PRELIMINARY RESULTS OBTAINED BY RIA DETERMINATION OF THE PROTEINS ASSOCIATED TO PREGNANCY (PAG) IN GOAT AND SHEEP

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Summary

In goat and sheep, the glycoproteins associated to pregnancy (PAG) are little studied. Their study in these species is very important in the evaluation of prediction of early pregnancy. Even though numerous research has been done in order to investigate the physiological functions of placental proteins, the exact biofunction of the glycoproteins associated to pregnancy is still unknown. The objective of research was the study of the dynamics of PAG in goats and ewes in the first part of pregnancy(1-35 days after mating) and the determination of correlations with the reproductive status of females and to earlier the pregnancy.The experiment was realized on 49 Saanen x Carpatina goats and 72 Merinos of Palas sheep from the biostation of the Research and Development Institute for Goat and Sheep, Palas Constanta. The females in normal reproduction season were monitored for the detection of estrus and mated naturally. The blood was collected by puncture of the jugular vein on days 0, 7, 14, 25 and 35 after mating (day 0). The serum was obtained after centrifuging at 1500 x g for 15 minutes and stored at -20^{o}C till the RIA determination. The RIA of the plasma with the purpose of detecting the concentration of proteins specific to pregnancy was realized in two experimental series in February and December 2008, at the Laboratory for Reproduction Physiology – The Faculty of Veterinary Medicine, Liege, Belgium. The RIA dosing was realized after the application of the method with the preincubation of the serums to test with specific serum (Atg$^{\circ}$) and then with the marked antigen (Atg*), considering the high sensitivity of this method to detect the smallest values of PAG from day 0 to day 35 after mating. The diagnosis of the pregnancy state was based on the principle of PAG antibodies binding to the specific antigen, establishing through RIA the quantity of free antigen. Non-pregnancy involves the attachment of the antigen marked by non-specific antibodies. The preliminary results have demonstrated that the sheep PAG were had values ranging between 4.197-15.985 ng/ml and 0.01-3.39 ng/ml in nonpregnant ewes. In the pregnant goats, the PAG concentrations ranged between 16.75±3.44-27.17±2.95 ng/ml, while in the nonpregnant goats, the PAG values were 1.38±0.35 – 2.03±0.51 6 ng/ml. The purpose of the experiments was to find one accurate method for early pregnancy diagnosis in goat and sheep. The conclusion was that ovine pregnancy can be reliably diagnosed on Day 25 after AI and goat pregnancy on Day 30, by using a heterologous radioimmunoassay of PAG.

Keywords: pregnancy diagnosis; PAG; ewe; goat.

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Introduction

The researches effected internationally regarding the study of glycoproteins associated to pregnancy (PAG) in cow and sow, have demonstrated that the presence of these glycoproteins in the circulatory system of females is evidence for pregnancy or non-pregnancy in the first stages of embryo development. Pregnancy-associated glycoproteins (PAG)

and pregnancy-specific protein B (PSPB) belong to the aspartic proteinase family, and are secreted by the trophoblastic binucleate cells (Beckers *et al* 1994; Buttler, 1982; Chart, 1989). They are detectable in the maternal blood around the time of definitive attachment of the fetal placenta when the trophoblastic binucleate cells start to migrate and fuse to the endometrial cells forming the fetomaternal syncytium (Xie *et al*, 1991, Wooding, 1984; Zoli, 1991). Therefore, these glycoproteins are good indicators of both pregnancy and feto-placental well being. In sheep, the glycoproteins associated to pregnancy (PAG) are little studied while in goat they have not been studied at all. The study of glycoproteins associated to pregnancy in sheep and goat is necessary because of their importance in the evaluation of fertilization and early pregnancy. Even though research has been done to investigate the physiological roles of placental proteins, the exact biofunction of glycoproteins associated to pregnancy is still unknown. In sheep, the identification of immunological antigens in the peripheral circulation in pregnant females led to the partial purification of certain proteins associated to pregnancy, which are found in large quantities in days 9-10 from fertilization and remain constant till parturition (Mialon, 1993; Zoli and Beckers, 1995; Willard *et al*, 1995). In goat, they are partially characterized from the biochemical point of view but they reach a maximum level in the eighth week of pregnancy (Currie, 1990; Gonzales, 1999). Martal (1985) characterized a protein called trophoblastin, present in pregnant sheep from day 5 to day 30 of pregnancy. The presence of certain specific proteins in the first 10 days after the fertilization of sheep ovules allowed Cerini and Findley (1976) to prepare a polyclonal anti-embryo serum used for the quick identification of the presence or absence of these proteins through the agglutination test. The emphasis of glycoproteins in the circulatory system of mated sheep and goats, of these proteins associated to pregnancy through techniques of molecular biology and immunological techniques, provides data regarding the sure presence in the uterus of the embryo or fetus in the first stages of development.

