cows receiving exogenous P4 supplementation.
Key Words: Bovine somatotropin, progesterone, cows

2703

Treatment of repeat breeder cows with intrauterine infusion of platelet rich plasma (PRP)

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Repeat breeding can be a major factor involved in infertility. Repeat breeders are cows that are cycling normally, with no overt clinical abnormalities, and which have failed to conceive after at least three successive inseminations. The identification of all the causes of a repeat breeding problem can be difficult since the repeat breeder syndrome can be a problem due to management or to a variety of individual cow problems. Strengthening the estrus detection programs, the use of embryo transfer during summer heat stress, the administration of GnRH at insemination, and the administration of hCG following AI could reduce the number of repeat breeders, but not all cases respond to treatments. The PRP is an important source of growth factors and chemokines that promote angiogenesis, fibroplasia, matrix deposition and ri-epithelialization, and could be a growth factors-rich supplementation also for the embryo. In our *in vitro* experience, the culture of bovine embryos with 5% PRP and 5% fetal calf serum (FCS) resulted in significantly higher blastocysts production compared to control with 10% FCS. For this reason, the purpose of this study was to evaluate the effect of intrauterine infusion of PRP on the conception rate in repeat breeder dairy cows. To produce PRP, 450 ml of whole blood from the mammary vein of different cows were collected into bags containing CPDA-1. The whole blood was centrifuged at 100 *g* for 30 min and the supernatant was further centrifuged at 1500 *g* for 10 min. The resulting platelet pellet was resuspended with poor platelet plasma to obtain a concentration of 1×10^9 platelet/ml. The PRP was frozen at -70°C and thawed at room temperature for three times to promote the release of platelet-derived growth factors. One hundred and thirty repeat breeder cows, free of any significant detectable pathologic disorders associated with the reproductive tract, were included in the study. The cows were divided into two groups. Group A (n = 65) was treated with 5 ml of heterologous PRP 48 h after artificial insemination (AI). Group B (n = 65), control group, did not receive PRP after AI. Pregnancy diagnosis was performed by ultrasound 36-days after AI. Conception rates were 64.62% (42/65) in group A and 32.31% (21/65) in group B and the difference was statistically significant ($p < 0.05$). At ninety days after AI, no statistically difference in embryo mortality between treated and control groups was evident (4.78% in group A compared to 9.5% in group B). These preliminary data suggest that intrauterine infusion of PRP may improve the conception rate, making the uterine environment more appropriate for pregnancy. PRP contains growth factors and serotonin that could be involved in the release of prolactin, in the enhancement of luteal function and in the P4 increase during early pregnancy. P4 would favor the maintenance of pregnancy in repeat breeders that usually are susceptible to early abortions.
Key Words: Platelet rich plasma, repeat breeder, intrauterine, conception rate

2704

Reproductive efficiency and possible risk factors for poor reproductive performance in cattle herds bred naturally in the Lake Chad basin of Nigeria

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A survey was conducted in the Lake Chad basin in north-eastern Nigeria during 2006–2008 to determine the reproductive efficiency of cattle herds bred naturally under traditional husbandry system in this part of the country. It is hypothesized that the reproductive performance of these cattle is low and the risk factors for this lowered performance is not known. This study was achieved via questionnaire interviews administered to farmers. Out of the 500 questionnaires administered to herd owners, 350 (70%) responses were received. Data collected from 350 herds comprising of 7930 breeding cows and heifers in government and private ownership, managed by either semi-intensive, intensive or pastoral-nomadic systems were analysed using Epidata (Epidata association, Denmark) to test the hypothesis. Calving interval in 55% of these herds was 480–600 days, with a calf crop of 44%. Most cases of abortion (69%) occurred in mid-gestation, with 10% being recorded during early pregnancies. The occurrence of abortion was more under the pastoral than the semi-intensive system. It is therefore concluded that the reproductive efficiency of cattle herds in the lake Chad Basin of Nigeria is low; and it is characterized by the occurrence of abortions, extended calving interval and low calf crop yield. The risk factors associated with this poor performance include poor level of nutrition, harsh climatic conditions, poor genetic content, poor level of education of farmer and underlying reproductive diseases; but farmer education was considered the major risk factor. Increased farmer awareness and veterinary care, improvement in nutrition and cross breeding with exotic cattle of superior genetic content is recommended for increased reproductive efficiency.

Key Words: Cattle, efficiency, Lake Chad basin, Nigeria, reproduction

2705

Prepartum supplementation with rice bran and crude glycerine improves the productive and reproductive perfomance of beef cows in pastoral system

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In extensive rangeland cow-calf systems, the forage availability and quality of native pastures limits the prepartum energy intake with negative effects on postpartum performance of cows. The aim of this work was to evaluate the effect of prepartum supplementation with crude glycerine and rice bran, a readily available alternative for farmers, on early postpartum (60 day) production responses of beef heifers. The experiment was conducted in the Experimental Station Bernardo Rosengurtt, Uruguay (32°21′SL; 54°26′W) from winter 2011 to summer 2012. Fifty-seven pregnant Hereford, Angus and their crosses, heifers with 5.0 ± 0.1 U of body condition score (BCS, scale: 1–8) and 416 ± 5 kg of body weight (BW) were used in a randomized block design according to genotype, expected calving date, BCS and BW. At 52 ± 2.3 day before expected calving date, cows were assigned to two nutritional treatments with two replicates in each group: (i) SUP (n = 29) supplementation until calving with 500 ml of crude glycerine (biodiesel industry, ALUR, Uruguay: glycerol: 85%; methanol: 2%) and 1 kg of dry matter/cow/day of rice bran (13% of crude protein) and (ii) CON (n = 28) no supplementation. All cows grazed a native pasture paddock divided in four (forage allowance: 10% of the BW during all the experiment). Every 20 day cows BCS was recorded. After calving, milk production, milk composition and calves weight were registered. Ultrasounds studies were performed at 65 day after mating period. Data were analyzed in a mixed model using repeated measures. Reproductive data were analyzed using generalized model specifying the binomial distribution and logit transformation of data. Supplementation did not affect the birth weight of the calves (p = 0.9) nor calving difficulty (p = 0.6). BCS at calving was greater in SUP than in CON cows (4.8 vs. 4.1 ± 0.1 units; p = 0.002). The means of milk production and fat content during the first 60 day of postpartum were greater in SUP than in CON (7.9 vs. 7.0 ± 0.3 kg; p = 0.02; 3.9 vs. 3.6 ± 0.1%; p = 0.04, respectively). No differences between SUP and CON were observed in protein or lactose milk content. Milk production was greater in crossbreed than in pure breed cows (7.9 vs. 7.0 ± 0.3 kg; p = 0.03) and in those sucking male than female calves (7.8 ± 0.2 vs. 7.00 ± 0.3 kg; p = 0.04). The growth rate of SUP calves was greater than CON (0.85 vs. 0.77 ± 0.02 kg; p = 0.01). A correlation between milk production and growth rate was found (r = 0.26; p = 0.003). At 60 day of age, SUP calves were 2 kg heavier than CON, but this difference was not significant (p = 0.1). Supplementation did not affect the pregnancy rate (CONT: 47 vs. SUP 57%; p = 0.5) but more (p = 0.0047) cows had resumption ovary cyclicity in SUP (93%) than in CONT (47%). The use of crude glycerine and rice bran as a pre partum supplementation improved the productive and reproductive performance of primiparous beef cows grazing native pastures and could be an alternative for farmers and for the biodiesel industry.

Key Words: Beef cow, glycerine, milk production

2706

Factors influencing the chance of cows being pregnant 30 days after the voluntary waiting period

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Most reproductive performance indicators are affected by management decisions such as the voluntary waiting period (VWP). We have previously studied a herd reproductive performance indicator that is adjusted for the herd VWP; i.e. the proportion pregnant cows after the herd WVP plus 30 days. The objective of this study was to verify if the established factors affecting reproduction also affects this adjusted indicator at the cow level, i.e. pregnant or not at the herd VWP plus 30 days. Cows that calved between 1 July 2008 and 30 June 2009 and originated from herds that had more than 50 milking cows on average were available for inclusion in the study, in total 132 721 dairy cows. VWP was calculated for each herd as the days postpartum by which 5% of the cows in the herd had received a first insemination. The associations between the outcome variable and the predictor variables were analyzed by generalized estimating equations models. The analysis was stratified by parity and in this abstract we are reporting the analysis on first parity cows only. Results are given in the table below and the odds ratio for pregnancy at VWP plus 30 days are shown. We also tested if dystocia, insemination type, milk urea (mM), organic management and the use of total mixed rations affected the probability of pregnancy, but none turned out to have significant effects on first parity cows. Our results show that well-known associations apply also for this reproductive performance indicator that adjusts for a management factor (VWP). Accordingly, this makes the indicator a suitable tool in the preventive herd health work.

Key Words: Performance indicators, dairy cow, voluntary waiting period

Table 1 Associations for pregnancy at VWP plus 30 days

Factor	OR	Factor	OR
S. Red breed vs. S. Holstein	1.15	High MY[2] vs. Low MY	0.71
Disease repr. vs. No repr. disease	0.81	Autumn vs. Spring	1.19
Disease vs. No disease	0.71	High SCC[3] vs. Low SCC	0.87
High FPR[1] vs. Low FPR	0.79	Twin birth vs. No twin	0.69
Free stalls vs. Tie stalls	1.44		

[1]Milk fat/protein ratio, [2]Milk yield, kg, [3]Somatic cell count.

2707

Effect of age at weaning and plane of nutrition before weaning on progesterone secretion and uterine gene expression at first service in beef heifers

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Different weaning ages and planes of nutrition may have long-term effects on reproductive performance. We test the hypothesis that luteal function and uterine gene expression are affected by age at weaning and plane of nutrition before weaning by assigning Hereford females calves (n = 21) to three different treatments between 2 and 5 months of age: (i) early weaning at 2 months of age (EW, n = 8); (ii) late weaning at 5 months of age (LW, n = 6) and (iii) LW plus creep feeding (LW + CF, n = 7). After five months of age all calves were exposed to the same management system. The number of luteal phases were calculated based on the presence of a corpus luteum and progesterone (P4) concentrations >1 ng/ml. At the age of first service (23 months) estrus was synchronized (Day 0) and uterine biopsies were taken, to study by SYBR-Green quantitative real time PCR the expression of IGF-I (days 7 and 16) and IGF-II (day 16) mRNA using HPRT and RPL-19 as control genes. Blood samples were taken daily to measure P4 from Day 0 to the day of biopsies in two consecutive cycles. Data were analyzed using a linear mixed model and means were considered to differ when p < 0.05. The numbers of luteal phases before cycle synchronization were similar for LW + CF (3.4 ± 0.6), LW (2.7 ± 0.5) and EW (2.4 ± 0.5) heifers, but mean P4 concentrations were higher in LW + CF than in LW and EW during the synchronized cycles (Table 1). Expression of IGF-I and IGF-II mRNA (Table 1) was greater in LW + CF than the other two groups of heifers (EW and LW). On Day 16, all heifers expressed more IGF-I mRNA than on Day 7.Our results suggest that the plane of nutrition may have a long-term effect on the luteal function and the expression of uterine

IGF-I and IGF-II at first service with potential implications for embryo development.
Key Words: Nutrition, progesterone, IGFs, uterus

Table 1. Progesterone (P4) concentrations and uterine gene expression of IGF-I and IGF-II in EW, LW and LW + CF heifers on Days 7 and 16 of the cycle (LSmeans ± SEM)

	EW	LW-CF	LW+CF
P4 (ng/ml)	3.1 ± 1.1[a]	4.5 ± 1.1[a]	7.8 ± 1.2[b]
	Day 7		
IGF-I	0.56 ± 0.07[a]	0.67 ± 0.07[a]	0.93 ± 0.08[b]
	Day 16		
IGF-I	0.60 ± 0.11[a]	0.60 ± 0.13[a]	1.67 ± 0.13[b]
IGF-II	1.18 ± 0.22[a]	0.86 ± 0.26[a]	1.93 ± 0.23[b]

[a] vs. [b]$p < 0.05$.

2708

Effect of age at weaning and the plane of nutrition before weaning on growth and somatotropic axis gene expression in the liver of beef heifers

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Endocrine IGF-I is produced by the liver through the union of growth hormone to its hepatic receptor (GHR), and with its binding proteins (IGFBP) acts systemically to stimulate the development of several body tissues. Different age at weaning and planes of nutrition before weaning result in differential daily gain but may also cause permanent changes in the functioning of the somatotropic axis, through alterations in the hepatic expression of the GH-IGF axis. To test this hypothesis Hereford females calves (n = 21) were assigned to three different treatments between 2 and 5 months of age: (i) early weaning at 2 months of age (EW, n = 8); (ii) late weaning at 5 months of age (LW, n = 6), and (iii) LW plus creep feeding (LW + CF, n = 7). After 5 months of age all calves were exposed to the same management system. Body weight (BW) was recorded from 2 to 23 months of age. At the age of first service (23 months) estrus was synchronized (day 0) and liver biopsies were taken on days 7 and 16, to study by SYBR-Green quantitaive real time PCR the mRNA expression of GHR, IGF-I, IGFBP3 and IGFBP2 using HPRT and ß-actin as control genes. Data were analyzed using a linear mixed model and means were considered to differ when $p < 0.05$. Average daily gain from two to five months of age was greater for LW+CF (1.05 ± 0.09 kg/day) than for LW (0.76 ± 0.10 kg/day) and EW (0.68 ± 0.11 kg/day) calves. Therefore, at 5 months of age, LW + CF calves (168 ± 6.5 kg) were heavier than EW calves (142 ± 6.7 kg) and tended to be heavier than LW calves (150 ± 7.0 kg, $p = 0.07$). By the time of first service all heifers had similar BW (LW + CF: 375 ± 11.7 kg; LW: 382 ± 12.6 kg, and EW: 380 ± 10.9 kg). The hepatic expression of the GHR mRNA tended to greater on Day 16 than on Day 7 (0.44 vs. 0.33 ± 0.05, $p < 0.10$). On Day 7, LW + CF heifers tended to have a higher GHR mRNA expression than LW heifers (0.46 ± 0.08 vs. 0.21 ± 0.09, $p = 0.06$). The hepatic expression of IGF-I, IGFBP3 and IGFBP2 did not differ among treatments. Data show that these pre-weaning differential managements had no effect on BW at first service, but, CF imposed during early development had a long-term effect on the hepatic expression of GHR mRNA with potential implications for their performance.
Key Words: Nutrition, weaning, GH-IGF-I, liver

2709

Hormonal and metabolic profile of crossbred Holstein Zebu cows in the transition period

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Crossbred cows (Bos taurus × Bos indicus) are a great part of Brazilian dairy herd. Production systems vary in presence of calf in milking parlour, pasture management, concentrate supplementation and sanitary and reproductive management. Although there are differences between crossbred Holstein Zebu and Holstein cattle, many times management measures adopted are based on Holstein cattle research because of few researches conducted with crossbred animals. These study objectives were to evaluate mobilization of body reserves and metabolic and hormonal profile during transition period in crossbred Holstein Zebu cows, as well as measure milk production and composition and progesterone concentrations postpartum to indicate return to ovarian activity (active corpus luteum). Seventeen crossbred Holstein Zebu cows (parity three to eight; 4130 kg milk/lactation), with calving date between June and July 2010, that is the dry season in Brazil, were used in this experiment (ethical committee UFMG – 40/2007). Blood samples were collected 4 h after feeding to measure glucose, insulin, IGF-1, NEFA and progesterone concentrations. Sampling was at days ten and five prepartum and two, five, ten, and then weekly postpartum until 60 days in milk (DIM). Weight and body condition score (BCS) were also recorded on the same days. Milk production measure and sampling for composition were performed weekly until 60 DIM. A randomized block design was utilized for statistical analysis, with analysis of variance and means tested with SNK ($p = 0.05$). Milk production was 18.7 ± 3.6 kg/day (4.1% fat, 3.2% protein, 4.6% lactose, 12.8% total solids). Lowest weight and BCS occurred on day two postpartum, with loss of 72.2 kg and 0.66 point of BCS, representing mostly loss of calf and placenta. NEFA concentrations were highest between two and 17 days postpartum (0.484 ± 0.425 mM), what shows this was the most intense period of negative energy balance. Even with high NEFA concentration, metabolic diseases weren't observed in the transition period. After day 24 NEFA concentrations were not different from prepartum concentrations ($p > 0.05$). Lowest insulin concentration was observed in day five postpartum (6.6 ± 3.0 μU/ml) ($p < 0.05$) and highest glucose concentration at day two postpartum (66.1 ± 10.6 mg/dl) ($p < 0.05$). IGF-1 concentration was not different between pre and postpartum periods (133.4 ± 132.0 ng/ml) ($p > 0.05$). After calving, progesterone concentrations were low until 38 DIM, when it reached 1.03 ± 0.77 ng/ml, indicating existence of functional corpus luteum. This delay in ovarian activity postpartum when compared to Holstein cows is probably not exclusively related to negative energy balance, as concentrations of glucose, insulin, NEFA and IGF-1 were equal to prepartum values after 24 DIM. Research supported by FAPEMIG - APQ 01005/08.
Key Words: Glucose, insulin, NEFA, IGF-1, progesterone

2750

Effect of meloxicam on pregnancy rate of recipient heifers of *in vitro* produced embryos with different degrees of cervical manipulation difficulty

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The aim of this study was to evaluate the influence of meloxicam, a prostaglandin synthesis inhibitor, on pregnancy rate of recipient heifers of *in vitro* produced embryos with different degrees of cervical manipulation difficulty. The experiment was performed using 207 *in vitro* produced Nelore embryos and 207 recipient heifers (Bos

taurus × Bos indicus), nuliparous, which were evaluated to the body condition score (BCS) from 1 to 5, and those which presented BCS between 2.5 and 3.5 were selected and randomly allocated in two groups, G1 (control) and G2 (treated with meloxicam). During inovulation, recipients were classified according to the degree of difficulty in passing the inovulator through the cervix, in Grade I (easy) and Grade II (difficult). Immediately after inovulation, recipients of G2 received an i.m. injection of 200 mg of meloxicam (Maxican® 2%, Ouro Fino®, Brazil). Statistical analysis was made using Chi-square test and results are summarized on Table 1. There was no difference on pregnancy rate considering animals which presented grade I cervix independently If the treatment was performed or not ($p = 0.22$). There was statistical difference ($p < 0.01$) between pregnancy rate of G1 (49.0%) and G2 (66.7%). Animals which presented Grade II cervix, pregnancy rate was superior for the group that received the treatment ($p < 0.01$).These results reinforce the hypothesis that probably due to the increase of endometrial prostaglandin production in response to prolonged cervical/uterine manipulation required in females with a higher degree of difficulty in passing the inovulator; meloxicam administration is highly recommended during inovulation process.

Key Words: Heifer, meloxicam, embryo, inovulation, prostaglandin

Table 1. Pregnancy rate between animals classified as Grade I and Grade II, considering G1 (control) and G2 (treated with meloxicam 2%)

	Cervix Grade I	Cervix Grade I	Cervix Grade II	Cervix Grade II
Group	n	Pregnancy rate n (%)	n	Pregnancy rate n (%)
1	72	39 (57.3)[a]	30	11 (21.1)[b]
2	45	29 (42.6)[a]	60	41 (78.8)[c]
Total	117	68 (56.7)	90	52 (43.3)

Different letters in the same column indicate statistical significance (Chi-square test, $p < 0.01$).

2751

Influence of progesterone and progesterone associated with hCG on pregnancy rate of Nelore cows used as recipient of *in vitro* produced embryos

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There is evidence for dairy cows that progesterone supplementation of animals at risk of low embryo survival rate as a result of progesterone insufficiency improves embryo survival rates. These animals represent 44% to 25% of the population. The aim of this study was to evaluate the influence of progesterone supplementation and progesterone supplementation associated with hCG on pregnancy rate on day 35 using of Nelore cows as recipient of *in vitro* produced embryos. Multiparous Nelore cows (n = 54) were evaluated to the body condition score (BCS) from 1 to 9, and those which presented BCS between five and eight were selected and randomly allocated in three groups, named GP4 (n = 17), GP4 + hCG (n = 18) and control group (n = 19). All protocols and treatments initialized on the same day. Animals were allocated in the same property and were raised extensively. On D0, synchronization of estrous cycle was achieved with aid of the following protocol: on D0, all animals were treated with a progesterone vaginal implant with 1.9 g of progesterone combined with an i.m. injection of 2 mg of estradiol benzoate. On D9, implants were withdrawn and the animals received an i.m. injection of 12.5 mg of dinoprost, an i.m. injection of 0.6 mg of estradiol cipionate and an i.m. injection of 750 IU/mg of eCG. On D13 a 1.9 g progesterone vaginal device was inserted on G1 animals; on G2, a progesterone vaginal device was inserted on D13 associated with an i.m. injection of 2500 IU of hCG. On D18 progesterone vaginal devices were withdrawn and inovulation was performed. Twenty seven days after embryo inovulation, pregnancy was determined with real-time ultrasonography using a 7.5 MHz transrectal transducer. Data were analyzed using completely randomized design of ANOVA. There was no statistical difference between pregnancy rate and treatment ($p < 0.05$) as summarized on table 1. Results are presented as means ± SEM. In this preliminary study, results demonstrate that progesterone supplementation or progesterone supplementation associated with hCG does not increase pregnancy rate of Nelore cows used as recipient of *in vitro* produced embryos. Although these results do not agree with those experienced by dairy cows, other experiments need to be performed to clarify the effect of different animal husbandry systems on pregnancy rate associated with the supplementation of progesterone and hCG.

Key Words: Progesterone, hCG, embryo, cow, Nelore

Table 1. Pregnancy rate of Nelore cows recipient of *in vitro* produced embryos ($p < 0.05$)

Group	Pregnancy rate ± SEM
Control	36.66 ± 0.49
P4	17.64 ± 0.39
P4 + hCG	22.22 ± 0.42

2752

Effect of estrus synchronization on interval to estrus and pregnancy of bull-bred, post-partum beef cows

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The study was designed to compare the intervals to estrus and pregnancy between estrus synchronized and control cows after natural bull service. The beef herd consisted of 144 animals; 31% primiparous cows, 36% between 3 and 4 years of age and 33% ≥5 years of age. Animals were blocked by calving date and sire group, and randomized to one of three treatment groups: an untreated control (n = 49), two doses of prostaglandin (PGF, Estroplan; Vétoquinol, QC, Canada) 14 days apart (n = 48) or a progesterone releasing intravaginal device (PRID; Vétoquinol) for 7 days followed by PGF at removal (n = 46). Cows were turned out with bulls on the day of second PGF injection or PRID removal. All cows were examined by transrectal ultrasonography three times at weekly intervals prior to turn-out to determine the presence of a corpus luteum (CL). In addition, body condition score (BCS) was recorded using a 5-point scale and a blood sample was collected. The interval from calving to turnout ranged from 32 to 102 days. A total of six single, mature sire groups were used with a bull to cow ratio of 1:24. All bulls were subjected to a breeding soundness examination and were classified as satisfactory. Heat detectors (K-mar) were applied at turnout and monitored daily for the first 21 days to determine the time of first estrus. After a breeding period of 60 days, cows were examined by ultrasonography for pregnancy. Fetal age was estimated by combining the date of estrus and fetal crown-rump length or trunk diameter. The cows were re-examined for pregnancy 50 days later. Cox proportional hazards models were used to compare the interval to estrus and pregnancy, using SAS software. Age, BCS, days from calving to turnout and serum copper and vitamin E concentrations were evaluated as potential confounding variables. Cows were clustered according to sire group. Pregnancy rates did not differ between treatments ($p = 0.86$) or sire groups ($p = 0.41$). Cows treated with a PRID showed estrus 2.4 times sooner than the control or PGF-treated cows ($p < 0.01$). In addition, age, serum copper levels and the calving to turnout interval were associated with the interval to first estrus ($p < 0.05$). Although the interval to pregnancy did not differ among treatments ($p = 0.38$), the median interval from turn-out to pregnancy was 14, 16 and 6.5 days for the Control, PGF and PRID groups, respectively. Cows with a BCS ≥2.5 were four times more likely to have a CL at turnout and became pregnant three times earlier ($p < 0.05$). An increase in serum copper of 0.5 ppm resulted in cows becoming pregnant 1.25 times earlier. Although cows treated with PRID + PGF showed estrus earlier in the breeding season, there was no difference in the interval from turnout to pregnancy. Further research is needed to increase the number of cows per group and to determine if a higher bull ratio is needed during the first cycle for synchronized cows.

Key Words: Estrus, synchronization, bull, breeding

2753

Progesterone determination in milk in grazing Holstein cows as a predictive tool for reproductive efficiency

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Holstein cows (n = 884; maximum consanguinity = 2%, 1–3 lactations) of seven commercial farms in a grazing dairy production were selected to conduct a field study to estimate phenotypic associations between commencement of postpartum luteal activity after calving (PPLA) and luteal function, cow category (parity and age or calving interval, CI), body condition score (BCS) at calving and change of BCS from calving to 60 days postpartum (DPP; ΔBCS), milk yield and reproductive parameters. Primiparous cows were categorized according to age (20–28 months, 28–35 months, >35 months) and multiparous cows according to CI length. All cows were, within each farm, classified in low, medium or high (33rd percentiles) milk yielding cows. Cow BCS was registered twice a month from -30 to 120 DPP. Milk samples were taken twice a week from 10 to 90 DPP for progesterone (P4) determination. A luteal sample was considered when P4 concentrations in skim milk were ≥1 nM and PPLA was considered as the interval between calving and the first luteal sample. Means were considered to differ when $p \leq 0.05$. Probability of pregnancy within 100 DPP was affected by farm, cow category, BCS at calving and ΔBCS, and luteal function (number of luteal samples/total milk samples), but did not differ due to milk yield. The PPLA tended ($p = 0.066$) to be longer while the probability of pregnancy within 100 DPP was less in younger than older primiparous cows. Multiparous cows with longer CI (380 < CI < 540 days and CI >540 days) had longer PPLA than cows with shorter CI (<380 days). Multiparous cows with CI >540 days had a lower probability of pregnancy within the first 100 DPP than CI <380 days and 380 <CI <540 days. Multiparous cow data suggest that fertility is a repeatable trait. Concentrations of P4 in skim milk luteal samples did not differ among cow categories, but increased with BCS at calving (2.4 nM per unit) and ΔBCS (1.8 nM per unit), and tended to be affected ($p = 0.08$) by the interaction between cow category and milk yield. In older primiparous and multiparous CI <380 days cows, concentrations of P4 were lower for low than high milk yielding cows whereas in multiparous CI >540 cows, concentrations were greater for low than high milk yield cows. Cow BCS at calving had a major effect in all reproductive parameters: calving to first service interval decreased 36 days and pregnancy rates within 100 DPP increased from 4% to 43% as BCS at calving increased from 2.25 to 3.25 unit. The luteal function also affected reproductive parameters: an increase from 0 to 1 of luteal samples/total milk samples,samples implied a decrease of 67 days in calving to first service interval. To our knowledge, this is the first field study that analyzes fertility based on P4 determinations in milk, BCS at calving and change of BCS during the first 60 DPP. This may allow a better prediction of the reproductive performance of dairy cows.

Key Words: Dairy, postpartum anestrus, pregnancy

2754

Early weaning in primiparous autumn-calving cows in low body condition score: effects on body condition, nefa and insulin concentrations and ovarian cyclicity

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In rangeland, when age at first mating is 18–20 m old, heifers generally calve during late summer-early autumn and calves suckle during winter when native pastures have low availability and quality, reducing milk yield and weaning weight of calves. Also, these cows are weaned and rebred in the following spring, since they have low probability to reinitiate ovarian cyclicity during autumn-winter. However, an early weaning (EW) may advance the reinitiation of ovulation allowing cows to rebred in autumn. The aim of this experiment was to evaluate the effect of an EW on different productive parameters. At 70 days postpartum sixteen primiparous autumn-calving cows (crossbred Angus × Hereford) with a body condition score (BCS, scale 1–8) of 4.1 ± 0.1 unit, were assigned to two treatments: calves suckling ad libitum (S, n = 7) and calves that were weaned from their dams (EW, n = 9). All cows were in anestrous at the time treatments commenced (Day 70; Day 0 = calving) and they were managed together on native pastures with a forage allowance of 10%. Calves from S group were weaned on Day 210 (end of winter). BCS was measured every 14 days from Day 70 (April-22) to 285 (December-1, onset of AI). Cows were blood sampled weekly from Day 56 to 140 and biweekly from Day 140 to 294. Presence of corpus luteum (CL) was recorded every 14 days by ultrasonography from Day 70 to 285. BCS, NEFA and insulin were analyzed by repeated measures using the MIXED procedure with time as the repeated effect. Probability of cycling cows was analyzed using generalized linear models using the GENMOD procedure. Cows BCS decreased in both groups from the beginning of the experiment until Day 169 reaching an average of 3.4 u. Then it increased until the end of the experiment, but cows from EW group presented greater ($p < 0.05$) BCS than C cows from Day 225 to 385 (average 4.0 ± 0.1 vs. 3.6 ± 0.1 u for W and S, respectively). NEFA concentrations increased ($p < 0.0001$) between Day 126 and Day 182, decreasing after that, until the end of the experiment. Insulin concentrations varied along the experiment ($p < 0.0001$) but no differences were found between treatments (average along the whole experiment: 7.31 ± 0.34 and 7.61 ± 0.30 for S and EW, respectively). EW cows had more probability to cycle at Day 211 than S cows (44 vs. 14%, $p < 0.05$) and at day 285 (100 and 71% cows had CL in EW and S respectively; $p < 0.005$). During winter (from Day 113 to 210) NEFA concentrations increased and BCS decreased, reflecting a mobilization of body reserves in all cows. EW in primiparous autumn-calving cows with low BCS was not successful in inducing ovulation during the early postpartum period. However EW cows had greater BCS and more probability to ovulate at the end of the spring when AI started that would give them more chances to be pregnant compared with S cows.

Key Words: Autumn calving, early weaning, ovarian cyclicty, nefa, urea

2755

EFfect of body condition and supplementation during postpartum on the onset of ovarian activity and pregnancy rate in crossbred cows (*Bos taurus* × *Bos indicus*) under tropical conditions

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The availability of nutrients in the tropics is a limiting factor on reproductive variables in livestock. The reproductive behavior of the cow during postpartum (PP) has been linked to body condition (BC), which at the same time, responds to changes in energy metabolism. A research was conducted to evaluate feeding level (FL) at PP and BC on blood glucose concentration (GLUC), uterine involution (UI), ovarian activity (OA), and pregnancy rate (PR) on postpartum cows under tropical conditions. Thirty-two crossbred cows (Bos taurus × Bos indicus) fed with graze pasture (Erlochloa polystachya, Cynodon nlemfuensis and Cynodon dactylon) were randomly assigned to the following treatments (T), using a combination of BC at calving and FL during PP, as follows: T1: HBCHFL, high BC to PP (BC: ≥2.5) and high FL PP (HFL); T2: LBCHFL, low BC to PP (BC: <2.5) and HFL; T3: HBCLFL, HBC and low FL PP (LFL); and T4: LBCLFL, LBC and LFL. The BC was measured every 15 days PP (DPP) until 120 DPP. The feed ration was made by a supplement based on sorghum, maize, cotton seed, urea, salt, molasses, bi-calcium phosphate plus a mineral block (BM).The expression of glucose transporters (Glut-1) in adipose and muscle biopsies was assessed by Western blot analysis. Reproductive activity and UI were evaluated by transrectal palpation and ultrasound (Aloka SSD 900 Co. Ltd., Tokyo, Japan) from 15 to 90 DPP, once a week before onset of heat and twice a week after heat, using a 7.5 MHz linear probe. The UI,

characteristics of cervical mucus (CCM), symmetry of uterine horns (SUH), uterine position (UP), and cervix diameter (CD) were studied. Ovarian structures were studied by ultrasound. Ovarian follicles were classified according to their diameters in: Class 1 (≤5 mm); Class 2 (6–9 mm), and Class 3 (≥10 mm). The PR was determined at 120 DPP. The GLUC was analyzed by both a General Lineal Model (GLM) for repeated measurements in time and ANOVA, evaluating T effect on GLUC. Reproductive variables were analyzed by MANOVA, where Class 3 follicles (FOL3) and CL were dependent variables and T was a factor. The survival analysis (SA) for FOL3, CL, and PR appearance was made at 30, 45, 60, 75, 90, 105, and 120 DPP. The GLUC concentration was higher in T2 (49.3 ± 5.2 mg/dl) and T1 (47.4 ± 5.3 mg/dl) groups compared to T4 (41.8 ± 4.3 mg/dl) and T3 (48.3 ± 5.3 mg/dl), respectively. The CD caused a significant ($p < 0.01$) effect on FOL3 and CL and T on FOL3. The eighty porcentge of the cows in T3 and T1 groups had shown FOL3 at 60 and 80 DPP. In more than 50% of T3 and T1 groups, the CL tended to appear 60–90 DPP. The PR was higher in T3 (60%) and T1 (80%) groups compared to T4 (50%) and T2 (50%). The Glut-1 expression was higher in animals that received a low FL. These results show that body condition is a determinant factor on reproductive performance in crossbred cows in grazing pastures under tropical conditions.

Key Words: Crossbred cows, reproduction, body condition, nutrition

28. Reproductive management - other ruminants & pigs:

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Nutritional supplementation with 140 g of mesquite together with the male effect improve reproductive performance of anoestrous ewes

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In the arid Hidalgo region of central Mexico, sheep have poor fertility because they are managed under extensive conditions on pastures of poor nutritive value. The development and transfer of simple and inexpensive technologies to improve their fertility will bring significant economic benefits to these subsistence farmers. Nutritional supplementation using regional leguminae and the male effect are simple techniques that can increase ovulation rates and induce synchronized ovulation in anestrous sheep. The aim of the present trial is to evaluate using nutritional supplementation with 140 g of bean pods from Mesquite (Prosopis spp). This is an alternative to soy bean, a proven, but unaffordable alternative. This together with the male effect could improve fertility of anoestrous ewes. From February 24th to March 24th eighty one mixed breed (Dorset × Hampshire × Suffolk) adult anoestrous ewes (median BCS = 2.5) were exposed to three sexually-active rams (treated with 16 h light from November 1st to January 15th) and were fed maize straw supplemented with (i) a traditional diet: of 730 g of rolled maize, 15 g of soy bean, 10 g of Lucerne, 0.20 of marine salt and 0.10 g of a commercial mineral mix (n = 27); (ii) a Maize-Soy diet: of 950 g of Lucerne, 290 g of rolled maize and 140 g of soy bean (n = 27) or (iii) a Mesquite diet: consisting of 950 g of Lucerne, 290 g of rolled maize and 140 g of mesquite pods (n = 27). The diets were offered to the animals at 9:00 h and 17:00 h beginning at 9:00 h on the day the males were introduced to the flocks and ending at 17:00 h 2 days after mating. Ovulation rate was determined by trans-rectal ultrasound 7–10 days after mating. The ewes in the Mesquite group responded sooner ($p < 0.05$) with oestrous at 7.1 ± 0.06 (mean ± SEM) after the introduction of sexually-active males compared with 16 ± 0.15 and 20.0 ± 0.05 days for maize-soy and traditional diets respectively. There were no differences ($p > 0.05$) in ovulation rate (1.3 ± 0.02; 1 ± 0.02 and 1 ± 0.02 for the traditional, Mesquite and Maize-Soy diets respectively). These results show that the inclusion of 140 g of Mesquite is an effective alternative to soy bean for the nutritional supplementation of anoestrous ewes. Mesquite advanced the day of oestrous but had no effect on ovulation rate and at the same time the use of Mesquite reduced by 23% the cost of the nutritional supplementation. Supported by Universidad Autónoma Metropolitana Iztapalapa 1440905 and PROMEP 14411146 Mexico.

Key Words: Anoestrous ewes, nutritional supplementation, male effect, ovulation rate, mesquite

2801

Reproductive variables of Murrah buffaloes according to different level of milk production

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World milk production has increased in the last decades, and the buffalo milk industry has accompanied this increase, so some aspects such as keeping of a herd animal husbandry is of crucial importance for growth and interventions when necessary to improve the productive and reproductive rates of herds. This study was conducted in a dairy buffalo farm located at the district of Taipu-RN, Brazil Latitude -05°37′18″ and Longitude 35°35′48″ during 2011. Fertility and milk yield data, comprising 177 lactation records, over a period of 1 year, were used to assess the interrelationship between calving interval (CI) and level of production. Lactating buffaloes were placed into low (≤5 l/buffalo/day; n = 25), medium (> 5 ≤ 10 l/buffalo/day; n = 108) and high (> 10 l/buffalo/day; n = 44) yielding groups. Data (average ± SE) were analysed by analysis of variance (ANOVA) and the means were compared by the *T*-Test using the statistical program SPSS 16 for Windows. Fertility parameters such as calving interval (CI) and serves per conception for buffaloes conceiving (S/C) were significantly lower ($p < 0.001$) for low yielding buffaloes than medium and high yielding buffaloes, 391.33 ± 2.06 days; 420.83 ± 6.17 days and 457.00 ± 8.79 days, respectively. High yielding buffaloes presented a calving interval 66 days longer than low yielding buffaloes while medium yielding presented 29 days longer. Low yielding buffaloes had higher first service conception rate (FSCR) when compared to medium and high yielding buffaloes, 60.7 ± 2.68; 60.8 ± 2.27; 57.21 ± 2.6, respectively, ($p < 0.002$). High yielding buffaloes required 0.36 extra inseminations per conception when compared to low yielding buffaloes. The interval calving first service was significantly lower for the group of low yielding (48.3 ± 2.68) when compared to medium (55.8 ± 1.17) and high yielding (54.21 ± 1.32) buffaloes ($p < 0.001$). Overall, the high yielding buffaloes needed 07 extra days after calving to present the first service than low yielding herd-mates. Peak lactation for high, medium and low yielding were 15.2 ± 0.16; 13.6 ± 0.16 and 10.7 ± 0.06 l/day ($p < 0.001$). From the data presented it is clear that high-producing buffaloes have grater impairment of reproductive parameters, so further monitoring is necessary to minimize losses and maximize economic production indices.

Key Words: Buffalo, milk production, calving interval

2802

Number of fetuses influences the accuracy of pregnancy scanning in ewes

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Pregnancy scanning is considered an important tool in sheep breeding, and the number of sessions performed in Sweden has increased continuously, using transabdominal (B-mode) ultrasonography. Most

breeds used in the Nordic countries produce on average approximately 2 lambs/ewe which is generally higher than in breeds used in other countries. The recommendation is that the examination should be performed at day 40–80 in gestation. The aim of this study was to examine the accuracy of pregnancy scanning in ewes in Sweden, i.e. the correspondence between number of fetuses determined at scanning and the actual number of lambs born, and to study factors that could influence a possible discrepancy found between those. This study was based on data obtained from the database Elitlamm, a recording system for sheep owners. A total of 44 783 observations were registered during 3 years (2008–2010) and the number of fetuses were counted in 39 724 ewes diagnosed as pregnant. The ewes aged between 0.5 and >6 years (four age classes) and belonged to six breed groups: Gotlandic, Finnsheep, Texel, beef crossings, landrace crossings and other crossings. The effects of various factors on the difference between number of lambs born and number of fetuses diagnosed at the time of scanning were analyzed by analysis of variance (SAS, proc GLM) and the accuracy of the scannings (% with exact number of fetuses diagnosed) were analyzed by log-linear models (SAS, proc LOGISTIC). The accuracy of determination of number of fetuses at scanning decreased with the number of fetuses diagnosed at time of examination ($p < 0.001$). The highest accuracy 93.7% (n = 10 245) was seen in ewes with only one fetus diagnosed at scanning. The accuracy in ewes with two fetuses at scanning was 91.9% (n = 21 465), three fetuses 82.4% (n = 7 222), and in ewes with ≥4 fetuses 70.8% (n = 792). From a subset of complete data (n = 23 677), breed, age of ewe, and gestation stage, significantly affected the accuracy. Finnsheep ewes gave birth to the largest number of lambs and also showed the lowest accuracy ($p < 0.001$). Accuracy decreased with age of the ewe ($p < 0.001$). The accuracy was 45.9%, 91.2% and 88.2% when ewes were examined <40 days, between 40 and 80 days and >80 days of gestation, respectively ($p < 0.001$). It was more common that the numbers of fetuses were overestimated, especially when there were ≥2 fetuses. The number of fetuses diagnosed at the time of scanning was overestimated in 21.6% of the cases when ≥4 fetuses were diagnosed. In conclusion, pregnancy scanning with ultrasonography is a technique with high accuracy and thus, a useful tool to improve management of pregnant ewes and at lambing. However, our study shows that the accuracy is influenced by number of fetuses, age and breed of the ewe, as well as gestation stage. Such results should be taken into consideration by the sheep owner when interpreting and implementing the scanning results.

