Influence of cortisol and leptin in the follicular fluid on leptin markers in the endometrium

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Leptin is a multifunctional peptidic hormone, mainly produced by adipose tissue, having receptors (Ob-Rs) in the reproductive organs, hypothalamus and pituitary. Glucocorticoids, such as cortisol, also directly influence leptin production. The importance of these two hormones in reproductive activity has been described by several authors, who observed the involvement of both, in the improvement of reproductive indices, either by their direct influence on the hypothalamic-pituitary-gonadal axis, oocyte maturation or the uterine preparation to receive the concept. However, when in excess in circulation, they can negatively influence the reproductive system. Markers such as leptin and its long-chain functional receptor are being studied in the endometrium of species such as humans, cattle and pigs, presenting results that correlate the decrease of these with infertility or embryonic loss, but to date it has not been possible to find studies to address this issue in horses. In this study we evaluated: 1) the presence of leptin (Ob) and its receptor (Ob-Rb) in the endometrium of mares, observing the influence of obesity and estrous cycle on these markers and 2) in an adverse and stressful management condition, the influence of intrafollicular cortisol, leptin levels in the follicular fluid (FF), and the Ob and Ob-Rb markers in the endometrium during the estrous cycle phases. The results showed that leptin and its receptor (Ob-Rb) are present in the equine endometrium, with immunostaining in the luminal and glandular epithelium in all stages of the estrous cycle evaluated, however, showing a more intense immunological labeling in Ob -Rb (142.68 ± 4.97, $P < 0.0001$) in the glandular epithelium during the diestrous in mares of moderate body score. It was not possible to observe the influence of intrafollicular cortisol (FF) on the variables evaluated, because cortisol remained within the physiological values for the species, however a positive correlation can be observed between intrafollicular cortisol and leptin levels, being the cortisol increased (30.1 ± 0.07ng / ml, $P < 0.05$) in the follicles closest to ovulation. It can also be noticed that the immunological labeling of the leptin receptor in the glandular epithelium was more intense (144.52 ± 3.17, $P < 0.0001$) in the animals that presented follicles up to 22 mm, and the immunostaining of both Ob and Ob-Rb correlated negatively (r: -0.7836; $P < 0.0001$, r: - 0.7343; $P < 0.0001$), with cortisol levels in FF.

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Evaluation of the zebu cow pregnancy rate with the use of estrus detection device

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Bovine farming is increasingly dependent on technological innovations to increase productivity and improve herd genetics. The Fixed Time Artificial Insemination (FTAI) allows the insemination of a larger number of animals in a shorter time without the need for detection of estrus. However, some authors report that the detection of the estrus can improve the use of the FTAI technique, since the animals that present behavioral changes during the estrus, they are fitter and have a larger follicular diameter, thus increasing the pregnancy rate of the herd. Currently, there is a commercial estrus detection device (Estrotect®) where its operation is related to the color change according to the acceptance or not of mounts by other animals. This device has initial gray color, which is changed to fluorescent color, according to the friction and friction during the acceptance of mounts, thus indicating the intensity of the estrus of the animal and if it is able to be inseminated adhered transversely to the vertebral column near the transitional region of the sacral vertebrae, being classified in scores of 1 to 4, where score 1 is completely unchanged, score 2 has color change in less than half of the device, score 3 shows color change in more than half of the surface of the device, and score 4 presents total color change. The objective of this work was to evaluate the efficiency of Estrotect ® as an aid in the detection of the estrus of the animals, correlating the herd pregnancy rate. This work was approved by ethics committee on the use of animals (CEUA) number 37/2017 of Salesian Catholic University Center Auxilium of Araçatuba/SP. In the present study were used 48 animals with a mean age of 24 to 36 months, zebu mestizo, with body condition score (ECC) of 3 to 3,5. The animals were submitted to the protocol of the ovulation synchronization associated to FTAI in the following moments: 1) on day zero (day 0): application of 2 mg of estradiol benzoate (Sincrodiol®, Ouro Fino, São Paulo, Brazil) intramuscularly and placement of intravaginal device containing 1.9 g of progesterone (Cronipress®, Biogenesis Bagó, São Paulo, SP, Brazil), 2) after 8 days (day 8): application of 12.5 mg of prostaglandin F2α (Lutalyse®, Pfizer Animal Health) by intramuscular route, withdrawal from the intravaginal device and application of 300 eCG - Equine Chorionic Gonadotrophic (Novormon®, Schering-Plow Co., São Paulo, Brazil) by intramuscularly. Then, the device was placed for detection of estrus (Estrotect®); 3) after 24 hours (day 9): application of 1 mg of estradiol benzoate (Sincrodiol®; Ouro Fino, São Paulo, SP, Brazil); and 4). On day 10, the evaluation of the estrus detection device (Estrotect®) was performed according to the staining and graded according to the manufacturer's recommendation (score of 1 to 4). The animals were inseminated and after 28 days an ultrasound examination was performed to diagnose the pregnancy. The data were submitted to descriptive statistical analysis. The pregnancy rate total was 79.06% (34/43), similar to the results found (1), where it evaluated the efficiency of the estrotect ®, thus confirming that it can be used both as an aid in the detection of the estrus in conjunction with visual observation as well as the unique tool in the detection of herd estrus. Regarding to the evaluation of the Estrotect® score, 3 (8.82%) animals presented a score of 1 with a pregnancy rate of 6.97%; 4 (11.76%) animals presented score 2 with 9.30%; 11 (32.35%) animals presented a score of 3 with 25.58% and 16 (47.05%) animals presented score 4 with 37.20%, respectively.With this, a higher pregnancy rate was observed in animals with Estrotect® score 4. From the results obtained, it is noticed that the higher the Estrotect® score, the higher the estrus intensity, the greater the pregnancy rate of the herd. With this, we can conclude that the estrus detection device is a useful tool to aid in FTAI protocols without the need to evaluate estrus or animal behavior. (Bonato G. L. et al, Braz. J. Vet. Res. Anim. Sci., Sao Paulo, v. 49, n. 1, p. 19-23, 2012)