Material and methods

1. Blood Sampling

The blood samples from goats (n=35), were collected from goats in July 2008, in induced estrus on Days 0 (day of the insemination), 7, 14, 21, 25 and 30 after natural mating. The blood samples from ewes (n=61) were collected in natural estrus, on Days 0 (day of the insemination), 7, 14, 25 after natural mating. The plasma was preserved at -18°C till analyzed for PAG by Radioimmunoassay method (RIA).

2. PAG Radioimmunoassays

PAG concentrations were determined by RIA (without preincubation of the antiserum) in the Phisiolgy of Reproduction Laboratory from The Veterinary Faculty Liege Belgium, according to the method of Beckers and Zolly (Sanyal, 1989; Zoli, 1991; 1995). PAG concentrations were determined with a heterologous, double-antibody radioimmunoassay (RIA) using the bovine PAG 67 kDa subunit as tracer and standard, and rabbit antiserum raised against a mixture of caPAG 55 and 59 kDa subunits (R708) as the first antibody. The purified boPAG 67 KDa subunit was radiolabelled by chloramine T using ^{125}I (Greenwood *et al*, 1963). The antiserum used in this assay has been proved to be specific for PAG molecules against other members of the aspartic proteinase family (pepsinogen, pepsin, chymosin, rennet, cathepsin D and renin) . The inhibition of binding of the tracer to the antiserum was observed with the sera of the pregnant goats and ewes, while it was not observed with the sera of nonpregnant females. The procedures of the assay were similar to those of Perényi et al. (Perenyi *et al*, 2002)

who used the same assay for early pregnancy diagnosis in cows. The radioactivity of the sediment was counted by using a gamma counter (PerkinElmer 2470 automatic Multigamma counter, USA) with a counting efficiency of 75 %. The standard curve ranged from 0.2 to 58 ng/mL.

The discriminatory value for diagnosis of pregnancy by the PAG-RIA tests was ≥ 1 ng/ mL. Based on nonreturn data, the accuracy for diagnosing pregnant (sensitivity) and non-pregnant goat and ewes (specificity) and predictability of both tests were calculated.

The ovarian activity on a group of 15 goats was watched by laparotomy as they were subject to a MOET treatment (Multiple Ovulation and Embryo Transfer), which permitted the diagnosis of ovarian cysts which determined the continuous repetition of estrus in goats. The return to estrus of sheep and goats was watched in 3 estrus cycles.

3. Analysis of data

The pregnancy state was appreciated based on the non-returns to estrus for a period of 3 estrus cycles, and 3 months later based on the clinical analysis of pregnancy. The PAG values were statistically analyzed using a Student's *t*-test (Armitage *et al*, 1998).

Results and Discussion

Table 1 shows the PAG values in pregnant and non-pregnant goats. These were evaluated on a number of 43 plasma samples from certainly pregnant goats and 65 samples from uncertainly pregnant goats.