Key Words: Ultrasonography, accuracy, transabdominal, reproduction, ultrasound

2803

Flushing with extruded of soybean and crude glycerine increased the percentage of twins in ewes under extensive system conditions

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Nutrition is the environmental factor that exerts the greatest influence on fertility, ovulation rate and prolificacy in ewes. Diets rich in energy and protein previous to the mating period increase the ovulation rate. The aim of the present work was to evaluate the effect of a short period of supplementation with extruded of soybean and crude glycerine previous the mating period, a readily alternative for farmers, on pregnancy rate and the percentage of ewes lambing twins in grazing condition. The study was conducted during April 2011 (breeding season) at the Experimental Farm Bernardo Rosengurtt, Uruguay (32°21′ SL). One-hundred-and-eighteen adult, Corriedale, body weight (BW): 45.2 ± 0.38 kg (mean ± SEM) and body condition score: 4.11 ± 0.03 BC units (BCS, scale: 1–5), were used in a randomized block design according to BCS and BW and assigned to two treatments: (i) supplemented ewes (SUP, n = 59) with extruded of soybean (BIOPROST®, BIOGRAN, Uruguay, 40.5 g crude protein, 2.9 Mcal/kg) and 300 ml of crude glycerine (biodiesel industry, BIOGRAN, Uruguay: glycerol: 31%, methanol: 20%) individually early in the morning during 6 days of the late luteal phase (9–15 day from estrous cycle) and (ii) non supplemented ewes (NSUP, n = 59). Animals grazed together a native pasture paddock (1484.5 kg/dry matter/ha forage mass available, crude protein 57 g/kg DM, 57% of digestibility, and a relation green/dry: 60/40%). During all the experiment, the forage allowance was 6% of dry matter/100 kg of BW. The estrous cycle was synchronized with two intra-muscular doses of 40 μg of prostaglandin (PG, Dalmaprost®, Lab. Fatro, Uruguay) 8 day apart. After the second dose of PG, estrous was detected every 12 h, using vasectomized rams. Twelve hours after detection of estrous, cervical AI was performed using fresh semen. Pregnancy diagnosis was performed by ultrasound (Aloka SSD 500; Corp. Ltd, with a 7.5 Mhz transducer) 30 and 60 day after AI. Reproductive data were analyzed using generalized model specifying the binomial distribution and logit transformation. Data of BC and BW were analyzed in a mixed model using repeated measures. Supplementation did not affect the BC of the ewes (4.19 ± 0.03 vs. 4.16 ± 0.03 units, NSUP and SUP, respectively; $p = 0.5213$) nor the BW (46.6 ± 0.5 vs. 46.3 ± 0.5 kg, NSUP and SUP, respectively; $p = 0.7435$). The pregnancy rate at first service was no different between treatments (86 vs. 83%, SUP and NSUP, respectively, $p = 0.7714$) but the percentage of ewes lambing twins was greater in SUP than in NSUP ewes (27 vs. 12%, $p = 0.0455$). No triplet lambing was observed. No clinical effects were observed during supplementation period. Supplementation of days 9–15 of estrous cycle with extruded of soybean associated with crude glycerine increased (15%) significantly the number of ewes lambing twins.

Key Words: Flushing, ewes, nutrition, protein

2804

Adding lupins to pre-mating diets improved potential litter size of gilts mated during the seasonal infertility period

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Reduced litter sizes are a common manifestation of seasonal infertility, and are likely due to a suppressed pattern of episodic luteinising hormone (LH) release prior to ovulation. Dietary sugar beet pulp increased LH pulse frequency and embryo survival in gilts (Ferguson et al., 2007. Reproduction 133:433–439), and we have previously demonstrated improved oocyte developmental competence in gilts fed a lupin fibre rich diet during the seasonal infertility period of summer and early autumn (Weaver et al., 2011. Manipulating Pig Production XIII; 168). The objective of this study was, to determine whether feeding gilts a lupin-fibre based diet during summer and early autumn would improve potential litter size. From 160 days of age and 107.2 ± 0.69 kg live weight (LW) until artificial insemination at their second oestrus Large White cross terminal line gilts received 3 kg/day of one of two dietary treatments (n = 31 gilts per diet). The two diets were a standard female finisher diet (Control) supplying 13.25 Megajoules (MJ) digestible energy (DE) per kg, 15.3% crude protein (CP), and 3.9% crude fibre (CF) and a lupin based high fibre diet (Lupin; 20% whole lupins and 15% lupin hulls) supplying 13.22 MJ DE/kg, 18.6% CP and 11.8% CF. Commencing at 169 days of age, 20 min of daily, physical boar contact was used to stimulate puberty attainment and maintain oestrous cyclicity, with gilts artificially inseminated at their second oestrus. Following their second oestrus, gilts received 2.2 kg/day of the control diet and were housed individually until slaughter 28.7 ± 0.19 days after their first insemination. There was no effect of treatment on mean days to puberty (10.3 ± 0.86 days), the proportion of gilts expressing puberty (80%) or LW at second oestrus (134.0 ± 1.41 kg). The number of corpora lutea (ovulation rate) was similar for Control and Lupin gilts (15.4 ± 0.42 and 15.4 ± 0.48). However, gilts fed the lupin diet had significantly ($p < 0.05$) more embryos (13.8 ± 0.75 vs. 11 .2 ± 0.65) and a higher embryo survival rate (0.91 ± 0.04 vs. 0.77 ± 0.04) than their control counter parts. The observed increase in embryo survival in gilts fed the lupin fibre diet prior to mating supported our previous data demonstrating that lupin fibre based diets improve oocyte developmental competence. These data support the relationship between improved oocyte developmental competence and embryo survival *in vivo*. Further, it is evident that adding lupins to gilt rearing diets during summer improved potential litter size.

Key Words: Seasonal infertility, litter size, dietary fibre

2805

Effects of pre-mating conditions on subsequent piglet birth weight and uniformity

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Two important factors affecting piglet survival are piglet birth weight and piglet uniformity. It has been shown that pre-mating nutrition, i.e. feed intake and feed composition, can affect piglet birth weight and uniformity. We studied effects of pre-mating conditions which affect the metabolic state of the sow, such as lactation length, lactational backfat loss and length of the weaning-to-estrus interval (WEI), on subsequent piglet birth weight and uniformity. At the IPG research farm, mean birth weight and uniformity of piglets (birth weight SD and CV of total born) of 2128 litters (>4 total born, WEI ≤7 day, non-repeat breeders) was measured, and analyzed with the MIXED model procedure of SAS 9.2. The statistical model included parity (1, 2, 3 + 4 and ≥5), genetic line (Topigs20 and Topigs40), total number of piglets born, and either lactation length (≤24 day, 25–27 day, >27 day) or lactational backfat loss (≤2 mm, 2–5 mm, >5 mm) and its interactions with parity and genetic line as explanatory variables, and the factors sow and farrowing batch as random effects. In a separate dataset, piglet uniformity of sows with a WEI of 8–21 day and a WEI >21 day (incl. repeat breeders) was compared with sows having a WEI ≤7 day, using the same model. Piglet birth weight was not affected by lactation length, lactational backfat loss or WEI (Table 1). Lactation length did not affect subsequent piglet uniformity. Only in Topigs20 sows, birth weight SD and CV increased with previous lactational backfat losses (for Topigs20 sows with backfat losses of ≤2 mm, 2–5 mm, and >5 mm, resp., SD was 285, 297 and 310 g ($p = 0.04$) and CV was 20.6, 21.6 and 22.4% ($p = 0.05$)). Furthermore, birth weight SD and CV were lower for sows with a WEI >21 day (incl. repeat breeders) compared with sows having a WEI ≤7 day (Table 1), even when not corrected for litter size. We conclude that pre-mating conditions can affect subsequent piglet uniformity. Severe backfat losses during lactation may have negative consequences for subsequent piglet uniformity, although consequences may differ between breeds. A prolonged WEI (incl. repeat breeders), i.e. a recovery period after weaning, seems beneficial for subsequent piglet uniformity.

Key Words: Piglet birth weight, piglet uniformity, pre-mating conditions, sow

Table 1. Effects of a prolonged WEI (incl. repeat breeders) on litter characteristics of total born piglets at birth

	WEI 7 day	WEI 8–21 day	WEI >21 day + repeat breeders	SEM	p-value
Number of litters, n	1584	72	182		
Total number born, n	13.7[a]	14.9[b]	14.4[b]	0.3	<0.01
Mean birth weight, g	1428	1438	1431	17	0.83
SD of birth weight, g	310[b]	291[ab]	287[a]	7	<0.01
CV of birth weight, %	22.2[b]	20.8[ab]	20.5[a]	0.5	<0.01

[ab]Within rows, values lacking a common superscript differ ($p \leq 0.05$).

2806

Lactation weight loss in mildly feed restricted sows and its relation with embryonic survival and metabolic blood parameters during lactation and gestation

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From literature it is known that feed or protein restriction during lactation negatively affects metabolic hormones and metabolites during lactation and also affects embryonic survival. This study aims to describe relations between embryonic survival and metabolic blood parameters during lactation and early gestation in sows with a high or low weight loss during lactation. At weaning, 40 pregnant first parity sows were divided into one of two weight loss classes, based on the median weight loss; (i) low weight loss (LWL, ≤13.8%, n = 22) and (ii) high weight loss (HWL, >13.8%, n = 18). From day 10 before weaning till day 35 of gestation, blood samples were taken from 29 sows at 3–4 days interval. Two sows were omitted from the analyses on blood parameters due to faulty catheters. Embryonic survival at day 35 was 12% lower for HWL than for LWL sows (65.6 ± 3.4% vs. 77.4 ± 3.0%, $p = 0.001$, respectively), resulting in 1.9 fewer vital embryos (14.9 ± 0.7 vs. 16.8 ± 0.7, $p = 0.007$, respectively). Plasma IGF-1 profiles were similar for HWL and LWL sows during lactation and gestation. Plasma IGF-1 concentrations were highest on day 4, 8 and 11 after weaning, then decreased up to day 20 after weaning, after which they stabilized to values slightly lower than during lactation. Average IGF-1 concentration during lactation was positively correlated with IGF-1 concentration during gestation ($r = 0.46$, $p = 0.01$). Average NEFA concentrations during lactation tended to be higher in HWL than in LWL sows (989 ± 75 vs. 816 ± 67 μm/l, $p = 0.10$, respectively) and were higher in HWL than in LWL sows on day 5 before weaning (1180 ± 81 vs. 702 ± 81 μm/l, $p = 0.01$, respectively). After weaning, NEFA concentrations sharply declined and were similar for HWL and LWL sows. NEFA concentrations during lactation were positively correlated with NEFA concentrations during gestation ($r = 0.51$, $p = 0.07$). Plasma urea profiles were similar for HWL and LWL sows during lactation and gestation. Concentrations of Urea were highest on day 4 after weaning and then decreased to day 10 after weaning to values slightly lower than during lactation. On day 4 after weaning, plasma urea concentrations were lower in HWL than in LWL sows (5.3 ± 0.2 vs. 6.3 ± 0.2 mM, $p = 0.0001$). Average urea concentration during lactation was positively correlated with average urea concentration during gestation ($r = 0.67$, $p = 0.0001$). NEFA, IGF-1 and urea levels during lactation or pregnancy were not related to embryo survival ($p > 0.10$). This study showed that lactation weight loss affected embryonic survival at day 35 and slightly affected lactation NEFA concentrations and plasma urea concentrations on day 4 after weaning. However, embryonic survival was not related with metabolic parameters during either lactation or early gestation.

Key Words: Pigs, lactation, metabolism, weight loss

2807

Seasonal influence on the culling pattern of gilts and sows in Thailand

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The aim of the present study was to evaluate the seasonal influence on the culling pattern of gilts and sows in commercial swine herds in Thailand. Data of 33 366 gilts and sows removed from seven swine herds between 2006 and 2009 were included. The collected data included animal identities, culling date, parity number at culling and reason for culling. Seasons were classified as hot (15 Feb to 14 Jun), rainy (15 Jun to 14 Oct) and cool (15 Oct to 14 Feb). The females were sent to be slaughtered in parity number 0 (14.1%), one (14.7%), two (8.2%), three (7.9%), four (8.1%), five (9.2%), six (12.9%), seven (13.6%) and 8–14 (11.3%). Of these pigs, 93.2% were sent to slaughter

house, 6.8% were found death and 0.02% were euthanized. The reasons for culling consisted of reproductive disorders 38.1%, old age 34.7%, illness/sudden death 10.2%, lameness 10.1% and miscellaneous causes 6.9%. Reproductive disorders included no heat (10.5%), abnormal vaginal discharge (8.9%), not being pregnant (8.6%), abortion (6.3%), dystocia (1.6%) and others (2.1%). The culling of gilts and sows due to reproductive disorders varied among herds from 27.6% to 55.1%. Reproductive disorders accounted for 75.0% of the culled gilts, 55.7% of the culled primiparous sows, 39.4% of the culled sow in parity 2–6 and 4.3% of culled sow in parity 7–14. The frequency of gilts and sows culled due to reproductive disorders in cool (41.0%) and rainy (40.5%) seasons was higher than that in hot season (33.4%, $p < 0.001$). In conclusion, the gilts and primiparous sows were culled mainly due to reproductive disorders. Season significantly influenced the frequency of gilts and sows culled due to reproductive disorders in Thailand.

Key Words: Pig, season, culling, reproduction, management

2808

Development of species-specific vaccines for population control of wild pigs using recombinant phage

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The world-wide expanding populations of wild pigs have created economic, health, and welfare problems. Humane alternatives that are practical, effective, inexpensive, and safe are needed to augment current control methods. Our focus is on development of contraceptive vaccines for wild pigs using filamentous phage as a delivery vector for immunogenic peptides with contraceptive properties. In this study, multiple phage-peptide constructs [where the peptides mimic sperm epitopes that bind to zona pellucida (ZP) proteins] were generated via selection from a phage display library using a novel approach. The selection procedures were designed to allow for identification of ZP-binding phage clones with potential species-specific properties. Species specificity is an important feature for a wildlife vaccine as the goal is to control overpopulation of the target species only and not to affect non-target species reproduction. Six phage-peptide antigens were injected intramuscularly into domestic pigs (three animals/antigen) and corresponding immune responses evaluated. Administration of the antigens into pigs stimulated production of anti-peptide antibodies, which were proven to act as anti-sperm antibodies by immunostaining of boar semen samples. Potentially, such anti-sperm antibodies could interfere with sperm delivery or function in the male or female genital tract, leading to contraceptives effects. The treatment groups were compared using one-way ANOVA at a significance value of $p \leq 0.05$. Although antibody responses varied in the first part of the experiment (months one, two and three), no statistically significant differences were found among groups of pigs immunized with different antigens for the last three bleedings (months 4, 5, and 6). Staining of semen samples collected from different mammalian species, including pig, cat, dog, bull, and mouse, with anti-sera from pigs immunized with ZP-binding phage allowed identification of phage-peptide constructs with different levels of species specificity. Based on the intensity of the immune responses as well as specificity of these responses in different species, two of the antigens with fusion peptide sequences GEGGYGSHD and GQQGLNGDS were recognized as the most promising candidates for development of contraceptive vaccines for wild pigs. The ability of the phage-peptide antigens studied here to stimulate production of antibodies which bind to sperm cells suggests that their administration to wild pigs might lead to contraceptive effects.

Key Words: Recombinant phage, population control, wild pigs, anti-sperm antibodies, species-specific vaccine

2809

Effect of egg weight on embryonic development and hatchability of Naked neck chickens

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This study was conducted to determine the effect of egg weight on egg weight loss, fertility, embryonic mortality, hatching yield and hatchability of Naked neck chickens (Gallus gallus domesticus). A total of 900 Naked neck chicken eggs collected from the same breed and age, under the same feed and management practices were used for this study. The eggs were classified according to three weight groups (A : >50 g, B: 45–50 g, and C: <45g). A complete randomized design of three treatments, five replicates with each replicate having 60 eggs was used for the experiment. Total, early, mid and late embryonic deaths were significantly ($p < 0.001$) affected by the weight of the chicken eggs. Egg weight loss, hatching yield (percent of fertile eggs) and hatchability (percent of total number of eggs incubated) were also influenced ($p < 0.001$). However, egg weight had no effect ($p > 0–05$) on egg fertility rate. The small size eggs (group A) had the least mortality (10.79%), the highest hatching yield (87.76%) and hatchability (88.16%). This might be attributed to egg weight loss of this group during incubation. This may imply that sorting indigenous Naked neck chicken eggs prior to incubation might be advantageous in production operation aimed at improving the productivity of these chickens.

Key Words: Embryonic deaths, hatchability, hatching yield, naked neck

Table 1 Embryonic development and hatchability parameters

	A	B	C	Mean	SE
Egg weight (g)	55.16[c]	47.88[b]	39.62[a]	47.55	2.654
Fertility rate (%)	98.22	98.62	98.41	98.22	0.172
Total embryonic deaths (%)	31.32[a]	27.91 [b]	10.79[c]	21.67	2.980
Early embryonic deaths (%)	60.64[a]	50.87[b]	46.89[c]	52.71	2.060
Mid embryonic deaths (%)	14.14[c]	17.60[b]	18.70[a]	16.81	0.687
Late embryonic deaths (%)	22.82[c]	30.77[b]	34.33[a]	29.31	1.701

[a,b,c]Means in the same row not sharing a common superscript are significantly different ($p < 0.05$). SE: Standard error.

2850

Species variations in success of treatment of endometritis and conception rates following infusion of Lugol's iodine in buffalo (*Bubalus bubalis*) and cows

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Infusion of Lugol's iodine intrauterine for treatment of uterine infections is an established practice, however, excess volume and concentration of the drug may damage the uterine epithelium. The present study compares the effect of infusion of Lugol's iodine intrauterine in cows and buffaloes. Data was collected retrospectively from 201animals (137 buffaloes and 64 cross-bred cows) kept under similar management conditions at an organized dairy farm, suffering from clinical endometritis. Lugol's iodine (av. vol. 30 ml; conc. 0.25%) was infused once or more times in these cases. Following first infusion, 36.49% buffaloes and 42.19% cows conceived at first observed oestrus. Seven (5.10%) buffaloes and three (4.68%) cows conceived at second service, whereas further 5.10% (07) buffaloes and 15.63% (10) cows conceived subsequently. An average of 5.6 and 5.8 services for

buffaloes and cows, respectively were required giving an overall conception rate of 46.71% in buffaloes and 62.5% in cows. A major complication observed in 27% (37) buffaloes and 4.68% (03) cows was development of ovaro-bursal and/or uterine adhesions after Lugol's iodine infusion. None of the cows whereas 7.29% (10) buffaloes developed uterine adhesions after first infusion. Another 6.66% cows and 38.46% buffaloes developed uterine adhesions after second infusion. Thereafter number of animals developing adhesions increased with each infusion, buffaloes ranging from 45 to 100% up to seventh infusion while cows ranged from 20 to 33% only. With higher number of infusions, services per conception also increased. The cows subjected to second, third and fourth infusion had 5.72, 10.7, 6.0 services per conception which was 4.4, 5.08, 6.0 respectively in buffaloes. Though many factors may be responsible for development of uterine adhesions in buffaloes, inherent susceptibility of buffalo genitalia to irritant drugs can be the reason. Coiled genitalia in buffalo might have led to prolonged stay of Lugol's iodine for longer period in the uterus and hence development of inflammation and adhesions. It is thus concluded that Lugol's iodine though effective to control uterine infections in dairy animals can be harmful if infused repeatedly. Buffaloes are more susceptible to develop genital adhesions after infusions of Lugol's iodine warranting its controlled use in this species.
Key Words: Lugol's iodine, buffalo, cattle, adhesions

2851

Male goat testosterone secretion is stimulated in February-April after exposure to only 1.5 months of long days in December

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The sexual activity of local male goats from subtropical Mexico (latitude, 26°23′N) can be induced during the non-breeding season (February–April) by exposure to 2.5 months of long days from November 1st followed by natural photoperiod. The objective of the current study was to determine whether a reduction of long days from 2.5 to 1.5 months stimulates the testosterone secretion during the non-breeding season. All males were allocated to open pens. The control group was under natural photoperiodic variations (n = 5); the second group (n = 5) was submitted to 2.5 months of long days (16 h of light by day) from November 1st; the third group (n = 5) was subject to 1.5 months of long days from December 1st. From January 16th onwards, males from these two light-treated groups were kept under natural photoperiodic variations. Testosterone plasma concentrations were measured once a week from October to May. The mean date (± SEM) of increase in testosterone secretion (> 5 ng/ml) did not differ between males exposed to 2.5 or 1.5 months of long days (2.5: February 14 ± 4 days; 1.5: February 17 ± 1 days; $p > 0.05$). In control group, the increase in testosterone secretion occurred later at the onset of natural breeding season (May 16 ± 4 days; $p < 0.01$). The mean date of decline in testosterone secretion occurred later in males exposed to 2.5 than in those exposed to 1.5 months of long days (2.5: April 23 ± 4 days; 1.5: April 7 ± 3 days; $p < 0.01$). The maximal plasma testosterone concentrations did not differ between males exposed to 2.5 or 1.5 months of long days (2.5: 11 ± 1 ng/ml; 1.5: 10 ± 1; $p < 0.05$). These results allow us to conclude that 1.5 months of long days followed by natural photoperiod stimulate onset and maximum of testosterone secretion, as do 2.5 months of long days. However, the duration of high testosterone plasma concentrations is shorter when males are exposed to 1.5 than in those exposed to 2.5 months of long days.
Key Words: Caprine, photoperiod, androgens, subtropics

2852

Improving the artificial insemination management in Mediterranean sheep: a field trial in Comisana and Massese breeds

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In Mediterranean countries artificial insemination (A.I.) in sheep is usually performed using cervical insemination with ram semen diluted in milk based extender and stored at 15°C due to its simplicity and satisfactory results. The fertility of ewes after A.I. is affected by many factors such as intervals between lambings, season, age of ewe, heat stress, nutrition state or breed. The ram may greatly influence fertility after cervical insemination, particularly when cooled semen is used. In this study, two field trials were conducted during breeding and non-breeding season to evaluate various factors affecting the fertility in artificially synchronized Comisana and Massese ewes after cervical insemination with semen refrigerated at 15°C or 4°C. Fertility was assessed by pregnancy rate evaluated ultrasonographically 50 days after insemination. A total of 132 ewes (80 Comisana and 52 Massese) and 13 rams (seven Comisana and six Massese) were used in this study. In a first trial, two timing of insemination (50 and 55 h after sponge removal) and two extenders for semen refrigeration (milk and milk + egg yolk) were evaluated. In a second trial, three semen refrigeration storage protocols (15°C for 4 h and 4°C for 24 and 48 h) were compared. Treatment for inducing oestrus and ovulation was the same in both the trials. Sperm quality was evaluated by semen analysis (concentration, motility and membrane integrity) after storage in different conditions. Results of the first trial showed no significant differences in pregnancy rate with respect to time of insemination and extenders (51% and 51.5% as average, respectively). Concerning the experiment 2, when the semen of Massese breed was stored at 4°C a significant ($p \leq 0.05$) lower motility (30%) and membrane integrity (17.7%) was reported as compared to the semen stored at 15°C (65% and 48% respectively). On the other hand, no significant difference in semen quality was reported in Comisana breed under the same condition. In Massese breed no pregnancy was reported after the semen was stored at 4°C, while in Comisana breed no significant differences in pregnancy rate was reported among semen stored at 15°C for 4 h or 4°C for 24 and 48 h. In conclusion, our results showed the possibility to store ram semen at 4°C for 24 or 48 h using extender with milk and egg yolk without compromising the pregnancy rate after cervical insemination. This can suggest new strategies for improving genetic programs for milk yield and composition in Mediterranean sheep breeds.
Key Words: Sheep, artificial insemination, fertility, cooled semen, sperm quality

2853

Estrogen and progesterone receptors in the vagina of cycling ewes and anoestrous ewes trated with GnRH with or without progesterone priming

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Estrogen and Progesterone receptor (ER, PR) concentrations were investigated in the vagina of cycling and anoestrous Corriedale ewes treated with GnRH with or without Progesterone (P) priming. Six cycling ewes were killed at Day 1 (n = 3) and 6 (n = 3) after oestrus detection. Twenty two anoestrous ewes were assigned to two groups: GnRH (n = 11) and P+GnRH (n = 11). The GnRH ewes were treated every 2 h with 6.7 ng of GnRH (i.v) for 16 h, followed by bolus injection of GnRH (4 μg) at 18 h on Day 0. The P+GnRH ewes were treated with 0.33 g of P (CIDR) for 10 days and after CIDR removal they were treated according to the same protocol than in the GnRH ewes. Ewes were killed on Day 1 (n = 6, for each treatment) and Day 5 (n = 5, for each treatment) after bolus injection. Samples of vagina and blood were taken for receptors and P determinations when the ewes were killed. The ER and PR determinations were performed by ligand binding assay. The ligands used were 3H-E2 for ER or 3H-

ORG-2058 for PR, while nonlabelled ligands were diethylstilbestrol and ORG-2058 respectively. The ER, PR and P concentrations (mean ± polled SE) were analyzed by ANOVA. In cycling ewes, the P concentration was lower on Day 1 than to Day 6 (7.1 ± 0.9 vs. 0.71 ± 0.6 nM respectively, $p \leq 0.001$). There was a significant effect of day on the ER and PR concentration ($p \leq 0.05$). On Day 1 the ER and PR concentration were higher than Day 6 (267 ± 33, 310 ± 19 and 130 ± 33, 127 ± 19 fmol/mg protein). In anoestrous ewes, the P concentrations on Day 5 were higher in the P + GnRH ewes than in GnRH ewes (6.5 ± 0.54 vs. 3.1 ± 0.61 nM respectively, $p \leq 0.001$). There was a significant effect of treatment, day and interaction between them on the ER concentrations while for PR only interaction between treatment and day was significant ($p \leq 0.05$). The ER and PR concentration on Day 1 were similar in P + GnRH and GnRH ewes (159 ± 10, 123 ± 16 and 116 ± 10, 98 ± 16 fmol/mg protein). On Day 5 ER and PR were lower in the P + GnRH ewes than in the GnRH ewes (55 ± 11, 60 ± 18 and 155 ± 11, 152 ± 18 fmol/mg protein), and in the P + GnRH ewes ER and PR decreased from Day 1 to Day 5. In GnRH ewes was increased from Day 1 to Day 5.The decrease in ER and PR from Day 1 to Day 6 in cycling ewes may be due to the inhibitory effect of higher circulating levels of P in agreement with those reported in the uterus, cervix, and pituitary gland of cycling ewes. Similar inhibitory effects were found at Day 5 in the vagina of P + GnRH ewes. In contrast, in the GnRH ewes alone trated ewes, ER and PR concentrations increased from Days 1–5, showing a different regulation of ER when P is not given previously. These results suggest that P primed anoestrus ewes exhibited ER and PR concentration profile similar to those of cycling ewes. However, in ewes treated with GnRH alone this is not observed, suggesting an alteration in the concentration of ER and PR might be involved in a different microenvironment unfavorable to their functions.

Key Words: Vagina, ewes, cycling, anoestrus, profile

2854

Effect of feed suplementation on conception rate and embryonic survival in alpacas

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Alpacas live in highland over the 4000 m with a deficient quality and quantity of pastures. Reproductive performance is deficient with a rate of birth less at 50% and different factors may be involved in these poor reproductive performance. The study was carried out with the objective of evaluate the effect of a feed supplementation before service on the conception rate and embryonic survival in alpacas. One hundred ninety four alpacas adults of 4–6 years were assigned to four treatments: T1 (n = 49): Alpacas lactating + supplementation; T2 (n = 48) Alpacas lactating without supplementation; T3 (n = 49): Alpacas non lactating + supplementation and T4 (n = 48): Alpacas non lactating without supplementation. Body weight and body condition score were registered before initiation of experiment and each two weeks. Animals of treatment 1 and 3 were supplemented with two kg of alfalfa hay and 500 00 UI/ml of Vitamin A, 75 000 UI/ml Vitamin D3 and 500 mg/ml Vitamin E four weeks before initiation of experiment. Animals were maintained under natural pasture. Ultrasound examinations with a rectal probe of 7.5 MHz were performed on mating day (D0) and D2, D9, D20, D30 y D60 post mating. Conception rate was evaluated with Fisher test. Follicular size before mating was 9.1 ± 0.7, 8.3 ± 0.5, 8.2 ± 0.6 and 7.9 ± 0.8 to T1, T2, T3,T4 with differences between T1 and T4 ($p < 0.05$); Corpus luteum size: 13.6 ± 1.1; 11.2 ± 0.9; 11.6 ± 1.3 and 10.8 ± 1.9 to T1, T2, T3, T4, different T1 and T3 and T4 ($p < 0,05$); conception rate D20: 65.6; 51.7; 52.9 y 51.1% to T1, T2, T3, T4 different between T1 respect T2, T3 and T4 ($p < 0.05$). D30: 62.5; 37.9; 47.1 y 33.3% to T1, T2, T3, T4 different T1 respect T2, T3 and T4 ($p < 0.05$); D60: 62.5; 34.5; 47.1 y30.3% different T1 respect T2, T3 and T4 ($p < 0.05$). Feed supplementation before mating is important to conception rate to D20 but would be more important to embryo and fetal survival.

Key Words: Alpacas, reproduction, nutrition

2855

The effect of a low prolactin dose administered per os on mortality and body gain of piglets

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Prolactin (PRL) is a multifuncional signaling hormone best known for its role in lactation and physiological adaptations during pregnancy. Its biological actions are not limited to reproduction, it also controls a variety of behavioral patterns and plays a significant role in homeostasis. In our recent study (Gajda et al., 2011. *Acta Biochim. Polon.* 58, suppl. 4) we demonstrated the positive effect of 0.75 or 1.5 mg of PRL administered per os to the piglets at the onset of parturition on their viability and body gain during the first 70 days after birth. Therefore, the objective of this study was to determine the effect of a low PRL dose administered per os on the viability and body weight of piglets during the first 10 weeks of their life. The prolactin (Biolactin, Biochefa, Poland) was isolated from freeze-dried pig hypophyses. A total number of 330 piglets was used in the experimental (n = 161 piglets, 14 litters) and control (n = 169 piglets, 14 litters) groups. The experimental group received 0.15 mg of PRL and the control group received a physiological salt solution administered per os. Body weight and mortality of piglets at birth and at 21, 28 and 70 days after birth in the experimental and control groups were calculated. The average body weight of piglets at birth and at 21, 28 and 70 days after birth were 1.45, 5.41, 6.85 and 23.50 kg in the experimental and 1.44, 5.21, 6.62 and 23.27 kg in the control group, respectively (differences statistically non-significant). The mortality of piglets at 0 to 21, 28 and 70 days after birth in the experimental group were lower in comparison to the control group (13.0 vs. 14.2%, 13.0 vs. 16.6% and 14.3 vs. 18.3%, respectively, differences statistically non-significant). Our preliminary results demonstrated a positive effect of a low prolactin dose on the mortality of piglets up to 70 days after birth. This study was funded by project no. NR12-0057-10 (NCBiR, Poland).

Key Words: Pig, prolactin, mortality, body gain

2856

Conditioning Murrah buffalo heifers prior to application of Ovsynch protocol improves the treatment response and fertility

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Delayed age at sexual maturity and low fertility with estrus induction protocols are the most significant problems in buffalo heifers leading to poor reproductive efficiency. We report that conditioning the heifers using vitamin and mineral supplementation and deworming prior to estrus induction increases the response to the treatment and fertility. Acyclic Murrah buffalo heifers (n = 30; confirmed using progesterone levels and ultrasound evaluation two times at 11 day intervals) were subjected to three treatment protocols (n = 10 for each treatment). Treatment I- Ovsynch protocol (Buserelin acetate 10 μg on day 0 and day 9, Cloprostenol sodium 500 μg on day 7); treatment II included administration of Vitamin A (30 lakh IU), D3 (10 lakh IU) & E (500 mg), phosphorus (2 g) per heifer and Ivermectin (0.2 mg/kg body weight) and in treatment III, heifers were first administered with Vitamin A, D3, E, phosphorus, Ivermectin and 1 month later Ovsynch protocol was applied. Heifers were inseminated twice at 12 h interval with frozen-thawed semen when they showed spontaneous estrus in treatment II and on 11th day in treatment I and III. In treatment I, only three heifers responded to the treatment but none conceived. In treatment II, only one heifer showed estrus signs within a month after treatment. In treatment III, 90.48% of heifers responded to the treatment and 47.61% heifers conceived in first insemination and 45.45% conceived after second insemination leading to an overall conception rate of 71.42%. These results demonstrate that estrus induction can be achieved in non-cyclic Murrah buffalo heifers and

conditioning the heifers before application of exogenous hormones improves the estrus response and fertility.
Key Words: Estrus induction, Ovsynch protocol, body conditioning, conception rate

2857

Follicular wave emergence after GnRH induced ovulation at the beginning of the breeding season in dromedaries (*Camelus dromedarius*)

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Dromedaries are considered to be seasonally polyoestrous animals. In the Arabic peninsula, the breeding season extends from the autumn until the spring reaching its peak between December and April. Some authors suggest that female camels in good body condition show regular ovarian activity throughout the year. Hence, seasonal reproduction seems to be related to nutritional and other factors rather then to photoperiod in this species. As Camelids are not ovulating spontaneously, the determination of anoestrus period is difficult compared to other seasonal breeders (sheep, horse). The aim of the present study was to monitor ovarian follicular wave emergence following GnRH induced ovulation in lactating and dry camels at the beginning of the breeding season. In October and November, a large group of camels were given a GnRH analogue (20 µg Buserelin i.v.; Receptal, Intervet, Holland) when a 1–2 cm follicle was present on the ovaries. Ovulation was confirmed by ultrasonography and progesterone determination on Day 5 after ovulation in 83 lactating and 17 non-lactating dromedaries. All of these camels were in average to good body condition. The development of the new follicular wave was monitored every second day until Day 21 after ovulation. The diameter of the dominant or that of the largest follicle was recorded. The proportion of animals with and without wave emergence and the characteristics of dominant follicle development were compared between lactating and dry camels. The proportion of camels with impaired follicular wave emergence was significantly higher in lactating vs. dry animals ($p < 0.001$). In 24 of 83 lactating camels (28.9%), the dominant follicle reached a mean (±SEM) maximum diameter of 1.04 ± 0.06 cm by Day 9, then it declined. In these animals, the size of the largest follicle was significantly smaller from Day 11 until Day 21 compared to other lactating camels ($p < 0.001$). In 59 (71.1%) lactating animals and in all 17 dry camels, follicular wave emergence had a normal pattern after GnRH induced ovulation. In lactating animals with normal follicular wave emergence, the dominant follicle reached 1.51 ± 0.04 cm in diameter by Day 13 and the mean maximum diameter was 1.87 ± 0.06 cm on Day 19. The daily growth rate of the dominant follicle was 0.75 mm. The development patter of the dominant follicle in non lactating dromedaries was similar to that of lactating animals. These data demonstrate that lactation has a negative influence on follicular development in dromedary camels at the beginning of the breeding season. It seems that the interaction between lactation and season enhances the manifestation of seasonal reproduction in female dromedary camels.
Key Words: Camel, GnRH, dominant follicle, lactation, season

2858

Follicular arrest in ovaries induced by Busereline in alpacas (Lama pacos)

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Several follicular stimulation protocols have been used in alpacas. This has been done without considering the initial size homogeneity of follicles to be stimulated. This approach has resulted in the recruitment of different size follicles. From this there are significant consequences on oocyte quality. This could be the reason that there are so many adverse results, and the poor protocol repeatability. Our objective was to develop a protocol to optimize and homongenize follicular arrest and have a better control of follicle growth. In a prospective randomized study, eight alpacas were used in the experiment. Both ovaries were evaluated by ultrasonography and we manually ablated the follicles. Busereline, a GnRH-agonist, was used at a dose of 50 ug per day, per animal, and administered subcutaneously for 10 days. Animals were monitored daily after treatment using a 6–8 MHz transducer, and Aloka 500 Scanner. Both ovaries were evaluated recording the number and diameter of follicles to evaluate follicular development. As a result the follicular development was not reinitiated after ablation during the 10 days of the treatment, in 75% ($n = 6$) of eight animals. The first day of total arrest in the six animals was in the sixth day of treatment. We conclude that Busereline treatment to induce follicular arrest in the alpacas is promising.
Key Words: Follicles, busereline, alpaca

2859

Characterization of follicular dynamics and corresponding hormone concentrations in multi- and nulli-parous mithuns (*Bos frontalis*)

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Characterization of follicular dynamics is important before applying biotechnological tools like superovulation and embryo transfer technology for production augmentation in Mithun (Bos frontalis), a rare ruminant of Southeast Asia. The present study was therefore designed to examine the follicular dynamics and circulating hormone concentrations in multiparous mithun cows and nulliparous heifers. Multiparous, cyclic mithun cows ($n = 13$) and cyclic heifers ($n = 9$) were examined during one estrous cycle. The examinations included transrectal ultrasound monitoring and daily blood sampling. Frequent blood samples (at every 4-h) were also collected during peri-estrus period to record the changes of plasma estradiol and LH. Distributions of two- and three-wave cycles were similar in the two groups: 74 and 26% in cows, 69 and 31% in heifers, respectively. Cycle lengths were shorter by 2.7 days in heifers than in cows, and 2.3 days in two-wave than in three-wave cycles. The ovulatory follicle was smaller in heifers than in cows (11.16 ± 1.16 mm vs. 15.94 ± 1.17 mm). The greater numbers of large follicles in cows than in heifers corresponded well to the higher concentrations of FSH in cows. The duration of dominance of the ovulatory follicle tended to be longer in cows than in heifers. Estradiol concentrations around estrus and the preovulatory LH surge were higher in heifers than in cows (11.63 ± 2.12 vs. 7.92 ± 1.97 pg/ml and 16.55 vs. 10.21 ng/ml). Progesterone concentrations were higher in heifers than in cows from Day 3 to Day 16 of the cycle. Circulating progesterone was similar between two-wave and three-wave cycles. The results revealed differences in ovarian follicular dynamics and in plasma concentrations of steroids and gonadotrophins in heifers and multiparous mithun cows.
Key Words: Ovarian dynamics, *Bos frontalis*, FSH, ovulation, estrus

29. Spermatology & andrology I:

2900

Fertility prediction in buffalo bulls on the basis of sperm motion traits, fertility associated antigen (FAA) and HSP70 expression

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Bull fertility depends upon fertilizing ability of the sperm and is best evaluated through fertility trial. However, conducting fertility trial is a time consuming and involves multiple variables. The selection of high fertility buffalo bulls based on subjective tests of sperm viz. motility, live count and morphology is a challenge due to variability in results. This warrants the identification of reliable traits to enhance the accuracy of fertility prediction. The possible candidates are CASA based sperm motion traits, Fertility associated antigen (FAA) assessment and heat shock protein70 (HSP70) expression. To predict the fertility of buffalo bulls using: (i) CASA based sperm motion traits, (ii) assessment of FAA and c) expression for HSP70. Six frozen semen straws each of the 20 buffalo bulls (6–8 year) were analyzed through CASA for sperm motion traits (individual motility, progressive motility, average path velocity, VAP; straight line velocity, VSL; curvilinear velocity, VCL; amplitude of lateral head deviation, ALH; Beat cross frequency, BCF; straightness, STR; linearity, LIN and sperm size). For HSP70 expression, total RNA was harvested, first strand cDNA was synthesized followed by quantification using a Real time PCR. Primer and probe for HSP70 (Acc. No. U02892) and endogenous control (18S) were designed using the Primer Express software package. In fresh semen of the same bulls, FAA was investigated using Reprotest (ReproTec Inc.). Fertility trial was conducted on 166 adult normal cycling buffaloes (7–10 buffaloes per bull). The data in percent values were subjected to arc sine transformation followed by ANOVA and Games Howell *post hoc* test. For all pairs, HSP70 was compared using Tukey-Kramer HSD. Based upon fertility trial, buffalo bulls were categorized into good (conception rates, CR > 50%), average (CR 30–50%) and poor (CR < 30%) groups. Individual motility was higher ($p < 0.05$) in good as compared to average and poor fertility groups. Progressive motility, VAP, VSL, VCL, ALH, BCF, STR, LIN and sperm size as well as HSP70 expression were similar ($p > 0.05$) between various groups. Out of nine good fertility buffalo bulls, seven were FAA positive. All the average fertility and three poor fertility bulls were also FAA positive, which indicates that FAA assessment cannot be used as a marker for fertility in buffalo bulls unlike cow bulls. Fertility in buffalo bulls can be predicted by CASA based individual sperm motility. However, assessment of FAA and HSP70 expression may not be reliable for fertility prediction in buffalo bulls.

Key Words: Fertility, buffalo bull, fertiliity associated antigen, HSP70

2901

Exploring *in vitro* sperm characteristics and their importance in the prediction of conception rate

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It is well recognized that bull fertility can be better estimated when a combination of several *in vitro* semen analysis is performed. The aim of this study was to assess *in vivo* fertility as well as *in vitro* sperm characteristics of different sires and to identify important sperm variables in the prediction of conception rate in a timed-AI (TAI) program. Suckled Nelore cows from a commercial farm were used. All cows (n = 191) received the same TAI protocol. On day 0, cows received estradiol benzoate and intravaginal progesterone releasing device. On d 8, the device was removed and PGF2α, eCG and estradiol cipionate were administered. On day 10, cows were TAI. Frozen semen doses from three Angus bulls and three batches from each bull were used. The semen handling protocol was according to routine procedures of the farm. The same thawing procedure was repeated in the lab to mimic field conditions. The following *in vitro* sperm analysis were performed: Computer Assisted Semen Analysis, Sperm Thermal Resistance Test (TRT at 2 h), Hiposmotic Swelling Test (HOST), assessment of plasma and acrosomal membranes integrity by PI/FITC-PSA, plasma membrane stability by Hoechst 33342/Yo-Pro/M540, lipid peroxidation by C11-BODIPY581/591, sperm morphometry and chromatin structure by Toluidine Blue staining. Partial Least Squares (PLS) was the statistical analysis utilized. The cutoff was determined according to Wold in Umetrics (SAS, 2001) which considers a value < 0.8 to be 'small' for VIP (Variable Importance for Projection). The following procedure was performed: after to run PLS analysis, variables presenting VIP value lower than 0.8 were excluded. Then, PLS was performed for a second time without the deleted variables. Again, variables with VIP < 0.8 were excluded. Then, PLS was performed for the last time. According to field results, conception rate of TAI was 50.8% (97/191). According to PLS results, the following sperm variables were selected as important predictors of conception rate: total motility (TM), progressive motility (PM), average path velocity (VAP), beat cross frequency (BCF), rapidly moving cells (RAP), TM_TRT, PM_TRT, VAP_TRT, BCF_TRT, RAP_TRT, HOST + cells, IPIA (sperm with intact plasma and acrosomal membranes), HBD (sperm with high level of lipid bilayer disorganization), IPP (sperm with intact plasma membrane suffering lipid peroxidation), major defects, total defects, ellipticity, fourier 0, fourier 2 and chromatin heterogeneity. We concluded that the TAI protocol was efficient and that PLS analysis was a suitable statistical method to explore and summarize the relationship between *in vitro* sperm characteristics and fertility results of a TAI program.

