Influence of transvaginal ultrasound-guided follicular punctures in the mare on heart rate, respiratory rate, facial expression changes, and salivary cortisol as pain scoring

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ABSTRACT

Transvaginal ultrasound-guided follicular punctures are widely used in the mare for diagnosis, research, and commercial applications. The objective of our study was to determine their influence on pain, stress, and well-being in the mare, by evaluating heart rate, breath rate, facial expression changes, and salivary cortisol before, during, and after puncture. For this experiment, 21 pony mares were used. Transvaginal ultrasound-guided aspirations were performed on 11 mares. After injections for sedation, analgesia, and antispasmodia, the follicles from both ovaries were aspirated with a needle introduced through the vagina wall into the ovary. In the control group, 10 mares underwent similar treatments and injections, but no follicular aspiration. Along the session, heart rate and breath rate were evaluated by a trained veterinarian, ears position, eyelid closure, and contraction of facial muscles were evaluated, and salivary samples were taken for evaluation of cortisol concentration. A significant relaxation was observed after sedative injection in the punctured and control mares, according to ear position, eyelid closure, and contraction of facial muscles, but no follicular aspiration. Along the session, heart rate and breath rate were evaluated by a trained veterinarian, ears position, eyelid closure, and contraction of facial muscles were evaluated, and salivary samples were taken for evaluation of cortisol concentration. A significant relaxation was observed after sedative injection in the punctured and control mares, according to ear position, eyelid closure, and contraction of facial muscles, but no difference between punctured and control animals was recorded. No significant modification of salivary cortisol concentration during puncture and no difference between punctured and control mares at any time were observed. No significant modification of the breath rate was observed along the procedure for the punctured and the control mares. Heart rate increased significantly but transiently when the needle was introduced in the ovary and was significantly higher at that time for the punctured mares than that for control mares. None of the other investigated parameters were affected at that time, suggesting discomfort is minimal and transient. Improving analgesia, e.g., through a multimodal approach, during that possibly more sensitive step could be recommended. The evaluation of facial expression changes and heart rate is easy-to-use and accurate tools to evaluate pain and well-being of the mare.

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1. Introduction

Transvaginal ultrasound-guided follicle aspiration was initially developed for oocyte recovery in women in association with in vitro fertilization (IVF) [1,2]. The technique was then adapted to the cow [3] and is nowadays conducted routinely in women and cows for oocyte collection
for IVF. In the mare, transvaginal ultrasound-guided follicular puncture (ovum pickup, OPU) has been developed in the early 90s [4–6] and is widely used for diagnosis [7,8] and research purposes [9–22]. Moreover, commercial applications have been developed [23–26]. As each OPU session involves multiple needle punctures into the vagina, bladder, peritoneum, and the ovaries and because the same mare may be used again for later sessions, identification and alleviation of pain is essential for animal welfare. Previous studies have analyzed the effect of repeated transvaginal aspiration of immature follicles on mare health and ovarian function and morphology [27,28] or fertility [21,29]. However, to our knowledge, the effect of transvaginal follicle aspiration on stress, pain, and well-being has never been studied in the mare. Our hypothesis was that transvaginal aspiration of follicles may induce stress and pain that could affect well-being. Therefore, our aim was to determine the influence of transvaginal ultrasound-guided follicle aspiration on stress, pain, and well-being in the mare.

Stress induces two different physiological responses: a rapid reaction characterized by an increase in catecholamine levels, which in turn increases heart rate, and a long-term adjustment response, which results in an increased glucocorticoid level, cortisol being the dominant glucocorticoid in horse plasma [30]. For example, during transport, which includes multifactorial stressors, increased salivary cortisol concentrations and changes in heart rate have been observed [31,32]. A positive correlation between equine serum and salivary cortisol concentrations has been established [33]. Moreover, serum cortisol includes both free and bound inactive fractions, whereas salivary cortisol represents a part of the free cortisol fraction, which is the biologically active form [33]. The analysis of salivary cortisol is a validated noninvasive technique avoiding stress induced by repeated intravenous blood sampling [33].