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Role of Estradiol in Prolactin-induced suppression of Luteinizing Hormone pulsatile secretion

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It is known that chronic hyperprolactinemia inhibits fertility by suppressing luteinizing hormone (LH) pulsatility and that oestradiol (E2) is a key regulator of LH secretion. However, the involvement of the E2 in the inhibition of LH secretion caused by acute hyperprolactinemia remain unknown. Prolactin (PRL) effects on the brain are considered to be E2 dependent. However, infertility induced by hyperprolactinemia would be self-limiting if required E2, because secretion of gonadal steroids would eventually decline following LH inhibition. The present study evaluated the role of E2 in PRL-induced suppression of pulsatile LH secretion in a model of acute hyperprolactinemia. Two weeks after ovariectomy (protocol CEUA-UFMG 83/2014), adult Wistar female rats (250–300 g) were treated with oil (OVX) or E2 (OVX+E2; 1 μg/0.2 mL/rat, s.c.) daily for 3 consecutive days. On the fourth day, ovine PRL, at doses of 0.5 mg/rat (experiment 1) or 2 mg/rat (experiment 2), or vehicle (Veh) (n = 7–10 per group) were administered subcutaneously 30 minutes before tail tip blood sampling for evaluation of pulsatile LH release. Thirty sequential 10-μL blood samples were collected over 180 min. LH was measured in the whole blood by ELISA. E2 reduced the frequency of LH pulses and mean LH levels in OVX+E2 rats. The moderate dose of 0.5-mg/rat oPRL further reduced the frequency of LH pulses in OVX+E2 but had no efect in OVX rats. The high dose of 2-mg/rat oPRL decreased pulse frequency equally in OVX+E2 and OVX rats, whereas lowered pulse amplitude and mean LH levels only in OVX+E2 rats. The efects of 2-mg/rat oPRL on kisspeptin neurons of the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei were also investigated. Kisspeptin immunoreactivity and Kiss1 mRNA levels in the ARC were lower in OVX+E2 compared with OVX rats. oPRL decreased both kisspeptin peptide and gene expression in the ARC of OVX rats whereas did not further reduce them in OVX+E2 rats. In the AVPV, oPRL had no efect on kisspeptin immunoreactivity, although increased the stimulatory efect of E2 on Kiss1 mRNA levels. Additionally, Gnrh mRNA levels were suppressed by oPRL regardless of E2. The present findings reveal that E2 modulates the responsiveness of gonadal axis to PRL but is not essential to hyperprolactinemia-induced suppression of ARC kisspeptin and LH pulsatile secretion.