Key Words: Conception rate, IATF, important predictors, PLS, semen analysis

2902

Seminal plasma proteins from Dairy Gir bulls in peripuberty and their relationship with sexual precocity and fertility

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Seminal plasma proteins of peri-pubertal (from 60 days before and 60 days after puberty) bulls, analyzed by two-dimensional electrophoresis (2D-E), can be used as markers of sexual precocity and fertility. The objective was to identify in Dairy Gyr bulls from 14 to 23 months of age, raised under high nutrition level, from five high milk production herds, selected for early sexual maturity (sired from 15 bulls) seminal plasma proteins analyzed by 2D-E, and identified by mass spectrometry. Body weight, scrotal circumference and physical and morphological semen evaluations were performed and the animals classified by CAP – Andrologic Classification by Points (with specific scores for zebu and taurine, similar to the Breeding Soundness Evaluation). At age of 15 months it was registered CAP > 60 points for a single animal (3.3%); CAP from 30 to 55 points for six animals (20.0%) and CAP < 30 points for 23 animals (76.7%). Twelve animals (super-precocious) reached puberty prior to the beginning of the experimental phase and two others that had not reached puberty by the end of the experimental phase were excluded from the experiment. Based on the median value of age at puberty (18 months) in the group of 16 remaining animals, it was carried out comparisons between seminal plasma protein profiles in the three moments (-60; 0; + 60). Out of these of 16 animals, four that reached puberty prior to 18 months (early maturing) and four that reached puberty after 18 months (regular maturing) were randomly selected for analysis, by software Image Master 2D Platinum 6.0, of protein spots expression in each group. For quantitative analysis of the spots, early maturing animals showed, respectively, for moments -60, 0 and + 60, averages of 'protein spots' of 77 ± 11a, 91 ± 14b and 104 ± 4c and regular maturing animals averages of 67 ± 11a, 72 ± 10b and ± 82 14c ($p < 0.05$ among groups, and $p > 0.05$, when comparisons were made between age at puberty within groups). However, the qualitative

analysis showed difference between puberty groups ($p < 0.05$). From the spots showing expression differences, digested by trypsin and analyzed (mass spectrometry in MALDI-TOF/TOF system), were identified the following proteins: Precursor of Spermadhesin 1; Spermadhesin Z-13; Inhibitor of metalloproteinase 2 (TIMP 2); PDC-109; Precursor of TIMP-2; Clusterin; Glutathione peroxidase; Albumin, Precursor of BSP-30 kDa; Precursor of the BSP A3. It is suggested that the Precursor protein of Spermadesin I and that found on spot of 57 kDa and pI 4.6 (not identified), registered in animals with higher fertility (based on the CAP), might be used as a positive marker for sexual precocity-fertility and the protein from spot of 88 kDa and pI 5.0, as a negative marker of these same characteristics.
Key Words: Dairy Gyr, fertility, peripuberty, seminal plasma proteins, sexual maturity

2903

Composition and digestibility of dietary mineral supplementation increase the semen quality of A.I. bulls

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High semen quality of A.I. bulls is an important factor for high pregnancy rates of dairy and beef cows, because more than 80% of cows will be inseminated. Twenty nine Holstein Friesian bulls, 1 Simmental, 1 Limousin and 1 Angus bull were used in the investigation. Eighteen of these bulls had problems with the production of high quality semen in the ejaculate or post-thawing and constituted the study group. Over a period of 8 weeks the study group was feed with a special mineral feed with the following composition per kg: 9% calcium, 6% Natrium, 8000 mg organic zink, 2000 mg organic manganese, 1000 mg organic copper, 35 mg selenium, 1 500 000 IE undegradable vitamin A, 40 000 IE vitamin D3, 20 000 mg vitamin E, 1000 mg vitamin C, 150 000 mg biotin, 1000 mg cholin chloride. This investigation was performed over a period of more than 24 weeks to capture the whole time of spermatogenesis. It was divided into: 8–12 weeks prior feeding the different mineral was used as basic value in control and study group; 'feeding interval' with different mineral together in control and study group over 8 weeks; observation period with the normal mineral feed from week 9 after start of feeding to week 24 after start of feeding. Semen was collected from each bull using artificial vagina and was evaluated for volume, density, motility (in ejaculate and post-thawing) and sperm concentration. Morphology of ejaculate was analyzed every half year for bulls with semen production. It will be differentiated in: normal sperms, not fertile or to young sperms, sperms with abnormal or pathologically deviations on head, intermediate section, tail and head cap as well as loose heads. The concentration of sperms in ejaculate was significant different between study and control group prior to feeding. During the time of feeding and after feeding there was observed a significant increase of 77 million sperms per milliliter in the study group. Mineral supplementation could increase the amount of living sperms in the ejaculate significant. In the time of feeding and after feeding we observed 14% of ejaculate with more than 70% amount of living sperms in the ejaculate. This was a significant increase of 5% compared to the time prior feeding. Straight forward motility in ejaculate could be increased by feeding the changed mineral supplementation only in young sire. Here we found a significant improvement from 74% to 87%. But here it is remarkable that the young bulls have also better physical developing and showed a continuous production. Morphologically changes were observed for an increase in the amount of normal sperms. There was an improvement of 3% in the time of feeding and after feeding compared with the basic value. We could found 91% of normal sperms in study group. Bulls of control group had an average amount of morphologically normal sperms of 95%. The present results indicate that the mineral supplementation can increase a lot of parameters to produce high quality semen of A.I. bulls.
Key Words: Semen quality, mineral supplement, A.I. bulls

2904

Season-induced variations in lipid composition are associated with semen quality in Holstein bulls

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Season-induced variations in fatty acid and cholesterol composition in bovine semen have been associated with semen quality. Given the specific roles of the various semen compartments in fertilization, we hypothesized that environmental stress-induced alterations in the lipid composition of a specific compartment (seminal plasma, spermatozoon head or spermatozoon tail) might impair semen quality and sperm function. Semen samples were collected from five mature Holstein-Friesian bulls during the summer (August to September) and winter (December to January). Semen was evaluated by computerized sperm-quality analyzer for bulls and centrifuged to separate the sperm from the seminal plasma. The cell fraction was sonicated to separate the sperm head and tail compartments. Cold lipid extraction was performed with chloroform:methanol (2:1, v/v), and fatty acids and cholesterol were identified and quantified by gas chromatography. The average volume of ejaculates did not differ between seasons but semen concentration was lower in winter than in summer ($p < 0.02$) with a higher proportion of spermatozoa defined as morphologically normal ($p < 0.02$). The motility, progressive motility and velocity of spermatozoa were higher in the winter vs. summer samples ($p < 0.02$). The concentrations of six major fatty acids (c22:6n3, c20:5n3, c20:4n6, c20:4n6, c16:0, c14:0) varied within fractions and between seasons, with prominent impairment in the tail compartment. For example, the content of docosahexaenoic acid (c22:6n3) in the tail compartment was lower ($p < 0.01$) in summer than in winter; the content of eicosapentanoic acid (c20:5n3) in the tail compartment was twofold lower in winter than in summer ($p < 0.001$). The relative concentration of palmitate fatty acid (c16:0) in the tail compartment was 10% higher in summer vs. winter ($p < 0.001$). The concentration of monounsaturated fatty acids in the tail was lower ($p = 0.005$) in summer than in winter. The proportion of polyunsaturated fatty acids (PUFA) in the tail compartment was lower ($p = 0.0006$) in summer than in winter; the concentrations of omega-6 and omega-3 PUFA subfamilies differed between seasons, with lower concentrations of omega-6 ($p = 0.005$) and omega-3 ($p = 0.0007$) in the summer compared to winter samples. Nevertheless, the ratio between omega-3 and omega-6 fatty acid concentrations did not differ between seasons. In addition, the tail compartment in the winter samples was richer in cholesterol than the corresponding summer samples ($p = 0.01$) whereas its concentration in the seminal fluid was higher in the summer. Findings indicate a seasonal variation in both physiological and structural parameters. Given the association between alterations in lipid composition and reduced sperm motility and velocity, summer semen seems to be of inferior quality.
Key Words: Season, sperm quality, lipid composition

2905

Effect of vitamin E on epididymal sperm motility in water buffalo (*Bubalus bubalis*) post-mortem

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Vitamin E (Vit. E) is believed to be the primary component of the antioxidant system of the spermatozoa and is one of the major membrane protectants against reactive oxygen species. The objective of this study was to determine Vit. E effective levels on improving the computer-assisted sperm analyzers (CASA), parameters of buffalo€™s epididymal sperm. Sperms were collected from tail of epididymes in slaughtered animals. Seven levels of Vit E (1, 1.5, 2, 2.5, 3, 4 and 5 mM), were added into human tubal fluid media containing sperms

with 10%, bovine serum albumin and were incubated for 6 h in 37 Â°C. Sperm motility was examined at 30, 60, 120, 180, 240, 300 and 360 min after incubation with CASA. Simultaneously, viability percentage of sperm was measured using one step eosin-nigrosin staining. The results showed that CASA parameters; rapid progressive motility (Class A, %), straight line velocity (VSL, Âµm/s), average path velocity (VAP, Âµm/s), in 1, 1.5, 2, 2.5 levels of Vit. E and in the case of 180, 240, 300 and 360 min after incubation were significantly higher than control ($p < 0.05$). Other parameters; curvilinear velocity (VCL, Âµm/s), amplitude of lateral head displacement (ALH, Âµm), in 1, 1.5, 2, 2.5 levels and in the case of 240, 300 and 360 min were also significantly higher than control ($p < 0.05$). The viability percentage in 1, 1.5, and 2 mM was significantly higher than control ($p < 0.05$). In conclusion, Vit. E proved to improve motility pattern and viability percentage of epididymal sperm in water buffalo.

Key Words: Vitamin E, epididymis, buffalo, CASA

2906

Causes of rejection of rams in the breeding soundness evaluation

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This work comprehends two studies aiming at determining the causes of rejection of rams in the breeding soundness evaluation in the period prior to the breeding season and the mean scrotal circumferences of the breed examined. In study I, 1119 young (one year old) and adult (2–6 years) rams were examined and evaluated in order to determine both the causes of rejection and the scrotal circumference variation in five races, in 21 farms, between December 2010 and January 2011. Study II consisted of the evaluation of the records of the breeding soundness evaluation of 1015 young (1 year) and adult (2–6 years) Corriedale rams at the same farm from 2003 to 2010 aiming at ascertaining the impact of the adoption of the breeding soundness evaluation as a routine. To investigate the relationship between the binary variable approved or rejected at the stages of the breeding soundness evaluation and the age groups of (young and adult) rams, chi-square tests (PROC-FREQ) were used. The pathologies that caused the rejection of rams at each stage of the breeding soundness evaluation were subjected to multivariate cluster analysis (PROC CLUSTER and TREE). Data related to the scrotal circumference were subjected to variance analysis (PROC GLM), and means were compared through Tukey's test at 5%. In Study I, the general rejection rates of young and adult rams of all the races considered ranged from 14.91% to 21.62%, respectively. There was no significant difference between mean scrotal circumferences (SP) of young rams when compared to the adult rams assessed. Poll Dorset rams had the largest mean scrotal circumference (36.62 cm), while Australian Merino, Corriedale and Ideal rams had lower values (31.33, 31.50 and 30.37 cm, respectively). Cumulative rejection rates of 19.57% observed in Study I are within the range expected; rams were rejected either because they did not reach the seminal quantity or quality desired, or because they had physical defects. Study II evidenced that by adopting the breeding soundness evaluation as a routine it is possible to reduce the general rejection rates to values near 10%, as it was seen from 2004 to 2010, since in 2003, when the technology was implanted, those values were 19.41%. Therefore, the importance of the adoption of the breeding soundness evaluation as a routine should be highlighted.

Key Words: Breeding soundness evaluation, ovine, scrotal circumference

2907

First report of dietary L-carnitine and fish oil effects on sperm characteristics in ram

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The separate positive effects of L-carnitine and fish oil (FO) on male reproductive performance have been reported in some species. Our objective was to determine effects of feeding L-carnitine alongside with fish oil on ram's sperm characteristics by Computer Assisted Semen Analysis (CASA). Nineteen Zandi rams were randomly assigned to experimental groups and offered an isoenergetic and isonitrogenous ration. The treatments consisted of control (CTR; $n = 4$); fish oil (FO; 35 g/day per ram; $n = 4$); L-carnitine (LCAR; 220 ppm/day per ram; $n = 4$) and FO + LCAR ($n = 4$) with constant level of Vitamin E. Also, three rams in farm were used as control without Vitamin E (data not shown). Diets were fed to the rams for 70 days (10 day as adaptation period) during the physiological breeding season and semen was collected from each ram once per week. Data were analyzed by SPSS and differences were declared significant at $p < 0.05$. Supplemented diet with FO, LCAR and FO + LCAR improved volume, concentration as well as total sperm output (5.50, 5.40, 5.06 vs. 3.38×10 9 for FO + LCAR, FO, LCAR and CTR, respectively) compared with CTR. Adding FO in ram's diet increased sperm viability (90.8 and 90 vs. 82.3 and 81.9% for FO + LCAR, FO, LCAR and CTR, respectively) as well as rapid (40, 39 vs. 32 and 28%) and medium (24, 19, 17 and 16% FO + LCAR, FO, CTR and LCAR, respectively) progressive sperm motility. Interestingly, L-carnitine supplementation had significant effects on non-progressive sperm motility (38.4 vs. 31.3, 30.5 and 27.8% for LCAR vs. FO, CTR and FO + LCAR, respectively) as well as hyperactive sperm percent (22.4 vs. 28.5, 33.4, and 33.7 for LCAR vs. CTR, FO + LCAR, and FO, respectively) compared with other groups. In conclusion, unique fatty acids of fish oil alone or alongside with L-carnitine improves semen quality and sperm progressive motility. Furthermore, L-carnitine supplementation has beneficial effects on total sperm output with no positive effects on sperm motility characteristics.

Key Words: L-Carnitine, fish oil, sperm characteristics, ram

2908

The effects of oxytocin and PgF2alpha injections on libido sexualis and semen quality in warmblood stallions

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In recent years, studies have been made on to improve the quality of ejaculates in animal species (buffalo, ram, bull, rabbit and stallion). Oxytocin and PgF2alpha have been identified from these studies. But the role of oxytocin and prostaglandin hormones are not well known in males. In this study, the effects of exogenous oxytocin (Hormonipra, HIPRA) and PgF2alpha (Gestavet-Prost, HIPRA) on seminal quality and libido sexualis were studied in 12 warmblood stallions aged 15–22 years. Based on pretreatment datas, stallions were randomly assigned to one of four groups each containing three animals. One member of each group received oxytocin 10 IU, iv (Group 1), oxytocin 20 IU, iv (Group 2), PgF2alpha 5 mg, im (Group 3) or PgF2alpha 10 mg, im (Group 4) 30 min before each collection. At the beginning of the study, semen samples were collected using a Missouri model artificial vagina from each stallion. Their libido sexualis and spermatological parameters (semen volume, sperm concentration, total sperm motility, abnormal sperm, membrane integrity, and pH) were recorded to create a control group. According to obtained results, semen volume (95 ± 25 ml), sperm concentration ($270 \pm 15 \times 106$/ml) and total sperm motility ($66 \pm 4\%$) were higher in group 2 (20 IU of oxytocin) than the other groups ($p < 0.05$). Sperm concentration per ejaculate were decreased in group 3 and 4 ($180 \pm 13 \times 106$/ml, $175 \pm 13 \times 106$/ml resp.). The percentage of abnormal sperm cells and membrane integrity did not differ between the groups ($p > 0.05$). The stallions,

which were injected PgF2alpha, showed decreasing libido and were reluctant for mating, while sexual behaviours were not changed in the in group 1 and 2. The side effects were noted following PgF2alpha administrations in all stallions. PgF2alpha administrations caused thrilling, tremor and perspiration. Early erection and premature ejaculation were observed, although duration of ejaculation was longer in the stallions, which were injected PgF2alpha, than the other groups. As a result, the study indicate that administration of oxytocin 30 min prior to semen collection in the stallions can improve not only the ejaculate quality, but also the time of sample collection. We concluded that PgF2alpha and oxytocin, at this dosage of administration, may change the reproductive capacity of stallion.

Key Words: Stallion, oxytocin, prostaglandin F2alpha, semen, libido

2909

Impact of computer-assisted sperm morphology analysis for quality assurance of AI centers

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Sperm morphology has a great influence on fertility. Analysis of morphology is time-consuming and, therefore, often not realized during semen processing in AI centers. A new generation of computer-assisted semen analysis (CASA) systems offers automated, fast assessment of sperm morphology abnormalities in parallel to motility measurements. Their impact for quality assurance in AI studs clearly depends on accuracy and precision of morphology assessment. The aim of this study was to validate the CASA system SpermVision™ Automorphology (Minitube, Verona, USA) for its use to identify distinct sperm morphological abnormalities, i.e. proximal and distal cytoplasmic droplets, and bent tails in boar semen samples. Single spermatozoa (n = 12 568) of 48 samples were assessed visually on the screen by an experienced lab technician and automatically by CASA. Of 83% of the defect spermatozoa (n = 3414) were classified as defect by CASA (sensitivity). Of 96% of the intact spermatozoa were recognized as intact (specificity). Of 89% of the sperm tagged as 'defect' by CASA were defect (positive predictive value). Of 94% of sperm tagged as 'intact' were intact (negative predictive value). Furthermore, 210 semen samples were assessed by CASA and by phase-contrast microscopy examination of a fixed aliquot. Results of both methods were judged according to standards of the German Pig Producers Association (ZDS). The CASA Automorphology detected 83% of the samples that exceeded threshold values with either a proximal droplet, distal droplet or a bent tail (sample-based sensitivity). Of 88% of samples with below threshold morphology were correctly classified as 'passed' by Automorphology (sample-based specificity). Repeatability was tested in 10 consecutive CASA analyses of each of 13 samples. Six from seven samples with above threshold morphology were in all repeated CASA analyses correctly identified as 'failed'. Likewise, five from six samples with below threshold morphology were correctly assessed as 'passed' in all repeated CASA analyses. In conclusion, SpermVision™ Automorphology provides a valuable tool for improving quality control in AI centers by real-time assessment of sperm morphology during semen processing.

Key Words: CASA, sperm morphology, quality control

2910

Separation of apoptotic boar spermatozoa using magnetic activated cell sorting with annexin V-conjugated microbeds

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One of the features of apoptosis is the externalization of phosphatidylserine (PS) which could be used to remove apoptotic cells from semen preparations. Magnetic-activated cell sorting (MACS) using annexin V-conjugated microbeads which bind to PS was already successfully used to enhance human semen quality for assisted reproductive techniques, whereas boar spermatozoa were separated by MACS for the first time in the present study. MACS (miniMACS, Miltenyi, Biotech GmbH) of 12 boar semen samples after 3 days of liquid storage at 16–17°C delivers two fractions: unbound (non-apoptotic/live spermatozoa) and bound (apoptotic/dead spermatozoa). Flow cytometry after staining spermatozoa by Annexin V-conjugated with Alexa Fluor 488 (A) and propidium iodide (PI) revealed four subpopulations: live (A-/PI-), early apoptotic – live (A+/P-), late apoptotic and early necrotic – dead (A+/PI+), late necrotic – dead (A-/PI+). Frequency of early apoptotic and late necrotic (dead) spermatozoa was significantly higher in bound (14.1 ± 10.6% and 24.1 ± 10.2%, respectively) in comparison to unbound fractions (3.4 ± 2.1% and 12.7 ± 3.1%, respectively) and to control samples (3.5 ± 1.6% and 12.0 ± 5.0%, respectively) ($p < 0.05$). The lowest level of live spermatozoa was found in bound fractions (10.6 ± 8.0%) and it differs significantly in comparison to control samples ($p < 0.05$). MACS for separation of apoptotic cells caused damage to the boar spermatozoa in terms of motility obtained by computer-assisted semen analysis (CASA), viability according to Hoechst staining and morphology assessed by Giemsa-stained samples. An unusual anomaly on the head of spermatozoa was observed in both fractions. Although in bound fractions there was significantly lower level of normal spermatozoa (5.9 ± 7.3%), than in unbound (31.8 ± 12.6%) ones ($p < 0.05$), a significant decrease in proportion of morphologically normal spermatozoa is observed in both fractions in comparison to control samples (67.2 ± 17.0%) ($p < 0.05$). Spermatozoa subpopulation with detached acrosome in unbound fractions at 12.7 ± 7.2%, in bound fractions at 57.8 ± 23.0% and in control samples at 5.7 ± 4.8% confirm an increase of mentioned abnormality in both fractions after MACS in comparison to control samples ($p < 0.05$).

Therefore, the results of our study show that MACS seems to be inappropriate for boar semen separation under tested conditions, although it has been successfully used for excluding apoptotic spermatozoa from human semen.

Key Words: Apoptosis, annexin V, magnetic activated cell sorting, boar semen, flow cytometry

2911

Sequence of tyrosine phosphorylation pattern during boar sperm capacitation *in vitro*

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Tyrosine phosphorylation has been detected in boar spermatozoa during capacitation; however the specific patterns or the sequence of phosphorylation has not been described. We have investigated how sperm tyrosine phosphorylation is affected by incubation with estrus oviductal fluid (EODF), metestrus oviductal fluid (MODF), positive and negative controls i.e. capacitating (CM) and non capacitating medium (NCM). Frozen-thawed and washed spermatozoa from four boars were incubated with EODF, MODF, CM or NCM 6 h. Tyrosine phosphorylation during incubation was studied by confocal microscopy at hourly intervals using an immunofluorescent technique. Six types of immunofluorescent patterns were observed: (i) no fluorescence; (ii) fluorescence over the acrosomal region-pattern A; (ii) triangular or tongue-shaped fluorescence in the equatorial segment-pattern E; (iv) fluorescence at both the acrosomal region and the equatorial segment-pattern AE; (v) fluorescence in the tail-pattern T, (vi) fluorescence over the entire spermatozoon-pattern AET. There was no significant difference in the proportion of spermatozoa displaying pattern A at any given time during incubation but a tendency to get higher during incubation. The proportion of pattern E spermatozoa decreased significantly ($p < 0.05$) during the first hour of incubation and then maintained relatively constant during the rest of the incubation. A majority of the spermatozoa showed AE pattern at the start of the incubation, and then this proportion decreased significantly ($p < 0.05$) in the CM and EODF groups after 3 h, while there was no significant change in the NCM and MODF groups. Almost no spermatozoa displayed T pattern after thawing, and during incubation up to 4 h no significant changes were observed. Thereafter, the percentage of spermatozoa showing T pattern increased significantly ($p < 0.05$) in the CM, EODF and MODF groups compared to the NCM group. Almost no spermatozoa showed AET pattern

immediately after thawing but there was a steady increase during incubation. After 4 h, the proportion of spermatozoa displaying AET pattern in the EODF group was significantly ($p < 0.01$) higher than in all other groups. At 1 h of incubation the proportion of spermatozoa displaying AET pattern was only 1.09% in the EODF treated group, which increased to 23.69% at 6 h of incubation. At start, there were a large proportion of non-fluorescent spermatozoa. The dynamics of different patterns during incubation indicate that tyrosine phosphorylation started at the equatorial segment then shifted and/or progressed to the acrosomal area over time. Tail phosphorylation appeared to be a late event. The phosphorylation occurred first in the principal piece and end-piece, and then progressed to the mid-piece. The appearance of the AET pattern is also a late event. This is the first time a specific pattern of tyrosine phosphorylation during capacitation is shown in boar spermatozoa.

2912

Optimal inclusion level of butylated hydroxytoluene in semen extender improves the quality of post-thawed canine sperm

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The study was conducted to evaluate the potential cryoprotective effect of butylated hydroxytoluene (BHT) through post thaw evaluation of canine semen and its optimal inclusion level. Ejaculated canine semen was extended in TRIS-glucose-egg yolk extender containing various concentrations of BHT (0.5, 1.0, 1.5, 2.0 and 2.5 mM). Semen was frozen at -196°C using 200×10^6 spermatozoa per 0.5 ml straws and post-thawed evaluation was carried out in terms of sperm motility, viability, plasma membrane integrity and acrosomal integrity through phase-contrast microscope, supravital staining, hypo-osmotic swelling test and normal acrosomal ridge, respectively. BHT was found to improve ($p \geq 0.005$) all post-thawed semen quality parameters at an inclusion level of 1.0 mM in the extended semen. However, higher concentrations than this were found to have detrimental effects. This study on canine semen is first of its kind being reported from Pakistan and will be helpful for practicing veterinarians of developing countries where the pet ownership is gaining strong footing and so is the demand of cryopreserved canine semen.

Key Words: Canines, butylated hydroxytoluene, cryopreservation, hypo-osmotic swelling test

Table 1. Effect of BHT inclusion on post thaw canine semen quality cryopreserved in a TRIS-citrate-egg yolk extender

BHT (mM)	Motility %	Acro. integrity %	HOS positive %	Viab. %
0.0	$38.6^{f} \pm 2.1$	$20.0^{d} \pm 1.2$	$54.2^{e} \pm 1.5$	$50.1^{e} \pm 2.7$
0.5	$45.5^{c} \pm 2.1$	$21.3^{c} \pm 1.0$	$56.4^{c} \pm 1.1$	$58.5^{b} \pm 1.2$
1.0	$52.2^{a} \pm 2.4$	$23.40^{a}1.3$	$59.5^{a} \pm 1.0$	$60.07^{a} \pm 1.5$
1.5	$49.2^{b} \pm 2.1$	$22.5^{b} \pm 1.3$	$58.0^{b} \pm 1.2$	$58.2^{b} \pm 1.9$
2.0	$44.4^{d} \pm 1.9$	$22.3^{b} \pm 1.3$	$55.1^{d} \pm 1.2$	$56.1^{c} \pm 1.0$
2.5	$42.2^{e} \pm 1.9$	$20.5^{d} \pm 1.2$	$53.6^{e} \pm 1.2$	$54.9^{d} \pm 1.0$

$^{a, b, c, d, e, f}$ denote difference ($p < 0.05$) within columns.

2913

DNA damage assessment of canine freeze–dried sperm using Acridine Orange and alkaline Comet Assay

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The conservation of species using biotechnology to preserve canine sperm cells is already in use, but still distances, transportation and biological barriers may hinder the use of such aid. The freeze–drying of sperm cells using chemically defined media shall be a way to overcome those difficulties. Hence, the objective of this study was to no only verify the level of DNA damage caused by the freeze-drying process, but also compare and contrast the two DNA damage assessments (Acridine Orange staining and Comet Assay). Sperm samples of four adult domestic dogs were collected by digital manipulation. Then samples were aliquoted and divided into three disctinct groups (G1 = fresh semen; G2 = using Human Tubal Fluid as extender; G3 = using Synthetic Oviductal Fluid as extender) and taken to the freeze–drier. After freeze–drying samples were re-hydrated with their respective 'freeze–drying media' and analyzed using both Acridine Orange staining (AO) and Comet Assay (CA). Both DNA damage assessments proved that the freeze-dried samples could be used for Intracytoplasmic sperm injection since sperm cells were immotile after rehydration. However, samples differed in DNA integrity quality according to the AO and CA results ($p < 0.0001$). G1 showed greater DNA damage (25.7%) if compared to G2 (22.7%) and G3 (14.7%) in the AO assessment. The CA did not show much of a difference concerning the order of samples that contained more DNA damages, in this evaluation G1 showed 18.3% of DNA damages comparing to G2 and G3 that showed 14.9% and 3.7%, respectively ($p < 0.0001$). As far as we are concerned this is the first report of canine freeze–dried sperm DNA damage assessment and thus may provide support to the application of freeze–dried sperm samples. Such technique might be an alternative for the preservation of wild canine gene pool in conservational programs.

Key Words: DNA damage assessment, canine freeze-dried sperm, acridine orange staining, comet assay

2914

Different separation methods for removal of blood from dog semen – preliminary study

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Haematospermia in dogs usually occurs secondary to benign prostatic hyperplasia (BPH) or trauma to the penis during semen collection. It is already known that the presence of blood is detrimental to cryopreservation of spermatozoa. However, cryopreservation cannot always be postponed to the time after treatment of a dog affected with BPH. Moreover, a number of conservative procedures to treat BPH are harmful to sperm quality with consequent negative impact on suitability of semen for freezing. The aim of the study was to find a suitable method for separation of blood from canine ejaculates. The sperm-rich fraction of the ejaculates from two dogs was pooled. Canine blood [washed with the TRIS-fructose-citric acid extender to achieve a pure suspension of red blood cells (RBC)] was added to semen to mimic contamination (5%, v/v). An aliquot of the admixture (200×10^6 spermatozoa with 325×10^6 RBC) underwent a swim-up procedure and gradient centrifugation at $500\times g$ for 20 min in 4 commercially available density gradient separation media: 40/80 PureSperm®, 40/80 CaniPure® (both Nidacon International AB; Sweden), 50/90 Isolate® (Irvine Scientific; CA, USA) and 45/90 Percoll® (Pharmacia; Sweden). Semen was analyzed before and after separation. Sperm count and RBC concentration were evaluated in a Bürker chamber and sperm motility under a light microscope, at 200× magnification. Semen samples were fractionated by a density gradient column. This resulted in six sperm fractions: A, B, C, D, E and sperm pellet [F]. All media except Isolate® and Percoll® presented a pellet which included the maximum number of total and motile spermatozoa

and the lowest number of RBC. Isolate® did not produce any sperm pellet. Isolate® and Percoll® yielded the highest percentage of sperm with the lowest RBC number in fraction E. The highest number of RBC was found in fraction D for all media. Fresh sperm motility was 78%. Motility after separation was 70% for swim-up, 83% for PureSperm® pellet, 77% for CaniPure® pellet, 70% for Isolate® fraction E and 35% and 50% for Percoll® fraction E and pellet, respectively. Total and motile sperm and RBC yields were 62%, 56% and 50% in semen samples after swim-up; 48%, 51% and 2% in PureSperm® pellet; 41%, 40% and 3.8% in CaniPure® pellet; 15%, 13% and 5% in Isolate® fraction E; 7%, 3% and 1.2% in Percoll® fraction E and 2.5%, 1.7% and 4% in Percoll® pellet, respectively. In this preliminary study, according to the coincidence test for relative frequencies, PureSperm® and CaniPure® were superior to the other gradient centrifugation media for separation of motile spermatozoa from red blood cells. Percoll® and Isolate® centrifugation and swim-up procedure resulted in poor separation. The use of gradient separation is a more suitable solution for severe haematospermia than the swim-up technique. Besides more efficient separation of RBC from ejaculates, it also allows to eliminate bacterial contamination that is often connected to prostatic disorders. This study was supported by GACR 523/08/P561 and MZE 0002716202.

Key Words: Dog spermatozoa, sperm separation, blood, density gradient centrifugation

2950

No link between sperm morphology and velocity across maternal mouthbrooding cichlid fish from Lake Malawi

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Sperm competition theory predicts that males optimise sperm traits to maximize fertilisation rates when sperm from more than one male compete to fertilise a given set of eggs. In addition to this it is assumed that longer sperm swim faster and therefore increase fertilisation rates for males which produce them. However many studies report conflicting data which does not always support this theory and even fewer report links between velocity and morphology. We examined sperm characteristics from 29 species of maternal mouthbrooding cichlid fish from Lake Malawi for an association between sperm morphology and velocity. Sequential polyandry is common in mouthbrooding cichlids where broods of mixed paternity have often been demonstrated. Mouthbrooding could be viewed as an intermediate between external and internal fertilisation, allowing insights into the relative importance of different selection pressures depending on point of fertilisation. We analysed two speed measures (curvilinear velocity and straight line velocity) and five morphology measures (head length, head width, flagellum length, total length and head:flagellum ratio) and accounting for the shared ancestry implied by phylogeny, no relationship between sperm swimming velocity and any element of morphology was detected. These results are in contrast to other studies using closely related cichlids from Lake Tanganyika (cichlids from both lakes shared a common ancestor ˜30 million years ago). Here, polygamous species were found to have longer, faster sperm when compared to closely related monogamous species. Malawi cichlids have much less variation in their reproductive modes when compared with Tanganyikan cichlids and the data from this study provides an indication that there may be little or no sperm competition within cichlids from Lake Malawi. Details of reproductive behaviours are largely unknown for many Lake Malawi cichlids, however it is interesting to note that the only lekking species in this study were found to have short fast sperm when compared to the other species. Males in lekking species invest a great deal of energy constructing and defending a bower and may therefore invest less in sperm. Understanding what drives selection of sperm traits is important as sperm selection can be a key aspect of breeding programs both for livestock and endangered species. Furthermore the speed with which sperm evolve within a taxon could be an important consideration in establishing viable breeding populations of some species.

Key Words: Sperm competition, sperm size, sperm swimming speed, sexual selection

2951

Effect of melatonin implants on scrotal circumference and seminal quality parameters of dohne merino rams during seasonal anestrus at 43°s

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Melatonin implants have been revealed as an interesting treatment to increase lamb production, improving both ram and ewe reproductive performances. Focusing on ram, melatonin has been used in semen production stations to maintain high quality semen characteristics throughout the year. However, data concerning the effect of this hormone on South American sheep production systems are scarce. Thus, this experiment was designed to evaluate the effect of melatonin implants inserted during the anestrous season on September on scrotal circumference, semen quality, and live weight (LW) and body condition (BC) of Dohne Merino rams at 43°S. Eight–teen rams were allocated into two groups balanced for LW, BC and age, receiving (group M, n = 9) or not (group C, n = 9) three s.c. melatonin implants (3 × 18 mg). Scrotal circumference (cm), LW (kg) and BC were recorded fortnightly from thirty days after implantation, and volume (ml), masal motility (1–5), sperm concentration (number of spermatozoa per ml) and percentage of live spermatozoa were measured from ejaculates collected during eight weeks. At the end of the experimental period, no statistical differences were observed between groups for LW (M: 94.85 ± 2.59; C: 93.18 ± 2.42 kg), BC (M: 3.07 ± 0.15; C: 3.02 ± 0.17), volume (M: 0.87 ± 0.12; C: 0.85 ± 0.13 ml), masal motility (M: 2.80 ± 0.13; C: 3.10 ± 0.11), sperm concentration (M: 4.79 ± 2.93; C: 4.02 ± 2.52 × 10^9/ml) or percentage of live spermatozoa (M: 78.7 ± 3.9; C: 76.8 ± 4.2%). Only scrotal circumference was significantly affected by treatment (M: 37.1 ± 0.9; C: 34.2 ± 1.0 cm; $p < 0.05$). In conclusion, melatonin implants produced a significant effect on scrotal circumference of Dohne Merino rams, although it was not accompanied by an improvement of seminal characteristics. More experiences have been designed to determine whether or not treatment with melatonin could be introduced in Argentina as a means of improvement sheep production, especially to enhance seminal doses of genetic-improver rams.

Key Words: Melatonin, semen, rams, scrotal circumference, Dohne Merino

2952

Effect of adding soybean or salmon oil and vitamin E the diet on the ultrastructure of boar spermatozoa after cryopreservation

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Cryopreservation induces many stresses to cells, including plasma membrane damage, causing loss of cell function and even cell death. Sperm sensitivity to cooling damage is determined by membrane phospholipid composition, which can be affected by diet. The structural integrity of spermatozoa, after treatment, can benefit artificial insemination by elucidating factors which improve or damage cells after cryopreservation protocols. These structural evaluations can help select methodologies to improve the reproductive efficiency and utilization of semen from endangered as well as economically important species. The aim of this study was to evaluate the effect of adding different oils and levels vitamin E to the diet of boars on the ultrastructure of their spermatozoa after cryopreservation. Semen samples were obtained from 24 mature Dalboar 85 (Perdigao S/A) boars, 12 months of age, kept in a climate controlled environment. The experiment was a 2 × 3 factorial with treatments in a randomized complete block design. Boars

were fed diets supplemented with either soybean or salmon oil (3.5 kg/100 kg of diet) and three levels of vitamin E (150, 300 and 450 mg/kg). After feeding these diets for 1 month, the semen was collected one time per week for 10 weeks by gloved hand technique. The semen was processed and frozen by the method described by Fürst et al. (2005), modified by Moraes et al. (Brazilian Journal of Animal Science, v.40, p.1204, 2011). The samples were thawed a 37°C to 20 s and then prepared for transmission electron microscopy (TEM) analysis by first diluting each sample with phosphate buffer saline (pH 7.2) and centrifuging five times at 1500 rpm for 3 min at room temperature. The pellet was then fixed in 2.5% buffered glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Spurr ERL 4026 resin. The thin sections obtained were contrasted with uranyl-acetate and lead-citrate and observed using TEM. Acrosomes, nuclei plasma membranes and mitochondrial structures were evaluated from longitudinal sections and flagella axoneme was examined for number, orientation, and integrity of the microtubules from cross-sections. Vitamin E did not affect spermatozoal structure. The salmon oil diet did not affect the acrosome or nuclei of spermatozoa. Spermatozoa with cytoplasmic droplets were more numerous, and mitochondrial alterations and loose apposition of the plasma membrane occurred when boars were fed soybean oil. Longitudinal sections of spermatozoal flagella demonstrated more alterations in the tail midpiece when boars were fed soybean oil. Salmon oil lipids may benefit boar sperm plasma membranes prior to cryopreservation that resulted in less ultrastructural damage after thawing and may reduce the number of sperm with cytoplasmic droplets. The soybean oil diet did not benefit boar spermatozoa. Similarly Vitamin E had no effect on sperm cell structural abnormalities but probably serves as an antioxidant in the cell. Supported by FAPEMIG, Minitub and Perdigao.

Key Words: Pigs, nutrition, salmon oil, spermatozal, transmission electron microscopy

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Effect of milk extender supplemeted with vitamin E analog in chilled canine semen

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The aim of the present study was to investigate the protective effects of vitamin E analog (Trolox) in canine semen after 96 h of chilling. Ejaculates of the nine Beagles dogs, 6–8 years old, maintained under the same conditions, were collected weekly, during ten weeks. Ejaculates were pooled in three experimental units, each experimental unit, consisting of a pool of ejaculate from three dogs (total nine animals) and separated in two samples, one was incubated with Tris milk extender (control-CE Tris: 30.25 g/dl; citric acid: 17 g/dl; frutose: 12.5 g/dl; milk: 38 ml/dl; streptomycin: 1000 μg/ml and penicillin: 1000 IU/ml), while the other was submitted to a Tris milk extender enriched with 0.25 mM/ml of Trolox (antioxidant-AE). One sample of each extender was immediately evaluated (fresh) while the others were evaluated 24, 48, 72 and 96 h after chilling in a cool storage container (Botutainer®). Total motility, vigor, hyposmotic and thermal resistance tests and free radicals quantification (TBARS) were performed in all samples. The results were analyzed by Tukey test, with significance level 5%. Sperm morphology did not differ among collections ($p > 0.05$). In fresh samples, the control group and antioxidant extender group presented motility, vigor, thermal resistance and hyposmotic test values of 81.6% vs. 85.0%, 3.95 vs. 4.21, 65.5% vs. 69.8% and 81.9% vs. 85.3%, respectively. Thus, the enrichment with vitamin E did not affect sperm parameters ($p > 0.05$). Chilled samples supplemented with vitamin E analog improved motility (62.8% vs. 58.6%), and hyposmotic test (75.9% vs. 71.7%) compared to control group, respectively ($p < 0.05$), althought did not differ during 24 (72% vs. 66.5%; 76.9% vs. 72.4%), 48 (62.2% vs. 56.2%; 74.3% vs. 68.9%), 72 (52.3% vs. 49.6%; 72.9% vs. 69.2%) and 96 (42.5% vs. 39%; 70% vs. 66.4%) hours ($p > 0.05$), in the same order, for motility and hyposmotic test parameters, respectively. Free radicals production measured also did not differ between groups ($p > 0.05$). In conclusion, the milk extender containing 0.25 mM/ml of vitamin E improved motility and plasmatic membrane integrity of canine semen after 96 h of chilling.

Key Words: Antioxidant, diluent, free radicals, plasmatic membrane, viability sperm

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Correlation of semen fertility with ROS levels and motility in a multiparametric analysis

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Fertility is a multiparametric phenomenon that relies on the use of semen of sufficient quality and quantity. Motility is one of the most important characteristics believed to be associated with the fertilizing ability of spermatozoa. Reactive oxygen species (ROS) are also known to play an important role in the capacitation process when spermatozoa are exposed to mild physiological concentrations but at high concentrations, ROS cause many negative effects leading to many pathologies including infertility. In addition, cryopreservation procedures have been shown to increase ROS concentrations contributing to decreased fertility. Therefore, the main objective of this study was to determine if detection of ROS levels can be used in a multiparametric approach with CASA to predict fertility of bovine semen. Washed frozen-thawed semen was incubated with C11-BODIPY$^{581/591}$ to quantify lipid peroxidation (LPO) by flow cytometry (FC). Semen motility was evaluated by CASA immediately after emptying the straw and after washing the semen. FC and CASA results were correlated with the fertility of the semen (FERTSOL) obtained from the Canadian Dairy Network. FERTSOL is an adjusted 56-day non-return rate where semen with a FERTSOL≤ -1 is considered to have low fertility (n = 30), semen with a FERTOL between -1 and + 1 are considered to have medium fertility (n = 32) and semen with FERTSOL ≥+1 is considered to have high fertility (n = 32). Semen with a green/(green + red) fluorescence ratio [Ball and Vo, 2002. *J Androl.* 23(2):259–269] above 0.4 is considered to have high level of LPO (significantly different than low level LPO; $p < 0.05$, n = 13; one way ANOVA) and semen having < 60% of total motility and 30% of progressive motility is considered to have low motility (significantly different than average to high motility semen; $p < 0.01$, n = 21; one way ANOVA). No significant correlation was found between LPO ($r^2 = 0.029$, $p = 0.097$, n = 94; Pearson correlation) and semen fertility. However, six of the low fertility lots exhibited a high level of LPO. CASA analyses also revealed that 10 of the low fertility lots presented low motility. Since fertility can be affected by motility or ROS damage and that both phenomena can be mutually exclusive, a multiparametric approach combining CASA and FC demonstrated a total of 46.7% (n = 14) of the low fertility semen having lower motility and/or higher ROS level. Addition of FC to CASA analyses allowed to identify 13.4% additional low FERTSOL semen lots compared to CASA analyses alone. In conclusion, a multiparametric approach for the evaluation of frozen-thawed semen using ROS and CASA is a powerful tool to identify low fertility semen and could be applied in semen production centers in better quality control procedures.