As in other animal species, pain in horses is difficult to assess, but pain-related behaviors have been identified [34,35]. However, many of these behaviors have been observed in response to severely painful conditions and may not be induced by mild to moderate pain. Facial expressions are used to assess pain in humans [36,37], mice [38], rats [39], and rabbits [40]. Recently, a standardized pain scale based on facial expressions in horses (Horse Grimace Scale) has been developed and validated [41]. The Horse Grimace Scale offers an effective and practical method of identifying painful conditions.

Our objective was to determine the influence of transvaginal ultrasound-guided follicular punctures on pain, stress, and well-being in the mare, by evaluating heart rate, respiratory rate, facial expression changes, and salivary cortisol before, during, and after puncture.

2. Materials and methods

All procedures on animals were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the French Ministry of Agriculture and with the approval of the ethical review committee (Comité d’Ethique en Expérimentation Animale Val de Loire) under number 02701.02.

2.1. Transvaginal ultrasound-guided follicular punctures

For this experiment, 21 adult cyclic pony mares from our experimental study, from 3 to 21 year old and from 265 to 390 kg were used in May and June. The mares were familiar with the staff, the OPU premises, and the material used. Their ovarian activity was assessed by routine rectal ultrasonography, using a 7.5-MHz transrectal probe (Aloka, Wallingford, USA) for choosing mares with several follicles from 5 to 25 mm.

Transvaginal ultrasound-guided aspirations were performed on 11 mares (ovum pickup, OPU group). Mares entered the puncture room and were restrained in stocks. They received a first injection of detomidine (Medesedan, 10 µg/kg intravenously, detomidine chlorhydrate; Centravelt, Plancoet, France) for sedation and analgesia. After evacuation of feces from the rectum, the perineal area was cleaned with povidone-iodine scrub and a urinary probe was introduced in the bladder. Just before the OPU procedure, mares were injected detomidine (Medesedan, 15 µg/kg intravenously), butorphanol (Dolorex, 10 µg/kg intravenously, butorphanol tartrate; Centravelt) for analgesia, and butylscopolamine (Estocelan, 0.2-mg/kg butylbromide scopolamine and 100-mg/kg sodium metamizole [dipryrone], intravenously, Centravelt) for analgesia and antispasmodia, to induce smooth muscle relaxation. Ultrasound-guided transvaginal follicular aspiration was performed following a routine procedure in our laboratory as previously described [10,42]. Briefly, the ultrasound transducer was introduced into the vagina, whereas the ovary was manipulated per rectum to position the follicles to be aspirated in line with the needle-guide on the screen. Then, the needle was introduced through the vaginal wall into the follicle. The content of the follicle was aspirated with a double-lumen needle (length 700 mm, outer diameter 2.3 mm, internal diameter 1.35 mm; Casmed, Cheam, Surrey, England) by use of a vacuum pump at 150 mm Hg. Flashes of the follicle with heparinized PBS (phosphate-buffered saline, Dulbecco A; Oxoid, Basingstoke, Hampshire, England and heparin, Choay, Sanofi Aventis 5000 IU/mL) were repeated 10 times. The procedure was repeated for all follicles above 10 mm on both ovaries. All collected fluids were examined for oocyte recovery. Mares received an injection of antibiotics (Depocilline, 15000–IU/kg benzylpenicillin, intramuscularly, Intravet, Beaucouze, France) at the end of the puncture session.

In the control group, 10 mares underwent similar treatments (emptying of the rectum, cleaning of the perineum, and urinary probe) and similar injections (10–µg/kg detomidine, 10–µg/kg butorphanol, 0.2–mg/kg butylbromide scopolamine, and 100–mg/kg sodium metamizole) but without introduction of the ultrasound transducer into the vagina or subsequent follicular puncture. No antibiotics were injected.

Each day, after ovarian-activity assessment, mares with the largest number of follicles joined the OPU group, whereas those with fewer follicles were used as controls.