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Luteal function and embryo mobility in mares with distinct ages and degrees of endometrial degeneration

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The migration of the conceptus throughout the entire uterus is critical for the success of the maternal recognition of gestation in mares. However, an impaired interaction between the uterus and the travelling embryo was recently found in old mares and mares with severe endometrial degeneration. In the present study, the effect of age on embryonic development and luteal function was evaluated during the first 20 days of gestation using two groups of age: Young and Old groups (5.6±0.2 years and 17.2±0.9 years, respectively; n=6 pregnant mares/group). Moreover, pregnant mares were further assigned into two experimental groups (n=7 mares/group) according to the histopathological classification of the endometrium: Mild group (categories I and IIA) and Severe group (category III). Transrectal Doppler ultrasonography examination and blood collection were performed every 24 hours during the first 20 days of gestation (D0 = day of ovulation). Total area of the corpus luteum (CL), luteal vascularity, CL area with blood signals, progesterone concentrations and embryonic vesicle diameter were evaluated from D0 to D20. Additionally, the number of embryonic location changes were evaluated every five minutes during two consecutive hours from the first day of visualization of the vesicle until the day of embryonic fixation by transrectal B-mode ultrasonography. The Old and Severe groups had greater total CL area ($P \leq 0.04$) and reduced luteal vascularity ($P \leq 0.04$) than the Young and Mild groups, respectively, during the first days after ovulation. On the other hand, progesterone concentrations and CL area with blood signals were not affected ($P \geq 0.8$) by age and degree of endometrial degeneration. A negative effect of age ($P < 0.01$), but not of endometrial degeneration ($P = 0.6$), was found for the embryonic vesicle diameter. The diameter of embryos from young mares was statistically greater ($P < 0.05$) between D15 and D18, when compared to old mares. The conceptus mobility was high ($P > 0.1$) until D14 in the Severe group, while a reduced number of changes of the embryo location was detected earlier ($P < 0.05$) in the Old group. Our results suggest compensatory structural adjustments of the CL to ensure the uninterrupted supply of progesterone concentrations until day 20 of pregnancy in old mares and mares with severe endometrial degeneration. Advantaged age of the mare was associated with premature reduction of embryonic mobility and the delayed development of the conceptus. The location of conceptus fixation and the pregnancy maintenance were not affected by age and degree of endometrial degeneration. This research was financially supported by FAPESP (#2009/52575-1).

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Intrauterine infusion of Ozone in susceptible mare

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Endometritis are important cause of reduced fertility in mares and the third most common pathology in this species. Can lead to serious complications such as: inability to take the gestation to term, early embryonic loss, abortion, placentitis, neonatal sepsis and postpartum metritis. Antimicrobial resistance is a growing veterinary health concern globally. When a mare is infected with resistant bacteria, not only treatment of that animal becomes more difficult, but also the diagnosis can be masked and the antibiotic-resistant bacterium may spread to other animals at farm. The treatment failure with antibiotics results in prolonged illnesses with, more complications, more veterinarians visits and economic losses. In mares has been reported that chronic uterine infections that are resistant to antimicrobials can be caused by biofilm production, which consists of a matrix that adheres the microcolonies preventing penetration of antibiotic. Formation of biofilm in the equine reproductive tract is theorized to be a significant cause of chronic endometritis in the mare. The objective of this work was to test a intrauterine infusion of sunflower ozonized oil in susceptible mare in order to recover from bacterial and fungal endometritis. A 19 year old mare with history of no pregnancy or embryo collected in the last 3 breeding seasons was diagnosed with fungal infection by Aspergillus and bacterial infection by Escherichia coli both forming biofilm in vitro (proved by crystal violet technique). Mare was treated parentally with 6.6 mg/kg q 24 h of Gentamicin for 7 days and a uterine infusion with 66 µg/mL in 60 mL ozonized sunflower seed oil in a diestrus mare. Forty eight hours later, uterine lavage was performed in order to promote evacuation of uterine fluid and after 6 litters lavage mare was considered clean. At the first 2 liters we could observe presence of debris which could suggest disruption of biofilm *in vivo*. Ozone may help in stimulation of lymphocytes and monocytes to assist releasing several cytokines which enhance the tissue regeneration mechanism and initiate the process of epithelial formation. In this study apparently ozone disrupted some microorganism such as bacteria and fungal by diffusing through the protein coat in the nucleic acid core. In conclusion, treatment of infectious endometritis due to biofilm produced by bacteria, fungal organisms can be difficult and Ozone might be an alternative option without generate any type of antimicrobial resistance.