Key Words: Semen fertility, flow cytometry, ROS, multiparametric approach

3001

Comparison of fixable and non-fixable fluorescent stains for bovine spermatozoa viability assessments

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Fluorescent stains and techniques that have potential application to flow cytometric evaluation of spermatozoa are continuously being developed (Gillan et al., 2005. *Theriogenology* 63:445–457). Sperm viability assessments are normally based on plasma membrane integrity (Graham, 2001. *Anim. Reprod.* Sci. 68:239–247). The choice of a fluorochrome is influenced by the application and excitation wavelengths available on the flow cytometer. Fixable viability dyes are

an emerging option because they are retained after fixation, allowing permeabilization and delayed analysis (Perfetto et al., 2006. *J. Immunol. Methods* 313:199–208). The aim of this study was to evaluate sperm viability tests on flow cytometry using two different excitation spectra non-fixable viability fluorescent stains and one fixable protocol. Frozen semen (TRIS-egg yolk extender, 7% glycerol, 250 µl French mini-straws) of ten Nellore bulls from the same insemination centre were thawed and combined to produce a heterospermic pool of semen. Semen was divided in six samples reaching a final concentration of 2×10^6/ml. Three samples were immediately stained and three aliquots were submitted to three continuous cycles of flash frozen in liquid nitrogen and thawed before staining to cause membrane damage. Six replicates were analyzed for each protocol: Propidium iodide, which is a membrane impermeable probe that only binds DNA of unviable cells. PI is excited by argon ion laser (488 nm) and red emission (640 nm long pass); SYTOX® Green based on membrane integrity and DNA stain that is excited with 488 nm argon ion laser, but with green emission (530/30 nm) and LIVE/DEAD® fixable aqua that stains intracellular amines when the membrane is damaged. The LIVE/DEAD stain is available in several emission wavelengths. In this protocol, spermatozoa was stained and fixed by 4% paraformaldehyde, being excited by the 405 nm violet laser and 522/31 nm emission; 10 000 events per sample were evaluated (Attune® acoustic focusing flow cytometer – Life Technologies). Results were analyzed by ANOVA and means submitted to Tukey test ($p < 0.05$). No differences in the percentage of non-viable sperm were found between PI, SYTOX Green and LIVE/DEAD® fixable aqua before (51.26 ± 3.21%; 48.42 ± 4.54% and 49.36 ± 6.61%, respectively), neither after membrane damage induction (76.53 ± 12.75%; 77.60 ± 10.20% and 80.95 ± 6.09%, respectively). These results indicate the ability of stain protocols to identify non-viable sperm cells, facilitating the adequate choice of a specific fluorochrome for an experiment like, multiple color analysis, avoiding wavelengths emission spillover and in case of assays requiring sperm fixation or permeabilization.

Key Words: Semen analysis, sperm viability, bovine, flow cytometry, fluochrome stain

3002

Content and activity of Na/K-ATPase in high- vs. low-fertility holstein bulls

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We recently observed that frozen-thawed sperm from high-fertility (HF) bulls had increased phosphotyrosine content and were in transition to a hyperactivated motility pattern compared to low-fertility (LF) bulls. These functional modifications are hallmarks of bovine sperm capacitation. The objective of this study was to compare HF vs. LF bulls for the content and activity of Na/K-ATPase, as this protein is involved in regulation of sperm capacitation. The HF and LF bulls (n = 5 each) were selected based on their non-return rate (NRR; range: 71–74 and 54–60 for HF and LF bulls, respectively). Membrane proteins extracted from frozen-thawed sperm of these bulls were used for quantifying the alpha4 isoform of Na/K-ATPase content (immunoblotting) and Na/K-ATPase activity (colorimetric assay). There was no difference in the content of alpha4 isoform of Na/K-ATPase between HF vs. LF bulls. The specific activity of Na/K-ATPase (pmoles/µg of total protein/min) did not differ ($p = 0.15$) between the HF (20.09 ± 2.76; mean ± SD) and LF bulls (28.87 ± 12.31). There was no significant correlation between Na/K-ATPase activity and NRR ($r = -0.42$; $p = 0.23$) in HF and LF bulls. In summary, HF and LF bulls did not differ in the content and activity of Na/K-ATPase. Perhaps the Na/K-ATPase-mediated signal transduction leading to tyrosine phosphorylation and hyperactivated motility in sperm are differentially regulated in HF vs. LF bulls, through a mechanism independent of the content and activity of this protein.

Key Words: Na/K-ATPase, bull, sperm, capacitation, fertility

3003

Effects of extender and equilibration time on post-thaw motility of cryopreserved bull semen at 0, 30, 60 and 90 min after thawing

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The objective of the study was to determine the effect of using two extenders [egg yolk-based (Triladyl®) and soybean lecithin-based (AndroMed®)] at three equilibration times (2–3 h, 4–5 h, and 8–9 h) on post-thaw motility at 0, 30, 60 and 90 min after thawing of cryopreserved bull semen. The different equilibration times were evaluated to determine the best time for semen freezing under field conditions. One semen sample was collected from each of 18 Simbrah bulls. After collection, semen volume and concentration were evaluated for purposes of dilution and sperm motility was evaluated subjectively under brightfield microscopy (×400). Each semen sample was divided into two fractions that were diluted either with Triladyl® or Andromed®. Each fraction diluted with Triladyl® and Andromed® was subdivided into three aliquots that were maintained to equilibrate at 5°C for: (i) 2–3 h; (ii) 4–5 h; or (iii) 8–9 h. Semen was packed into 0.5 ml straws and frozen by exposing the straws to liquid nitrogen vapors for either 20 min (Triladyl®) or 10 min (Andromed®), and plunging them in liquid nitrogen for storage. Two weeks later, two straws of semen from each treatment were thawed in a water bath at 37°C and sperm motility was evaluated immediately (0 min), at 30, 60 and 90 min after thawing. Data were analyzed using ANOVA and the Tukey test for difference of means. When the interaction between two factors was analyzed, the highest semen motility after thawing was obtained with: (i) equilibration time of 8–9 h and Triladyl® (40.65 ± 1.04%), (ii) equilibration time of 8–9 h at 0 min after thawing for both extenders (40.27 ± 2.05%), and (iii) Triladyl® at 0 min after thawing (36.48 ± 1.2%) ($p < 0.05$). However, when evaluating the interaction among the three factors no statistical difference was found for any of the combinations ($p > 0.05$). In conclusion, both Triladyl® and Andromed® extenders had the same effect on the post-thaw motility of bull semen.

Key Words: Semen, bull, equilibration time, extender, tropics

3004

Capacitation affects the location of Na^+/K^+ ATPase in bull sperm

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Na^+/K^+ ATPase is a transmembrane protein concentrated in the head plasma membrane of bull sperm. Na^+/K^+ ATPase is important for fertility and capacitation, and when bound to its ligand ouabain acts as a direct signalling molecule stimulating tyrosine phosphorylation during capacitation. Na^+/K^+ ATPase is a dimer; several isoforms of its alpha (α: 1, 2, 3, 4) and beta (β:1, 2, 3) subunits exist. We hypothesize that specific α and β isoforms in the head are more likely to be involved in sperm capacitation than those in the midpiece and tail, which would more likely be involved in motility. To test this hypothesis, fresh bull sperm was incubated in capacitation media. The presence and specific localization of Na^+/K^+ ATPase isoforms in the sperm head was determined by immunocytochemistry with monoclonal antibodies to the α1-3 isoforms and the β1-3 isoforms previously proven for bull sperm (Hickey and Buhr, Theriogenology, 2011, doi:10.1016/j.theriogenology.2011.10.045). Fresh semen was collected from seven bulls and aliquots were incubated in capacitating media (control) ± 0.02 µg/µl heparin ± 0.1 mM ouabain. Following incubation at 39°C for 0 or 5 h, sperm head membranes were permeabilized with methanol to expose the inner leaflet of the head plasma membrane and intracellular membranes, or left intact prior to incubation with antibodies to each individual isoform. Patterns of fluorescence, correponding to intensity and location (cell compartment) were defined and used to qualify the antibody binding over time. No or very little fluorescence was observed

when intact sperm were initially incubated with the α1 and β1-3 antibodies in capacitating medium, any initial signal disappeared after 5 h capacitating incubation. Neither heparin nor ouabain affected these isoforms. This suggests that α1, β1, β2 or β3 isoforms are not detectable on the outer membranes of intact sperm, and their location is not affected by capacitation. In intact sperm, α2 was initially present uniformly over the head. Incubation for 5 h reduced this fluorescence, but in the presence of ouabain or heparin fluorescence indicative of α2 presence appeared in the tail. Permeabilising non-capacitated sperm (0 and 5 h) revealed different α2 locations not evident in capacitated sperm. Sperm permeabilized after 5 h in heparin or ouabain showed an overall increase in intensity with some concentration of α2 to the apical ridge; non-capacitated sperm did not. Intact sperm in five bulls had α3 over the entire post-acrosomal area or concentrated below the equator; two bulls showed only the equatorial concentration. An increased number of very narrow equatorial α3 bands were observed in intact capacitated sperm. Permeabilized sperm at 0 h had α3 uniformly throughout the post-acrosomal area. With incubation (±heparin or ouabain), more sperm showed equatorial concentration of α3. These results support the hypothesis that the location of Na^+/K^+ ATPase isoforms in bull sperm change with capacitation.

Key Words: Bull sperm, Na^+/K^+ ATPase, capacitation, immunocytochemistry

3005

Egg yolk-induced head-to-head agglutination of bull spermatozoa is prevented by α-ketoglutarate

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Bull sperm head-to-head agglutination is induced by egg yolk components present in the egg yolk-citrate diluent, Caprogen. Previously, we have shown that the agglutination process involves calcium and other components in the citrate soluble fraction of egg yolk. Streptomycin is currently used as an anti-agglutination agent despite its toxicity. The objective of the present study is to develop an alternative anti-agglutination method leading to improved semen diluents by eliminating the toxic effect of streptomycin. Effect of an antioxidant, α-ketoglutarate (α-KG), on sperm agglutination was investigated ***in vitro*** using semen collected from nine bulls. The percentage of agglutinated sperm after 72 h incubation at 22°C in Caprogen with 0, 4, 5 and 6 mM α-KG was 88.3 ± 2.2%, 6.1 ± 2.9%, 3.9 ± 2.3% and 1.7 ± 1.2% ($p \leq 0.001$), respectively. The percentage of motile sperm at 72 h was 73.3 ± 1.7%, 72.2 ± 1.7%, 72.8 ± 1.5% and 72.8 ± 1.5% in Caprogen with 0, 4, 5 and 6 mM α-KG, respectively. The samples were then transferred into an incubator at 37°C and evaluated every 24 h until end-point (≤10% of motile sperm in each sample) to determine the sperm survival time. The total survival time of sperm incubated in Caprogen with 0, 4, 5 and 6 mM α-KG was 146.3 ± 4.1 h, 141.3 ± 3.1 h, 141.2 ± 3.1 h and 139.2 ± 2.9 h, respectively. Furthermore, the effect of diluent pH (6.59 vs. 7.18) on the anti-agglutinating function of 6 mM α-KG was investigated. After 72 h incubation at 22°C, the percentage of agglutinated sperm was 2.2 ± 1.5% at pH 6.59 and 71.1 ± 5.6% at pH 7.18 ($p \leq 0.001$). In conclusion, results from this study indicate that α-KG is a promising anti-agglutination agent that can be included in Caprogen.

Key Words: Head-to-head agglutination, bull spermatozoa, α-ketoglutarate, egg yolk, semen diluent

3006

A preliminary assessment of the ram spermatozoa glycoproteome using 2DLC-MS/MS

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The fertility of frozen thawed ram spermatozoa is limited following cervical artificial insemination (Maxwell et al., 2007. *Soc. Reprod. Fertil.* 64:13–38). There is evidence to suggest that seminal plasma proteins bound to the sperm membrane during ejaculation are altered or modified during cryopreservation and that this may contribute to the impaired ability of ram spermatozoa to traverse the cervix (Maxwell et al., 2007. *Soc. Reprod. Fertil.* 64:13–38). However, relatively little is known about the identity of these proteins. As such, the current study aims to determine the identity of glycoproteins in ram spermatozoa. Spermatozoa were obtained from Merino rams (n = 6), pooled and washed using density gradient centrifugation (PureSperm) prior to protein extraction (8% urea, 1% SDS) and digestion (trypsin). Glycoproteins were isolated and enriched using N-linked hydrazide chemistry (Sun et al., 2007. *Mol. Cell. Proteomics* 6:141–149). Briefly, N-linked glycolysated peptides were captured and bound to an Affi-prep HZ resin (BioRad, Australia) while unbound non-glycosylated peptides were removed. Glycosylated peptides were then enzymatically released using PNGase F, separated using two-dimensional liquid chromatography (2DLC) and analysed by tandem mass spectrometry, MS/MS (QSTAR®Elite; AB SCIEX, USA). Identified spectra and resultant proteins were then analysed using Protein Pilot v3.0 (AB SCIEX, USA). LC-MS/MS identified a total of 56 glycoproteins from the extracts of ram spermatozoa, among which, 13 have been previously identified in an LC-MS/MS analysis of ram seminal plasma (Druart et al. unpublished results). To our knowledge this is the first study to utilise hydrazide chemistry and advanced proteomics to assess the glycoproteome of ram spermatozoa. These preliminary results show the potential of using this technique in future experiments to compare the glycoproteome of epididymal and ejaculated spermatozoa, in addition to spermatozoa that have been chilled or cryopreserved. Investigation of these potential differences may highlight proteomic modifications that occur to the sperm membrane during cryopreservation and help understand their effect on sperm function and fertility.

Key Words: Spermatozoa, glycoproteome, seminal plasma, mass spectrometry, sheep

3007

Comparison of sperm motility kinetics and membrane phospholipid translocation between cryopreserved ejaculated sperm and epididymal sperm from stallions

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The development of a reliable technique to freeze epididymal semen would provide a unique opportunity to preserve valuable genetic material from unexpectedly lost stallions. The aim of this work was to compare the apoptotic indices of sperm obtained from ejaculate (Group 1, G1) and sperm from the cauda epididymis (Group 2, G2). For G1, two ejaculates from each of seven stallions were collected and subjected to cryopreservation using a BotuCrioTM extender. One week after the last semen collection, the stallions underwent bilateral orchiectomy, and sperm from the cauda epididymis was harvested immediately after castration (G2). The sperm harvesting from the epididymides was performed by retrograde flushing of the caudal portion of the epididymis using a skim-milk extender (BotuSêmenTM1). The recovered sperm was then cryopreserved using the BotuCrioTM extender. The sperm motility parameters were analyzed by CASA (Computer-Assisted Sperm Analysis), and apoptosis was estimated using epifluorescence microscopy for membrane phospholipid translocation. The samples were evaluated immediately (0 h) and 8 h (8 h) after thawing. The sperm parameter results for the G1 and G2 samples were 62.3.±12.9a vs. 72.6 ± 8.4a, 31.6 ± 9.2a vs. 35.3 ± 10.32a and 49.3 ± 14.33a vs. 59.7 ± 13.59a at 0 h and 26.0 ± 21.6b vs. 54.7 ± 12.2[a], 6.1 ± 6.4b vs. 17.4 ± 8.54[a] and 13.7 ± 14.85b vs. 37.6 ± 14.15[a] at 8 h, for the total motility, progressive motility and percentage of rapid cells, respectively. Concerning the membrane phospholipid translocation of the G1 and G2 samples, the results for 0 h were 25.1 ± 3.89a vs. 28.7 ± 4.15a, 10.8 ± 3.53a vs. 13.0 ± 3.11a, whereas the results for 8 h were 22.86 ± 5.11a vs. 21.0 ± 4.79a and 6.4 ± 2.07b vs. 12.1 ± 3.76[a], 11.7 ± 1.80a vs. 14.0 ± 3.26a and 30.8 ± 5.72[a] vs. 24.6 ± 5.83b for the percentage of viable cells, percentage of viable cells with translocation of phospholipids and percentage of damaged cells with translocation of phospholipids, respectively. At 0 h, no differences in

the sperm parameters were observed between the groups, whereas significant statistical differences were observed in the sperm motility parameters between the treatment groups after 8 h. In the translocation of phosphatidylserine, no difference was found for the 0 h time point between the groups. However, at 8 h after thawing (8 h), the G1 exhibited a lower percentage of viable spermatozoa and a higher percentage of damaged cells, as evaluated by the translocation of membrane phospholipids, when compared to G2. These results support the conclusion that frozen-thawed epididymal sperm has similar or higher motility parameters than ejaculated sperm after thawing. In addition, incubating sperm at 20°C for 8 h after thawing increased the motility parameters and reduced apoptosis in the epididymal sperm, suggesting that epididymal spermatozoa are more resistant to the cold shock caused by cryopreservation than ejaculated sperm.

Key Words: Stallion, sperm, apoptosis, epididymal, spermatozoa

3008

Influence of time within the breeding season on ROS production and stallion sperm quality

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There are anecdotal reports that fertility declines towards the end of the equine breeding season. Although mares not conceiving earlier in the season may be over-represented in the numbers bred at the end of the season resulting in skewed fertility data, fertility may genuinely decline in both mares and stallions towards the close of the breeding season. Objective: to determine whether sperm quality and ROS-production change between the beginning and the end of the breeding season. Standard cooled semen doses (up to four per stallion) were collected from nine stallions at a commercial stud at the start of the Swedish breeding season (April/May) and at the end (August). The semen was extended in INRA96 (IMV Technologies, lÂ´Aigle, France). After transport overnight to the laboratory at the Swedish University of Agricultural Sciences (SLU), aliquots were evaluated for motility by computer assisted sperm analysis (SpermVision; Minitube, Teifenbach, Germany), chromatin integrity (sperm chromatin structure assay), morphology, membrane integrity (SYBR14/PI) and production of reactive oxygen species by staining with hydroethidine and dihydrodicholorofluorescein diacetate. The overall mean values for spring and autumn were compared by Least Squares Means using a mixed Anova (SAS 9.2). Mean values of the different parameters in spring and autumn were as follows: total motility 80 ± 9% and 89 ± 6%, $p < 0.05$; progressive motility 55 ± 12% and 59 ± 8%, not significant (NS); DNA fragmentation index (%DFI) 13 ± 7 and 17 ± 7, NS; normal morphology 56 ± 13% and 61 ± 13%, NS; intact sperm membranes 75 ± 9% and 77Â ± 7%, NS; living, not superoxide producing 45 ± 13% and 60 ± 6%; $p < 0.01$; living, superoxide producing 22 ± 16% and 9 ± 6%, $p < 0.05$; dead, superoxide producing 33 ± 8% and 31 ± 6%, NS; living, no peroxide producing 66 ± 7% and 67 ± 7%, NS; living, peroxide producing 1 ± 1% and 2.6 ± 3%, NS; dead, no peroxide producing 32 ± 18% and 32 ± 6%, NS; dead, peroxide producing 0.2 ± 0.2% and 0.5 ± 0.7%, NS. The variation between stallions and ejaculates was significant for all parameters ($p < 0.01$–0.001). Menadione stimulation significantly increased the production of both superoxide and hydrogen peroxide but the effect was not different between the seasons (data not shown). Mean superoxide production by stallion spermatozoa was higher at the start of the breeding season than at the end of the breeding season. Differences in sperm quality also occurred for individual stallions but did not affect the overall mean values. Funded by the Swedish Foundation for Equine Research, Stockholm, Sweden.

Key Words: Stallion, ROS production, sperm quality, superoxide

3009

Contribution to assessment of cat kinetics sperm motility using computer-assisted sperm analysis

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The challenge of measuring the fertilizing potential of semen samples with Computer-Assisted Sperm Analysis CASA- and understanding the correlation between variables and fertility of animals, are preliminary questions to their appropriate application in clinical practice and research with cats. Studies in humans had demonstrated the applications and the impact that the CASA system, through the correlation of some values of CASA and the rate of oocyte fertilization, the pregnancy rate after *in-vitro* fertilization, intrauterine insemination and the percentage of motile sperm after thawing. Individual recognition and precise definition of sperm cell values and stringent cut-offs are essential to generate proven scientific data. The calibration and validation of CASA with a well-defined setup for cats would enable a better understanding of aspects from sperm function and would accelerate advances in researches. Stachecky et al. (1993) noticed that abnormal sperm morphology was negatively correlated to the values of VCL, VSL and ALH in domestic cat semen, but the equipment used had few parameters in common with the current setups. Therefore, the aim of this study was to generate information to calibrate the CASA setup for domestic cat epididymal spermatozoa. In the present experiment it was used the same values of Stachecky et al. (1993) to setup the CASA HTMA-IVOS 10 and collect precise measurements of 1000 sperm cells from the cauda epididymides of 10 normospermic fertile cats. The values of mean, standart deviation (SD) and percentage (25–75%) obtained were: head size pixel 3.91/0.74 and (6.10–7.35) head area pixel 6.58/1.21 and (3.79–4.15) and elongation 69.61/ 4.57 and (67–70, 30), respectively. The setup was established out of values of cellular recognition performed by the equipment with the partial Stachecky setup. All collected data were submitted to descriptive statistical analysis and Pearson correlation. Based on these results the CASA system was setup with the following values: Frames for second = 60 Hz; Number of frames = 50; Minimum contrast = 30; Minimum Cell Size = 3 pix; Cell Size = 6 pix; Intensity cell = 100; Dark field = 60×; Velocity Average Pathway (VAP) = 70/s; Straightness (STR) = 80%; VAP-Cuttoff = 30 μ/s; VSL-Cuttoff = 20 μ/s; Intensity = 3500; Magnification = 1.95. These values were tested with different cat semen samples (15 animals) and their capacity to recognize cell was measured. Villaverde et al. (2006) used these values and confirmed the accuracy of this generated analysis. A study conducted by Rijsselaere et al. (2004), noticed that many results widely varied when setup parameters were changed. M. Filliers et al (2008), research cat semen used one setup for CASA system, but without describing the methodology that was used to define it. The present setup allows a better comparison of the results obtained from different research centers and could optimize the integrated use of reproductive biotechnologies to support research on experimental.

Key Words: CASA, semen, cats, setup, calibration

3010

The effects of various diluents on sperm motility of rabbit semen stored at 5°C for up to 96 h

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The preservation of rabbit semen for several days after collection is a major limitation for artificial insemination programs of the rabbit meat industry. The low levels of fertility and prolificacy achieved with frozen semen do not allow its use at a commercial level, thus the improvement of liquid storage protocols is imperative. The aim of this study was to identify a suitable extender for the liquid storage of rabbit semen for up to 96 h at 5°C. Semen was collected from two adult rabbit bucks twice daily, two times a week, with an artificial vagina. Each ejaculate was initially evaluated for volume, sperm concentration and percentage of motile sperm. Samples containing at least 300×10^6

spermatozoa/ml and 70% motility were split into three equal aliquots. Each aliquot was diluted 1:10 with one of three different extenders: Extender A (313.79 mM TRIS, 103.07 mM citric acid.H_2O, 33.3 mM glucose and 80 mg/l kanamycin – Roca et al., 2000. *Anim. Reprod. Sci.* 64:103–112), Extender B (313.79 mM TRIS, 103.07 mM citric acid.H_2O, 33.3 mM glucose, 80 mg/l kanamycin and 14% gelatin – Lopez-Gatius et al., 2005. Theriogenology 64:252–260) or Extender C (250.04 mM TRIS, 79.76 mM citric acid.H_2O, 69.38 mM glucose, 75.0 IU streptomycin and 166.2 IU G-Penicillin – Boiti et al., 2005. *World Rabbit Sci.* 13:71–91). Diluted semen samples were stored at 5°C and sperm motility variables were assessed at 0, 24, 48, 72 and 96 h using a CASA system (HTM IVOS 12). A minimum of 300 cells were evaluated per sample. Data were analysed using a Linear Mixed Model in Genstat (VSN Int.). After 96 h of chilled storage, the percentage of total motility (±SEM) of spermatozoa diluted with Extender A (55 ± 5.2%) was significantly higher ($p < 0.05$) than spermatozoa diluted with either Extender C (34 ± 9.3%) or Extender B (27 ± 3.9%). There were no significant differences in the VAP, VSL, VCL, LIN or STR of spermatozoa diluted in Extender A, B or C. In conclusion, Extender A is the best diluent for the conservation of rabbit sperm motility during chilled storage at 5°C for up to 96 h. Further research will investigate the viability of the stored semen in rabbit artificial insemination programs.

Key Words: Liquid storage, rabbit, sperm

3050

Assessment of bovine sperm capacitation pathway L-Arginine/NO/GMPC through *in vitro* embryo production

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The aim of the study was to evaluate the effects of nitric oxide (NO) in *in vitro* capacitation induced by heparin (H) in cryopreserved spermatozoa from 3 bulls, by adding L-arginine (L-arg, precursor synthesis NO), 8-bromo-cGMP (Br-cGMP, cGMP analog main NO signaling pathway) and 1H-(1,2,4) oxadiazolo-(4,3-a) quinoxalin-1-one (ODQ, inhibitor of activity of soluble guanylyl cyclase -sGC). The control consisted of capacitated sperm in TALP medium-sp plus H (10 mg/ml). Different concentrations of L-arg (1, 2.5, 5, 10 and 50 mM), 5 and 10 mM Br-cGMP and 0.1 mM ODQ were added to the capacitation medium (200ÂÂ ÂL). The percentage of capacitated sperm was evaluated with chlortetracycline test (CTC) and the concentration of nitrate and nitrite (NO3-/NO2- stable metabolite of NO), in the capacitation medium, by the Griess method after 4 h. The quality of the *in vitro* capacitation was evaluated by the rate of blastocyst, after prior capacitation of sperm for 30 min. It was performed six replicates of each animal. Those data were analyzed by analysis of variance and means compared by the Tukey test, 5% probability. The addition of 1 mM L-arg in the capacitation medium increased ($p < 0.05$) the percentage of capacitated sperm (39.8%) compared to 10 mM L-arg (10.1%). These results may be due high NO synthesis in the culture medium after the addition of 10 mM L-arg (162 mM) compared to 1 mM L-arg (72.3 mM) reflected in lower production blastocysts rate (31.5 and 39.5% respectively, $p < 0.05$). Although 1 mM L-arg has increased the percentage of capacitated sperm, this was not reflected in the increased of capacitation quality because there was no difference ($p > 0.05$) in the blastocyst rate *in vitro* compared to control. The addition of 0.1 mM ODQ decreased the capacitation (25.6%) and NO synthesis (15.5%) reflected in decreased production of blastocysts (17.5%, $p < 0.05$). The addition of 1 mM L-arg + 0.1 mM ODQ was used to assess whether the L-arg have the effect of preventing the inhibitory action of ODQ. The percentage of capacitated sperm was increased after the addition of L-arg compared to ODQ (50.8%) and control (12.2%) but this was not reflected in sperm quality because the blastocyst rate did not differ to ODQ ($p > 0.05$). Treatment with 5 mM cGMP increased the capacitation (26%) and NO synthesis (26.5%) compared to control, reflecting the increased production of blastocysts compared to control (15.7%). The combination of 5 mM cGMP with 0.1 mM ODQ partially prevented the inhibitory effect of ODQ on capacitation, fully in the NO synthesis but theses events were not enough to reverse the decline in the production of blastocysts. These results indicate that: (i) NO is involved in the control of capacitation pathway cGMP, (ii) an optimal concentration of NO during the capacitation is needed fot the production of bovine embryos, but, NO is not the single determining factor, (iii) cGMP (5 mM) increased of capacitation quality *in vitro* reflecting the increased production of blastocysts.

Key Words: Capacitation, spermatozoa, bovine, nitric oxide, L-arginine

3051

Use of *in vitro* assessed semen quality criteria to predict fertility of bull semen

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Predicting *in vivo* fertility of bulls using *in vitro* assessed semen quality criteria remains a challenge for the breeding industry. Classical methods, as optical microscopy and CASA system are not able to predict *in vivo* fertilizing ability. Flow cytometry looks promising to achieve this goal. This study aimed to correlate *in vitro* quality assessment results performed on thawed bovine semen, to *in vivo* fertility estimated from field records. One single ejaculate from 72 young Holstein bulls was analyzed by flow cytometry (viability, acrosome status, mitochondrial potential, cellular oxidation and chromatin condensation), by optical microscopy (motility, morphological abnormalities) and by CASA (Velocity Average Path...). For each ejaculate, an adjusted Fertility Value (FV) was estimated from non-return rates at 28 days issued from 613 ± 99 inseminations per bull, ranging from 100 to 763, and adjusted on the same environment factors as the French breeding values model. FVs ranged from -12.8 to + 9.2 between bulls. Statistical analyses, combining cytometry parameters and microscopic evaluation were carried out to define different multivariate regression models aiming to predict FV. Individual correlations (R) between each quality parameter and fertility data were too low for an accurate prediction (Table 1). Multiple regressions using stepwise parameters selection led to two mathematical models presenting high correlations with FV and combining different parameters. The model A included six parameters issued from four different protocols of flow cytometry (viability, mitopotential and two cellular oxidation tests) and was highly correlated with FV ($R = 0.64$, $p < 0.0001$). The model B led to improved correlation results ($R = 0.69$, $p < 0.0001$), including the model A parameters together with two different morphological abnormalities percentages (Mid-piece partial disruption and total abnormalities). Combining different *in vitro* assessed parameters of the spermogram will pave the way of predicting bovine semen fertility. Most of the predictive criteria (75–100%) are assessed by flow cytometry, thus indicating that this promising technology will probably be added within a close future as a routine method to assess semen quality in semen production labs.

Key Words: Semen evaluation, bull fertility, flow cytometry, spermogram, CASA

Table 1. Descriptive statistics and correlation coefficient (R) between individual semen quality parameters and fertility values

Parameter	Mean	SD	Min	Max	R (p value)
Damaged acrosome in live sperm (%)	2.47	1.62	0.3	7.8	-0.030 (0.79)
Viability (%)	52.7	9.9	26.2	72.3	0.164 (0.17)
Mitopotential (mifu)	738	209	270	1272	0.322 (0.006)
Oxidation (%)	66.1	8.28	41.6	79.9	0.294 (0.01)
Morphological abnormalities (%)	21.5	5.8	11	40.5	-0.262 (0.03)
Chromatine condensation (mifu)	114.1	8.7	96	134	-0.331 (0.004)
CASA/VAP (μm/s)	111.4	7.5	91.4	124	0.142 (0.233)

3052

Correlations between testicular hemodynamic and sperm characteristics in rams

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In domestic mammals, pampiniform plexus is responsible for testicle blood supply and has a great importance in the heat radiation. Since spermatogenesis requires a temperature between 2 and 6°C below body temperature, vascular changes in the testis may lead to marked negative effect on sperm production, reflecting, especially, on sperm motility and morphology. This study aimed to evaluate the presence of correlations between changes in testicular blood flow by color Doppler ultrasonography and sperm characteristics. Eight Santa Ines adult rams aged between 1 and 6 years old, with different reproductive histories, showing a variation of the testicular parenchyma status since normal to different degeneration grades were submitted, fortnightly, over a 90 days period, to Doppler ultrasonography analyses and semen collect and evaluation. Each animal was evaluated for testicular hemodymanic by Doppler ultrasound (M5vet; Mindray®), using the spectral mode to evaluate resistance index (RI) and pulsatility index (PI) of pampiniform plexus and vascular color Doppler mode to evaluate vascularization score of the pampiniform plexus and testicular parenchyma by a subjective analysis of colored image (scored of 0–4) and objectively, on average number of pixels, after analysis of the image by the computer analysis software Image J. The semen was evaluated on sperm motility (%), by light microscopy evaluation (100× amplification) and sperm morphology (%), by phase contrast microscopy (1000× amplification). The data were submitted to Pearson′s linear correlation using the SAS (Statistical Analysis System) software. No correlations were found between motility and hemodynamic characteristics of testicles evaluated ($p > 0.05$). Positive correlations were found between the percentage of total sperm defects with left parenchymal score ($r = 0.32$, $p = 0.0235$), right parenchymal score ($r = 0.29$, $p = 0.0441$), left RI ($r = 0.31$, $p = 0.029$) and left PI ($r = 0.30$, $p = 0.0351$). There were no correlations between pampiniform plexus vascularization with testicular parenchyma vascularization ($p > 0.05$). On the other hand, the left pampiniform plexus score was positively correlated with the average pixels from the left pampiniform plexus ($r = 0.51$, $p = 0.0002$) and negatively correlated with RI ($r = -0.44$, $p = 0.0015$) and PI ($r = -0.41$, $p = 0.0031$) on the left pampiniform plexus. A similar situation was observed for the right side, with the right pampiniform plexus score positively correlated with the average pixel from the right pampiniform plexus ($r = 0.63$, $p < 0.0001$) and negatively correlated with RI ($r = -0.49$, $p = 0.0003$) and PI ($r = -0.40$, $p = 0.0039$) on the right pampiniform plexus. This study showed that the increase of sperm defect can be related to increase of testicular parenchyma vascularization, there is not a relationship between pampiniform plexus and testicular parenchyma vascularization, and the characteristics evaluated by Doppler ultrasound are related.

Key Words: Ovine, Doppler, vascularization, testis, semen

3053

Effect of antioxidants supplementation in equine semen extenders on semen acrosomal integrity and mitochondrial membrane potencial after cryopreservation

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The objective of this study was to evaluate post–thaw quality of frozen equine semen, processed with diluents containing different concentrations of α–tocopherol and ascorbic acid antioxidants and to determine a concentration capable of improving its cryopreservation.

Semen was collected from three stallions (two ejaculates per stallion) by artificial vagina, pooled and evaluated for routine parameters of semen quality. After centrifugation, the semen was divided in seven different samples and extended to a final concentration of 100×10^6 sperm/ml using Ghent® extender containing no supplements (control) and the following supplements with three different concentrations: α–tocopherol (0.5, 1, 2 mM) and ascorbic acid (0.45, 0.9, 1.8 g/l). Afterwards, the semen was packed in 0.5 ml straws and cryopreserved in a controled rate freezer.

After thawing, all samples were maintained at 37°C, while analyzes were performed at time 0(T0), 60(T1) and 120(T2) minutes. The evaluation of plasmatic and acrosomal membrane integrity (FITC–PSA and PI fluorescence) and mitochondrial membrane potencial (JC–1 fluorescence) of each samples was determined by flow cytometry. Sperm cells were analyzed at a rate of 500–1000 cells/s using FACS flow® as sheath fluid and 10 000 events for each sample were recorded and analyzed with Cell-Quest® software. The data was submited to homogeneity test, followed by variance analyzis (ANOVA one–way–$p \leq 0.05$), with post–hoc LSD test by IBM SPSS® Statistics Program. The measurement of plasmatic and acrosomal membrane integrity showed no statistical significance differences between the three incubation times measured. Although, the medium with ascorbic acid in a concentration of 0.45 g/l showed a higher percentage of sperm cells with intact acrossome, at all incubation times. It was observed that the acrosomal integrity membrane decreased over time; however the addition of antioxidants in the freezing medium slowed this process. Concerning the mitochondrial membrane potential after cryopreservation, the addition of 0.45 g/l ascorbic acid in the medium, showed a significant ($p \leq 0.05$) higher percentage (T0: 49.46 ± 7%; T1: 47.53 ± 4%; T2: 28.01 ± 4%) of sperm cells when compared to the control group (T0: 31.20 ± 4.28%; T1: 38.98 ± 4%; T2: 25.10 ± 5%). The α–tocopherol supplemented samples showed no statistical differences. Our results clearly indicated that adding antioxidants in stallion sperm freezing media, can improve the quality of the semen, in spite of not having performed others studies about its action in membrane′s lipid peroxidation (LPO). Further studies will be needed to understand the mechanism of how the addition of antioxidants can benefit semen cryopreservation, specially ascorbic acid, which is not only effective as a non–enzimatic antioxidant but also a reactive oxygen species scavenger with a high potency against to LPO.

Key Words: Equine, semen, cryopreservation, α-tocopherol, ascorbic acid

3054

Do spermatozoa from fertile Thoroughbred stallions live fast and die young?

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As ART are not permitted in Thoroughbreds, identification of fertility markers in Thoroughbred stallions depends upon investigating the relationship between post-coital dismount sperm quality and pregnancy outcomes. As stallion sperm utilise oxidative phosphorylation, it is hypothesised that more fertile spermatozoa with higher metabolic rates will display increased levels of oxidative damage upon arrival to the laboratory. The aim of this study was to ascertain whether the predictive ability of semen assessments is confounded by an increased rate of damage to higher quality cells during transport to the laboratory. Dismount samples ($n = 67$) from Thoroughbred stallions were assessed for motility (CASA), vitality (eosin), oxidative DNA damage (8-hydroxy-2-deoxy Guanosine; 8-OH-dG) and aldehyde dehydrogenase levels (Aldefluor®) within 8 h. Early pregnancy (14 day transrectal ultrasound) data was collected and the parameters of samples which achieved pregnancies (fertile; $n = 38$) were compared with the parameters of samples which did not achieve pregnancies (infertile; $n = 29$) using a Student's *t*-test. Fertile samples had fewer vital (57 vs. 66%; $p = 0.05$) and rapidly motile (14 vs. 22%; $p < 0.05$) spermatozoa, fewer live spermatozoa without oxidative DNA damage (58 vs. 68%; $p = 0.03$) and fewer Aldefluor positive cells (13 vs. 20%) than non-fertile samples. To study the relationship between motility and mitochondrial ROS, semen was collected ($n = 3$) and motility (CASA) and mitochondrial ROS production (MitoSOX™ Red) were assessed after incubation for 24 h at 37°C. A significant negative correlation was observed between the percentage of spermatozoa with low mitochondrial ROS and velocity (VCL; $R^2 = -0.80$, VAP; $R^2 = -0.77$). In conclusion, though fertility is dependent on mare and stallion factors, ′fertile′ spermatozoa undergo higher rates of oxidative phosphorylation, leading to increased ROS, oxidative DNA damage, aldehyde generation and an accelerated rate of cell death.