2.2. Heart rate, respiratory rate, and facial expression changes

Heart and respiratory rate were evaluated by a trained veterinarian using a stethoscope. Ears position (turned
forward, middle, and backward), eyelid closure (open, middle, and almost closed), and contraction of facial muscles (contracted, middle, and relaxed) were evaluated according to the Horse Grimace Scale [41] as a pain assessment tool.

For the OPU group, these observations were performed (1) before the mare entered the puncture room, (2) when the mare was restrained in stocks in the puncture room after the first injection of detomidine, (3) after the introduction of the probe into the vagina, (4) after the introduction of the needle through the vagina wall into the first ovary, (5) after the introduction of the needle through the vagina wall into the second ovary, (6) at the end of the puncture session after antibiotic injection just before the mare goes out, and (7) eight days after the puncture session at the same time.

For the control group, these observations were performed (1) before the mare entered the puncture room, (2) when the mare was restrained in stocks in the puncture room after the first injection of detomidine, (3) ten minutes after the first injection of detomidine, (4) twenty minutes after the first injection of detomidine, (5) eight days after the puncture session at the same time.

2.3. Salivary cortisol

Salivary samples were taken with cotton swabs (Salivette; Sarstedt) grasped by a clamp. Swabs were gently placed and kept under the tongue for 60 seconds.

For the OPU group, the saliva was collected (1) before the mare entered the puncture room, (2) when the mare was restrained in stocks in the puncture room after the first injection of detomidine, (3) ten minutes after the first injection of detomidine, (4) twenty minutes after the first injection of detomidine, (5) eight days after the puncture session at the same time.

For the control group, the saliva was collected (1) before the mare entered the puncture room, (2) when the mare was restrained in stocks in the puncture room after the first injection of detomidine, (3) 10 minutes after the first injection of detomidine, (4) twenty minutes after the first injection of detomidine, (5) eight days after the puncture session at the same time.

Samples were centrifuged for 5 minutes at 3000 × g at 4 °C and stored at −20 °C.

Salivary cortisol concentration was measured using the Cortisol Luminescence Immunoassay (IBL International, Hamburg, Germany) according to the manufacturer’s recommendations.

2.4. Statistical analysis

Comparison of heart rate, respiratory rate, facial expression changes, and salivary cortisol at the different steps of the procedure was performed by the nonparametric permutation test using the R software. The comparison between the OPU and control group for each step was performed by the nonparametric Mann–Whitney test. Statistical significance was established at P less than 0.05.

3. Results

3.1. Heart rate and respiratory rate

Mean heart rate of the mares from the OPU and control group is presented in Figure 1. In the OPU group, heart rate increased significantly when the needle was introduced in the ovary and decreased at the end of the puncture session (P < 0.05). In the control mares, no significant modification of the heart rate was observed along the procedure. Moreover, at the time of the introduction of the needle into the first ovary, heart rate was significantly higher for the punctured mares than that for the control mares (P < 0.05).

Mean respiratory rate of the mares from the OPU and control group is presented in Figure 2. No significant modification of the respiratory rate was observed along the different steps of the procedure. Moreover, no difference of the respiratory rate was observed between punctured and control mares at any step of the procedure.

3.2. Facial expression changes

Facial expression changes (ear position, eyelid closure, and contraction of facial muscles) from the OPU and control group are presented in Figure 3. We observed a significant relaxation according to ear position (turned forward to backward), eyelid closure (from open to almost closed), and contraction of facial muscles (from contracted to relaxed) after the first sedative injection, both in the OPU and

![Fig. 1](image-url) Mean heart rate (heartbeats/min ± standard error of the mean) of the mares from the ovum pickup (OPU) and control group. abValues with different superscripts differ significantly within a group.
control group. No difference between punctured and control mares was observed at any step of the procedure.

3.3. Salivary cortisol

Salivary cortisol concentration of the mares from the OPU and control groups is presented in Figure 4. No significant modification of the salivary cortisol concentration along the different steps of the procedure was observed. No difference of the salivary cortisol concentration was observed between punctured and control mares at any step of the procedure.