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Morphometry, cell quantification and immunolocalization of AMH in bovine testis fetuses

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The anti-Mullerian hormone (AMH), also known as Muller Inhibitory Substance (MIS) is expressed by Sertoli cells of male fetus. AMH play an important role in inducing ducts Muller regression in the male fetus, hence sexual differentiation. Also, in the gonadal development, though still requires investigation. Herein, the seminiferous tubules morphometry and cell quantification also AMH immunolocalization were performed in bovine fetuses of different ages to investigate AMH role during gonadal development. Thirteen pairs of testicles of bovine fetuses ages from 4 to 8 months (24-98 CRL) were collected in a slaughterhouse, fixed in 10% formaldehyde for 24 hours, processed for conventional histology and included in paraffin. AMH immunolocalization was performed on histological sections of 5µm deparaffinized using anti-AMH antibody (SC 28912) according to manufacturer's instructions, DAB-stained and HE-stained. Slides were visualized using the Eclipse Ci-E photomicroscope (Nikon Corporation, Tokyo, Japan) and NIS-Elements Basic Research software - NIKON Version 4.0. Statistical analysis were performed using ANOVA with significance level of 5%, Tukey post-test in Bioestat 5.0 software. The results were showed in mean ± s.d. for tubular diameter 47.5(±5.1), 47.1(±4.5), 43.7 (±3.9), 48.2(±4.2) and 42.1(±3.2) µm; Sertoli cells 7.4(±6.2), 11.7(±2.2), 12.8(±1.9), 12.6(±1.7) and 11.8(±1.9) and number of gonocytes 1.4(±0.8) 1.1(±0.7), 0.6(±0.7), 1.5 ± 0.7 and 0.9(±0.6) visualized in 4, 5, 6, 7 and 8 months, respectively. As a result, the tubular diameter had no increase between 4 and 5 months, however at 6 and 8 months the smallest diameters were observed. Regarding the Sertoli cells quantification, a gradual and significant increase between 4 and 7 months ($p < 0.05$) was observed. Moreover, fetal ages showed significant difference in the number of Sertoli and germ cells ($p < 0.05$). However, there are no studies in the literature relating fetal age and proportion of Sertoli cells and germ cells numbers. Immunolabeling was observed in the Sertoli cells (pre-Sertoli) within the sexual cords (developing seminiferous tubules) at all ages and was not observed in gonocytes and interstitial tissue. Considering that AMH role in gonadal development is not well understood, this is the first report of AMH immunolocalization in testis of bovine fetuses of gestational age more advanced and showed AMH during male fetal development. Future studies might elucidate the molecular mechanisms of AMH protein in testicular development and physiology in bovine fetuses.

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PKA and AMPK activities and relation with motility in cryopreserved spermatozoa of Atlantic salmon

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Sperm motility in fish with external fertilization is a critical factor in aquaculture, in order to achieve high reproductive efficiency. In this context gametes preservation techniques, such as cryopreservation, provides extended semen availability, conservation o high value strains and reduce the need for maintenance of large broodstock. However, it also reduces sperm motility in percentage and duration. Despite this, cryodamage has been scarcely studied from a cell signalling point of view. In this study, the activities of cyclic AMP-dependent protein kinase (PKA) and AMP-activated protein kinase (AMPK) in fresh and cryopreserved spermatozoa of Atlantic salmon (*Salmo salar*) were compared, and their potential relation with reduction of post-thawed sperm motility was assessed. PKA has been related to motility activation in salmonids, likely through phosphorylation of axoneme proteins. AMPK participates in energy regulation and sperm motility in mammals and poultry; and has been shown to improve sperm function and antioxidant defences after freezing-thawing. A significant reduction in membrane integrity and motility was observed in cryopreserved spermatozoa compared to fresh samples, similar to previous reports of cryodamage. Both PKA and AMPK kinase did not exhibited differences in their activity after motility activation, whereas showed significant reduction after cryopreservation ($P < 0.05$), which are likely to be associated with the reduction of sperm quality. Significant correlation between PKA and AMPK activities was found (Pearson's $P = 0.9462$; $P < 0.0001$), however, *in-silico* docking analysis indicated that AMPK activation by PKA is unlikely. Additionally, both kinases activities strongly correlated with motility, suggesting that they may play a relevant role in cryopreservation induced motility reduction. No previous reports of this phenomena in fish was found, making these findings interesting and worthy of further study. Moreover, potential biotechnological applications may be developed based on this and further research.

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Intrafollicular oocyte transfer (IFOT) in ewes – initial study