This 'live fast, die young' phenomenon means that assessments must be performed close to ejaculation for a true indication of sperm quality.
Key Words: Stallion, ROS, motility, mitochondria, fertility

31. Stem cell biology in reproduction:

3100

Bovine endometrial cells express markers of pluripotence and display osteogenic and chondrogenic differentiation potential *in vitro*

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There are no reports of resident stem cells in the bovine uterine endometrium. This research was aimed to identify the presence of stem cell markers in the bovine endometrium during the oestrus cycle and to evaluate the differentiation potential to osteogenic and chondrogenic lineages of endometrial cells *in vitro*. We isolated eight cell lines from endometrium of cycling cows; four from early luteal phase (days 3–5 post-oestrus; ELF) and four from late luteal phase (days 12–15; LLF). Some gene and protein markers for stemness were studied both in the cells and in the fresh tissue from which they were derived. All the qPCRs were normalized for ACTB expression; P4r was used as a marker of endometrial origin. The markers studied by RT qPCR were OCT4, SOX2, Nanog and CD44 and immunohistochemistry (antibodies against OCT4, SOX2, CD44, cytokeratin and vimentin). For differentiation, cells in passage 2 were seeded in 6-well dishes allowed to get confluent and changed to differentiation media (DM) based on DMEM low glucose, to induce to osteogenic lineage [OL = DM + 10% SFB, 10–7M dexamethasone, 0.01 mM β glycerophosphate, 0.3 mM vitamin C), or chondreogenic lineage [CL = DM + 1X insulin-selenium-transferrin mix; Gibco, NY, USA, 35 ug/ml vitamin C, 100 nM dexamethasone, 10 ng/ml TGF β). Control time-matched cells were not induced. Media was changed every third day. At days 0, 10, 14 and 21 cells were stained for the appropriate lineage (alizarin red for OL, alcian blue for CL and RNA extracted for RT-PCR). We found mRNA expression of OCT4, SOX2 and CD44 in all fresh samples, and their encoded protein in immunohistochemistry, with expression mainly confined to some cells in the glandular (GE) and luminal epithelium for OCT4 and SOX2, CD44 showed a membranous pattern in the GE. In cell cultures, CD44 expression was detected in all samples, but OCT4 and SOX2 only in two lines of the LLF. Nanog expression was not detected in any samples. The four LLF cell lines displayed osteogenic and chondrogenic potential upon induction with appropriate inducers, none of the ELF cell lines did. *in vitro* osteogenesis was evident at day 7, but stronger at day 14, it persisted to day 21 of induction. A similar pattern was found for CL although a little more delayed in time. ELF cells did not differentiate into any of the lineages tested. In qPCR, gene markers Sox9 (OL) and collagen $\alpha 2$ (CL) were expressed in the LLF induced cells, but not in the ELF or in the controls. These findings suggest that endometrial cells express markers of stemness and might be implicated in a cellular response to nest a potential embryo, especially cells from late luteal phase which displayed markers of stem cell related to pluripotency, as well as osteogenic and chondrogenic differentiation potential *in vitro*. This work was supported by FONDECYT 1110642 grant from Ministry of Education of Chile.
Key Words: Oct4, endometrium, osteogenesis, chondrogenesis, stem cells

3101

Long-term culture and identification of bovine spermatogonial stem cells

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Manipulation of Spermatogonial Stem Cells (SSCs), which includes enrichment/culture of donor SSCs and transplanting the SSCs into the testes of recipient males, is an alternative approach for animal assisted reproduction. SSCs are the only stem cell type in the adult that contributes genetic information to the next generation. *in vitro* culture and transplantation of SSCs help to study SSCs and take advantage of their applications in transmitting genetic information from donor of SSCs to next generation. Long-term culture of SSCs is the core technique for the manipulation of SSCs described above. However, except for rodents, long-term culture of SSCs from large animal has not been very successful. The purpose of the present study was to establish a technique for long-term culture of bovine SSCs. Initially, using immunofluorescence staining of frozen sections, we determined that CDH1 and PLZF are specific markers for bovine SSC. After obtaining preparations of single cell suspension from bovine testis three steps of differential attachment were required for enrichment and purification of SSCs as determined by the high rate of positive immunofluorescent staining of the cultured cells with PLZF and CDH1. Cells were cultured on mitomycin C treated STO cells as the feeder layer in serum-free cell culture medium and differentiation was inhibited by the addition of proper growth factors including GDNF. RT-PCR revealed that cultured cells expressed CDH1 and Thy1 transcripts. Previous research had illustrated that expression of Thy1 by SSCs is a conserved phenotype between rodents and bovine. Analysis showed that the cultured SSCs maintained their characteristics for more than 20 passages (> 1 year) *in vitro*. Bovine SSCs could be cryopreserved in liquid nitrogen for over one year, maintaining the expression of SSC specific marker genes after thawing. We established a set of techniques are for long-term culture of bovine SSCs which lay a solid foundation for bovine SSC manipulation.
Key Words: Bovine, spermatogonial stem cells, culture, identification

3102

The role of GalNAc glycan residues associated adhesion and self-renewal in cultured bovine gonocytes

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Gonocytes are primitive germ cells that have a germline stem cell potential, and can be maintained their potential in culture condition. Cell surface molecules of germ cells that are essential in cell adhesion and survival of germ cells are available to enrich and identify an objective population. In domestic animals, N-acetylgalactosamine (GalNAc) that is found on the surface of gonocytes and/or spermatogonial stem cells may affected in their physiological characteristics. The lectin DBA (Dolichos biflorus agglutinin) recognizes the GalNAc residue on the surface of germ cells. Therefore, enriched gonocytes by using DBA are a useful model of understanding a biological function of cell surface glycans. The present study found that the population of bovine gonocytes in the testis of 3.5-month-old calves was significantly higher on the DBA-coated plate (4.21% $\pm$ 1.09) than those on the gelatin- (GN, 2.03% $\pm$1.02) and laminin- (LN) coated plates (0.85% $\pm$0.73), but not statistically significant on the poly-L-Lysine- (PLL) coated plate (2.08 $\pm$ 0.9) at 4 h after cell plating. Interestingly, the average numbers of colonies in culture significantly increased on the DBA (126.5 $\pm$ 7.5) with compared to other ECM components (GN 72.5 $\pm$ 0.5, $p < 0.05$), PLL (33 $\pm$ 13.0, $p < 0.01$) and LN (0, $p < 0.001$). The DBA plate was effectively restricted the growing of somatic cells, resulting in a beneficial effect for colony formation. In

addition, the expressions of pluripotency-related transcription factors were dynamically changed on the lectin DBA in culture. The levels of POU5F1 transcripts were markedly up-regulated on the DBA -coated plate, and SOX2, REX1 and c-MYC transcripts also considerably increased. These results suggest that a structural change of carbohydrate chains by forming GalNAc-DBA complex on germ cells may effect on cell growth and cell survival in culture. These glycan complexes may provide microenvironment for cell adhesion and colony formation of spermatic stem cells and provide a possibility to maintain bovine gonocytes for a long-term culture.

Key Words: Gonocytes, spermatogonial stem cells, bovine testis, glycoconjugates, lectin

3103

Isolation and chondrogenic differentiation of bone marrow mesenchymal stem cells (MSC) from bovine fetuses

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The potential for isolation of bone marrow mesenchymal stem cells (MSC) and the plasticity to differentiate into bone, cartilage, and adipose tissues have been characterized in many mammal species. However, limited information regarding bovine MSC is available, despite the wide relevance of the bovine experimental model and the potential application of adult stem cells in bovine veterinary medicine. Here we present preliminary data on the isolation and *in vitro* chondrogenic differentiation of bone marrow MSC obtained from bovine fetuses. Bone marrow was aspirated from the femur of two bovine fetuses (8–9 months of gestation) collected at a local abattoir. The bone marrow sample was centrifuged in PBS and in basal medium consisting in DMEM (high-glucose; GIBCO BRL, Grand Islands, NY, USA) supplemented with 100 μg/ml streptomycin, 100 IU/ml Penicillin and 2.5 μg/ml Amphotericin. Following the determination of cell viability and number, nucleated cells ($5 \times 10^4/cm^2$) were plated in culture flasks in basal medium and incubated at 38°C in a humidified atmosphere containing 5% CO_2. After being cultured for 4 days, non-adherent cells were removed by changing the culture medium. After four or five passages, cells were detached using 0.25% trypsin in 0.1% EDTA and resuspended in basal (control) or differentiating medium consisting in basal medium supplemented with 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 1 mM pyruvate, 50 μg/ml ascorbate 2-phosphate and 8 ng/ml TGF-β1. Aliquots of 1×10^6 cells in 1 ml of each medium were spun down at 500 g for 5 min in a 15-ml polypropylene conical tube. Pellets were cultivated at 38°C in 5% CO_2 for 21 days. During the entire 21-day experimental period, cells were sampled on day 0 and at seven-day intervals thereafter for analysis of expression of cartilage-specific genes Agrecan, Collagen-2 and Sox-9, pluripotent gene Nanog, and housekeeping gene Gapdh by quantitative-PCR (Q-PCR). After 21-day of differentiation, pellets were fixed in 2% paraformaldehyde, paraffin-embedded, sectioned and processed for histologic evaluation with Toluidine blue staining. After 4 days of culture, fibroblast-like cells were observed attached to the plastic culture flasks. Clearly identifiable pellets were observed after 7-days of culture in differentiation medium; whereas, no pellet formation was observed in control tubes. Expressions of Agrecan, Collagen-2 and Sox-9 in day-21differentiation pellets were 169.6, 28.3, and 19.2 fold-relative to day 0. Nanog expression in day-21 differentiation pellets was 17.5 fold-relative to day 0. Staining with Toluidine blue and Alcian blue indicated cartilaginous matrix formation in pellets at day 21 of differentiation. MSC were isolated from bovine bone marrow based on characteristic adhesion property to the plastic culture flasks. Expression of cartilage-specific genes and cartilaginous matrix formation suggest *in vitro* differentiation of MSC into the cartilage lineage.

Key Words: Mesenchymal stem cell, differentiation, bovine fetuses, chondrogenic, *in vitro* culture

3104

Analysis and comparison of the expression of SOX2 in blastocyst and hatched blastocyst

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The present experiment aimed to compare the level of expression of SOX 2 in bovine blastocysts (D7) and hatched blastocysts (D10), because it is known that around hatching, the inner cell mass (ICM) differentiates into hypoblast and epiblast. Cumulus oocyte complexes were matured in TCM 199 for 24 h and fertilized with frozen thawed sperm. Presumptive zygotes were cultured in SOFaaci for seven days (group 1) and for ten days (group 2). All embryos were washed three times in PBS, pooled and frozen at -80°C until RNA extraction. For quantification of SOX2 mRNA levels, total RNA was isolated from pools of seven embryos per assay (n = 3) and for each examined developmental stage using RNeasy Micro kit (Qiagen). The reverse transcriptase Superscript III (Invitrogen) was used for the synthesis of complementary DNA (cDNA) and the polymerase chain reaction (qPCR) was performed with the Gotaq qPCR Master Mix (Promega, MA, USA). As negative control, cDNA was replaced by nuclease free water in the qPCR reaction. Quantification of expression was determined by the relative standard curve method and normalized to the housekeeping gene YWHAZ. Standard curves for SOX2 and YWHAZ were derived from 10 fold serial dilutions of bovine DNA and gave correlation coefficients >0.99 and efficiencies >94%. The data from three assays were analyzed by ANOVA and it was found that the level of SOX2 transcription is 9.8 times higher in blastocyst (D7) cells than in hatched blastocysts (D10) cells. As SOX2 is a pluripotency marker its expression is higher in the earlier stage of embryonic development. Acknowledgement: FAPESP

Key Words: SOX 2, bovine, pluripotency, PIV, stem cell

3105

Possible factors for engraftment of monkey embryonic stem cells after in utero transplantation into sheep fetuses

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We previously developed monkey/sheep hematopoietic chimeras by in utero transplantation (IUT) of monkey embryonic stem (ES) cells in sheep fetuses. In this study, we examined the possible factors affecting ES cell engraftment in both the recipient sheep and donor cells. Undifferentiated monkey ES cells and their derivatives at early hematopoietic differentiation stage were transplanted into sheep fetuses; the latter cells had been allowed to differentiate by culturing on OP9 cell layers for 6 days. The two types of cells were transplanted into the liver or subcutaneous tissue of recipient sheep fetuses at 43–49 or 50–73 days of gestation (full term = 147 days) using ultrasound to identify the site for transplantation. After birth, monkey hematopoietic engraftment in the bone marrow was analyzed in 29 lambs using colony-PCR with cells grown in methylcellulose in the presence of defined cytokines, and teratoma formation was analyzed by biopsy and immunohistochemistry. We found that hematopoietic engraftment was only observed when ES cell-derivatives at the early hematopoietic differentiation stage were transplanted into fetal livers at 50–73 days of gestation. However, teratoma formation with mature monkey tissue structures was observed following transplantation of undifferentiated ES cells into the fetal subcutaneous tissue, but not into the liver, and at

43–49 days of gestation, but not at 50–73 days. These results demonstrate that the differentiation status of the donor cells, the transplantation site, and the age of the fetus at transplantation are important factors in the successful hematopoietic engraftment or tumor formation in sheep fetuses. It is also possible that the immunological status of the recipient fetus might influence the success of xenotransplantation of ES cells.
Key Words: Sheep, fetus, stem cells, differentiation, in utero transplantation

3106

Induced pluripotent stem cells derived from equine keratinocytes

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The generation of induced pluripotent stem cells (iPSCs) from domestic species offers potential not only in veterinary regenerative medicine but also for disease modelling and *in-vitro* toxicological studies. Most animal iPSC lines generated to date have been derived from fibroblasts, however, studies in human and mice have indicated that keratinocytes and other epithelial cell types may represent a superior cell source for reprogramming. This study reports the generation of iPSC lines from equine keratinocytes. Skin biopsies from a 5-month old foal were used to derive keratinocyte cultures. These cells were then transduced with retroviral constructs coding for murine Oct-4, Sox-2, C-myc and Klf-4 sequences. Following transduction, tight cell colonies with sharp boundaries resembling previously reported human iPSCs were generated. The reprogrammed cells were cultured on SNL feeders in serum-containing media with bFGF and LIF. Several iPSC lines were successfully expanded over >10 passages and they expressed pluripotency markers including alkaline phospahates, Oct-4, Sox-2, SSEA-1 and Nanog. These were able to form embryoid bodies and to differentiate into derivatives of the three germ layers *in-vitro* as was indicated by expression of α-Fetoprotein, Gata-4, Nestin, β-tubulin and smooth muscle actin. Some of the cell lines had a tendency to spontaneously produce well-differentiated neurons expressing both Nestin and β-tubulin. Other equine iPSC lines spontaneously formed thin-walled, fluid-filled vesicles in culture which were steroidogenically active and resembled structures reportedly produced by induced trophoblast cells from other species. This is the first study reporting the generation of iPSCs cells from equine keratinocytes. These cells may offer distinct advantages over fibroblast-induced iPSC and represent a step forward towards future veterinary biomedical applications of equine pluripotent stem cells.
Key Words: Induced pluripotent stem cells, keratinocytes, equines, regenerative medicine

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Cultivation of mesenchymal stromal cells from cryopreserved mononuclear cells isolated from equine umbilical cord blood

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The therapeutic potential of mesenchymal stromal cells (MSC) for cellular therapy has generated an increasing interest in this type of research. In human, as well as in veterinary medicine, considerable research has been performed on the cryopreservation of expanded MSC, but little information is available on the possibility to cryopreserve the original mononuclear cell fraction (Lee et al., 2004. *Biochem. Biophys. Res. Commun.* 320:273–278; Casado-Diaz et al., 2008. *Cytotherapy* 10:460–468). The present study describes a protocol to successfully expand equine MSC after cryopreserving the mononuclear cell fraction of umbilical cord blood (UCB). To this end, the mononuclear cell fraction was isolated from 7 UCB samples as previously described (De Schauwer et al., 2011. Tissue Eng. C 17(11):1061–1070) and cryopreserved in standard 1.8 ml cryotubes at a concentration of $1–2 \times 10^6$ cells per ml cold freezing solution (high glucose DMEM, 10% FBS, 20% DMSO). Cells were kept frozen for a minimum of 6 months before thawing. Frozen cryotubes were thawed in an alcohol bath at 37°C. Cell viability after thawing remained around 98%. Approximately 2×10^6 cells were seeded per well of a 6-well culture plate in culture medium containing low-glucose DMEM, 30% FCS, 10^{-7} M low dexamethazone, 50 μg/ml gentamycine, 10 μl/ml antibiotic antimycotic solution, 250 ng/ml fungizone and 2 mM ultraglutamine. In six out of seven samples, adherent spindle-shaped cell colonies occurred within 6.8 ± 2.1 days and were passaged after 11 ± 2 days of incubation at 37.5°C and 5% CO_2. After three passages, undifferentiated MSC were immunophenotyped using multicolor flow cytometry, as previously described (De Schauwer et al. in press. Cytometry). Cultured MSC are positive for CD29, CD44, and CD90, and were negative for CD45, CD79α, MHC II and the monocyte marker. This mAb recognizes the calprotectin molecule L1, which has a restricted distribution within monocyte-derived cells. A variable expression was found for CD105. In conclusion, equine MSC can be cultured successfully after cryopreservation of the isolated mononuclear cell fraction, an approach that is both time- and cost-efficient.
Key Words: umbilical cord blood, mesenchymal stromal cells, cryopreserved mononuclear cells, equine

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Plasticity in pig induced pluripotent stem cells modulated by small molecules

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Small molecules targeting specific signalling pathways have the potential to improve the efficiency of somatic cell reprogramming. The use of mitogen-activated protein kinase pathway (ERK) and glycogen synthase kinase 3 (GSK3) inhibitors (2i) in combination with LIF has been shown to promote the generation of germline competent embryonic stem cells (mESC) in mice. The aim of this study was to test whether pig reprogrammed cells respond to different signalling inhibitors by altering their gene expression profile without losing their undifferentiated state. Pig induced pluripotent stem (piPS) cells were generated from foetal fibroblast by lentiviral transduction of doxycycline inducible OCT4, SOX2, KLF4 and c-MYC. Two culture conditions were used to derive 12 piPS cell lines: eight cell lines cultured in medium with LIF only, were characterized by FGF5 expression (a marker of mouse epiblast stem cells). These cells are known as primed cells. Another four cell lines cultured in medium with 2i + LIF expressed STELLA (a marker of mESC and human stem cells). These cells are known as naïve cells, and express low levels of FGF5. The addition of FGF receptor inhibitor to 2i cells (3i) induced the expression of REX1, an additional marker of naïve pluripotency. Primed and naïve stem cells were able to form embryoid bodies, which spontaneously differentiated into three somatic germ layers upon removal of small molecules. Interestingly, only naïve cells reactivated the expression of the germ cell specific markers Vasa and Dazl after 15–23 days of differentiation. Furthermore, naïve piPS cells labelled with red fluorescent protein implanted in the primitive streak of chicken embryos contributed to multiple embryonic and extraembryonic tissues, demonstrating *in vivo* differentiation capability. These experiments show that different states of pluripotency are interconvertible by modulation of the culture conditions using small molecules. Somatic cell reprogramming in pigs can be improved in 2i/3i conditions to obtain naïve piPS cells with potential to differentiate into germ cell precursors. The gene expression dynamics of the pluripotency markers REX1, STELLA and FGF5 are valuable readouts for studying the plasticity of piPS cells in response to culture conditions.
Key Words: Pig, iPS, stem cell, germ cell markers, small molecules

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Comparison of differential plating methods to isolate bovine spermatogonial stem cells

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Spermatogonial stem cells (SSC) have important application in animal reproduction, from establishment of treatments for infertility to more advanced biotechnologies such as animal transgenesis. However, the isolation of bovine SSCs is still less efficient than other species. Therefore, the use of molecular markers such as alpha six integrin (A6Int) to identify them is of great interest in order to improve their isolation. Goal of this study was to verify which differential plating (laminin, lectin from Datura Stramonium (DSA), Matrigel, bovine serum albumin (BSA) or PBS/control) is the most efficient for isolation of bovine SSCs. In this way, 3 g of parenchyma of testicles (n = 6) from adults Nelore bulls were minced with a scissor and consecutively digested with two solutions: colagenase (1 mg/ml) for 30 min at 37°C and trypsin (2.5 mg/ml) for 5 min at 37°C. Cells from each testicle were plated (5 × 106 viable cells) in one 100 mm Petri dish covered with 1 ml of laminin (20 μg/ml), DSA (20 μg/ml), Matrigel (5%), BSA (0.5 mg/ml) or PBS (all prepared 12 h before the onset of differential plating). Cells were cultured for 18 h in high humidity atmosphere with 5% of CO2 at 37°C. All cells from the supernatant were centrifuged (600 × *g*/5 min) and Tripan Blue exclusion method was used to determine concentration and viability. Approximately 1×10^6 viable cells from each sample were fixed with cold ethanol 70% and incubated with antibody anti A6Int labeled with Alexa Fluor 488 (BioLegends®) for 30 min at 4°C. All samples were washed three times (600 × g/ 5 min) with PBS, incubated with 0.5 μg of propidium iodide for 10 min and washed two times more. The percentage of cells marked with anti-A6Int antibody was determined by flow cytometry. The effect of each differential plating method on the presence of positive SSC for A6Int was evaluated by Tukey's Studentized Range (SAS®) and the effect of each testicle in this percentage by *T*-test (SAS®). No effect of treatment was observed on the percentage of A6int positive cells ($p = 0.277$). However, it was observed and effect of testicle ($p \leq 0.001$). These results show that there is a high variability between testicles from adult bovines regarding isolation of SSC. The differential plating is frequently used to obtain an enriched population of SSC from pre-pubertal bulls, but the same method is not so efficient for isolation of SSCs from adults. In conclusion, more studies are needed to improve the isolation of bovine SSCs.

Key Words: Spermatogonial, bovine, stem cell, differential plating, alpha 6 integrin

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Effect of different FCS concentrations on matured bovine spermatogonial stem cells coculture with Sertoli cells

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In the present study, we examined the *in vitro* effects of co-culture with Sertoli cells and of different FCS concentrations on the efficiency of spermatogonial cells colony formation. Both Sertoli and spermatogonial cells were isolated from adult bull testes by enzymatic digestion. The purification of spermatogonia was done after isolation of interstitial cells, spermatid, sperm, spermatocytes and Sertoli cells. Isolation of Sertoli cells was done with 5 Î¼g/ml Dathura Stratmonium Aglutinin (DSA) in PBS. In addition to morphology, the identity of spermatogonia was confirmed by OCT4 immunocytochemistry. Isolated spermatogonia were cultured with various concentrations of FCS (0, 2.5, 5, 10%) with or without a Sertoli cells feeder layer for three weeks; cell were passaged at the end of first week. Colony number and diameter were determined under a light microscope at the end of the each week. The statistical significance between mean values was determined using one sample Kolmogorov- One-way ANOVA and $p < 0.05$ was considered to be significant. Results indicated that 10% FCS was the best concentration for *in vitro* culture of adult bull spermatogonial cells. Coculture with Sertoli cells showed a significant increase in number and diameter of colonies in all FCS concentrations. Positive results were observed also when spermatogonia were co-cultured on Sertoli cells with 5% FCS with number and area of colonies higher ($p < 0.05$) than those obtained in medium supplemented only with 2.5% or no FCS. It is concluded that co-culture with Sertoli cells in the presence of 10%FCS increases adult spermatogonial cell colony formation and proliferation compared to the other treatments, however it is also possible to adapt spermatogonial cells to culture conditions with only 5% FCS.

Key Words: Spermatogonia, sertoli, bovine, culture

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Treatment of mares with chronic uterine injury using stem cells obtained from bone marrow

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Stem cells (SC) have the capacity to self-regenerate and differentiate into various cell types. These cells have an important role in the development of living beings and an enormous potential for use in regenerative medicine. The aim of this study was to evaluate the efficiency of bone marrow stem cell therapy in the treatment of the endometrium of mares with chronic degenerative process characterized by the presence of severe uterine fibrosis. Fifteen mares aged 14–23 years, weighing 400–600 kg, with a reproductive history of infertility and with a severe degree of uterine fibrosis diagnosed by histology, were used. After asepsis and sedation of the mares, the bone marrow was aspirated from fifth sternebrae with an 8 gauge × 12 cm length Jamishidi six needle and a 20 ml syringe containing 5 ml of DPBS and 1 ml of heparin 1000 UI/ml. The cell culture of mesenchymal cells was performed according to the methodology described by Boyum (1968). The administration of stem cells in the uterus was performed with a flexible videoendoscope using a sclerosis needle. A total of 8×10^6 cells in a volume of 0.5 ml were injected in 20 different points spaced 1 cm, following a horizontal line from the tip of one uterine horn to the other. Uterine biopsies were performed before and at 30 and 60 days after treatment for histopathologic evaluation. The results showed a significant decrease ($p < 0.05$) in the number of glandular nests (D0: 15 ± 2.04; D30: 8.09 ± 1.54; D60: 6.45 ± 1.04). In relation to the glandular distribution (1 – homogeneous; 2 – homogeneous/mixed; 3 – mixed; 4 – mixed/ heterogeneous; 5 – heterogeneous) it was also observed a significant improvement ($p < 0.05$) on grade classification (D0: 5; D30: 3; D60: 1). Glandular density (1 – low; 2 – low/high; 3 – medium; 4 – medium/normal; 5 – normal) and glandular activity (1 – active; 2 – inactive; 3 – mixed) were also better after SC treatment (D0 3/2.5; D30 3/1; D60 1/1). Based on these results we can concluded that the treatment of mares with chronic degenerative process of the endometrium with MSC derived from bone marrow was effective in improving the endometrial histological architecture regarding the activity, distribution and glandular density. Further studies are warranted to determine the effect of this novel therapy on a large number of mares and its impact on fertility rates.

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Key Words: Stem cell, mares, uterine injury, equine, bone marrow

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Isolation and nuclear transfer of germ-line stem-like cells from adult pig testes

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Testes-derived male germ-line stem (GS) cell, the *in vitro* counterpart of spermatogonial stem cell (SSC), has potential biomedical applications in reproductive biotechnology, genetic engineering, transgenesis and infertility treatment. However, little success has been achieved in the isolation of GS cells from adult pig testes. This study was designed to identify an optimal isolation and culture method for establishing GS cell lines from adult pig testes. Testes from abattoir-derived crossbred adult pigs of unknown age were decapsulated and seminiferous tubules were mechanically teased to release the differentiating and differentiated germ cells. Putative SSC were then isolated by differential plating and cultured in a feeder-free condition in high glucose DMEM supplemented with 20% (v/v) fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, 2 mM 2-mercaptoethanol, 1% nonessential amino acids, 20 ng/ml epidermal growth factor and/or 10 ng/ml human recombinant glial cell line-derived neurotrophic factor at 39°C in a humidified atmosphere of 5% CO2 in air. Three dimensional colonies appeared after 6–8 days of *in vitro* culture and stained weakly positive for alkaline phosphatase similar to mouse GS cells. The colonies could be maintained with undifferentiated morphology for more than 90 days and were positive for the markers of undifferentiated spermatogonia (PGP9.5), proliferating germ cell (Pig VASA), pre-meiotic germ cell (DAZL) and pluripotency (OCT4, NANOG, and SOX2) genes. The purity of these colonies was confirmed by negative expression of the markers for sertoli cell (GATA4 and SOX9), peritubular myoid cell (αSMA) and differentiating spermatogonial and germ cells (c-KIT). Upon nuclear transfer into enucleated recipient cytoplast, the putative GS-like cells could cleave (67.4 ± 3.8 vs. 76.0 ± 8.1) and form blastocyst (17.5 ± 2.6 vs. 19.5 ± 2.7) at a rate similar to those observed with fibroblast cells ($p > 0.05$). Taken together, our results suggest that GS-like cells could be isolated from adult pig testes and they can be reprogrammed to embryonic state by nuclear transfer. Further studies should be directed to establish long-term *in vitro* culture system and characterization of isolated GS-like cells.

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Key Words: Germ-line stem cells, SSC, GSC, embryonic development, adult testes

32. Stress, nutrition and reproduction:

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Hair cortisol concentrations and semen production of Bos taurus bulls

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Persistent increase on cortisol concentrations due to chronic stress can have detrimental effects on reproductive performance of animals. Blood cortisol concentrations have been used to investigate the stress-mediated influence of the adrenocortical system on the hypothalamic-pituitary-gonadal (HPG) axis. To date, cortisol assessment in hair samples has been reported as a validated method to evaluate hypothalamic-pituitary-adrenal (HPA) axis activity in humans and animals. The objectives of this study were to evaluate changes in hair cortisol concentrations relative to the beginning of semen production and the effects of hair cortisol concentrations on semen quality of bulls. Hair samples were obtained from 12 Holstein Frisian bulls (BW = 475 ± 57 kg; age = 13 ± 1 months) with a razor from the scapular region. Hair samples were collected at the time of arrival (P1) at the artificial insemination centers. After 30 days (P2), at the start of semen production, samples of new hair growth were collected from the same area. Hair samples were stored in dry tubes at room temperature until analysis. Hair was extracted by methanol and cortisol concentrations were determined by RIA. Mean hair cortisol concentrations in bulls at P1 and P2 were 4.5 ± 0.9 and 18.6 ± 6.5 pg/mg, respectively. The difference between the two sampling periods were significantly ($p < 0.05$) and the increase at P2 could be due to environmental and management changes. Moreover, it was observed that bulls with hair cortisol concentrations > 10.0 pg/mg at P2 produced ejaculates with a significantly ($p < 0.05$) lower spermatozoa concentrations than bulls with hair cortisol concentrations < 5 pg/mg; thus, producing lower seminal doses. These results indicate that hair cortisol monitoring could be a useful tool for the evaluation of HPA axis activity in relation to the beginning of semen production in bulls. Evaluating the effect of HPA activity on the HPG axis could be important not only for their potential economic implications but also for implications concerning animal welfare.

Key Words: Hair, cortisol, bull, semen

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Relationship between body condition and rump fat thickness in Zebu cows with different reproductive states

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The body condition score (BCS) is a useful indicator of energy status and breeding potential, because of the effect on follicular dynamic, ovarian activity, endocrine function and pregnancy rate in beef cows. However, the subjectivity, repeatability, and validity of BCS have been questioned. The aim of the present study was to determine the relationship among body weight (BW), BCS and rump fat thickness (RFAT) measured by ultrasonography, and to evaluate the relationship between BCS and RFAT in different reproductive states. Ninety two Zebu (Brahman) cows had their BW, BCS and RFAT evaluated in different reproductive states: (i) non pregnant; (ii) first gestation third; (ii) second gestation third; and (iv) third gestation third. A BCS value was attributed for each cow following a 1–5 points scale. Ultrasonographic images for RFAT measurement were obtained using a 3.5 MHz linear transducer. Body condition scores and ultrasound measurements were collected on the same day. The relationship between BCS and RFAT values was investigated using correlation and regression models from SAS. The BCS was a relatively good predictor of RFAT ($R^2 = 0.69$; $p < 0.01$), but only for non pregnant Zebu cows. Similarly, BCS was correlated ($r = 0.77$; $p < 0.01$) with RFAT in non pregnant cows. Also, it was shown that BCS presented intermediate correlation ($r = 0.45$–0.69; $p < 0.05$) with RFAT in pregnant cows. However, both BCS and RFAT showed low correlation ($r = 0.36$–0.48; $p < 0.05$) with BW in pregnant cows. The BCS classification using a 1–5 point scale, can be used to predict the RFAT in non pregnant Zebu cows.

Key Words: Beef cattle, body weight, pregnancy, subcutaneous fat

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Effect of rumen-inert fat enriched with polyunsaturated fatty acids (ω–3 and ω–6) on the reproductive and productive performance of crossbred dairy cows

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Rumen-inert fat enriched with polyunsaturated fatty acids (PUFA; ω–3 and ω–6) has been used to feed dairy cows to improve milk production and the reproductive efficiency. In order to evaluate the effects of supplementation with rumen-inert fat enriched with PUFA (ω–3 and ω–6) on milk production and some reproductive parameters of crossbred dairy cows (Holstein by Brown Swiss), a total of 14 cows, from a commercial herd, was divided into three groups: Group 1 (G1, n = 6) received 400 g of rumen-inert fat/cow (17% ω–6; 0.5% ω–3; Energras®); Group 2 (G2, n = 5) received 400 g of rumen-inert fat/cow (17% ω–6; 5% ω–3; Energras-ω–3®) and Group 3 (G3, n = 3) or control group, which did not received fat supplementation. Every week, from day 20 ± 2 day until 90 ± 2 day postpartum (PP), ultrasonography (US) of the ovaries was performed to measure number of class 3 (C3) follicles (≥10 mm) and area of corpora lutea (CL) and from d 10 PP milk production was measured during the afternoon milking. Every 15 day body condition score (BCS) was measured. All groups were fed according to the farm's schedule, receiving a total of 4 Kg/day of a commercial concentrate food/cow with 20% of protein, twice a day, during the milking time. Data for number of accumulated C3 follicles was analyzed using the GENMOD procedure assuming a Poisson distribution, whereas accumulated CL area and milk production were analyzed using the MIXED procedure and the BCS and interval from parturition to first observed CL by US were analyzed using the General Linear Model procedure from SAS. The statistical model for number of accumulated C3 follicles, accumulated CL area, BCS and milk production included the effects of group, day postpartum or day of lactation and the interaction group by day, whereas the model used to analyze the interval from calving to first observed CL only included the effect of group. There were not differences among groups for number of accumulated C3 follicles on d 90 PP (10.7 ± 2.6; 12.8 ± 3.1 and 13.1 ± 3.1 follicles for G1, G2 and G3, respectively) neither for accumulated CL area: G1 = 26.0 ± 2.1; G2 = 24.9 ± 2.3 and G3 = 27.1 ± 3.1 cm^2). Cows in G2 had a higher milk production (9.6 ± 0.4 l; $p < 0.02$) until d 90 compared with cows in G1 (7.9 ± 0.4 l) and G3 (7.0 ± 0.4 l), with a significant interaction group by day ($p < 0.008$). There were not significant differences among groups for the interval from calving to first CL (G1 = 23.5 ± 4.8; G2 = 36.4 ± 5.2 and G3 = 35.7 ± 6.8 day) probably as a consequence of the number of cows per group. These results show that cows supplemented with enriched PUFAs (G2) produced more milk during the first 90 day of lactation. Cows in G1 seem to have a shorter interval from calving to first CL, however intervals were not statistically different.

Key Words: Crossbred cows, polyunsaturated fatty acids, fat supplementation, rumen-inert fat, milk production

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Incorporation of various dietary omega-3 fatty acids into granulosa cells and oocytes and the effects on *in-vitro* fertilization performance in dairy cows

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The objectives of this study were to determine the incorporation of various omega-3 (n-3) fatty acids (FA) supplemented to dairy cows into granulosa cells and oocytes, and the effects on *in-vitro* fertilization (IVF) performance. Forty-two dry cows (256 day pregnant) were assigned to three groups and were fed a basal diet and supplemented with encapsulated fats: (i) SFA – supplemented with saturated fat at 240 and 560 g/day per cow prepartum and postpartum (PP), respectively; (ii) FLX – supplemented prepartum at 300 g/day per cow with fat providing 56.1 g/day C18:3n-3 (ALA), and PP at 700 g/day per cow providing 131.0 g/day ALA from flaxseed oil; and (iii) FO – supplemented prepartum at 300 g/day per cow with fat providing 5.8 g/day C20:5n-3 (EPA) and 4.3 g/day C22:6n-3 (DHA), and PP at 700 g/day per cow providing 13.5 g/day EPA and 10.0 g/day DHA from fish oil. Ovaries were monitored for follicular status, and following estrous synchronization >7 mm follicles were aspirated. Ovum pick up (OPU) was performed from 50 day PP twice weekly (20 sessions; five cows per group) and *in-vitro* maturation and fertilization were conducted. The FA profile was determined in follicular fluid (FF) and granulosa cells from aspirated follicles. The data were analyzed as repeated measurements using the PROC MIXED procedure of SAS. The proportion of ALA was 2.9 and 2.7-fold greater in FF and granulosa cells, respectively, of FLX cows than in both other groups ($p < 0.001$). The proportion of C22:5n-3 (DPA) was 2-fold greater in granulosa cells of FO than in SFA ($p < 0.003$), and the proportion of DHA was 6.7-fold greater in FF of FO than in both other groups ($p < 0.001$). In cumulus-oocyte-complexes (COC), the proportion of ALA was higher in FLX than in SFA and FO (0.93, 0.30 and 0.40, respectively; $p < 0.007$). Also, DPA and DHA were detected in FO COC (1.18 and 1.12%, respectively), whereas they were not found in both other groups. A total of 915 oocytes were collected across treatments. The average number of follicles aspirated during OPU was higher in FLX and FO than in SFA (10.7, 9.6, and 8.0 follicles/cow/session; $p < 0.05$), and the number of recovered oocytes tended to be higher in FLX and FO than in SFA (4.8, 3.9, and 3.3 oocytes/cow/session, respectively; $p < 0.06$). Number of oocytes chosen for IVF was higher in FLX than in SFA (3.84 and 2.88 oocytes/cow/session, respectively; $p < 0.05$). Oocytes cleavage rate was higher in FLX and FO than in SFA (52, 48 and 35%, respectively; $p < 0.01$), and the number of cleaved oocytes were increased in FLX and FO than in SFA (1.97, 1.66 and 1.06 oocytes/cow/session, respectively; $p < 0.04$). Percentage of oocytes developed to blastocysts tended to be higher in the FLX and FO than in the SFA cows (15.2, 16.4 and 8.8%, respectively; $p < 0.1$). In conclusion, various dietary n-3 FA were incorporated into FF, granulosa cells and COC. Both n-3 FA sources (flaxseed and fish oil) similarly improved IVF performance; therefore, feeding botanical n-3 source rich in ALA seems to be a satisfactory approach to improve oocyte quality in dairy cows.

Key Words: Omega-3, oocytes, IVF

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Involvement of free cholesterol and high density lipoprotein in development and resistance of the preimplantation bovine embryo to heat shock

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Development of the mammalian preimplantation embryo is susceptible to disruption by elevated temperature. The molecular and biochemical basis for developmental, genetic and other differences in embryonic resistance to heat shock is largely not known. Here we tested the hypothesis that increasing free cholesterol content could improve embryonic resistance to heat shock. In Experiment 1, embryos were cultured without treatment, with methyl-β-cyclodextrin (MBCD), or with cholesterol loaded to MBCD (0.41 mM cholesterol) for 3 h before exposure to heat shock (41.0°C for 15 h) or no stress (38.5°C continuously) beginning at 30 h after insemination (one-to-two cell stage). For all treatments, heat shock reduced ($p < 0.05$) development to the blastocyst stage. For control embryos, percent of oocytes that became blastocysts was 29.9% for non-stressed embryos and 15.9% for heat-shocked embryos (SEM = 2.4, n = 4 replicates). Culture in methyl-β-cyclodextrin (MBCD), the carrier for cholesterol, reduced ($p < 0.05$) development (19.8% for no stress and 1.8% for heat shock). Development in the presence of cholesterol-loaded MBCD (0.41 mM cholesterol) was similar to controls (34.1% for no stress and 15.0% for heat shock). For Exp. 2, embryos were not treated or received cholesterol-loaded high density lipoprotein (HDL = 0.07, 0.27 or 2.74 mM cholesterol equivalents) for 3 h before heat shock as in Exp. 1. There was a heat shock × treatment interaction ($p < 0.01$) that

reflected the fact that (i) the deleterious effects of heat shock were reduced by HDL and (ii) high concentrations of HDL reduced development in the absence of heat shock only. Heat shock reduced development to the blastocyst stage from 39.3% to 13.5% in the absence of HDL, from 35.4% to 23.1% for 0.07 mM, 21.6% to 21.3% for 0.27 mM and from 28.3 to 26.7% for 2.74 mM cholesterol equivalents (SEM = 3.5%, n = 4 replicates). In Exp. 3, cholesterol assays confirmed that, as compared to untreated embryos (0.3 pmol/embryo), MBCD (0.15 pmol) and 2.74 mM cholesterol-equivalent HDL (0.23 pmol) reduced cholesterol content while cholesterol-MBCD increased cholesterol content (0.42 pmol; SEM = 0.02, n = 6). Results were interpreted to mean that other actions of HDL (for example, protection from free radicals) was responsible for the thermoprotective properties of this molecule. In conclusion, raising cholesterol content does not improve embryonic survival in response to heat shock. Depletion of cholesterol, in contrast, reduces competence of embryos to develop to the blastocyst stage. High density lipoprotein is thermoprotective to embryos and probably acts through a mechanism independent of its actions on embryonic content of free cholesterol.

Key Words: Embryos, cholesterol, heat shock, stress, blast development

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Follicular environment and embryo development of dairy cows fed diet containing low concentration of fatty acids supplemented with different sources of fatty acids

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Objectives were to evaluate the impacts of supplementing diets containing low amounts of long chain fatty acids (FA, <1.8%) with either mostly saturated free FA (SFA) or Ca salts enriched with polyunsaturated FA (PUFA, 27% C18:2n6 and 3.5% C18:3n3 of the total FA) on follicular fluid composition and embryo quality in lactating Holstein cows. At 60 day from the expected calving date, cows were blocked by parity and assigned randomly to 1–3 treatments (% of DM): no supplemental FA (Ctrl, n = 26), 1.7% SFA (n = 25), and 1.7% as Ca salts of PUFA (EFA, n = 25). At 40 DIM, cows had their estrous cycle synchronized by an injection of 25 mg of prostaglandin-$F_{2\alpha}$ followed 3 day later by an injection of 100 μg of GnRH. The first wave dominant follicle was aspirated on d 6 after the GnRH. Concentrations of progesterone (P4), estradiol (E2), total and free insulin-like growth factor (IGF-1) were measured in the follicular fluid (Table 1). Cows were subjected to a standard FSH-based superovulation protocol following follicle aspiration. Uteri were flushed on d 7 after AI. Recovered embryos-ova were evaluated for fertilization and grade quality according to the IETS protocol (1998). Cows had their estrous cycle re-synchronized for single ovulation. Uteri were flushed on d 15 after AI. Interferon-tau (IFN-τ) concentration was quantified in uterine flushes. Plasmatic P4 concentration was measured between the AI and the embryo collection (day 15). Data were analyzed by the Glimmix procedure of SAS. Orthogonal comparisons were performed to determine the effect of feeding fat (Ctrl vs. SFA + EFA) and source of fatty acid (SFA vs. EFA). Pregnant cows fed fat had greater ($p < 0.01$) plasmatic concentration of P4 compared with Ctrl (2.3, 3.1 and 3.6 ng/ml, respectively for Ctrl, SFA and EFA). Although cows fed EFA diet had larger first wave dominant follicles, the composition of the follicular fluid was not affected by treatments. Furthermore, fatty acid supplementation did not improve embryo quality in lactating dairy cows.

Parameter	Ctrl	Treatment				p	
		SFA	EFA	SE	TRT	Fat	FA
		Follicle					
Diameter, mm	14.1	14.0	16.4	0.9	0.08	0.42	0.03
E2, ng/ml	459	232	349	259	0.87	0.66	0.69
P4, ng/ml	98	67	152.5	122	0.92	0.99	0.73
Total IGF-1, ng/ml	37.2	42.4	44.8	7.2	0.80	0.55	0.79
Free IGF-1, ng/ml	0.59	0.64	0.54	0.06	0.44	0.98	0.20
Embryo d 7, %							
Fertilization	59.8	75.3	74.5	12.6	0.68	0.38	0.96
Blastocyst	10.0	16.4	6.3	7.3	0.51	0.89	0.25
Embryo d 15							
Elongated, % (n/n)	71.4 (5/7)	66.7 (4/6)	50.0 (2/4)	*	0.86	0.77	0.63
Length, mm	7.1	11.1	5.2	4.4	0.68	0.93	0.51
INF-τ, ng/ml	0.14	0.55	0.11	0.27	0.45	0.72	0.49

Key Words: Essential fatty acids, embryo quality, dairy cow

3206

The vaginal prostaglandin E (PGE) or hyaluronan (HA) base impregnated sponges did not alter the serum malondialdehyde concentration in Thai goat (*Capra hircus*) during oestrus

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Estrous synchronization in goats is well established, thus using this technique in Thailand is questioned due to the potential for oxidative stress. Oxidative stress represents an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Lipid peroxidation (LPO), a well-established mechanism of cellular injury in animals, is used as an indicator of oxidative stress in serum and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition, and the measurement of MDA and HAE has been used as an indicator of lipid peroxidation. The aim of this study was to investigate concentration in goats. Forty-five goats were equally assigned to four groups. They were synchronized to estrus using progestagen sponges and 250 IU PMSG at sponge removal. At 50 h after progestagen sponge removal, Prostaglandin E (PGE) or Hyaluronan (HA) sponges were vaginally impregnanted: Group 1 (sponge with 1000 μg PGE); Group 2 (sponge with 40 mg HA); Group 3 (sponge with 600 μg PGE) and Group 4 (sponge with 300 μg PGE). Prostaglandin E or HA sponge were impregnated for 24 h and then removed. Serum was collected twice at PGE or HA sponge removal and at 24 h following PGE or HA sponge removal. Serum MDA concentration was determined by ELISA. Data was analyzed by ANOVA. There was no difference of serum MDA concentration between goats treated by PGE or HA sponge ($p > 0.05$). In both treatments, there were no significant differences of serum MAD concentrations before sponge removal and at 24 h following PGE or HA sponge removal. The mean ± SD of serum MAD concentration before PGE or HA sponge removal and at 24 h following PGE or HA sponge removal were 18.441 ± 0.42 nM and 18.078 ± 0.49 nM in group 1, 18.923 ± 0.49 nM and 18.234 ± 0.52 nM in group 2, 20.789 ± 0.49 nM and 17.589 ± 0.49 nM in group 3, 19.767 ± 0.52 nM and 18.656 ± 0.49 nM in group 4, respectively.The presence of MDA following the administration of impregnated sponges indicates the cellular injury that may cause the oxidative stress in goat. The results shows the impregnated sponge had no affect on the serum MDA concentration. Administration of a vaginally impregnated implants containing PGe or HA did not appear to induce oxidative stress in goats; however, additional studies are required to clarify on the fertility following impregnated sponges.