4. Discussion

Ovum pickup allows repeated oocyte and fluid collection from ovarian follicles in live females. Through the use of this technique, more in-depth studies may be conducted

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**Fig. 2.** Mean respiratory rate (breaths/min ± standard error of the mean) of the mares from the ovum pickup (OPU) and control group. No significant modification of the respiratory rate was observed within each group.

**Fig. 3.** Ear position, eyelid closure, and contraction of facial muscles of the mares from the ovum pickup (OPU) and control group. Ear position (3: turned forward, 2: middle, and 1: turned backward, ± standard error of the mean [SEM]), eyelid closure (3: open, 2: middle, and 1: closed, ± SEM), and contraction of facial muscles (3: contracted, 2: middle, and 1: relaxed, ± SEM). a,bValues with different superscripts differ significantly within a group.
Transrectal palpation and introduction of an intravaginal device has been shown to induce moderate stress in the cow [44]. The most questionable aspect of OPU is the multiple transvaginal and transovarian punctures which, based on our results, constitute the more invasive part of the OPU. As each OPU session involves multiple needle punctures into the vagina wall, peritoneum, and the ovaries and because the same mare may be used again for later sessions, identification and alleviation of pain is essential for animal welfare.

Previous studies have analyzed the effect of repeated transvaginal aspiration of immature follicles on mare health and ovarian function and morphology [27,28] or fertility [21,29]. They showed that follicle aspiration carries a small possibility of ovarian abscess formation or rectal abrasion but preserves normal ovarian function defined as the ability to develop normal follicles and corpora lutea and has no adverse effect on fertility. To our knowledge, the effect of transvaginal follicle aspiration on stress, pain, and well-being has never been studied in the mare. In our study, mares underwent treatments for sedation, analgesia, and antispasmodia so that the OPU can be performed in good conditions. These treatments have been first established according to the dosage recommended by the manufacturer and refined thanks to our 20-year-old experience with equine OPU [5,10,15–19,45].

Stress is defined as a nonspecific response of the body to a stimulus. One response is that the autonomic nervous system causes and increase in catecholamine levels, which in turn increases the blood pressure and heart rate [46]. In our study, we measured heart rate at several time points, but evaluation of heart rate variability would have been an additional welfare indicator. During follicular punctures, the manipulation of the ovary, and in particular pulling the broad ligament, which is a very sensitive structure, may increase the heart rate. In our conditions, the analgesia seems to indeed adequately prevent the potential pain of that part of the procedure as no increase in heart rate was observed at the time of insertion of the probe into the vagina, when the ovary is simultaneously brought toward the probe per rectal manipulation. In contrast, heart rate increased significantly in the OPU group when the needle was introduced into the ovary. This step could be a sensitive one, and particular attention should be brought to analgesia to maintain the well-being of the mares. In the control group, the injections have no significant effect on respiratory and heart rate of the mares, whereas detomidine is known to decrease respiratory and cardiac rate. This is probably due to the low dose of detomidine used in our study. The dose of detomidine could be increased to decrease pain when the needle was introduced into the ovary, but an increased dose may have some side effects. The procedure could be improved using a local anesthesia of the ovary, but it is difficult to perform and may induce unwished effects on oocyte quality. The management of pain may require a multimodal approach, employing drugs with different mechanisms of action. The most commonly used analgesic medications in horses include the alpha-2-adrenergic agonists, nonsteroidal anti-inflammatory drugs, and opioids. In standing horses, the combination of detomidine and opioids, like butorphanol, results in synergistic analgesic effects, reliable sedation, and stable cardiorespiratory function with reduced side effects [47]. Finally, a multimodal analgesia including epidural anesthesia (using local anesthesia drugs like lidocaine, mepivacaine, or bupivacaine) could be recommended.