Table 1. PAG concentration in pregnant and nonpregnant goats (2008)

	Days		Statistics				
			N	x±sx	Min-Max	Median	CV%
Pregnant goats	Day 0	B/Bo	6	87.4±8.72	50.4 - 106.07	94.72	24%
		PAG ng/ml	**6**	**1.1±0.53**	**0.099 - 3.509**	**0.58**	**119%**
	Day 7	B/Bo	9	65.95±7.09	41.28 - 106.07	60.65	32%
		PAG ng/ml	**9**	**2.95±0.42**	**1.372 - 5.155**	**2.61**	**43%**
	Day 14	B/Bo	7	52.29±2.12	44.95 - 58.56	51.64	11%
		PAG ng/ml	**7**	**4.39±0.87**	**2.771 - 9.45**	**3.548**	**53%**
	Day 21	B/Bo	10	36.12±5.64	18.27 - 62.23	30.665	49%
		PAG ng/ml	**10**	**8.85±2.42**	**2.467 - 25.287**	**6.6215**	**87%**
	Day 25	B/Bo	7	14.36±4.92	1.92 - 36.43	6.9	91%
		PAG ng/ml	**7**	**16.75±3.44**	**5.16 - 29.833**	**20.088**	**54%**
	Day 30	B/Bo	4	2.81±0.84	1.06 - 4.85	2.655	60%
		PAG ng/ml	**4**	**27.17±2.95**	**20.985 - 34.792**	**26.4605**	**22%**
Nonpregnant goats	Day 0	B/Bo	17	85.32±3.54	64.25 - 107.28	86.11	17%
		PAG ng/ml	**17**	**1.38±0.35**	**0.066 - 6.2**	**0.907**	**105%**
	Day 7	B/Bo	18	75.8±4.13	40.91 - 107.03	75.445	23%
		PAG ng/ml	**18**	**1.68±0.31**	**0.15 - 5.168**	**1.463**	**77%**
	Day 14	B/Bo	12	74.53±6.01	45.82 - 108.07	71.29	28%
		PAG ng/ml	**12**	**2.03±0.51**	**0.054 - 6.036**	**1.6905**	**87%**
	Day 21	B/Bo	6	76.92±6.17	61.45 - 96.39	75.22	20%
		PAG ng/ml	**6**	**1.47±0.39**	**0.347 - 2.542**	**1.473**	**64%**
	Day 25	B/Bo	6	94.11±5.01	80.7 - 108.13	93.51	13%
		PAG ng/ml	**6**	**0.54±0.18**	**0.078 - 1.08**	**0.468**	**83%**
	Day 30	B/Bo	6	91.56±4.78	81.41 - 106.34	86.93	13%
		PAG ng/ml	**6**	**0.63±0.19**	**0.032 - 1.045**	**0.8**	**76%**

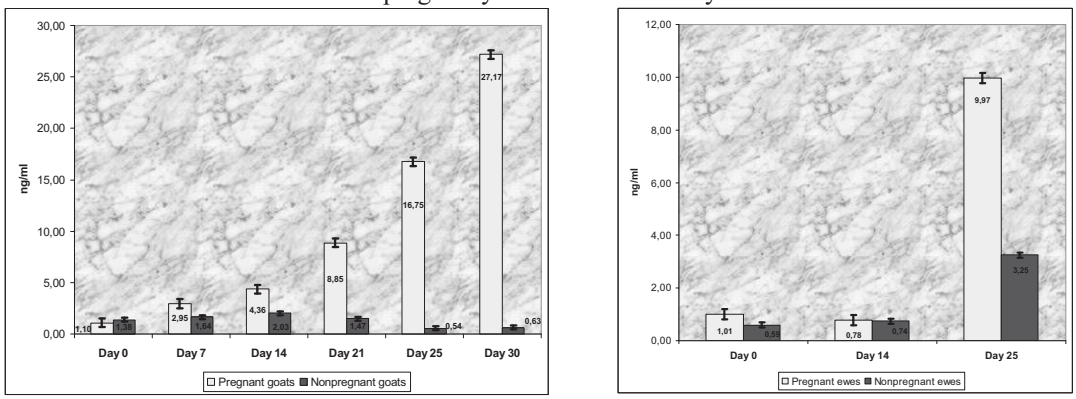
The goats were watched carefully for estrus return on the day of the sexual cycle when the collection was done. On day 0 (estrus), the PAG values were between 1.1 ±0.53 – 1.38±0.35 ng/ ml. The quantitative variability of PAG was very high (CV% - 119-105), which justifies the use of the average, which indicates a value of 0.58-0.90 ng/ ml on day 0 (estrus). As expected, the PAG values in the pregnant goats rose constantly till day 30 thus: 2.61 ng/ml on day 7, 3.54 ng/ml on day 14, 6.62 ng/ml on day 21. On days 25 and 30 the PAG values increased to 20.088 ng/ml and 26.46 ng/ml, respectively (table 1). In the non-pregnant goats, the PAG values are between 1.463-1.690 ng/ml, with very low values in pro-estrus and estrus and higher in metestrus and diestrus. In the non-pregnant goats, the PAG values were higher than 6.036 ng/ml. in 6 goats, the ovarian activity was watched directly by laparotomy, as these females were included in an experiment for the induction of multiple ovulations, being treated with eCG and Folligon.