Key Words: Malondialdehyde, Thai goat, oxidative stress, impregnated

3207

When two rat litters – in utero and suckling – are overlapped, the second litter tends to be smaller, but newborn pups have bigger body and testicular weight and higher Sertoli cell numbers

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Sertoli cell numbers can mediate fetal programming of sperm production by intrauterine undernutrition, both in laboratory and farm animals. In order to study the effects of overlapping gestation with lactation of a previous litter, on rat pups litter size and testicular development, nine Sprague–Dawley female rats (age = 4 months, weight = 244.8 ± 13.1 g) were mated to the same adult male (same age) twice. From Day 21 on (first gestation), rats were monitored for parturition every 3 h. The 2nd mating occurred 4–6 h after the first litter was born. While the same male was caged with every mother, the pups (litter A) were withdrawn transitorily from the cage. Litters A (1st litter) were suckling while litters B were in utero. Gestation was diagnosed by colpocitology. From Day 1 on, (first day with sperm in smears) dams were caged individually with standard rat chow and water ad libitum. Both rats bodies and feed consumption were weighed daily. Once coitus occurred, pups were returned to their mother. Every Litter A were weaned at 20 days of age, when litter B from the same mother was on Day 20 of gestation. One male pup/litter was sampled (thus, n = 9 for both litters A and B) for standard testicular quantitative histology at 2 days of age. Data are presented as mean ± SD. Both the effects of litter (either A or B) and age (either at coitus or at parturition) were tested by anova ($p < 0.05$). There was no effect of age on any of the dependent variables measured. Litters B tended to have fewer pups than litters A but litter total weight was not different. However, body and testicular weight was bigger in litters B, as well as Sertoli cells numbers at 2 days of age. Briefly, at least in rats, it seems that the tendency to bigger litter size seen in litters A was more detrimental to pups' body and testis weight, and to testis histology than spending uterine life (litters B) while the mother was lactating litters A. This may have fetal programming effects on sperm production in case Sertoli cell numbers keep smaller after puberty.

Key Words: Gestation overlapping, histology, testis, litter size, fetal programming

Table 1. Clinical and histological variables in overlapping rat litters

	Litter		
	A	B	p
Mother weight, d 2 (g)	272.9 ± 16.4	279.0 ± 12.8	0.39
Mother weight, d 20 (g)	321.6 ± 19.0	281.9 ± 13.5	9×10^{-5}
Mother weight gain (d 2–20, g)	48.7 ± 20.3	2.9 ± 6.5	8×10^{-6}
Nr pups/litter	11.8 ± 1.9	9.1 ± 3.7	0.072
Litter weight (g)	81.6 ± 5.7	73.0 ± 29.8	0.41
Mean pup weight (g)	7.0 ± 0.9	8.0 ± 1.1	0.07
Sampled pup weight (g)	7.3 ± 0.8	8.5 ± 0.9	0.006
Testis weight ($gx10^{-3}$)	2.7 ± 0.5	3.9 ± 0.4	0.0001
Testis cords diam (μm)	366.5 ± 9.6	368.2 ± 18.9	0.81
Sertoli cells nr ($x10^5$)	3.0 ± 0.8	4.2 ± 0.90	0.01

3250

Influence of two kinds of handling on pregnancy rate and on reactivity score of Nelore cows using fixed time artificial insemination (FTAI)

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Although fixed-time artificial insemination (FTAI) is a relatively simple process, cattle need to be handled several times and the contact with feedlot operators is intensive. For this purpose, low stress handling is a useful tool to minimize the undesired effects of FTAI handling. The aim of this work was to evaluate the influence of the low stress handling on pregnancy rate on day 35 after FTAI and on reactivity score of Nelore cows raised extensively. Multiparous cows (n = 126) were randomly allocated in two groups, G1 (n = 66) and G2 or control group (n = 60). Cows were acclimated two weeks before the beginning of the treatment that lasted 10 days. The G1 group was submitted to low stress handling, in which animals were handled in calm and quiet manner, without loud noises and/or using physical aggression, using the point of balance, respecting the fight zone and using flags for handling. The G2 group was handled as usual in the operation, with yelling, kicking animals and use electric prods and sticks. On D0, all animals were treated with a progesterone vaginal implant combined with an injection of estradiol benzoate. On D8, implants were withdrawn and the animals received an injection of d-cloprostenol, estradiol benzoate and FSHp. On D10 (48 h after implant withdrawn), AI was performed in both groups by the same inseminator. On D0, D8 and D10, at the squeeze chute, reactivity was scored as R1, R2, R3, R4 or R5 and was calculated based on: (i) tension score (1 = relaxed – absence of abrupt movements of head and/or tail; 2 = restless – presence of abrupt movements of head and/or tail; 3 = very restless - muscular trembling); (ii) breathing score (1 = not audible; 2 = audible and deep; 3 = snorting/snoring – deep breathing with sound emission); and (iii) bellowing score (0 = absence of bellowing; 2 = presence of bellowing). Therefore, reactivity scores were defined thus: R1 (calm) = tension score of 1, breathing score of 1 or 2, and bellowing score of 0 or 1; R2 (low reactive) = tension score of 1 (if breathing ≥2), breathing score of 1, 2, or 3 (if bellowing score = 0) and bellowing score of 0 or 1; R3 (moderately reactive) = tension score of 1 (if bellowing = 1) or 2, breathing score of 1, 2, or 3 (if bellowing = 0), and bellowing score of 0 or 1; R4 (reactive) = tension score of 2 (if bellowing = 1) or 3, breathing score 1, 2, or 3 (if bellowing = 0) and bellowing score 0 or 1; and R5 (highly reactive) = tension score 3 (if bellowing = 1). Thirty-five days after artificial insemination, pregnancy was determined with ultrasonography. There was no significant correlation ($p < 0.05$) between reactivity score and pregnancy rate in each group and between pregnancy rate in the two groups. However, there was a significant statistical difference ($p < 0.05$) between the two groups considering reactivity score as shown on table 1. It could be concluded that although low stress handling has influenced reactivity, it did not affect pregnancy rate in Nelore cows submitted to FTAI and raised extensively.

Key Words: Cattle, handling, stress, Nelore, pregnancy

Table 1 Pregnancy rate and mean reactivity in Nelore cows submitted to FTAI

Group	Pregnancy rate (n°./n°.)	Mean reactivity ± SEM
Traditional	37/66	2.12 ± 0.07^b
Low stress	30/60	1.62 ± 0.05^c

Different letters in the same column indicate statistical significance (Tukey test $p < 0.05$).

3251

Effect of heat stress in the maturation rate, fecundation, and development of bovine embryos *in vitro* fecundated

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The climatic conditions are a major factor in the success of livestock explorations. Economic losses and operating costs in the industry attributed to heat stress are large, mainly due to the decrease in productive performance, increased mortality and decreased reproductive efficiency. There are indications that heat stress affects the physiological responses, damaging ovarian dynamics, and the quality and competency of oocytes in females. The aim of this study was to evaluate the effect of 2°C increase in temperature during the maturation, fecundation, and development of '*in vitro*'–derived embryos. For this purpose, cumulus oocyte complexes (COCs) were obtained by follicular puncture from slaughterhouse ovaries and after identification, were divided into four groups: control 1 (C1), control 2 (C2), exposed 1 (E1) and exposed 2 (E2). The oocytes in the control group (C1: n = 316 and C2: n = 447) were cultured at 38°C and the oocytes of exposed group (E1: n = 365 and E2: n = 337) cultured at 40°C during the maturation period (24 h) with 5% CO_2 and 95% humidity. After the maturation period, oocytes of group C1 and E1 were evaluated for their morphology under an optical microscope and, after removal of cumulus cells evaluated the maturation rate by the presence of polar body. After this period, the oocytes of group C2 and E2 were fecundated with semen treated by Percoll discontinuous gradient. The oocytes of group E2 were cultured at 40°C throughout the period of fecundation and embryonic development and the oocytes of group C2 were cultured at 38°C. The embryos were evaluated for cleavage rate, morula and blastocyst formation by optical microscopy. In group C1, the oocytes showed uniform expansion of cumulus cells, classified as moderate to high, with brown color and uniform appearance of the ooplasm, with a maturation rate of 69.6% (220/316). In the oocytes exposed to 40°C (E1), we observed a decrease in the expansion of cumulus cells, and the same showed rounded appearance and retraction of the ooplasm with dark coloration, verifying a maturation rate of 49.0% (179/365). For the statistical analysis, the chi-square test ($p > 0.05$) was used to determine significance. In the group C2, after the *in vitro* fecundation period, the rates of cleavage, morula and blastocyst were 68.2% (305/447), 50.1% (153/305) and 43.3% (132/305), respectively. For group E2, no zygotes were produced (0/337). These data suggest that the increase in temperature negatively influences maturation and fecundation of bovine oocytes.

Key Words: Reproduction, cattle, heat stress, oocyte, embryo

3252

Effect of dam size and nutrition during pregnancy on fetal ovary development of offspring

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This study examined the effects that maternal size and nutrition during pregnancy have on the development of fetal ovary at different stages of gestation, based upon the hypothesis that maternal uterine environment can affect the growth and reproductive development of offspring. Romney ewes were selected at the time of mating for heavy (H; mean: 60.8 ± 0.18 kg) or light (L; mean: 42.5 ± 0.17 kg, n = 450) liveweights, then were oestrus-synchronized and artificially inseminated. Ewes were allocated to either ad lib (A) or maintenance (M) nutrition from Days 21–140 of pregnancy, resulting in four treatment groups: HA (n = 151), HM (n = 153), LA (n = 155) and LM (n = 153). On Days 65, 100 and 140 of pregnancy, twin-bearing dams were slaughtered and fetal ovaries were collected, weighed and placed in Bouin's fixative. Fetal ovaries were then sectioned (5 μm) and stained (H&E) for morphological assessment. Numbers of dividing and non-dividing oogonia (Day 65) and primordial and primary follicles (Days 100 and 140) were counted in four 100 × 100 μm^2 and 500 × 500 μm^2 areas per section, respectively. Follicle cross-sectional areas were measured for each follicle counted from Day 100 and 140 fetuses, using Image J software. On Day 65, HM fetuses had more dividing oogonia than LM fetuses, whereas LM fetuses had more non-dividing oogonia than other groups. On Day 100, LM fetuses had fewer primordial follicles, and more primary follicles, than other groups (see Table 1). Primordial follicles in LM fetuses were larger (1238 ± 19 μm^2) than in other groups (overall mean: 842 ± 7 μm^2). On Day 140, HM and LM fetuses had more primordial follicles than HA and LA fetuses. Primordial follicles in HA and LA fetuses (1045 ± 12 μm^2; 1094 ± 9 μm^2, respectively) were larger than in HM and LM fetuses (846 ± 9 μm2; 862 ± 9 μm^2, respectively). HM fetuses had the fewest primary follicles, but HA and HM fetuses larger primary follicles (13 919 ± 966 μm^2; 13959 ± 1075 μm^2, respectively) than LM fetuses (10400 ± 859 μm^2). These results indicate that both low dam liveweight at the time of conception and a low plane of nutrition during pregnancy exert pressure upon the development of the fetal ovary to develop more quickly than in a dam that is heavier at conception or is on a higher plane of nutrition during pregnancy.

Key Words: Sheep, fetal programming, ovary, germ cells, follicle

Table 1. Follicles in fetuses from heavy (H) or light (L) dams, on ad lib (A) or maintenance (M) nutrition during pregnancy

	Day of gestation					
	65		100		140	
Follicles (n/field)	Dividing oogonia	Non-dividing	Primordial	Primary	Primordial	Primary
HA	5.1 ± 0.4^{bc}	16.7 ± 0.6^{a}	13.5 ± 0.6^{b}	0.6 ± 0.1^{b}	7.2 ± 0.4^{a}	0.6 ± 0.1^{b}
HM	6.7 ± 0.5^{c}	17.3 ± 0.8^{a}	18.1 ± 0.7^{c}	0.3 ± 0.1^{b}	12.8 ± 0.4^{b}	0.4 ± 0.0^{a}
LA	4.2 ± 0.3^{b}	16.9 ± 0.5^{a}	19.5 ± 0.5^{c}	0.1 ± 0.1^{a}	8.1 ± 0.3^{a}	0.7 ± 0.0^{b}
LM	2.0 ± 0.7^{a}	23.1 ± 1.1^{b}	7.5 ± 1.1^{a}	1.9 ± 0.2^{c}	12.8 ± 0.4^{b}	0.6 ± 0.1^{a}

$^{a, b}$ differ ($p < 0.05$).

3253

Short- or long-term hypobaric hypoxia decreases corpus luteum size and insulin-like growth factors in cycling sheep

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Fertility in sheep flocks at high altitude is lower than that at sea level, likely associated with hypoxia and oxidative stress on ovarian metabolism. Under normal conditions, insulin-like growth factors (IGF) are involved in the growth of follicles and corpus luteum (CL), with a direct correlation between IGF expression and CL size and function. Our aim was to establish the effects of the hypoxia/oxidative stress induced by high altitude (HA) environments and of the antioxidant vitamin supplementation on CL size and CL expression of IGF-I and IGF-II, in high altitude native (HH group) or naïve (LH group) ewes reared at an altitude of 3600 m. Ewes native to low altitude (LA) and kept at sea level were the control (LL group). Half of the animals of each group were daily supplemented with vitamin C 500 mg and vitamin E 350 IU, per dose, from 30 days before initiation of the observed cycles (groups HHV, LHV and LLV, respectively). Corpus luteum dynamics were evaluated by ultrasound during the reproductive cycle. At day 5 after ovulation, CL tissue samples were taken from half of the animals of each group for determination of IGF-I and IGF-II mRNA expression by RT-PCR. The results showed that high altitude decreases CL growth, independently of the origin of the animals (maximal ultrasound CL area: HA = 0.76 ± 0.18 vs. LA = 1.22 ± 0.28 cm^2; $p < 0.001$). No effects of the vitamin supplementation or of the origin of the animals on CL area were observed ($p > 0.70$). Corpus luteum expression of IGF was also decreased by the effect of high altitude. Expression of IGF-I at HA was

approximately 5.3-fold lesser than those at LA ($p = 0.003$) while IGF-II expression at HA was about 2.1 fold lesser than that observed at LA ($p = 0.004$). As in the case of CL area, no effect of altitudinal origin or vitamin supplementation was observed for IGF expression. No significant correlation between CL size and IGF expression was detected, however, the effect of high altitude hypoxia on both variables showed a similar trend. In conclusion, our results indicate that long- or short-term exposure to high altitude reduces the CL expression of IGF and consistently the CL size. Changes in IGF and CL size among elevations may be responsible for the decreased fertility observed in ewes raised in high elevation environments. In addition, the lack of effect of antioxidant vitamins indicates that the ovary is protected from the oxidative stress induced by high altitude hypoxia.

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Key Words: Corpus luteum, sheep, hypoxia, oxidative stress, high altitude

3254

Reproductive performance of Ouled Djellal ewes in arid area of Algeria

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Sheep farming in arid and semi-arid regions of Algeria faces large fluctuations in the availability of fodder. This deficiency is particularly burdensome for pregnant ewes whose needs are the maximum and is a major constraint to the development of this sector. The objective of this study was to determine the influence of these difficult conditions on the reproductive status of sheep. Three hundred Ouled Djellal, clinically healthy, multiparous and primiparous ewes, between 2 and 5 years of age, with an average body condition score of 2.5 ± 0.5 were selected.

For each season (wet = november to april and dry = may to october), ewes were divided into three batches according to their physiological stage of reproduction: (i) ewes were pregnant (G; n = 70); (ii) ewes were lactating (L; n = 70); and (iii) ewes were non-pregnant and non-lactating (V; n = 60) to assess the influence of soil conditions and climate on reproductive parameters. The results of changes in reproductive performance according to the season indicated significantly ($p < 0.05$) higher rates of prolificacy, fertility, fecundity and digital productivity during the wet season compared to the dry season, with respective values of (162 vs. 147%), (77 vs. 68%), (125 vs. 110%), (130 vs. 117%). The average age at first lambing was 16 months. The average interval between lambing averaged 10 months, and the average interval from lambing to mating was 3.2 months. These two parameters were not significantly ($p > 0.05$) affected by season. Analysis of data on reproductive performance indicates that Ouled Djellal ewes adapt well to conditions associated with arid climates.

Key Words: Arid area, Ouled Djellal ewe, reproductive performance, season

3255

Effects of nutritional restriction on metabolic and ovarian function in llamas

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The aim of the study was to determine the effect of energy restriction on metabolic and reproductive indices in llamas. Non pregnant, non lactating adult llamas were assigned randomly to two groups (n = 7 per group) according to diet. Llamas in the respective groups were fed 40% (restricted) and 100% (control) of recommended maintenance energy requirements by altering the proportion of rye–grass hay and commercial concentrate in the diet. After two months of the imposed diet, ovarian follicular wave emergence was synchronized among females by transvaginal ultrasound–guided ablation of follicles ≥5 mm in diameter using a 17 g needle attached to a 5 MHz convex–array transducer. Ten days after follicle ablation, llamas were given 50 μg gonadorelin acetate im to induce ovulation. Llamas were examined daily by transrectal ultrasonography for 30 days after ablation to record serial changes in ovarian follicles and the CL. Blood samples were taken every two days to determine plasma concentrations of leptin. Metabolic status was monitored weekly by assessing body mass, body condition score (1 = thin; 5 = obese), and plasma metabolites (insulin, β hydroxy butirate, total protein, cholesterol and NEFA). Data were examined for normality and homoscedasticity, and values were compared between groups by student *t*-tests and analyses of variance for repeated measures. Results are summarized in Table 1. Compared to llamas on the control group, those in the restricted energy group had lower body condition scores ($p \leq 0.001$), lower body mass ($p \leq 0.001$), lower plasma concentrations of leptin ($p \leq 0.001$) and higher concentrations of cholesterol, NEFA and tryglicerides ($p \leq 0.001$), throughout the 30–day study period (effect of ration, $p \leq 0.05$; effect of time, $p \leq 0.001$; interaction, $p \leq 0.01$). In addition, maximum follicle and CL diameters were smaller and the CL life–span (i.e., duration of detectability by ultrasonography) was shorter in the restricted–energy group than in the control group ($p \leq 0.01$). We conclude that long–term nutritional restriction to 40% of maintenance energy requirements induces metabolic changes resulting in distinctive alterations in ovarian follicular and luteal gland development in this species. Norambuena is a postdoctoral Fellowship supported by Fondecyt:3110095, Chile.

Key Words: Nutrition, ovarian function, corpus luteum, camelids

Table 1. Effect of energy restriction on body condition, metabolism and ovarian function in llamas (mean ± SEM)

	Low energy group (n = 7)	Control group (n = 7)
Body mass	114 ± 10[a]	153 ± 16[b]
Body condition score (1–5)	2.4 ± 0.3[a]	3.9 ± 0.2[b]
Follicle size (mm)	8.4 ± 1.5[a]	11.7 ± 1.2[b]
CL size (mm)	11.7 ± 0.7[a]	14.1 ± 0.5[b]

Values with different superscript are different ($p \leq 0.05$).

33. Testis - spermatogenesis:

3300

The role of testicular biopsy and ultrasonography in diagnosis of infertility in camels (*Camelus dromedarius*)

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Male camel infertility is a heterogeneous disorder. A variety of factors may adversely impact sperm production and function and impair fertility. In that regard, there is a great need for a precise diagnosis of infertility disorders related to failure of conception in male dromedaries. Therefore, this study was designed for application of testicular biopsy in the infertile male camels and to compare between the different diagnostic tools of infertility. Eighty infertile male dromedary camels (5–15 years old) were used in this study during the rutting season (November to May). These animals were subjected to an algorithmic approach based on information collected during a careful breeding history, clinical examination, testicular evaluation, testicular ultrasonography, the results of the semen analyses, and testicular biopsy to diagnose the camel's infertility. The results of testicular ultrasonography and biopsy were compared with the semen analysis and revealed an accuracy of 92.5% and 90%, respectively. In addition, testicular ultrasonography was matched with the testicular biopsy of the infertile animals and resulted in 85% accuracy. Testicular biopsy is a promising method that, along with carefully performed history,

clinical examination, appropriate testicular ultrasonography procedure and semen analysis, can afford the veterinarian the opportunity for more precise diagnosis and treatment of many dromedary infertility disorders.

Key Words: Dromedary camels, infertility, testicular biopsy, testicular ultrasonography

3301

Effects of ripe papaya (*Carica papaya*) seed powder on the seminiferous epithelium of the boar testis

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The effects and reversibility of oral administration of papaya seed powder on boar germinal epithelium was evaluated. Fifteen pubertal Large White boars were randomly selected and divided into two groups. Each of the ten boars in the experimental group received a daily dose of 300 mg papaya seed powder mixed with 0.5 kg of conventional pig feed while each of the five boars in the control group received a similar dose of maize germ in conventional pig feed. The experiment was carried out for 56 days to trace gradual changes in the germinal epithelium through one-and-half cycles of spermatogenesis in the boar. After every 2 weeks, one boar from the control group and two from the experimental group were castrated and testicular tissue samples processed for histology. Blood was collected to assess hematological changes. At the end of 56 days the remaining entire boars were maintained for 14 days and 60 days respectively without the papaya powder to assess the reversibility of the testicular effects of the powder.

The papaya seed powder had no effect on hematological parameters. However, clear histopathological changes which appeared to be dependent upon duration of papaya seed powder consumption were noticed on the seminiferous epithelium of the experimental group of boars. It was observed that these changes were reversible after withdrawal of papaya powder consumption.

Although the mechanism for the effect of papaya seed extract cannot be explained by this study, it is concluded that papaya seed powder has a cumulative deleterious effect on the spermatocytes and spermatids by causing their disorganization, exfoliation and loss.

Key Words: Spermatogenesis, testicular histology

3302

Structural and functional proteins are differentially expressed in morphologically abnormal bovine sperm

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We previously reported that pyriform bovine sperm had impaired ability to interact with oocytes and yielded presumptive zygotes with reduced cleavage rates. The objective was to determine the molecular basis of abnormal morphology and impaired function in pyriform sperm. Since sperm functions are regulated by previously synthesized proteins, we hypothesized that expression of sperm proteins are altered in pyriform vs. normal (control) sperm. Ejaculates containing few (control) or numerous (≥90%) pyriform sperm were collected before and after scrotal insulation, respectively, and were cryopreserved. Extracted sperm proteins were subjected to 2-D gel electrophoresis. Overall, 131 protein spots were differentially expressed (≥2 fold; false detection rate ≤0.01), of which 25 with ≥4 fold change were identified by mass spectrometry. In pyriform sperm, expression of proteins associated with gamete interaction [sperm acrosome membrane-associated protein 3 (SPACA3) precursor; prohibitin (PHB); heat shock protein (HSPD1); seminal fluid protein A3 (BSP-A3)], cytoskeleton and cell morphology [F-actin-capping protein subunit beta isoform 1 (CAPZB)], sperm flagellar organization [calcium-binding tyrosine phosphorylation-regulated protein (CABYR); troponin C-like protein; spermatogenesis-associated protein 19 (SPATA19) mitochondrial precursor; heat shock protein beta-9 (HSPB9); dynein light chain roadblock-type 2(DYNLRB2)] were down regulated. Conversely, proteins involved in oxidative stress [clusterin preprotein (CLU); epididymal secretory glutathione peroxidase precursor (GPX5); peroxiredoxin-5 (PRDX 5)], apoptosis [histidine triad nucleotide-binding protein 2 mitochondrial precursor (HINT2)], and metabolism [L-asparaginase (ASRGL1); Chain H Bovine F1-ATPase (ATP5D)] were upregulated. We concluded that comparing morphologically normal vs. abnormal sperm induced by scrotal insulation was a suitable approach to identify important structural and functional proteins of bovine sperm.

Key Words: Bull, abnormal sperm, sperm proteins, 2-D gel electrophoresis, mass spectrometry

3303

Expression of anti-Müllerian hormone, cyclin kinase inhibitor, connexin 43, androgen receptor and steroidogenic enzymes in the normal and cryptorchid equine testis

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Cryptorchidism occurs at a frequency of 2–8% in male horses; however, the underlying molecular changes associated with disruption of spermatogenesis and steroidogenesis are not well understood in the cryptorchid equine testis. The objective of the current study was to compare the expression of anti-Müllerian hormone (AMH), AMH receptor (AMHR2), androgen receptor (AR), cyclin kinase inhibitor (CDKN1B), connexin 43 (Cx43), 3β hydroxysteroid dehydrogenase/Δ5-Δ4- isomerase (3βHSD), 17α-hydroxylase/17,20-lyase cytochrome P450 (P450c17) and aromatase cytochrome P450 (P450arom) in the undescended testes of cryptorchid stallions (n = 4) with that of normal stallions (n = 7) between two to three years of age. Testes were divided and fixed for immunohistochemistry (IHC) and quantitative real time PCR (q-PCR). Immunolabeling for AMH and AMHR2 was more intense in Sertoli cells from cryptorchid compared to normal testes, and mRNA expression was 2.1 × greater for AMHR2 in cryptorchid than in normal testes ($p = 0.02$). Immunolabeling for AR was more intense in Sertoli cells and Leydig cells of normal testes, and AR mRNA expression was 5.6x greater in normal than in cryptorchid testes ($p = 0.02$). Immunolabeling for CDKN1B was noted in the perinuclear area of Sertoli and germ cells, and immunolabeling for Cx43 between Sertoli and germ cells for normal but not for cryptorchid testes. Expression for mRNA for CDKN1B and Cx43 were greater in normal than in cryptorchid testes. For steroidogenic enzymes, mRNA expression for P450c17 and 3βHSD were not different between cryptorchid and normal testes; however, expression of P450arom was 6.4× greater in normal vs. cryptorchid testes ($p < 0.05$). Immunolabeling for P450arom and 3βHSD were reduced in cryptorchid vs. normal testes while immunolabeling for P450c17 in cryptorchid testes appeared to be variably reduced compared to normal testes. In conclusion, undescended testes of cryptorchid horses present a number of characteristics similar to the prepubertal testis suggesting that cryptorchidism is associated with a failure of Sertoli cell maturation and perturbation of steroidogenesis.

Supported by the UC Davis Center for Equine Health, the John P. Hughes Endowment, and the Albert G. Clay Endowment.

Key Words: testis, cryptorchid, anti-Mullerian hormone, Sertoli cell, steroidogenesis

3304

The effect of mildronate on morphology of testes, biochemical blood indices, sexual reflexes and semen quality in boars

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The purpose of this study was to investigate the effect of mildronate [3-(2,2,2-trimethilhydrazinium) propionate] (quaterin, meldonium, MET-88) on morphology of testes, a number of biochemical indices of blood, sexual reflexes and semen quality in boars. Experimental and control groups (six animals in each group) of conditionally analogue boars were used. The boars of the experimental group were treated orally 2.0 g of mildronate daily for 90 days. The semen was collected manually once every 10 days. Immediately after collection total volume of the ejaculates, percent motile spermatozoa and sperm concentration were determined. The semen (sperm) quality parameters were assessed before the administration of mildronate and further once in 39 days that corresponds to the length of spermatogenesis cycle in boars. The sexual behavior was detected for each boar. At the end of the experimental period, boars of both groups were euthanized. Testes were removed and their weight and volume were measured. Examples of testes were examined histologically. Slides were stained with hematoxylin-eosin, Van Gizon and Mallori tri-chrome technique. The histological slides were viewed under different magnification using an optical microscope (Leica DM500B) with programme Image-Plus 6.1. The obtained numerical data were processed using computer with Stata-9 and Microsoft Excel standard programme set. The arithmetic mean, dispersion, standard deviation, standard errors were calculated and *t*-test was applied. The spermatogenic epithelial thickness was significantly larger ($p < 0.01$) in mildronate treated boars, and proliferation of interstitial endocrine (Leydig) cells was observed. The concentration of plasma testosterone in boars of experimental group was statistically significantly higher ($p < 0.01$) than in animals of control group. The administration of mildronate in boars improves their sexual activity and the semen quality: time from approaching the dummy to the start of ejaculation was significantly ($p < 0.05$) reduced and the progressive motility of spermatozoa was significantly increased ($p < 0.001$). Administration of mildronate in boars of the experimental group did not affect the concentration of creatinine, total bilirubin, total cholesterol, glucose and aspartate aminotransferase/AST, alanine aminotransferae/ALT activity in comparison with animals of the control group, and at the end of the experiment, these parameters complied with normal values of this species. The study has found that the spermatogenic epithelial thickness, testosterone level in blood, sexual activity and spermatozoa motility are increased in the experimental (mildronate treated) group of boars.

Key Words: Mildronate, boars, testes histology, sexual reflexes, semen quality

3305

Identification of the inorganic pyrophosphate metabolizing, ATP substituting pathway in mammalian spermatozoa

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Sperm ATP is required for sperm motility within female reproductive tract, sperm viability and sperm-zona pellucida penetration during fertilization. Inorganic pyrophosphate (PPi) is generated by ATP hydrolysis in the cells and also present in extracellular matrix, cartilage and bodily fluids. Fueling an alternative pathway for energy production in cells, PPi is hydrolyzed by inorganic pyrophosphatase (PPA1) in a highly exergonic reaction that can under certain conditions substitute for ATP-derived energy. Recombinant PPA1 is used for energy-regeneration in the cell-free systems used to study the zymology of ATP-dependent ubiquitin-proteasome system, including the role of sperm-borne proteasomes in mammalian fertilization. Inspired by such experiments, this study reveals, for the first time, the presence of PPi, PPA1 and PPi transporter, progressive ankylosis protein ANKH in mammalian spermatozoa. Addition of PPi during porcine IVF increased fertilization rates significantly and in a dose-dependent manner (96.2–97.8% at 10–20 μM, $p < 0.05$). Boar spermatozoa stored for 3–4 days in 10 μM PPi-supplemented Beltsville thawing solution (BTS) showed higher fertilization rate than without PPi supplement in BTS (88.9% vs. 68.3%, $p < 0.05$). Fluorometric assay detected high levels of PPi in porcine seminal plasma, oviductal fluid and spermatozoa (relative intensity, no units: 111.5–117.7, $p < 0.05$). Immunofluorescence detected PPA1 in the postacrosomal sheath (PAS) and connecting piece of boar spermatozoa; ANKH was present in the sperm head PAS and equatorial segment. Both ANKH and PPA1 were also detected in human and mouse spermatozoa, and in porcine spermatids by Western blotting. Higher proteasomal-proteolytic activity (chymotrypin-like proteases activity, ZLLVY-AMC), indispensable for fertilization, was measured in spermatozoa preserved with PPi. In conclusion, the addition of PPi in IVF medium enhanced sperm penetration during porcine fertilization, increased sperm proteasomal enzymatic activities, and improved sperm viability during extended semen storage. The identification of an alternative, PPi dependent pathway for ATP production is spermatozoa elevates our understanding of sperm physiology and sets the stage for the improvement of the semen extenders, storage media and IVF media for animal biotechnology and human assisted reproductive therapies.

Key Words: Spermatozoa, inorganic pyrophosphate, inorganic pyrophosphatase, ANKH, fertilization

3306

Proteomic analysis of sperm anterior head plasma membrane: Unfolding the molecular components underlying sperm-egg interaction

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Capacitation is a process occurring in the female reproductive tract or *in vitro* in albumin containing medium, whereby mammalian sperm gain full ability to bind to the egg zona pellucida (ZP). This process likely involves reorganization and modification of sperm plasma membrane components, including those at the anterior sperm head plasma membrane (APM), the site for ZP binding. To understand the molecular basis underlying this process, we have performed quantitative mass spectrometry (MS) based proteomic analyses of APM vesicles isolated by nitrogen cavitation from non-capacitated and capacitated pig sperm. These analyses showed that a number of proteins, including ZP-binding molecules (i.e., SED1, ADAMs 1, 2 and 3) and chaperones (i.e. Hsp70), were present at higher amounts in capacitated APM vesicles. The immunoblotting results also confirmed the relative abundance of these proteins in non-capacitated and capacitated APM vesicles. Based on emerging evidence in the field and our proteomic data, it is possible that key molecules including ZP-binding molecules and chaperones are assembled into multi-molecular complexes at the APM during capacitation to facilitate sperm-ZP interaction. To verify this possibility, blue native gel electrophoresis (BN-PAGE) was used to detect high molecular weight (HMW) complexes, which were then assayed for their ZP binding ability by far-western blotting with biotinylated ZP glycoproteins. BN-PAGE analysis revealed increased levels of HMW protein complexes in the capacitated APM samples. Moreover, far-western blotting indicated

that the HMW protein complexes of capacitated APM vesicles selectively possessed affinity for ZP, a result implicating these complexes in endowing fertilizing ability of capacitated sperm. We are in the process of identifying proteins in these HMW complexes. These results will provide a better understanding of how these complexes are formed for ZP interaction. The information obtained from this study, and our future study in the human system, will be beneficial for the development of male fertility biomarkers, treatment of male fertility, as well as development of non-hormonal male contraceptives via interference of gamete interaction. Supported by CIHR and NSERC.

Key Words: Capacitation, zona pellucida, anterior sperm head plasma membrane (APM), high molecular weight protein complexes, proteomic analysis

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Morphological and ultrastructural assessment of giant panda spermatozoa

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The giant panda (Ailuropodae melanoleuca) is a critically endangered species and endemic to China. The total number of both wild and captive bred giant pandas is about 2300. Preserving this species and expanding its population are always the focus for researchers. The objective of this study was to investigate and characterize the morphology and ultrastructure of giant panda spermatozoa, providing detailed basic informations for the further development of reproductive science of giant panda. Semen samples of four adult male pandas maintained in the Beijing Zoo were investigated via scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The results show that the whole sperm consists of a head, neck, and tail, containing primary ultrastructures including acrosome (ac) which covers the anterior two-thirds of head, nucleus (n), centrioles (ce), mitochondrial sheath(MS), fibrous sheath(FS), outer dense fibers (ODF) and axenome (ax). The length of the head is 4.30 ± 0.27 μm, the width is 3.55 ± 0.21 μm, and the length of the remaining parts is 42.00 ± 2.27 μm. The total length is approximately 50 μm, which corresponds to the length of human and common domestic animal spermatozoa. The distinctive features of the panda spermatozoa are its head and neck. The head is flat and 'tennis racket' like, more round than other animals' heads. The neck, which is short and delicate, dose not attach to the head at the central site of the caudal head, but excentric, the width ratio of two sides is approximately 3/1.

Key Words: Giant panda, spermatozoa, morphology, ultrastucture

3308

Testicular weight and its correlation with body weight and testicular biometry in Japanese quail: implications for selection of breeders

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Currently, there is no reliable predictive parameter to estimate sperm production and breeding potential in Japanese quail. Despite the intra-abdominal location of the gonads, methods such as ultrasound may be used to assess testicular measures and indirectly estimate testicular volume, which is directly correlated to capacity of sperm production. Differences in testicular shape, size and position may also difficult a unique approach between right and left testes. Therefore, the objective of the present study was to determine which testicular measures better correlate with testicular weight, contributing to create a selection parameter for Japanese quail in breeding colonies. After weighing, 25 male Japanese quails were sacrificed by cervical displacement and testes were dissected, weighted and measured (length, width, thickness and circumference) with an electronic digital caliper. Parameters were correlated using Pearson's correlation coefficient (r) at 1% level of significance. Data are presented in Table 1. Even with gonadossomatic index near 4%, body weight was poorly correlated with testicular weight. Circumference revealed strong positive correlation for both testis, but may be difficult to assess by ultrasonography. Width and thickness showed a higher correlation coefficient, indicating that they could be used to estimate capacity of sperm production in both testes, rather than length. Although different in shape, the right testis was more elongated, and the left more rounded, and testicular weight was similar, suggesting equal contribution for sperm production. These findings may be important to develop a practical technique to select Japanese quail breeders, based on indirect assessment of sperm production capacity, by means of ultrasonography.

Key Words: Testicular weight, Testicular biometry, Japanese quail

Table 1. Pearson's correlation coefficient (r) between testicular weight and body weight, testicular length, width and circumference for right and left testes

	Body weight	Testicular length	Testicular width	Testicular thickness	Testicular circumference
Right testis weight	0.12	0.61*	0.75*	0.75*	0.81*
Left testis weight	0.14	0.81*	0.85*	0.90*	0.86*

*Pearson correlation significant at t test ($p < 0.01$).

3309

Replacement of soybean oil for flaxseed oil in the diet of Rhamdia quelen males: effects on sperm motility and membrane integrity

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Sperm membrane fluidity is important to sperm resistance to environmental conditions, and it is directly related to plasma membrane lipid composition. Dietary lipids can chance sperm lipid composition; flaxseed oil is rich in n3 polyunsaturated fatty acids, and its inclusion in the diets promotes increased fertility rates in various species. The objective of this work was to evaluate sperm characteristics of Rhamdia quelen males subjected to diets with different concentrations of flaxseed oil in substitution to soybean oil. Twenty eight males were randomly allocated to four tanks and fed the experimental diets for four weeks. Diets were composed of 100% soybean oil (diet 1 – control), 67% soybean oil + 33% flaxseed oil (diet 2), 33% soybean oil + 67% flaxseed oil (diet 3), or 100% flaxseed oil (diet 4). Approximately 100 μl of sperm was collected from each male, after 2 and 4 weeks on the diets, and evaluated for percent of motility (scale from 1 to 4; 1: 0–25%, 2: 25–50%, 3: 50–75%, and 4: 75–100% motile cells); duration of motility (from time to activation to time of vibratory movement), and membrane integrity (dual staining with SYBR-14 and PI). For both semen collections, there were no statistical differences ($p > 0.05$) among treatments in any sperm parameter analyzed. For all samples, percent motility ranged from 75 to 100% (scale 4). Means (±SE) of motility duration for diets 1, 2, 3, and 4, respectively, were 129.9 ± 17.3, 133.1 ± 27.0, 130.3 ± 24.1, and 178.2 ± 33.4 after two weeks, and 124.9 ± 11.1, 128.3 ± 14.5, 144.2 ± 18.4, and 122.5 ± 20.6 s after 4 weeks on the diets. Means (±SE) of membrane integrity for diets 1, 2, 3, and 4, respectively, were 83.9 ± 7.1, 71.4 ± 9.9, 93.3 ± 1.5, and 75.0 ± 9.1 after 2 weeks, and 70.0 ± 8.3, 71.1 ± 8.0, 59.2 ± 7.5, and 63.8 ± 8.0% after 4 weeks on the diets. Replacement of soybean oil by flaxseed oil in the diets of Rhamdia quelen males does not change sperm percent motility, motility duration, or membrane integrity after 2 or 4 weeks on the diets.

Key Words: flaxseed oil, fish sperm, motility, membrane integrity, Rhamdia quelen

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Changes in notch signaling pathway expression in the testis are associated with spermatogenesis along the mouse post-natal life

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In the adult male, the seminiferous tubules are continuously undergoing cellular proliferation and differentiation. The coordination of these cellular events and the start of spermatogenesis are still poorly understood. The Notch signaling pathway is a well known central regulator in several cellular events and comprises four receptors (Notch1,2,3,4) and 5 ligands (Jagged1, 2, Delta-like 1, 3, 4). Here we present preliminary data indicating the implication of the Notch pathway in the onset and maintenance of spermatogenesis in the mouse mammalian model. Four CD1 wild type male mice were humanely sacrificed at post-natal days 4 and 15, weeks 4, 10 and 20. The testes were collected, processed for immunostaining and the expression profiles of Notch1, 2 and 3, Jagged1 and Delta-like1 (Dll1) and 4 (Dll4) were analyzed through immunohistochemistry. At post-natal day 4, when seminiferous tubules only presented spermatogonia, we found expression of Dll4 and Notch3 in these cells. At post-natal day 15, expression of Dll4 and Notch2 was only present in sections of tubules in which meiosis had started. At week 4 post-natal, all Notch members except Notch3 were expressed in the seminiferous tubules. However, they showed different expression patterns in accordance with the phase of the cycle of the seminiferous epithelium. At weeks 10 and 20 post-natal, expression of Notch pathway members became cell type specific. Dll1 was only expressed in elongated spermatids. Dll4, Notch1 and Notch3 were expressed in spermatocytes and round spermatids. Notch2 was only expressed in round spermatids. Jagged1 was only expressed in the elongated spermatids' cytoplasmatic droplet, where Dll4 and Notch2 were also found. In Sertoli cells, expression was restricted to Dll4 and Notch3 only at day 4 post-natal. These results propose a role for Notch signaling in spermatogenesis, namely in the regulation of germline cell proliferation, differentiation, and stem cell state maintenance. As in the reported epithelial canonical function, expression of Dll4 at day 4 post-natal may restrict differentiation of type A spermatogonia, thus guaranteeing the stem cell pool. Selective expression of Dll4 at day 15 post-natal only in sections of tubules where germ cell proliferation and meiosis was already ongoing, prompt for a role of Notch signaling in the onset of spermatogenesis. At adulthood, specific expression of Notch members in specific cell types of the phase of the spermatogenic cycle indicates a central role of the Notch pathway in the regulation of spermatogenesis.