As in other animal species, pain in horses is difficult to assess because of their inability to communicate with humans in a meaningful manner. Several behavior-based assessments of pain in horses have been developed [35,48–50]. However, behavior-based assessments of pain are not without limitations that constrain their routine application. These include the need for trained and experienced observers, prolonged observation periods, difficulty to perceive mildly to moderately painful conditions. Facial expressions are commonly used to assess pain in humans, rodents, and rabbits [36–40]. These grimace scales are considered to give a number of advantages: they are

![Fig. 4. Salivary cortisol concentration (µg/dl ± standard error of the mean) of the mares from the ovum pickup (OPU) and control group. No significant modification of the cortisol concentration was observed within each group.](image-url)
less time consuming to carry out, observers can easily and rapidly be trained to use them, they can be used to effectively assess a range of painful conditions, from mild to severe pain. Recently, a standardized horse grimace scale that offers an effective and practical method of identifying painful conditions has been developed [41]. In our study, we used the horse grimace scale to evaluate facial expression changes related to pain and relaxation. After sedation, we observed a significant relaxation according to ear position, eyelid closure, and contraction of facial muscles. These observations suggest that sedation, analgesia, and myorelaxation were efficient.

A significant activation of the hypothalamo-pituitary-adrenal axis, as reflected by increased plasma cortisol concentrations, is usually observed during acute stress. Analysis of salivary cortisol concentrations is increasingly used to assess the adrenocortical response of horses to potentially stressful situations, and several cortisol assays have been validated for equine saliva [51]. Salivary cortisol mirrors the unbound biologically active fraction of total plasma cortisol, whereas plasma cortisol is largely bound to carrier proteins [33]. Cortisol concentrations follow a diurnal rhythm with the highest concentrations in the morning and a nadir in the late afternoon and evening [52]. This rhythm can be disrupted by even minor perturbations resulting in elevated cortisol concentration. The diurnal rhythm in cortisol release is well reflected by concentrations in saliva [53]. Cortisol release into blood or saliva may be physiologically influenced also by reproductive status and the sex of the individuals, as salivary cortisol was shown to increase in mares shortly before foaling [54]. It may be suspected that estrus in mares increases locomotion leading to higher cortisol concentrations [55]. In our study, salivary cortisol concentration did not differ between OPU and control groups, and no significant modification of salivary cortisol concentration was observed during transvaginal follicular puncture. When assessing stress, it is more useful and relevant to measure free cortisol than total cortisol in serum because the increase in serum cortisol during acute stress is largely made up of free cortisol [33,53]. Salivary cortisol represents a part of the free cortisol fraction, which is the biologically active form [33,53]. However, because salivary cortisol results from passive diffusion into the salivary glands, the increase in salivary cortisol concentration may be delayed. Thus, in our conditions, no acute stress was evidenced according to cortisol modifications. However, a delay in salivary concentrations’ increase may have occurred that would prevent us to detect this increase.

Ovum pickup is a routine procedure in our laboratory, with highly experienced staff and animals that are familiar to being manipulated by that staff. This probably accounts for the absence of any observed induced stress. In a previous study, transrectal ultrasound examination has been shown to induce a significant increase in salivary cortisol [46]. These data suggest that conditions for transrectal or transvaginal intervention, such as the skill of the staff or the familiarity of the mares with the staff and premises, may decrease acute stress of the mares.

In conclusion, following our hypothesis that transvaginal aspiration may induce stress and pain, we have established conditions for transvaginal ultrasound-guided follicular punctures with a significant relaxation thanks to a correct sedation and no acute stress and pain. Moreover, the pain induced by grabbing the ovary and pulling the ligament seems to be controlled in our conditions. However, the introduction of the needle into the peritoneum and the ovary induces a transient increase of heart rate and could be a sensitive step. Particular attention should be brought to analgesia, and multimodal analgesia could be recommended to maintain well-being of the mares. For example, a local anesthesia targeting the ovary or an epidural anesthesia with either local anesthetics may improve analgesia avoiding side effects of high doses of alpha-2-agonists. Finally, the evaluation of heart rate and facial expression changes have been shown to be easy-to-use, accurate, and reliable tools to evaluate pain and well-being of the mare. They may offer an effective and practical method of evaluating the efficacy of the methods used to ameliorate pain control in horses, in particular analgesia administration.

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