Table 2 . PAG in goats with embryo mortality and hyperestrogenic syndrome

Status		Statistics				
		N	PAG,ng/mL,x±sx	Min - Max	Median	CV%
Embrionary Loss	Day 0	3	2.84±0.67	0.757 - 4.265	3.51	58%
	Day 7	3	0.46±0.29	0 - 1.372	0.00	155%
	Day 14	3	3.68±0.14	3.383 - 4.1	3.55	9%
	Day 21	3	12.12±2.34	8.211 - 19.521	8.62	47%
	Day 25	3	18.95±4.91	3.921 - 29.833	23.09	63%
	Day 30	3	4.04±1.29	0 - 6.604	5.52	78%
Hyperestro-geny	Day 0	6	0.30±0.21	0 - 1.264	0.00	172%
	Day 7	6	0.90±0.31	0 - 1.759	1.00	86%
	Day 14	6	0.19±0.19	0 - 1.118	0.00	245%
	Day 21	6	2.09±0.43	1.117 - 3.99	1.73	51%
	Day 25	6	1.28±0.32	0 - 2.362	1.44	62%
	Day 30	6	0.73±0.2	0 - 1.384	0.83	68%

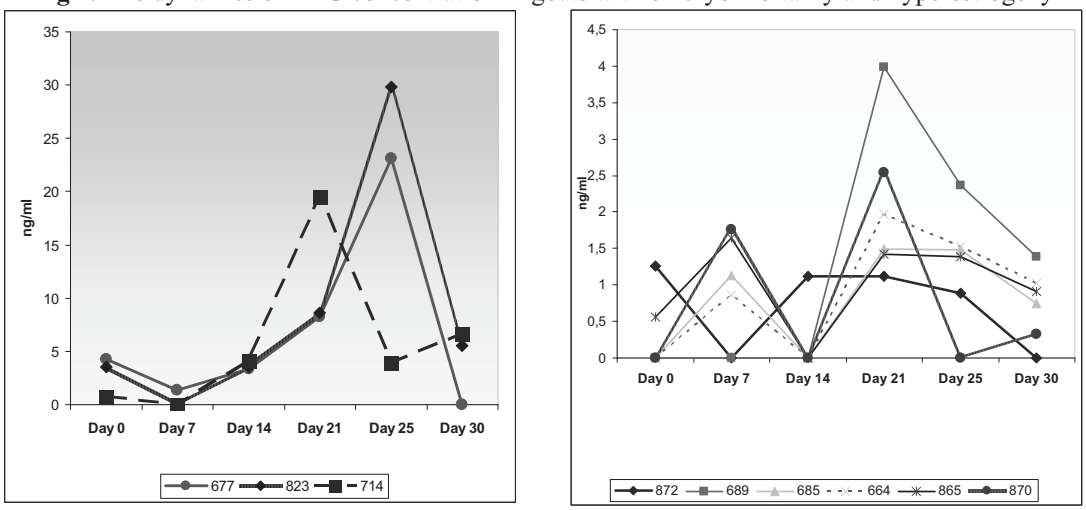
The laparotomy was done on day 7, after natural mating. A large number of non-ovulated follicles was found on the ovary, which later determined a hyperestrogenic syndrome, clinically manifested by repeated heat. In these females, the PAG values were non-detectable on days 0, 14 and 30, while in metestrus and diestrus the PAG values were 1.44-1.73 ng/ml (Table 2, Fig 2). The females which displayed embryo mortality, also showed considerable drops of the PAG values between days 25-30. In these goats (Fig. 2) the average PAG value was 23.09 on day 25, and 5,52 on day 30 (x±sx :18.95±4.91 and 4.04±1.29). The PAG values in goats obtained by us were according to those obtained by Folch J (Folch *et al*, 1993) Ranilla *et al* (1994), Sousa *et al,* (1999), Zamfirescu et al (2008), Mialon (1993) and Gonzalez *et al,* (1999). The corroboration of PAG values on day 30 after AI, with estrus return or not and the visible state of pregnancy at 4 months, may constitute a certain diagnosis test of precocious pregnancy, but also of embryo mortality.