Key Words: Notch pathway, spermatogenesis, mouse

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Comparison of 2D and 3D culture systems for murine male germ cell differentiation

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The study of spermatogenesis and spermatogonial stem cells (SSC) may result in the discovery of new knowledge about the biology of these and other types of adult stem cells (SC). In the future, could provide treatments for male infertility, understanding the origin and formation of testicular tumors, preservation of endangered species and genetic improvement of livestock and others importants issues in reproductive medicine. However, culture systems and composition of culture media used to induce spermatogenesis *in vitro* from the SSC are not fully understood. Thus, the goal of this experiment was to study the induction of the differentiation of those cells and spermatogenesis *in vitro* from the testicular digestion of Swiss mice (6–10 days postpartum, dpp) cultivated in different systems: the two-dimensional (2D) or conventional and three-dimensional (3D). For that, after testicular digestion, the cell suspension was transferred to plates coated with a thin layer of agar (2D system). While a thicker layer of agar (SACS – Soft Agar Culture System) or methylcellulose (MCS – Methylcellulose Culture System) were made to mimic the *in vivo* situation. In addition, we evaluated the effect of the addition of gonadotropins to the culture medium in both systems. It was possible to notice that the cluster formation and maintenance of spermatogonia was more visible on gonadotropins (+), in both systems. However, the 3D–MCS(+) was more advantageous compared to 2D culture system because it supported the formation of clusters of spermatogonia and also the newly formed cystic structures similar to lumen of the tubules seminiferous tubules. We concluded that the 3D–MCS(+) culture demonstrated better spatial organization similar as founded *in vivo*. However, the potential fertility of these round and elongating spermatids is unknown, requiring further experiments. Funding: Foundation for Research Support of Sao Paulo State (FAPESP), grant numbers 2009/13320–8 and 2010/14930–1.

Key Words: Murine, germ cells, spermatogenesis, spermatozoa, culture

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Characterization of a novel calcium binding protein of sperm: CABS2

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Though calcium ions are well-known to play fundamental roles in sperm, the exact mechanisms regulating calcium ion signalling in sperm remain to be elucidated. We first screened for the calcium binding proteins in silico. Namely, as the isoelectric points of many calcium binding proteins which have been already reported were apt to be lower than 4.5, we tried to screen the proteins, whose isoelectric points were lower than 4.5, from the mouse testis cDNAs registered in online datebase NCBI and found a candidate protein: Gene number 1700040L02Rik named as CABS2; Calcium binding protein sperm specific 2.
MW and pI of CABS2 protein were calculated to be 39 kDa and 4.4. CABS2 protein contained a coiled coil motif in the C-terminal domain. As there has been no infomation on the expression and function of CABS2, we analyzed them in the present study. We made the full-length recombinant protein CABS2 using mouse and porcine cDNA datebase. Recombinant full length CABS2 protein was found to bind calcium and to form oligomers. By Western blot analysis, CABS2 proteins extracted from mouse testis in the native form were detected as several bands. On the other hand, CABS2 proteins extracted with SDS were detected as a single band at 44 kD as denaturated form. Theses results indicate that CABS2 protein forms oligomer in the mouse testis. In order to identify the proteins which interacted with CABS2, we perfomed the pulldown assays and found several proteins. Immunohistochemical studies on mouse testis revealed that CABS2 proteins were firstly expressed in the round spermatids and retained in sperm in the cauda epididymis. It was also found that CABS2 protein was specifically localized in the flagella of matured sperm. These results suggest that CABS2 protein is involved in the flagellum formation and calcium ion signaling of the flagellar motility.

Key Words: Sperm, spermatid, calcium, testis, flagellum

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Effect of an injectable mineral solution on lipid peroxidation and semen quality in young Brahman bulls

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§%§ Oxidative stress has been linked to problems in sperm motility and viability due to biochemical and functional alterations of the sperm

plasma membrane, which eventually results in infertility. The objective of this study was to determine the antioxidant action of the commercial injectable mineral solution Calfosvit-Se®(Lab. Compañia California, Bogota-Colombia), whose components work as cofactors to enzymes that inhibit cell damage induced by oxidative stress, and its effects on semen quality. Twenty five healthy Brahman bulls, aged 15–18 months, body condition three (1–5) were randomly selected from a well known Brahman breeding center located in a tropical dry forest area of Lara State (Venezuela), and divided into two groups. Treatment group (n = 13) received a daily intramuscular dose consisting of 1 ml/20 KgBW of the product during five consecutive days. All 25 bulls were electroejaculated on day zero 0 (just before treatment), day 15, and day 30. Conjugated dienes (CD) and malondialdehyde (MDA) were quantified in milk-diluted semen samples in order to determine the effect of the injectable product on these indicators of lipid peroxidation. Also, a study of fresh semen quality was performed, including percentage of motile sperm (% mot), percentage of progressive motility (% progmot), sperm morphology (% abnormal), sperm vitality (% live), absent acrosomes (% acros). Data was analyzed through a Student t test for independent samples ($p > 0.05$), using SPSS v17.0 software for Windows. The results showed significant differences in MDA levels at 30 days post-treatment between the experimental group (1.09 ± 0.3) and the controls (1.58 ± 0.6), demonstrating the antioxidant protective effect of the product. The study of semen quality showed no significant differences ($p > 0.05$) for any of the variables determined; however, overall semen quality seemed better in bulls from the experimental group than controls: % mot 44 vs. 27.2; % progmot 34.1 vs. 16.2; % abnormal 45.8 vs. 45.2; % live 64 vs. 50; % acros 10 vs. 64, respectively, which could be related to the antioxidant effects of the product.

Key Words: Oxidative stress, Brahman, bulls, semen quality, minerals

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In vitro culture of equine testicle samples

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Spermatogenesis is one of the most complex and longest processes of sequential cell proliferation and differentiation in the body and recently it was described *in vitro* in murine testes (Sato et al., 2011). However, there are absences of reports regarding this technique on the equine. Then, the aim of the present study was to develop a method to culture testicular fragments obtained from adult horses. For that, a method similar to the one described by Sato et al. (2011) was employed. Briefly, testicle samples of approximately 1–3 mm^3 were placed in stands made of 1.5% agarose gel (Difco Agar Noble- BD 214230) on 6-well culture plates. Agarose gel stands were cut into hexahedrons of about $10 \times 10 \times 5$ mm in size and soaked in the culture medium for more than 24 h. Each agarose stand was loaded with approximately 3 testicle samples and between two to three stands were placed in each well. The amount of medium was adjusted to half to four fifth of the height of the agarose stands. Medium change was performed once a week. Culture medium was composed by MEM α (Gibco 32561-037), 10% KSR (Gibco 10828), 50 μg/ml of gentamicine (Sigma-Aldrich G1264), 3.0 μg/ml of Amphoterecin B (Gibco 15290-018), 80 μl/ml of ITS (BD 354352), 50 UI/l of rFSH (Puregon, Organon), 1 μM of testosterone and Equine Pituitary Extract (EPE). The culture system was maintained with 5% CO_2 in air at 32°C. The testicle samples were evaluated at day 0 (fresh, control), 7, 14, 28 and 43 under a light microscope and stained with haematoxylin and eosin. After 7 days in culture it was possible to observe the maintenance of the seminiferous tubules structure, the presence of some spermatozoa at the lumen, a reduction in germ cell number and eosinophilia and pyknosis in most of the Leydig cells. However many of the spermatogonia and Sertoli cells presented normal characteristics. The presence of multinucleated cells indicated abnormal cellular divisions. After 14 days in culture some seminiferous tubules showed signals of degeneration, but the majority is still preserved. The germ cells were substantially reduced, nevertheless Sertoli and Leydig cells remained present with characteristic morphology and more spermatozoa could be seen at the lumen of the seminiferous tubules. Multinucleated cells are still present. After 28 and 43 days in culture the signals of degeneration were still present in the majority of seminiferous tubules, as the deposition of proteic material inside the lumen. It's possible to see Leydig and Sertoli cells with adequate morphology, and germ cells were significantly reduced in number. However many spermatozoa could be seen at the lumen of the seminiferous tubules. These findings suggest that the cellular division characteristic of spermatogenesis continued to happen under our culture conditions. At the same time germ cells disappeared, an increase in the number of spermatozoa could be observed in the lumen of the cultured tubules. Thereafter, the functionality of the spermatozoa will be tested. Acknowledgements: FAPESP for financial support.

Key Words: Equine, testicle, culture, spermatogenesis, spermatozoa

3351

Sexual stimulus increases testosterone concentrations and testicular blood flow in dogs

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Testosterone concentration and testicular fluid content increases, after rams' sexual activity (Ungerfeld and Fila, 2011a. *Reprod. Domest. Anim.* doi:10.1111/j.1439-0531.2011.01926.x). Administration of small-dose multiple GnRH injections in rams provokes similar results, explaining the mechanism involved (Ungerfeld and Fila, 2011b. Reprod. Domest. Anim. 46(4):720–723). The objective of this study was to determine if contact with an estrous bitch and semen collection induces an increase in testosterone concentrations and testicular blood flow in dogs. Semen from 23 English Bulldog dogs was collected stimulating them with an estrous bitch. Initial friction movements were performed and the penile sheath was gently pulled back behind the bulbus as soon as penile erection started. Then constant pressure was maintained caudal to the bulbus, and erection and ejaculation was achieved. The first and second (sperm-rich) semen fractions were collected, but when the third fraction started to ejaculate, the collection stopped. Before the procedure, and 15 min after ejaculation, blood samples were obtained and testosterone was measured by RIA. Ultrasonographic examinations of the testes were performed with a B-mode ultrasound scanner connected to a 7.5 MHz linear array transducer at the same moments. Two ultrasound digital images of each testis were saved in ultrasound memory. Ultrasonograms were evaluated with a specific software (Image Proplus 3.01, Media Cybernetics, USA) analyzing in four spots with a diameter of 0.5 cm on the same place of the image with the spot metering technique. The pixel intensity of the testicular parenchyma was determined in each spot on a scale of 1 (white) to 255 (black). For each dog, means and SEM pixel intensity were determined for each testis and the average of both testicle was used for statistical analysis (paired t test). Testosterone concentrations increased from 232.3 ± 15.9 to 271.6 ± 17.1 ng/dl ($p < 0.0001$). Pixel intensity decreased from 168.7 ± 2.3 to 138.4 ± 2.8 ($p < 0.01$). The stimulus (contact with an estrous bitch and ejaculation) determined an increase of testosterone secretion, which was associated with an increase of testicular blood flow.

Key Words: Ultrasound analysis, testis, sexual stimulus, testosterone, dog

34. Transgenesis and cloning:

3400

The expression of pluripotency markers in donor cells increases gene expression variability in bovine cloned embryos

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Nuclear transfer (NT) in mammals is a complex process in which a differentiated cell is reprogrammed to induce embryo development. NT is associated with different degrees of nucleus epigenetic reprogramming of the donor cells. Expression of certain genes in these cells might facilitate its expression in the embryo produced after NT. This research was aimed to investigate the effect of constitutive expression of Oct 4 in the somatic cells used for nucleus transfer on the developmental potential of day 7 blastocysts generated with these cell lines and also to determine whether Oct 4 expression in the cell lines correlates with the expression of pluripotency genes in cloned blastocyst. For this, we selected five different somatic cell lines of cattle that expressed different levels of Oct 4. Cloned day-7 blastocysts were generated from each cell line, using hand made cloning. Blastocysts were pooled in groups of five and expression of Oct4 and Sox2 was measured by RT-qPCR, using ATCB as internal control. In vitro fertilized (IVF) time-matched blastocysts were produced and processed in the same manner. The development potential was assessed on the basis of blastocysts rate; grading and total cell counts at day 7. We found that previous (constitutive) expression of Oct 4 in the cell lines improved the percentage of blastocysts ($r = 0.92$; $p = 0.02$), rate of grade I blastocysts ($r = 0.96$; $p = 0.01$) and total cell number ($r = 0.98$; $p = 0.002$) and also increases the expression levels of Oct4 and Sox2 in grade I blastocysts. However there was a bigger variability in Oct 4 and Sox2 ($p = 0.03$) expression in the embryos generated from cells that expresses the highest levels of Oct 4 ($p = 0.02$). We also found a positive correlation between the blastocyst rate and the expression level of pluripotency markers in the embryos produced *in vitro* (IVF and cloning), however the correlation coefficient decreased when embryos were produced by cloning. In spite of the higher blastocysts rate and Oct 4 expression found here in cloned embryos, the implantation rates and ultimate cloning efficiency does not seems to be affected positively by these findings. This might indicate that the higher variability in Oct4 expression in cloned embryos is due to incorrect gene reprogramming and that the oocyte is unable to correct for higher Oct 4 levels. We concluded that Oct 4 expression in somatic cells is not a good prognosis marker for selecting cell lines, on the contrary it can be an explanation as to why using stem, ipS or stem cell-like cells as source of nuclei does not improve the actual cloning efficiency. This work was partially supported by Fondecyt grant No. 11100082 from the Ministry of Education of Chile.

Key Words: Cloning, Oct4, Sox2, pluripotency

3401

The aggregation of male cloned embryos with presumptive female *in vitro* fertilized embryos increases the fetal survival of predominately male chimaeras in cattle

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The cloning of cattle by somatic cell nuclear transfer (NT) results in low survival and high frequencies of abnormal placental and fetal development. We postulate that such anomalies may be overcome by complementing NT embryos with *in vitro* fertilized (IVF) embryos to form chimaeras. We aim to influence the gender and germline composition of chimaeras to produce cattle that are functionally male and produce sperm derived from the NT embryo. On Day 4, at the 12- to 16-cell stage, individual male NT embryos were aggregated with presumptive female IVF embryos derived from X-sorted sperm. On Day 7, suitable quality embryos were transferred individually to synchronized recipients. Pregnancy establishment on Day 35 was not significantly different between aggregate, IVF and NT groups (29/82 = 35% vs. 20/70 = 29% vs. 16/74 = 22%, respectively). By Day 100 of gestation there was still no difference in survival between aggregates and IVF, but both groups were significantly greater than NT (21% vs. 21% vs. 4%; respectively; $p < 0.005$). In the aggregate group, 14 of the 16 fetuses recovered around Day 100 were phenotypically male (88%). Even after accounting for the two females, the production of male fetuses following aggregation was still significantly greater than that from NT ($p < 0.02$). Expression of the female-specific mRNA for XIST (X-inactive specific transcript) was detected in either liver or heart from 11/14 aggregate male fetuses (79%), indicating somatic cell chimaerism. Chimaerism in the testes was determined by evaluating genetic variants of β-casein. The NT genotype was homozygous A2, whereas the sire used for IVF was homozygous A1 at the β-casein locus. Testis samples from all 14 aggregate males were positive for the A2 genotype. In addition, 9/14 males were also positive for the A1 genotype in the testes, indicating the presence of cells derived from the IVF embryo. Fetal testes from five confirmed chimaeras appeared histologically normal, with no evidence of ovarian-like structures. We have yet to genotype the germ cells (pre-spermatogonia), Sertoli cells and Leydig cells in chimaeric fetal testes and determine the colonization of the germline by derivatives of the two aggregated embryos. From the molecular analyzes of heart, liver and testis samples, chimaerism could not be detected in 2/14 male aggregate fetuses examined. Low level chimaerism cannot be excluded in these or other organs. Alternatively, they may be exclusively NT-derived. Although aggregation increased the developmental potential of NT embryos, abnormalities usually seen in clones also occurred in chimaeras. Four of the male chimaeras displayed evidence of hydroallantois and/or organ overgrowth. This might be minimized by reducing the relative number of NT cells in the aggregated embryo. Future work will also determine the minimum number of male cells required in each aggregate to influence the gender and germline transmission in the resulting chimaera.

Key Words: Embryo, bovine, nuclear transfer, chimaera

3402

Production of embryos cloned from beef purchased at the market

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Packaged high-quality beef could be a potential genetic resource if the cells in the tissue could be used for somatic cell nuclear transfer (SCNT). Bovine cloned embryos have been produced with cells obtained from cooled carcasses stored at chilled temperature for several days (Adams et al, 2004, Arat et al, 2005). Here, we investigated whether somatic cells taken from beef purchased at the market retain the potential to develop to blastocysts by SCNT.

In the first experiment, we investigated whether cells could be cultured from packaged beef and then used for SCNT. Packages of Beef from 19 cattle were purchased at a local butcher shop and a supermarket. Using coded information on the package labels, we were able to determine that the animals had been slaughtered 8–49 days before purchase of the meat. The beef was divided into muscle and adipose tissues. The tissues were minced, incubated at 37°C in DMEM containing 0.1% collagenase and 0.2% dispase, washed and cultured in MF-start medium (Toyobo, Osaka, Japan) at 37°C under 5% CO_2 in air with high humidity for several days. Live and actively dividing cells were obtained from the adipose tissue of the 8-day-old meat, but not from the muscle tissue or from either tissue from the older beef. Then, to examine whether these cells could be used for SCNT, the cells were electrically fused with enucleated bovine oocytes. Fibroblasts taken from a calf ear were used as control donor cells. The rates of fusion, cleavage and blastocyst development were not different between the beef and control cells (80 vs. 73%, 78 vs. 78% and 17 vs. 13%, respectively, $p > 0.05$). These results indicate that beef in the market contained live cells that have the potential to develop to blastocysts by SCNT.

In the second experiment, we examined whether non-growing cells from beef packaged 18, 19 and 24 days after slaughter could also be used for SCNT. Mononuclear cells were obtained from adipose tissue

of the beef. The viability of these cells was examined by live/dead staining using Hoechst 33342 and propidium iodide (PI), and TUNEL assay. The rates of dead (PI-positive) cells and TUNEL-positive cells were low and not different between beef and calf ear control cells (7 vs. 9%, $p > 0.05$, and 6 vs. 4%, $p > 0.05$, respectively). We were able to produce SCNT embryos using cells from the adipose tissues. The rates of fusion, cleavage and blastocyst development with beef and control cells were 52 vs. 77% ($p < 0.05$), 38 vs. 59% ($p > 0.05$) and 12 vs. 41% ($p < 0.05$), respectively. These results indicate that live and actively dividing cells could be obtained from packaged beef 8 days after slaughter and that these cells and even non-growing cells obtained more than 10 days after slaughter had the potential to develop to blastocysts by SCNT.

Key Words: Beef, somatic cell nuclear transfer, non-growing cell, blastocyst

3403

Embryonic patterning and extra-embryonic differentiation appear disconnected before implantation in bovine somatic cell nuclear transfer embryos

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Somatic cell nuclear transfer (SCNT) is the most efficient technology to reprogram cell fate, in cattle especially. To study the differentiation of Embryonic (E) and Extra-Embryonic (EE) tissues after reprogramming, we analysed the transcriptome of elongating EE tissues and the gastrulating pattern of E tissues from three groups of 10 bovine SCNT conceptuses at Day 18 of gestation compared to 10 IVP and 10 AI as controls. To explore the impact of reprogramming efficiency, we used three bovine fibroblast cell lines that provided similar blastocyst rates *in vitro* (Day 7) but resulted in different pregnancy rates at term (6%, 7%, 21%). Concerning the EE tissues, 72 genes were found to be differentially expressed between these groups, most - but not all – affecting the trophoblast. Concerning the E patterning, 5 gastrulating classes were used, from fully normal to strongly abnormal, and SCNT exhibited 20–60% of abnormalities. Using a set of six discriminative genes (CALM1, CPA3, CITED1, DLD, HNRNPDL, TGFB3) that we earlier described as an embryonic predictor on AI, new patterns were evidenced on SCNT. Finally, two genes (KLF4, ACVR2A) were identified as putative landmark for post-SCNT defective differentiations. Altogether this work raises questions on what controls re-differentiations beyond the blastocyst stage once somatic nuclei have gone through a successful embryonic reprogramming.

Key Words: Reprogramming, gastrulation, extra-embryonic tissues, gene profiling, cattle

3404

Scriptaid improves *in vitro* development of nuclear transfer bovine embryos but not early pregnancy rates in Nelore breed

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Several studies have revealed abnormal epigenetic reprogramming in somatic cell nuclear transfer. According to this, the use of artificial repairing factors, like histone deacetylase inhibitors (HDACi), was proposed as a possible solution to improve cloning efficiency. This study evaluated the effect of Scriptaid (HDACi) treatment into NT embryo on pre and post implantation development of reconstructed embryos in Nelore breed. Viable oocytes were matured *in vitro*. After 18 h, the first polar body extruded oocytes were used for enucleation and somatic cell injection. After reconstruction, the embryos were activated with ionomycin and 6-DMAP, randomly separated in two groups: Control (CONT, n = 1258) and Scriptaid (SCR, n = 1313). In SCR group, embryos were treated with 500 nM Scriptaid (concentration defined on literature review) for 20 h (4 h on activation and 16 h on initial culture medium), after this period the reconstructed embryos were transferred to culture medium without SCR, following the evaluation described below. Zygotes were cultured in SOFaaci and evaluated 48 hpa for cleavage rate (Cliv) and 168 hpa for embryo production. The embryos were transferred to synchronized recipients on D7 and pregnancy diagnosis was performed at 30 days (P30) and 60 days (P60). Analyses of cleavage at 48 hpa and blastocyst rates at 168 hpa were performed by chi- square. Data are shown as mean ± -SEM. Gestation rates (P30 and P60) were analyzed by Binomial Test. No difference on cleavage rates were found between CONT (83.6 ± 1.7%) and SCR (79.9 ± 2.2%). Blastocysts rates from CONT group presented lower ($p < 0.0001$) index than embryos from SCR (29.6 ± 1.8% and 39.8 ± 2.5%, respectively). A total of 171 embryos from CONT group and 219 from SCR group were transferred to recipients; with 31 (18.1%) and 49 (22.3%) pregnancies at P30, respectively. At 60 days, 15 (9%) pregnancies were maintained in CONT group and 17 (8%) in SCR group. The parameters between P30 ($p = 0.17$) and P60 ($p = 0.71$) were not significantly different. In conclusion, scriptaid improves pre implantation development of the reconstructed embryos, but no difference was observed in early pregnancy rates, P30 and P60. Continuous evaluation of pregnancy and birth rate needs remain to sustain the real benefit of scriptaid supplementation in the first hours of NT embryos.

Key Words: Nuclear transfer, embryo, histone deacetylase, inhibitor

3405

Dissecting functional oocyte enucleation by actinomycin D for bovine somatic cell nuclear transfer

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Oocyte enucleation by micromanipulation remains labor-intensive, and alternate enucleation methods hold low efficiencies. Oocyte treatment with 1 μg/ml of actinomycin D (AD), a DNA-damaging transcription inhibitor, for 14 h during *in vitro* maturation blocks parthenogenetic development, but allows blastocyst formation after nuclear transfer (NT). However, AD enucleation is detrimental to oocyte maturation and embryonic development. This study was aimed to investigate the toxicitiy caused by AD enucleation. The AD enucleation toxicity on bovine oocyte nuclear maturation was completely abolished by oocyte denuding (OD) (Control: 80.3%a, OD: 79.4%a, AD: 69.3%b, AD + OD:77.5%a) and by transcription inhibition (TI) (Control: 81.1%a, TI:81.4%a, AD:70.8%b, AD + TI: 84.1%a). The titration of AD concentration and shortening the incubation period of denuded oocytes increased enucleation effficiency (5 μg/ml of AD for 15 min). Moreover, it was possible to demonstrate that detrimental effects of AD enucleation on embryonic development are caused by damaged oocyte genomic DNA, because its physical removal from AD enucleated oocytes [NT(AD-C)] reduced caspase activity on day 3 embryos [NT:35.2%a, NT(AD):59.5%b, NT(AD-C): 37.1%a], recovered blastocyst development on day 8 [Pathernotes: 33.5%a, NT(AD):4.4%b, NT(AD-C):30.4%a] and pregnancy rate on day 90 [NT: 12.5%, 2/16; NT(AD-C): 7.4% 2/27]. These facts highlight a bimodal toxicity during functional oocyte enucleation by AD and further suggests that approaches to circumvent recognition of damaged DNA may increase embryonic development via NT.

Key Words: Cattle, reprogramming, cytoplast, nuclear transplantation, cloning

3406

Transmission and expression of a retrovirally introduced red fluorescent protein gene in successive generations of transgenic pigs

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We produced transgenic (Tg)-cloned pigs that systemically express a red fluorescent protein (humanized Kusabira-Orange: huKO) using a gene silencing resistant retroviral vector (Matsunari et al., 2008). The objective of this study was to determine whether the systemic red fluorescence phenotype exhibited by a founder Tg pig would be transmitted to successive generations of offspring. A founder Tg pig (female) produced by somatic cell nuclear transfer of retrovirally transduced cells was confirmed by fluorescence in situ hybridization to harbor a single copy of the huKO gene inserted into the q23 region of the chromosome 17. The founder Tg female was impregnated by artificial insemination (AI) using wild-type sperm, and gave birth to 19 (G1) offspring (11 females and eight males) including 11 (57.9%) transgenics in two separate deliveries. Epididymal sperm harvested from one of the G1 male were frozen stored and used to produce second generation offspring (G2) by AI to wild-type females or *in vitro* fertilization (IVF) with wild-type oocytes. Of the 77 offspring (34 females, 43 males) yielded in 12 separate litters, 39 (50.6%) were transgenic. All of the offspring in both the G1 and G2 generations inherited the systemic red fluorescence phenotype observed in the founder Tg clone. In addition, skin fibroblasts of the G1 and G2 offspring were confirmed by fluorescence-activated cell sorting analysis to exhibit the same fluorescence intensity as those of the founder Tg clone.

We confirmed that the huKO gene that had been retrovirally introduced to a somatic cell cloned pig can be transmitted to offspring according to Mendelian fashion and expressed even after two generations of sexual reproduction without undergoing gene silencing. We believe that proliferation by AI or IVF using frozen epididymal sperm is a practical means to utilize the huKO transgenic pigs as large animal models. This study was supported by JST, ERATO, Nakauchi Stem Cell and Organ Regeneration Project.

Key Words: Transgenic pig, retrovirus transduction, red fluorescent protein, Kusabira-Orange, germ line transmission

3407

Effect of Scriptaid treatment on the development of interorder porcine/bovine SCNT embryos

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Interorder cloning (IC) has been used in attempts to rescue highly endangered species, for studying mechanisms underlying nuclear-cytoplasmic interactions, and in biomedical research. However, the efficiency of IC is extremely low. Treatment with histone deacetylases inhibitors (HDACi) has been shown to improve nuclear reprogramming, as well as *in vitro* and *in vivo* development of somatic cell nuclear transfer (SCNT) embryos. In this study we investigated the effect of the HDACi 'Scriptaid' on the *in vitro* development of interorder porcine/bovine SCNT embryos. Cumulus oocyte complexes (COCs) aspirated from 3 to 6 mm diameter follicles were *in vitro* matured and then used as recipient cytoplasts for SCNT. Bovine recipient cytoplasts were obtained from 2 to 8 mm follicles and *in vitro* matured. Fibroblast cells were obtained from skin biopsies of a 3-months old gilt, and cultured in DMEM supplemented with 10% FBS and antibiotics. Embryos were reconstructed by hand-made cloning (HMC) as previously described (Ohlweiler et al., 2010). The experimental groups were as follow: (i) control: embryos were reconstructed using two halves of porcine cytoplasts; (ii) mosaic: embryos were reconstructed with one-half of porcine and one-half of bovine cytoplast; and (iii) interorder: embryos were reconstructed using two halves of bovine cytoplasts. Activation was performed using ionomycin (10 μM/5 min) followed by exposure to 6-DMAP (2 mM/3 h) in porcine zygote medium (PZM-3). Reconstructed embryos were exposed to 500 nM Scriptaid for 12 h starting after ionomycin treatment, and then washed and cultured in PZM-3 for 7 days. Data of cleavage and blastocyst were analyzed by the Chi-square test with $p \leq 0.05$. There was no effect of Scriptaid treatment on cleavage rates (Table 1). The blastocyst rate in the control and mosaic groups was increased by Scriptaid treatment ($p < 0.05$). However, Scriptaid treatment did not increase embryo development in the interorder group. These findings suggest that HDACi treatment is unable to promote cell reprogramming in interorder SCNT embryos.

Table 1. *In vitro* development of control, mosaic and interorder porcine SCNT embryos treated with Scriptaid

Cytoplast	Scriptaid treatment	Culture n	Cleavage n (%)	Blastocyst n (%)
Control (porcine + porcine)	-	130	122 (93.8)^{ab}	12 (9.2)^{b}
	+	156	149 (95.5)^{a}	27 (17.3)^{a}
Mosaic (porcine + bovine	-	97	92 (94.8)^{ab}	1 (1.0)^{c}
	+	98	88 (89.8)^{abc}	9 (9.2)^{b}
Interorder (bovine + bovine)	-	86	75 (87.2)^{bc}	1 (1.2)^{c}
	+	94	78 (83.0)^{c}	0 (0.0)^{c}

^{a,b,c} distinct letters in the same column differ ($p \leq 0.05$).

3408

In vitro development of intergeneric porcine/bovine SCNT embryos produced from distinct donor cell types of endangered swine breeds

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Embryo development in intergeneric cloning depends on proper nucleo-cytoplasmic interactions in the reconstructed embryo. It is also known that cloning efficiency is influenced by the donor cell type and species. This study investigated the effect of the donor cell type (fibroblastic-like cells - FIB vs. adipocyte-derived mesenchymal stem cells – ADMSC), and host cytoplast (control-porcine; mosaic-porcine + bovine; intergeneric-bovine), on development of somatic cell nuclear transfer (SCNT) embryos. Somatic cell cultures were established from two animals of different endangered pig breeds (Mulefoot – animal 1 and Moura – animal 2) by enzymatic digestion of tissue biopsies and culture in DMEM containing 10% FBS. Porcine and bovine oocytes used as recipient cytoplasts were maturated *in vitro* and embryos were reconstructed by hand-made cloning (HMC) method. Embryos were reconstructed by hand-made cloning (HMC) as previously described (Ohlweiler et al., 2010, Reprod Fert Dev. 22, 1; Mezzalira et al., 2011, Cel Reprog. 22, 1). Each donor cell type was fused with different cytoplasts as follows: (i) control: embryos were reconstructed using two halves of porcine cytoplasts; (ii) mosaic: embryos were reconstructed with one-half of porcine and one-half of bovine cytoplast; and (iii) intergeneric: embryos were reconstructed using two halves of bovine cytoplasts. Reconstructed oocytes were activated using ionomycin (10 μM/5 min) and 6-DMAP (2 mM/3 h),

and then cultured in PZM-3 for 7 days. Blastocyst rates were analyzed by the Chi-square test with $p \leq 0.05$ (Table 1). Mosaic and intergeneric groups produced lower blastocyst rates than controls. The group ADMSC-mosaic from animal two showed intermediate embryo development, evidencing that embryos reconstructed using half homologous cytoplasm are able to go through nuclear reprogramming. In embryos reconstructed with cells from the animal two development was higher in the ADMSC-mosaic group than the FIB-mosaic group. These findings suggest that development of intergeneric SCNT embryos are affected by the donor cell type and genotype.

Table 1. Porcine SCNT embryos reconstructed from distinct donor cells and recipient cytoplasts

Cell donor	Cell type	Cytoplast	N IVC	% Blastocysts
Mulefoot	FIB	Pig	123	17.1^{a}
		Mosaic	115	5.2^{b}
		Bov	122	3.3^{b}
	ADMSC	Pig	121	20.7^{A}
		Mosaic	126	3.2^{B}
		Bov	123	2.4^{B}
Moura	FIB	Pig	134	11.9^{x}
		Mosaic	117	1.7^{y*}
		Bov	139	0.0^{y}
	ADMSC	Pig	119	21.0^{X}
		Mosaic	129	11.6^{Y*}
		Bov	138	2.2^{Z}

a,b; A,B; x,y,z; X,Y,Z: distinct letters indicate a difference between cytoplasts within the same animal and cell type.

3409

Porcine transgenesis by intracytoplasmic injection of oolema vesicles co incubated with transgene

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Porcine transgenesis is an essential tool in agriculture, pharmacology and medicine. There is a wide variety of methods in pig transgenesis but they show some limitations. The aim of this work was to evaluate the use of oolema vesicles in the intracytoplasmic injection of a transgene for obtaining GFP+ porcine embryos, with the purpose of developing a simple and economic method in pig transgenesis. Recently, we described an efficient transgenesis system in bovines using co incubation with oolema vesicles with the transgene, and its subsequent intracytoplasmic injection. To test the use of vesicles technique in porcine activated oocytes we compared intracytoplasmic injection with (i) the plasmid alone or (ii) the plasmid co incubated with vesicles. The plasmid used is pCX-EGFP, linearized with HINdIII enzyme, at 30 ng/μl. Pig oocytes matured *in vitro* were activated by an electric pulse followed by treatement of 6-DMAP. Embryos were cultured in SOF medium at 39°C, 5% CO2. Experimental groups were: control (without injection; N = 109) – pCX-EGFP naked (injected only with pVp + plasmid; N = 58) – pCX-EGFP vesicles (injected with pVp + vesicles + plasmid; N = 76) – SHAM pVp (injected only with pVp; N = 16) – SHAM vesicles (injected with pVp + vesicles; N = 20). Rates of blastocysts at day 7 and eGFP expression under blue light were evaluated. Data were analyzed by Fisher's test ($p < 0.05$). Both groups injected with plasmid presented a lower % of cleavage respect to control [control 94%; pCX-EGFP naked 84% ($p < 0.05$) and pCX-EGFP vesicles 80% ($p < 0.005$)]; no differences in % of cleavage were observed between both SHAM groups and the other experimental groups. No differences were observed in blastocyst rates between the different groups. eGFP expression was higher in pCX-EGFP vesicles than in pCX-EGFP naked [pCX-EGFP vesicles 74% vs. pCX-EGFP naked 43% ($p < 0.005$)]. No differences were observed in the % of blastocysts expressing the transgene [pCX-EGFP vesicles 100%; pCX-EGFP naked 33% ($p > 0.05$)]. These results suggest that intracytoplasmic injection of pCX-EGFP linearized with HINdIII enzyme, both naked plasmid and co incubated with vesicles, affects early development of porcine parthenogenetic embryos; that is reflected in the lower % of cleavage of pCX-EGFP groups compared to control. Respect to eGFP expression, evaluation of genomic integration remains to be tested but these results suggest that the use of vesicles in the intracytoplasmic injection is a useful tool for obtaining GFP+ porcine embryos.

Key Words: Pig, transgenesis, parthenogenetic, vesicles

3410

β-actin is an optimal reference gene for gene expression analysis of mouse nuclear transfer preimplantation embryo

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Real time reverse transcriptase polymerase chain reaction (QRT-PCR) has become a widely used method for gene expression analysis due to its more accurate and sensitive features. Normalization of the assay is a critically important step in QRT-PCR analysis. Ideally, genes chosen should have stable gene expression among individuals, organs and cells during different developmental stages and various experimental treatments. Although housekeeping genes (HKGs) are often used as reference genes to normalize gene expression data, it was also reported that HKGs expression levels varied according to tissue types, cell-cycle phase and experimental treatments. Considering the stability of HKGs in the nuclear transfer (NT) has not been examined in different stages of preimplatation mouse embryos derived from different donor cells, the purpose of present study was to investigate the expression of β-actin, Histone H2A and 18s ribosomal RNA (18s rRNA) in pooled mouse preimplantation embryos derived from different donor cells, external luciferase control RNA was added to the reaction mixture to normalize the efficiency of the RT reaction in QRT-PCR procedures. The total RNA was extracted from mouse NT embryos by using embryonic stem cell, cumulus cell, pronuclear-exchanged, morula blastomere as donor cells, and *in vitro*-cultured and *in vivo* -recovered embryos. The β-actin gene had similar gene expression patterns in all kinds of embryos from the 1-cell stage to the blastocyst stage, four kinds of NT embryos had almost same β-actin gene expression level as the *in vitro* counterparts in each compared stage, except the β-actin mRNA expression level of the *in vitro* blastocysts was significantly lower than that of *in vivo* blastocysts. For Histone H2A and 18s rRNA transcripts, both mRNA expression patterns in mouse NT-derived embryos markedly differed from that of *in vivo* and *in vitro* counterparts, the common features of NT samples had significantly lower Histone H2A and 18s rRNA expression at blastocyst stage. These results suggest that β-actin is a stable housekeeping gene in mouse nuclear transfer embryos, it is an optimal reference gene for gene expression analysis of mouse NT preimplantation embryo.

Key Words: Gene expression, housekeeping genes, nuclear transfer, QRT-PCR, mouse

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The effect of aurintricarboxylic acid on the intergration of exogenous DNA with sperm-mediated gene transfer in mice

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Sperm-mediated gene transfer (SMGT) enables the production of transgenic animals by exploiting the ability of sperm cells to bind and

internalize exogenous DNA. Although SMGT has been used for at last 40 years the technique remains with low repeatability. A possible explanation to this high variation can be the activation of sperm endonucleases, leading the cell to a DNA fragmentation converting to an apoptosis like process (MAIONE et al., 1997; *Mol Reprod Dev*, 50: 406–409). The goal of this study was to evaluate the inhibition of sperm endonucleases by using the aurintricarloxilic acid (ATA) into enhancing of plasmid copies in sperm genomic DNA. For that, two concentrations of the plasmid PCX-EGFP were tested in the presence or ausence of ATA, according to the following experimental groups: 1) control group (0.5 × 106 sperm cells); 2) sperm cells + ATA; 3) sperm cells + 500 ng plasmid; 4) sperm cells + 500 ng plasmid + ATA; 5) sperm cells + 1000 ng plasmid; 6) sperm cells + 1000 ng plasmid + ATA. Samples were incubated for 30 min or without ATA, and then for more 30 min with (500 or 1000 ng) or without PCX-EGFP. Plasmid integration was assessed by qPCR according to Feitosa et al. (Theriogenology, 74: 563–568, 2010). Each reaction consisted of 5 µl of Platinum® SYBR Green, 2 µl of samples (1 ng/µ), 0.28 µl of primer sense (ATGGCCGACAAGCAGAAGAAC) and 0.28 µl of primer anti-sense (TGCCGTCCTCGATGTTGTG) (500 nM each) and water qsp 10 µl. Amplification was carried out at 90°C per 15 s and 64°C per 1 min for 40 cycles. Differences at plasmid amount among groups were estimated according to the Threshold Cycle (C_t) value compared and normalized by a straight-line equation obtained with the plasmid standard curve. Statistical analyses were performed using SAS system version 9.2. A variance test (PROC GLM) and TUKEY test for C_t means were used with a significance level of 5%. Differences in plasmid concentration did not affect the efficiency of internalization. This suggests that the increase in plasmid concentration during incubation was not enough to enhance the number of plasmid copies in sperm genomic DNA. The addition of ATA was not capable of increasing plasmid internalization in sperm cells (Percentage of integration: group 3 = 0.209%, group 4 = 0.001%, group 5 = 0.102% and group 6 = 0.001%). Based on these results we can conclude that ATA was not effective to increase the integration of exogenous DNA in mouse spermatozoa. Suggesting that the mechanism involved in integration of plasmid during SMGT was not influenced by the use of ATA, having no effect in SMGT protocol.

Key Words: Transgenesis, mouse, sperm, endonuclease, exogenous DNA

3412

Generation of transgenic buffalo offspring through lentivirus injection

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Pronuclear injection has been widely used in attempts to produce transgenic livestock, but transgenic rates achieved by this way in higher mammals are extremely low, up to date, transgenic buffalos still not success. Lentiviral vectors are now recognised as an efficient transgene delivery system and perivitelline space injection (PSI) has emerged as the standard delivery method. Thus, we wanted to set up the effective lentivirus transgene protocol to produce transgenic buffalo offspring by PSI.In the present study, buffalo cumulus-oocytes complexes were recovered by aspiration of buffalo follicles (diameter 2–6 mm), after *in vitro* maturation and *in vitro* fertilization, presumptive MII oocytes and zygotes were denuded by vortexing. LV-CMV-GFP was injected into perivitelline space of presumptive MII oocytes and zygotes. After cultured for 7 days, eight of EGFP positive transgenic blastocysts were transferred to four surrogate mothers, as results, two recipients (50%, 2/4) were pregnant and develop to term, three live transgenic buffalo offspring were born (37.5%, 3/8), after southern-blot, RT-PCR and immunofluorescence analysis, the results showed that GFP gene expressed in organs (heart, muscle etc.) of all three transgenic buffalo offspring (100%, 3/3). To our knowledge, this is the first report to produce transgenic buffalo by perivitelline space injection with lentivirus vector. And the results indicated that the lentivirus perivitelline space injection can efficiently produce the transgenic buffalo offspring.

Key Words: Buffalo, lentivirus

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Comparison of transfection methods effectiveness used to obtain transgenic pigs for xenotransplantation

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In the near future, transgenic animals with modified immunological systems will become 'living tissue and organ banks' used for human transplantation. Clinical findings indicate that the domestic pig best fulfills the criteria of organ suitability for xenotransplantation. In pigs, transgenesis efficiency using standard DNA microinjection does not exceed 2% in terms of the number of zygotes subjected to microinjection. Therefore, there is an urgent need to develop a less complex transfection method, whose efficiency will surpass or match the efficiency of DNA microinjection. Lipofection is a widely used technique to obtain stable transfection of somatic cells with high effectiveness. In case of zygote, surrounding it zona pellucida protects the cytoplasm from penetration of liposomes/DNA complexes. We developed DNA lipomicroinjection method which is based on injection of liposomes/DNA complexes into perivitelline space of pig zygotes. Lipomicroinjection is than much simpler to perform and causes lower loss of transfected material than standard DNA microinjection. Previously performed study on *in vitro* produced transgenic pig embryos indicated that both lipomicroinjection and standard DNA microinjection methods have comparable effectiveness. The objective of this study was to verify *in vivo* the efficiency of lipomicroinjection in comparison to standard DNA microinjection. Five hundred and nine CD59 microinjected and 480 CD59 lipomicroinjected pig zygotes were transferred into recipient. Fourty nine (one transgenic) and 25 piglets were born respectively. HLAe gene was microinjected into 928 zygotes and 115 zygotes were transfected via lipomicroinjection. Eighty six (one transgenic) and seven piglets were born respectively. Results of our study clearly demonstrated that standard DNA microinjection was more effective *in vivo* than alternative lipomicroinjection method and resulted production of two transgenic piglets: one with integrated CD59 gene and second with HLAe gene. Also number of pregnant recipients and produced piglets was almost twice higher in compare to that obtained after lipomicroinjection.

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Key Words: Xenotransplantation, transgenic, pig

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In vitro development of CMV-Fut transgenic and non-transgenic pig embryo

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Possible influence of a transgene on life functions of embryos make it reasonable to confirming or deny it in case of a particular gene construct. *In vitro* development of an embryo is a widely used criterion of its competence. The aim of the study was to compare *in vitro* development capacity of transgenic and non-transgenic pig embryos. Embryos from transgenic and non-transgenic pigs were isolated 24–26 h post-artificial insemination and cultured *in vitro* up to morula/blastocyst stage in NCSU-23 medium for 6 days in 5% CO2 in air at 39°C. Embryo quality were developmental competence: cleavage, morula and blastocyst rates. Group I embryos were obtained from CMV-Fut transgenic sows (n = 13) inseminated with CMV-Fut transgenic boar semen (TG1154). Group II embryos were collected from transgenic CMV-Fut sows (n = 8) inseminated with non-transgenic boar semen. Control group embryos were obtained from non-transgenic pigs (n = 7) inseminated with non-transgenic boar semen. All cultured embryos from group I and II, regardless of developmental stage reached, were frozen to do transgene test. The molecular tests are pending. Based on observations carried out to date following ratios of percentage of cleavage rate, morula and blastocyst were achieved: group I (156 cultured embryos) – 90.3, 80.7 and 70.0%,

respectively; group II (81cultured embryos) – 97.5, 95.0 and 86.4%, respectively; control group (73 cultured embryos) – 94.5, 90.4 and 80.8%, respectively. These preliminary results showed no difference in *in vitro* developmental capacity of embryo obtained from transgenic and non-transgenic pigs. However, the final ratios of developing embryos will be established after transgene test. Financial support: grant no NR12 0036 06
Key Words: Pig, transgene, embryo, *in vitro* culture

3452

Gene expressed in ram spermatid after delivered into germ cells

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Transgenic spermatozoa may become the most convenient carrier of the transgene and powerful tool in making transgenic offspring because they can be used in artificial insemination. However, data about making transgenic spermatozoa are scanty so far. Therefore, this study was to make transgenic spermatids. Testis samples were taken from prepuberty rams. The specimen was minced and digested using collagenase and trypsin in a two-step procedure. Spermatogonia were isolated in Percoll medium by gradiental centrifugation. The gradientally enriched male stem cells were seeded and grown on a feeder of ram Sertoli cells in a culture system supplemented with GDNF, EGF, FGF etc. Spermatogonial clones were formed after several weeks. The colonies were transfected using an eGFP-C1-IGF1 vector reconstructed in our laboratory. The transfected cells were kept in the culture for observation of eGFP and collected later for further investigation. Interestingly, a number of germ cells differentiated into transgenic spermatids after about four weeks in the culture. The reporter gene eGFP was expressed vividly in the multi-shape spermatids as observed under an inverted fluorescent microscope. The expression of the reporter gene was further confirmed by RT-PCR and Western blot, and the transgene IGF-1 by RT-PCR. In the RT-PCR analysis, total RNA was isolated from the transgenic spermatids and hexidimers were used for reverse transcription. Specific primers were used in amplifying the amplicans from the cDNA prepared in the previous step. Anti-eGFP antibody was used in Western blot. In both RT-PCR and Western blot, the specific bands of eGFP from electrophoresis were strong in the experimental group, but not visible in the control. In the RT-PCR for IGF-1, the transgene was increased by 30.77% compared with the control. In conclusion, male germ cells can be transfected *in vitro* to make transgenic spermatids. Undergoing a further step, spermiogenesis or maturation, they can become mature spermatozoa for natural fertilization. As *in vivo* gene delivery to male germ cells, either directly into the testis coupled with some techniques to promote transfer or integration, or by transplantation of transgenic spermatogonia, is difficult in general, making transgenic spermatozoa *in vitro* may provide a promising alternative method in producing transgenic offspring.
Key Words: Gene delivery, transgenic, spermatogonia, ram, GFP

3453

A cloned camel (*Camelus dromedarius*) calf produced from the skin fibroblasts of an elite bull

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Cloning by somatic cell nuclear transfer can be used to reproduce animals with the highest potential for milk production, the racing champions or conservation of endangered camelids. Optimization of the basic techniques for *in vitro* and *in vivo* oocyte maturation, ultrasonographic transvaginal ovum pick up, activation of oocytes and *in vitro* embryo culture in our lab during past few years was the basis for our success in production of world's first cloned camel, named Injaz, on April 8 2009 (Wani et al., Biol Reprod 82, 373–379). Injaz was produced from an embryo reconstructed with a cumulus cell obtained from a slaughtered animal. In the present study, however, we demonstrate use of skin fibroblasts as donor karyoplasts in somatic cell nuclear transfer to produce cloned dromedary camel calves. *in vivo* matured oocytes were collected from super-stimulated dromedary camels by an ultrasound guided transvaginal ovum pick-up. The ear skin biopsy was taken aseptically from an adult bull and a fibroblast cell line was obtained from the collected tissue and frozen after the second passage. For use as nuclear donors, the cells were thawed, passaged, and used from third to ninth passage after serum starvation for 72 h. Reconstructs were activated 1 h post-fusion with 5 μM ionomycine followed by exposure to 6-dimethylaminopurine for 4 h. The activated oocytes were cultured until Day 7. The proportion of oocytes that cleaved was recorded on Day 2, and those that reached morula and blastocyst stages were recorded on Day 7 of culture. Day 7 hatching/hatched blastocysts were transferred non-surgically into the left uterine horn of recipient camels at Day 5 or 6 after ovulation either singly or in pairs depending on the quality of the embryos. An initial pregnancy examination was performed by using transrectal ultrasonography between Days 14 and 16 (Day 0 = day of ovulation), followed by examinations at approximately weekly intervals until about Day 60 of gestation, and then at monthly intervals. Out of 92 embryos reconstructed, 83.7 ± 2.3% cleaved and 30.7 ± 2.8% developed to blastocyst stage. Out of 28-hatched blastocysts transferred to 19 recipients, four were diagnosed pregnant by Day 15. Three pregnancies were lost before Day 60 of gestation and one delivered a live calf after completing the normal gestation period. This study demonstrates the ability of camelid skin fibroblast cells to be reprogrammed and the development of cloned embryos, which in turn lead to the birth of a cloned calf following embryo transfer. In conclusion, the present study reports, for the first time, establishment of pregnancies and birth of live cloned camel by use of somatic cell nuclear transfer using skin fibroblasts as donor karyoplasts. This has opened doors for the amelioration and preservation of genetically valuable animals like high milk producers, racing champions, and males of high genetic merit in camelids.
Key Words: Camelids, somatic cell nuclear transfer, skin fibroblasts, cloning

3454

Outgrowths and capsule derived from aggregated cloned equine embryos

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The formation of the embryonic capsule seems to be necessary for pregnancy to be established in the equine. It is reported that an *in vivo* development is necessary to allow normal capsule formation, and remains unclear if cloned equine embryos cells are capable to produce capsular material on *in vitro* culture. Our aim was to establish culture conditions to allow *in vitro* development of equine cloned embryos beyond day 7, and to evaluate the ability of these embryos to produce outgrowths and capsule. Oocyte collection, maturation, cloning, and activation procedures were performed as described by Lagutina et al. (2007 Theriogenology 67, 90–98). After activation, zona free reconstructed embryos (REs) were cultured in DMEM/F12 with 5% of FBS in well of well system by the aggregation of three REs per well until day 7. Cloned equine blastocysts were maintained in DMEM/F12 with 10% of FBS from day 7 until their collapse (approximately day 17). Blastocysts development and morphological characteristics were observed every 24 h. After their collapse, they were picked up using 18G needles and then cultured in petri dish as tissue samples in DMEM/F12 medium containing 10% FBS, 1% ATB and 1 ul/ml ITS in 5% CO2 in humidified air at 39°C. Outgrowths were obtained from embryos, and they were allowed to grow for 20–30 days, then fixed. A total of nine cloned equine blastocysts were used for this experiment. Embryo size means per day are detailed bellow: Day 7, 110.50 μm ± 31.45; Day 8, 157.14 μm ± 44.16; Day 9, 230.32 μm ± 67.79; Day 11, 517.44 μm ± 276.10; Day 12, 777.81 μm ± 401.11; Day 13, 1218.20 μm ± 523.71; Day 14, 1702.22 μm ± 917.93; Day 15, 2242.27 μm ± 893.44 and day 16, 2884.84 μm ± 1068.31. All blastocysts were able to allow primary embryo outgrowth formation. We did not see, under light microscopy, a clear and confluent capsule as in *in vivo* embryos on *in vitro* zona-free equine cloned embryos neither at day 7, nor during culture until day 17. However, a structure morphologically identical to an equine

embryo capsule was found floating in the culture medium in six of nine explants, two to five days after embryo collapse. In conclusion, *in vitro* culture of equine embryos in DMEM/F12 allows embryo development beyond day 7. The high capability of day 17 collapsed embryos to produce outgrowths, show the elevated grade of proliferation of equine embryos cells in DMEM/F12 medium. Further experiments should be done to clarify if this capsule-like structure formed by cloned equines embryos has the same molecular characteristic of *in vivo* embryo capsule, and to know if the addition of uterus secreted molecules on *in vitro* culture medium it is necessary for a normal capsule formation at physiological times.

Key Words: Capsule, equine, embryo, outgrowth, explant

35. Other:

3500

Effects of by-pass fat supplementation on the productive and reproductive performance of first-calf Brahman cows

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Under tropical conditions pregnancy rates in beef cows is ≤30% and postpartum anestrus could vary between 150 and 210 day, as a consequence of a negative energy balance due to nutritional deficiencies, decrease in voluntary intake around parturition, low body condition score (BCS) and the effect of suckling. Therefore, the aim of this study was to evaluate the effects of by pass fat supplementation on the productive and reproductive performance of first-calf Brahman cows. A total of 30 first-calf Brahman cows with an average BCS of 7.0 ± 0.4 was assigned to two groups according with date of calving. One group (TG; n = 15) was supplemented with by pass fat (150 g/day; Energras® [0.5% ω–3; 17% ω–6]) beginning 30 day previous to parturition until 90 day postpartum and a control group (CG; n = 15) without supplementation. Both groups were pastured under the same conditions. Every 15 day, from 30 day before and 90 day after calving, body weight and BCS were measured and blood samples were taken to determine levels of cholesterol and triglycerides in serum. Twice a week, from 30 to 90 day postpartum ovarian activity (number of follicles and corpus luteum [CL] area) was evaluated using ultrasonography. Reproductive efficiency was evaluated at the end of a 3 months breeding season. Cows in TG had higher levels of cholesterol (33%; $p < 0.05$) than cows in the CG but there was not significant difference in triglycerides concentrations or in body weight ($p > 0.05$). However, BCS was higher ($p < 0.05$) for the TG than in the CG. Number of class 1 (≤5 mm) and two follicles (6–9 mm) did not differ between groups. Nevertheless, cows in TG had higher number of accumulated class 3 follicles (≥10 mm; $p < 0.06$; mean ± EE; TG = 29.6 ± 1.9 vs. CG = 24.3 ± 1.6), but there was not significant difference in diameter of class 3 follicles (TG = 12.0 ± 1.9 vs. CG = 11.5 ± 1.6 mm) or the area of CL ($p > 0.10$; TG = 2.9 ± 1.0 vs. CG = 3.2 ± 1.1 cm^2). Also, there was not significant difference ($p > 0.05$) between groups regarding reproductive efficiency indexes (interval calving to first postpartum CL [TG = 59 ± 19 day vs. CG = 56 ± 18 day]; interval calving to conception [TG = 90 ± 29 day vs. CG = 102 ± 30 day]; number of services/conception [TG = 1.3 ± 0.4 vs. CG = 1.6 ± 0.4] and pregnancy rate [66.7% for both groups). These results indicate that using by pass fat enriched with high levels of polyunsaturated fatty acids increased cholesterol levels and improved the BCS during the early postpartum period, decreased the deleterious effects of the negative energy balance.

Key Words: By-pass fat, first-calf, cholesterol, ovarian activity, Brahman

3501

Intergenerational fetal programming effects on lamb growth

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Potential intergenerational fetal programming effects of size and nutrition during pregnancy were investigated in sheep, since these factors have been shown to affect milk yield and composition in first-parity G1 offspring. Heavy (H) and light (L) pregnant ewes (G0) were fed either ad libitum (A) or maintenance (M) from d 21–140 of gestation. We measured milk production, milk composition and udder size of 24 twin-bearing grand-daughters (G2) at two-years of age, and the birth weights and body dimensions and growth of 48 great-grand-offspring (G3). Milk production, measured by the oxytocin method on one day each week for the first six weeks of lactation, was not significantly affected by the size or the nutrition of the grand dam. Live weight, condition score and udder dimensions did not differ among treatment groups. Milk lactose, fat and protein percentages and yields were similarly unaffected during the six-week trial. Birth weights and girths of lambs did not differ amongst groups but lambs whose great-grand-dams were fed maintenance (n = 24) had greater ($p = 0.02$) crown-rump lengths at birth (58.4 ± 0.8 cm) than those from ad libitum-fed dams (55.9 ± 0.6 cm). Furthermore, the great-grand-daughters (n = 6) of LM ewes grew faster ($p < 0.07$) than did other groups. Weights at 6 weeks of age were LM 19.0 ± 1.0; LA 15.7 ± 0.5; HM 15.9 ± 0.6; HA 16.1 ± 0.5 kg. Given the very small numbers of lambs in the LM group, these results may be a chance occurrence or they may be evidence of epigenetic intergenerational effects. These results are consistent with our previous finding that G2 lambs descended from M granddams were heavier at birth and grew faster to weaning than those from A granddams. We conclude that nutrition of the pregnant dam can have intergenerational effects on lamb growth, but that growth of the great-grand-lambs (G3) was programmed independently of lactational characteristics of their (G2) dams.

Key Words: Fetal programming, intergenerational effects, sheep, lactation, lamb growth

3502

Investigation of the presence of adhesin genes cna, fnbA and fnbB among Staphylococcus aureus isolated from clinical and subclinical sheep mastitis in Iran

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Different virulence factors are involved in *Staphylococcus aureus* pathogenesis. Surface proteins such as collagen- (Cna) and fibronectin-binding proteins (FnBPs) are important factors in adhesion and invasion of *S. aureus*. Therefore, the aim of this study was to investigate the presence of cna, fnbA and fnbB genes in a collection of *S. aureus* isolates recovered from clinical and subclinical sheep mastitis. In this study 45 isolates were identified as *S. aureus* by standard biochemical as well as amplification of species-specific thermonuclease gene (nuc). Then, isolates were analyzed for the presence of cna, fnbA and fbnB adhesion genes using specific primers by polymerase chain reaction (PCR). A 279-bp DNA fragment was synthesized from all of the *S. aureus* isolates following the amplification of species-specific gene (nuc). Interestingly, 43 (95.55%) isolates were found to be cna positive. From the 45 studied isolates, 39 and 35 were harbored the fnbA and fbnB genes, respectively. According to the large diffusion of cna, fnbA and fbnB genes among the studied isolates, it can be conclude that CBP, FnBPA and FnBPB encoded by the mentioned genes were presumably effective in pathogenesis of mastitis

caused by *S. aureus* and isolates likely express receptors for matrix proteins such as collagen and fibronectin. This encourages the development of new strategies to prevent mastitis, based on antagonist ligands able to interact with surface adhesions and block it specific binding with matrix proteins.

Key Words: *Staphylococcus aureus*, adhesin genes, mastitis, sheep

3503

Seasonal and ecological variations in the serum steroid hormone concentrations of one-humped male camel in Pakistan

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One-humped camel is a seasonal breeder, showing sexual activity during winter and early spring season in Pakistan. This study was conducted during four seasons of the year to establish the baseline reference values during the year and to evaluate the ecological and seasonal changes in the serum steroid hormones. A total of 24 adult sexually mature male one-humped camels from three districts of Punjab, Pakistan, namely Faisalabad (FSD, n = 12), Bhakkar (BKKR, n = 6) and Attock (ATTK, n = 6), were used. The blood samples were collected at regular monthly intervals. After centrifugation at 15 000 *g* for 10 min, serum samples were analyzed for the hormones by Radioimmunoassay (RIA) using commercially available kits. Means were compared by two-way ANOVA, Significance was calculated by Duncan's multiple range (DMR) test and Correlation was estimated by Pearson Correlation. The correlation between calculated parameters and climatic data was evaluated by linear regression correlation by using STATISICA 6.0 for windows. Mean (±SEM) serum testosterone concentration (ng/ml), was higher ($p < 0.01$) during the winter season at all ecological zones (FSD; 8.29 ± 0.54, ATTK 13.49 ± 1.37: & BKKR: 15.51 ± 1.15) and started to decrease during spring (FSD; 2.10 ± 0.28, ATTK: 6.19 ± 0.65 & BKKR: 6.58 ± 0.74) and reached baseline during summer (FSD; 0.66 ± 0.15, ATTK: 1.58 ± 0.30 & BKKR: 1.48 ± 0.29), maintained almost same during autumn at Faisalabad zone (0.66 ± 0.05 ng/ml); however increased again in autumn at Attock (8.03 ± 1.57 ng/ml) and Bhakkar (4.14 ± 1.03 ng/ml). Serum estradiol concentration (pg/ml) was higher ($p < 0.01$) during the cooler months including January (FSD:183.89 ± 15.24, ATTK: 165.17 ± 14.04&BKKR:133.00 ± 26.36), February (FSD:184.62 ± 16.33, ATTK:154.17 ± 19.41 & BKKR: 132.12 ± 41.21), it started to decline in the month of March (FSD;136.42 ± 8.35, ATTK:150.50 ± 24.80&BKKR:141.58 ± 28.01), AprilFSD; 116.83 ± 6.74,ATTK:108.63 ± 17.86&BKKR:112.67 ± 6.80 and May (FSD;114.92 ± 4.59,ATTK:95.67 ± 12.81 & BKKR: 86.50 ± 5.60), a slight increase was observed in the month of June but again decreased in July and remained on baseline in the months of August (FSD;78.33 ± 9.42,ATTK:74.00 ± 7.40 & BKKR:93.33 ± 2.79), September (FSD;54.50 ± 5.47, ATTK:66.67 ± 9.99 & BKKR: 66.00 ± 5.60), October (FSD;61.83 ± 5.53, ATTK:77.33 ± 6.74 & BKKR:56.05 ± 14.72) and November (FSD; 60.50 ± 8.75, ATTK: 63.24 ± 13.21 & BKKR:96.47 ± 15.76). This study revealed positive correlation between serum testosterone (T) and estradiol17- β (E) ($r = 0.454$). These hormones were negatively correlated with the average environmental temperature (T; FSD: R2 = 0.6883, ATTK: R2 = 0.946 & BKKR: R2 = 0.7487, E; FSD: R2 = 0.2565, ATTK: R2 = 0.3238 & BKKR: R2 = 0.0782) and rainfall (T; FSD: R2 = 0.0972, ATTK: R2 = 0.336 & BKKR: R2 = 0.1742, E; FSD: R2 = 0.0328, ATTK: R2 = 0.0178 & BKKR: R2 = 0.0033) however testosterone was positively correlated with the relative humidity (FSD: R2 = 0.0524, ATTK: R2 = 0.0165 & BKKR: R2 = 0.0457) and vice versa for estradiol (FSD: R2 = 0.0328, ATTK: R2 = 0.0178 & BKKR: R2 = 0.0033). In conclusion, this study provided baseline reference values during the year and to evaluate the ecological and seasonal changes in the serum steroid hormones for one humped camel.

Key Words: Steroid hormones, serum, seasonal breeder, camel

3504

Sperm factors influencing sow insemination outcomes

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Pregnancy loss, in which male factors are increasingly implicated, is an economic constraint to the pork industry. To further understand some of the factors involved, mating records (8309 sow artificial inseminations (A.I.) over 2 years from a commercial Australian unit were analysed. Of these, 1205 inseminations were conducted with extended, chilled semen concurrently assessed for a number of traits including arrival temperature, pH; CASA parameters - sperm motility, velocity, beat cross frequency, concentration; % live/dead (eosin-nigrosin), morphology (DIC @1000×), DNA integrity (DiffQuik) and relative bacterial load. Sow insemination outcomes (excluding abortion) included total piglets born (PBT), born alive (PBA) and stillbirths (PSB) and days to return to estrus (DRE). Statistical analyses utilised GLM procedures. Overall, average farrowing rate was 74% and all inseminated sows except the 1% which aborted were included in the analyses. Percent normal sperm was positively linked with PBT and PBA (both $p < 0.05$). In turn, PBT was negatively associated with % retained distal cytoplasmic droplets ($p < 0.01$) and % abnormal sperm heads ($p < 0.05$), whereas PBS was negatively linked with DNA integrity ($p < 0.01$). DRE was negatively associated with % abnormal sperm heads and % abnormal acrosomes (both $p < 0.01$). In conclusion, certain sperm traits (i.e. percent normal morphology, retained distal droplets and DNA integrity) were significantly linked with litter size, days to return to estrus and neonate viability. This indicates that considerable progress in pig reproduction can occur via increased attention to boar A.I. semen QC, particularly in relation to sperm morphology. The challenge is to improve boar semen quality control and quality assurance in a cost effective manner while retaining production efficiencies. References: Flowers, W. L. 2008. Genetic and phenotypic variation in reproductive traits of A.I. boars. Theriogenology 70:1297-1303. Safranski, T. J. 2008. Genetic selection of boars. Theriogenology 70:1310-1316. This work was funded by the Pork CRC Australia

Key Words: Sperm morphology, sperm traits, semen, return to estrus, insemination outcomes

3505

Administration of 4-vinyl-1-cyclohexene 1,2-epoxide diminishes ovarian follicles in dogs

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4-Vinylcyclohexene, an industrial chemical, is metabolised to epoxide derivatives in the body. These metabolites cause atrophy of the ovaries and reduce the number of pre-antral follicles. The aim of this study was to determine the effect of a single dose of 4-Vinyl-1-cyclohexene 1,2-epoxide (VCE) on the ovarian follicles. In present study 18 mongrel bitches (age 6–15 month and bw 9–15 kg) were randomly allocated into three groups. The VCE was injected intraperitoneally (160 mg/kg) to group 1 (n = 6) and group 2 (n = 6), and group 3 (n = 6) was left as untreated control group. Ovaries were collected by ovario-hysterectomy from group 1 and group 2 on fifth and eighth days after the treatment, respectively. The ovaries were evaluated histologically for

the determination of number of primordial and primary follicles. Briefly, ovaries were fixed with 10% buffered formalin, processed with ethanol and xylene, embedded in paraffin and sectioned (7 μm). Every fifth section was mounted on a glass slide in order to avoid double counting of the same follicle. The sections were stained with hematoxylin-eosin and evaluated. Comparisons between groups were performed with one-way ANOVA and Duncan test. Compared with the controls, numbers of primordial follicles in group 1 and group 2 decreased about 23% and 34% respectively (Control: 3417 ± 18.9; group 1: 2633 ± 33.1; group 2: 2261 ± 24.2; $p < 0.05$). The treatment groups showed a similar decline in the number of primary follicles (Control: 753 ± 9.5; group 1: 468 ± 13.7; group 2: 354 ± 36.4; $p < 0.05$). This study showed that VCE treatment decreases the number of ovarian follicle in the bitch. In conclusion, VCE may be used for chemical sterilization in female dogs.

Key Words: Vinyl cyclohexene epoxide, chemical sterilization, dog, ovary, follicle

3506

Therapeutic ultrasound as a potential male dog contraceptive: comparison of application protocols

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An ideal contraceptive for male dogs should be 100% efficient, irreversible, inexpensive and with no side effects. Ultrasound was used as a male contraceptive in several species, including dogs. Studies are needed to determine minimal number of treatments, interval between treatments, part of testes that requires treatment, frequency or power of ultrasonic wave exposure and many more need to be investigated before its application in practice. A previous study concluded that three treatments of 5 min/treatment per day (2.5 cm^2 transducer, 1 MHz, 1.5 W/cm^2) are ineffective. In this trial, effects of two testicular ultrasound exposure protocols (differing in ultrasound treatment length and number of applications) on testicular size, consistency and volume, and sperm concentration and motility were evaluated. Twenty dogs were divided in two equal groups- A and B. All subjects were exposed to 1.5 Wcm^2 of ultrasound on each testicle using Vetrison Portable ultrasound (Physiomed® Elektromedizin AG, Germany; 2.5 cm^2 transducer). Dogs in group-A received ultrasound for 5 min on alternate days for one week and dogs in group-B received ultrasound for 15 min inutes twice a day on alternate days. Sperm concentrations and motility evaluations were made before and 25 days after the end of treatments. Length and testicular width were echographically measured to calculate the volume. Semen collected was examined by using an integrated visual optical system for semen analysis for sperm concentration and for percentage of total and progressively motile sperms. All dogs were castrated at day 40 and gonads were collected for histological examination. Data concerning testicular volume were statistically analyzed with 'Wilcoxon matched pairs signed rank sum' test ($p \leq 0.05$); semen evaluation was statistically analyzed with ANOVA test ($p \leq 0.01$). After ultrasound treatment, all dogs showed no local or systemic adverse effects, and no pain or skin burns. However, dogs in group A exhibited marked tenderness of testicles at palpation. Group A dogs showed a statistically significant reduction of the volume of both testis (left 9.6 ± 3.7 vs. 5.5 ± 3.6/cm^3; right 9.6 ± 2.7 vs. 3.6 ± 1.4/cm^3; $p \leq 0.05$) while no reduction in testicular volume was noticed in dogs in group (left: 9.1 ± 1.4 vs. 9.2 ± 1.6/cm^3; right: 9.3 ± 1.5 vs. 9.4 ± 1.7/cm^3). Before the US treatment, mean volume of ejaculates was 10 ± 3.5 ml, sperm concentration was 300.8 ± 24.8 × 106/ml with an average percentage of total and progressive motile sperms of 88.2 ± 4.5 and 59.3 ± 5.3, respectively. After the ultrasound treatment, a zero sperm count was noticed in group A dogs ($p \leq 0.01$), and no variation in B group. Histology evaluation showed interstitial fibrosis, widespread tubular atrophy and hyalinization of the basement membranes in group-A dogs and no changes were observed in group-B dogs. Our results demonstrated that ultrasound treatment for 5 min on alternate days for one week leads to irreversible testis damage consistent with permanent sterilization, while reducing the number of applications-even with a longer treatment was ineffective on dog fertility

Key Words: Ultrasound, male dog, contraception

3507

Verification of a bioassay for measuring luteinising hormone (LH) in the Asian elephant (*Elephas maximus*)

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Current captive populations of Asian elephants are required to be self-sustaining if animals are not to be taken from the wild, thus captive breeding programmes often use artificial insemination and an accurate timing of ovulation is therefore required for the timing of the insemination. Elephants are unique amongst mammals in that their oestrous cycles exhibit a double luteinising hormone (LH) surge, the first an anovulatory (anLH) and the second an ovulatory (ovLH) approximately 3 weeks later, with only the second surge culminating in ovulation of a mature ovum. Current immunoassay technologies, developed for measuring LH in domestic species do not work well with exotic or native wildlife. Owing to the different tertiary structure of LH, the conventional assay for measuring LH in elephants uses an antibody that has significant cross-reaction with a range of moieties. However, suitable antibodies are not common, only available by application to a research lab, of limited quantity, and present a significant challenge to those wishing to set up the assay. The present study has investigated the potential value of a mouse Leydig tumour cell line (mLTC-1) as a generic bioassay for measuring LH in Asian elephants: the cell line produces progesterone in response to LH which can be measured using commonly available immunoassays. Cells were exposed to plasma samples taken from a single female Asian elephant (twice daily collection) around the time of the surges during two consecutive oestrous cycles. LH concentration profiles were analysed based on an algorithm using a non-linear statistical model where a surge was identified if its peak value exceeded the previous nadir by three standard deviations. The two anovulatory surges had baseline values of approximately 1000 pg/ml, with the first detectable rise being 7493 pg/ml and 5573 pg/ml respectively, whilst the ovulatory surges had the first detectable change to 9211 pg/ml and 6278 pg/ml from baselines of 617 pg/ml and 1045 pg/ml respectively. The bioassay has a good cross-reaction with LH from a wide range of exotic and native mammals, and utilises a commercially-available cell line. These are all good features for accessibility by laboratories. Shortened cell culture time and the short turnaround times of automated analysers give scope for the development of a more rapid and hopefully more meaningful result. These results were comparable to those obtained by a standard enzyme immunoassay (EIA), which confirms the bioassay as a potentially useful tool in reproductive management of this endangered species.

Key Words: Luteinising hormone, bioassay, asian elephant, cell line

3508

Semen characteristics and libido of rabbit bucks fed diets containing Garcinia kola seed meal

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In Nigeria, feeding of plant materials with known aphrodisiac properties has been used to improve reproductive performance. *Garcinia kola* has been reported to increase spermatogenic activity through its ability to increase peripheral testosterone. The purpose of the study was to determine the effect of feeding diets containing *Garcinia kola* seed meal on semen characteristics and sex drive of rabbit bucks. Three dietary treatments containing Garcinia kola seed meal (GKSM) at 0% (T_1), 2.5% (T_2) and 5.0% (T_3) were fed to three groups of six rabbit bucks aged 3 to 4 months. Nuts of Garcinia kola were bought from a local market and processed by removing the outer covering and chopping them into pieces. They were subsequently air-dried and ground before incorporation into the diets. The diets were formulated to be isonitrogenous and isocaloric containing 17% crude protein and 11.30 MJ/kg (2700 Kcal/kg) energy. The animals were fed

ad libitum. Sex drive was defined as reaction time (s) from introduction of the female into the buck's hutch and semen collection. Sex drive was measured fortnightly after the bucks attained puberty. After sperm collection, sperm concentration, sperm motility, and percentage of live and abnormal sperm were determined. Data were analyzed using the analysis of variance method and difference in means were tested using the Duncan's new multiple range test. The results showed that sperm concentration and total sperm differed significantly ($p < 0.05$) between the treatments. Sperm concentration was significantly higher ($p < 0.05$) in T_3 ($199.14 \pm 20.63 \times 10^6$/ml) than T_2 ($163.31 \pm 18.71 \times 10^6$/ml) and T_1 ($141.87 \pm 19.11 \times 10^6$/ml). Total sperm values obtained were $104.67 \pm 2.73 \times 10^6$ for T_1, $111.33 \pm 1.86 \times 10^6$ for T_2 and $115.67 \pm 2.33 \times 10^6$ for T_3. Sperm motility and live sperm proportion were however higher ($p < 0.05$) in T_2 than the other two groups. Sperm motility were $64.33 \pm 1.86\%$ for T_1, $76.33 \pm 0.88\%$ for T_2 and $73.33 \pm 1.55\%$ for T_3. Percentage of live sperm was $86.67 \pm 1.45\%$ for T_1, $93.33 \pm 1.67\%$ for T_2 and $81.67 \pm 3.33\%$ for T_3. Percentage of abnormal sperm did not differ ($p > 0.05$) between the treatment groups ($T_1 = 23.67 \pm 3.33\%$, $T_2 = 17.67 \pm 2.23\%$ and $T_3 = 18.67 \pm 3.01$), indicating that the feeding of the plant material did not have any deleterious effect on the spermatozoa morphology. A dose-dependent decrease was observed in sex drive. Reaction time was 27.57 ± 1.90 s for T_1, 20.75 ± 1.38 s for T_2 and 11.0 ± 0.38 s for T_3. This confirms that G. kola indeed has aphrodisiac properties. It is possible that the contents of G. kola may have excited the interstitial cells of Leydig causing them to release more testosterone which led to the improvement in male reproductive behavior. The results indicate that GKSM improves semen characteristics as well as sex drive (libido) in rabbit bucks. In conclusion, GKSM could be administered to low-performing male rabbits to enhance their reproductive performance.

Key Words: Rabbits, semen, libido, *Garcinia kola*

3509

Extensive review of assisted reproductive technologies for amphibian conservation

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The difficulties associated with breeding amphibians in captivity poses an important challenge which conservation institutions are attempting to address. Researchers focusing on the conservation of endangered amphibian species believe that by combining appropriate and successful assisted breeding techniques with captive management programs for threatened amphibians it would be possible to reduce or eliminate some of the major problems existing in a captive environment. Use of exogenous hormones for gamete acquisition, gamete storage techniques such as cryopreservation, artificial fertilization, and genetic banking are confirmed techniques in developing assisted breeding programs for amphibians (Kouba and Vance and Willis, 2009). However, each technique has its own difficulties and challenges. Moreover, due to a wide range of reproductive modes among different amphibian species, species-specific modifications are required for each assisted breeding technique or protocol (Kouba and Vance, 2009). Consequently, it is essential to develop further improvements and advances in reproductive technologies in order to achieve successful assisted breeding programs.

The purpose of this literature review is to investigate the amphibian extinction and captive breeding crisis; review technological achievements in amphibian assisted reproductive technologies and artificial fertilization and identify specific processes that need to be considered when developing assisted reproductive technologies and artificial fertilization techniques for amphibian species conservation. Finally, future concerns that require immediate prioritization will be addressed.

Key Words: Amphibian, assisted reproductive technologies, cryopreservation

3550

Relationship between single nucleotide polymorphisms of growth hormone receptor and reproductive performance in Holstein dairy cows

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Insulin-like growth factor-1 is one of the important factors for ovarian function, and mainly released from the liver in response to growth hormone (GH) via GH receptor (GHR) in cows. Recently, some SNPs were identified in the bovine GHR gene. The association of some GHR-SNPs with species or milk yield was examined; however the relationship between GHR-SNPs and reproductive performance remains unknown. The aim of the present study was to investigate the relationship between 4 GHR-SNPs and fertility in dairy cows. Primiparous Holstein dairy cows (n = 108), which were milked over 250 days, were used for analysis and their blood samples for genomic DNA extraction were collected as the occasion demands. To examine genotype, PCR products were digested with four specific restriction enzymes. SNP types for GHR were as follows; Fnu4HI (CC, CT and TT), NsiI (AA and AG), AccI (CC, CT and TT) and AluI (AA, AT and TT). The relationships between these SNPs type and reproductive performance (days to the first artificial insemination (AI), conception rate at the first AI, days open and the number of AI until conception) in primiparous cows were analyzed by ANOVA or Studentâ€™s *t*-test. Seventeen cows were not received AI because of mastitis. We could not analyze 22 samples for Fnu4HI, 38 for AccI and 19 for AluI and removed these samples from further analysis. In Fnu4HI-SNP, conception rate at the first AI in cows with CC (43.6%, n = 31) genotype was lower than cows with CT (60.0%, n = 30) or TT (75.0%, n = 8) genotype ($p < 0.05$), and days open in cows with AG genotype (126.4d, n = 23) of NsiI-SNP tended to be longer than cows with AA genotype (104.2d, n = 68, $p < 0.1$). However, there is no significant difference in AccI- and AluI-SNPs. Based on these results, additional analyses were performed between high fertility cows (H group) that have CT or TT genotype of Fnu4HI-SNP and AA genotype of NsiI-SNP (n = 38) and low fertility cows (L group) that have CC genotype of Fnu4HI-SNP and AG genotype of NsiI-SNP (n = 17). In H group, conception rate at the first AI was higher (63.2% vs. 29.4%, $p < 0.05$), days open was shorter (98.9d vs. 126.3 day, $p < 0.05$) and the number of AI by conception was fewer than L group (1.6 vs. 2.4, $p < 0.05$). Daily milk yield and 305 day - milk yield did not differ between two groups. From birth and 1st AI at heifer, monthly body weight and body height were similar between two groups; however, age at the first parturition tended to be younger in H group than L group (24.9 month vs. 26.7 month, $p < 0.05$). Degree of difficulty in parturition in H group (1.6) was lower than L group (2.4), whereas the incidence of peripartum disease was the same level between two groups. In conclusion, the present study indicates that SNPs of GHR might be associated with reproductive function but not growth and milk production in cattle.

Key Words: Growth hormone receptor, Holstein dairy cow, reproductive performance, single nucleotide polymorphism

3551

High-throughput and multiplexed UPLC-MS/MS quantification of melatonin and its metabolites in bovine ovarian follicular fluid samples

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The introduction of new analytical technologies in the animal reproduction area can improve knowledge on gamete and embryonic metabolism. Novel analytical tools are also relevant to the research progress of embryo biotechnologies related to embryo *in vitro* production, which are fundamental not only for *in vitro* fertilization, but also for nuclear cloning and transgenic strategies success. The objective of this study was to develop a method based on ultra-efficiency liquid chromatography and tandem mass spectrometry (LC-MS/MS) with high sensitivity and selectivity to detect and quantify simultaneously different compounds participating in the biosynthesis of melatonin and its metabolites in bovine follicular fluid (FF). Small (< 6 mm) and large (> 6 mm) bovine ovarian follicles were measured using a digital paquimeter and aspirated separately to form two pools from bovine ovaries collected at a commercial abattoir. For analysis, protein precipitation and MTBE protocol using 300 Î¼l (FF) was utilized. For LC-MS/MS analysis, each sample was diluted in 1.0 ml water and injected using a UPLC system (ACQUITY UPLC BINARY SYSTEM, Waters Technologies Inc.) coupled to a triple quadrupole mass spectrometer (Xevo TQ-S, Waters Technologies Inc.) with ESI (Electrospray) ion source (positive mode) using a chromatography column ACQUITY UPLC BEH C18 (2.1 × 5.0 mm) (Waters Technologies Inc.). The multiple reactions monitoring (MRM) method was optimized from a acetonitrile solution containing 200 ng/ml standards and infused at 10 Î¼L per min into the mass spectrometer. Two (02) MRM transition were used for each analyte. Calibration curves of melatonin (MEL), and the metabolites 6-hidroxymelatonin (6-HMEL), N1 -acetyl-N2 -formyl-5-metoxykinuramine (AFMK) and deuterated melatonin (MEL-D4) as an internal standard (IS) were obtained using eight serial dilutions (from 10 to 1000 pg/ml). The method showed linearity with a regression coefficient > 0.99 for the tree analytes. The detection limits were 3 pg/ml (MEL) and 5 pg/ml to 6-HMEL and AFMK. The samples of follicular fluid to MEL did not show a tendency on the best method of extraction, but 6-HMEL and AFMK showed better extraction using protein precipitation with acetone. To our knowledge, this is the first time that a high-throughput and multiplexed UPLC-MS/MS quantification of melatonin and its metabolites in bovine ovarian follicular fluid samples is reported. We are currently applying this method to the study of a significant set of bovine ovarian FF samples in order to quantify these analytes amounts and better understand the role of melatonin in oocyte maturation as well as its use in physiological amounts for greater success of *in vitro* production of bovine embryos.

Key Words: Melatonin, ovarian follicular fluid, bonive, metabolites, mass spectrometry

3552

The use of equine follicle stimulating hormone to increase equine chorionic gonadotropin in the pregnant mare

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Equine chorionic gonadotropin (eCG), obtained from pregnant mares, is used for assisted reproductive technologies in laboratory rodents and livestock. Many seasonal, paternal, fetal, and maternal factors influence eCG concentration in the pregnant mare. The objective of the current study was to use equine follicle stimulating hormone (eFSH) to superovulate mares to increase the incidence of twins with the intention of increasing eCG in the pregnant mare. Nineteen light horse type mares were enrolled in the study and divided into two groups. The first group, the control group (n = 9), did not receive eFSH. They were routinely checked, bred with fresh or cooled semen from the same stallion, and were given human chorionic gonadotropin (hCG) at time of breeding. The second group (n = 10) was given 12.5 mg of eFSH intramuscularly twice a day 5 to 7 days after ovulation was detected the previous cycle. Prostaglandins were administered intramuscularly the second day of eFSH treatment. Treatment with eFSH continued until follicles were > 35 mm in diameter, the mares were then given no treatment for 36 h. After 36 h, the mares were bred with fresh or cooled semen from the same stallion as the control group and given hCG. Blood samples were taken every seven days from day 35 to day 100 post-ovulation. Serum concentration of eCG was obtained using an assay as described by Thompson, et al. 1982 and was analyzed using the multivariate analysis using the mixed procedure as well as area under the curve (AUC). Significance was set at $p < 0.05$. The mares were divided into four groups: mares carrying twins to 100 days (n = 2), mares that lost one or both twins > 35 days (n = 7), mares that lost one twin < 35 days (n = 1), and mares carrying singletons (n = 9). The average peak values for each group were 66.29, 128.08, 80.76IU/ml, and 47.04 IU/ml, respectively. Due to the small number of mares, data were combined for the statistical analysis. Mares carrying twins to 100 days, past 35 days, and just before 35 days were combined (n = 10) and their concentration of eCG was compared to that of mares carrying singletons (n = 9). The group of mares carrying twins had higher peak concentrations of eCG and AUC compared to mares carrying singletons ($p < 0.05$). Inducing twins with eFSH could be a method used to increase eCG production.

Key Words: Equine follicle stimulating hormone (eFSH), equine chorionic gonadotropin (eCG), twins, mare