Fig.1 . The dynamics of the PAG concentration in sheep and goats with pregnancy and non-pregnancy confirmed clinically.



a)GOATS b)EWES

Fig 2. The dynamics of PAG concentration in goats with embryo mortality and hyperestrogeny



a) Embryo mortality b) Repeated heat syndrome

Table 3 . PAG concentrations in pregnant and non-pregnant sheep

	Days		Statistics				
			N	x±sx	Min-Max	Median	CV%
Pregnant ewes	Day 0	B/Bo	18	87.91±3.12	59.56 - 109.49	90.22	15%
		PAG ng/ml	**18**	**1.01±0.23**	**0.01 - 3.353**	**0.708**	**98%**
	Day 14	B/Bo	15	91.80±3.36	67.92 - 115.42	87.78	14%
		PAG ng/ml	**15**	**0.78±0.2**	**0.01 - 2.604**	**0.696**	**101%**
	Day 25	B/Bo	15	21.09±2.93	8.32 - 51.11	17.16	54%
		PAG ng/ml	**15**	**9.97±0.74**	**4.197±15.985**	**10.495**	**29%**
Nonpregnant ewes	Day 0	B/Bo	61	97.66±2.24	59.49 - 143	96.63	18%
		PAG ng/ml	**61**	**0.59±0.1**	**0.01 - 3.36**	**0.01**	**134%**
	Day 14	B/Bo	30	100.07±4.28	44 - 141.3	109.71	23%
		PAG ng/ml	**30**	**0.74±0.24**	**0.01 - 5.01**	**0.01**	**176%**
	Day 25	B/Bo	8	62.29±7.75	40.71 - 99.3	58.195	35%
		PAG ng/ml	**8**	**3.25±0.73**	**0.01 - 5.432**	**3.497**	**64%**

Blood was collected from 79 sheep on days 0 (estrus), 14 (diestrus) and 25 (diestrus in pregnant animals and proestrus or estrus in non-pregnant animals), the PAG determination was done according to the same protocol used in goats. Of the total 79 sheep, 18 was pregnant for sure after the first mating. The dynamics of the PAG values in certainly pregnant sheep has rising values, from 1.01± 0.23 ng/ ml on day 0 (average: 0.708ng/ml), to 0.78± 0.20 ng/ ml on day 14 (average: 0.696 ng/ml) and to 9.97 ± 0.74 ng/ ml, on day 25 (average: 10.495 ng/ml). In non-pregnant sheep, the PAG values on days 0 (estrus), 14 and 25 were 0.59±0.1 ng/ml, 0.74± 0.24 ng/ml and 3.25± 0.73 ng/ml (table 3, fig 2b).

Conclusions

The quantitative evaluation of PAG by RIA may constitute a sure method for the diagnosis of early pregnancy in sheep and goat;

The PAG values (average) in certainly pregnant goats were 20.88 - 26.460 ng/ml (x±sx: 16.75±3.44 / 27.17±2.95 ng/ml) on days 25-30 after natural mating;

The PAG values in certainly pregnant sheep were 10.495 ng/ml (x±sx: 9.97± 0.74ng/ml)

In estrus, goats and sheep displayed very low PAG values, often non-identifiable, between 0.580-0,907 ng/ml in goat and 0.01-0,708 ng /ml in sheep;

Embryo mortality was confirmed for sure by PAG determination on days 25-30 from mating. The incidence of embryo mortality was 8.57% (confirmed clinically).

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