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The greater demand for sheep meat has stimulated farmers to increase productivity, and reproductive biotechnology can play an important role to multiply animals with desired traits, e.g. embryo transfer. However, embryo collection in ewes is normally executed using surgical method, inducing post-surgical adhesions and reducing reproductive lifespan of high value ewe donors. Therefore, the development of intrafollicular oocyte transfer (IFOT) by laparoscopy can reduce the reproductive impacts of successive surgeries and improve donor ewe welfare. The IFOT technique is based on immature oocytes collected from donor females by follicular aspiration, followed by oocytes transfer to a preovulatory follicle of a pre-synchronized recipient female in estrous. After oocyte transfer, artificial insemination or natural service is accomplished and, around eight days later, the recipient is flushed to recover embryos. Currently, basic reports about IFOT in ewes were not found and this study was performed to determinate ideal intrafollicular injected volume and better needle size on IFOT practice, aiming future *in vivo* studies. Ovaries (n=25) from slaughtered ewes were used, despite of age, body condition score and reproductive status. Immediately after slaughter, ovaries were harvested, immersed in sodium chloride solution (NaCl 0.9%) at room temperature and transported to laboratory within 30 min. Arriving at the laboratory, follicles diameters were measured with pachymeter and the biggest follicle detected (between 2 and 5 mm) were selected to further tests. Pre-selected follicles were injected with eosin-methylene blue solution (0.1% in sodium chloride) and the efficacy of two volumes (10 and 20 µL) and two needle sizes (22 and 25G) were initially assessed. Injections were performed with a medical IV extension tube connected on each extremity to a 1 mL syringe and the tested needles. After follicle injection, the ovaries were again immersed in sodium chloride solution and kept at room temperature during 24 h, to determine if eosin-methylene blue solution remained within follicle. From 25 follicles injected, three disrupted during punction, before eosin-methylene blue solution injection (two with 2 mm and one with 3 mm). All follicles injected with 20 µL (n=4) presented loss of eosin-methylene blue solution, despite of diameter (2 to 4 mm) or needle tested. Therefore, it was decided to fix the volume to 10 µL on further injected follicles. Among follicles injected with 10 µL, 55.5% (10/18) maintained eosin-methylene blue solution within follicle, indicating that follicle wall remained intact using either 22G (50.0%, 5/10) or 25 G (50.0%, 5/10) needles in follicles that measured 3 (n=6), 4 (n=3) or 5 mm (n=1). Analysis of initial results indicate that ewe follicles < 2 mm should not be subjected to IFOT, and a volume of 10 µL can be injected in follicles > 3mm, despite of needle size (22 or 25G). Although further studies have to be implemented to refine technique before *in vivo* tests, using higher number of follicles with size similar to preovulatory follicles (5 to 7 mm), present results indicate the potential use of IFOT in sheep.

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Superoxide anion formation, high mitochondrial potential and the membrane integrity of stallion spermatozoa from phar-macologically-induced ejaculation

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Despite being the most common technique to collet semen from stallions, artificial vagina use is restricted in many cases including musculoskeletal and reproductive diseases. Due to this scenario, knowledge of alternative techniques, highlighting chemical ejaculation are needed in order to obtain and to preserve genetical material in these circumstances. One factor that can decrease the quality of the material collected is the formation of superoxide anion by the mitochondria. Therefore, the aim of the present study was to compare the volume, the concentration, the oxidative stress caused by the superoxide anion formation, the high mitochondrial potential and the membrane integrity between ejaculates collected by artificial vagina and two pharmacological inducing protocols. Seven sexually-experienced stallions from 3 to 25 years old were induced to ejaculation using two different protocols: Protocol 1 (IX) - imipramine hydrochloride (3mg/kg/v.o), followed 90 minutes later by the alpha-adrenergic agonist xylazine hydrochloride (0.66mg/kg/i.v); Protocol 2 (IDO)- imipramine hydrochloride (3mg/kg/v.o), followed 90 minutes later by detomidine hydrochloride (0.01mg/kg/i.v) associated with oxytocin hormone (20UI/ i.v). They also were collected twice using Botucatu® artificial vagina (Protocol 3 - AV) model (Botupharma Ltda., Botucatu, SP, Brazil), obtaining 14 base line ejaculates to compare to seven ejaculates from each protocol that were evaluated. Superoxide anion formation, high mitochondrial potential and the membrane integrity were measured by flow cytometry in a BD LSR Fortessa (Becton Dickinson, Mountain View, CA, USA) equipment using the protocol assay previously described by Freitas-Dell'Aqua et. al 2016. The statistical analysis were executed by GraphPad Prism 6.0 for Windows (GraphPad software, LA Jolla, California, USA; www.graphpad.com). The results are expressed in mean and standard deviation evaluated by Kolmogorov-Smirnov test. The membrane integrity values was AV (73,2 ± 3,391), IX (71,4 ± 14,5) and IDO (75,37 ± 8,298). The high mitochondrial potential found was in AV (72 ± 9,9), IX (65 ± 30,7) and IDO (67 ±12). The concentration (x106) found was AV (174,0 ± 107.2), IX (2.560 ± 3.222) and IDO (1.114 ± 1.104). The total volume (ml) collected was AV (61.3 ± 17.9), IX (15.2 ± 12.1) and IDO (45.8 ± 29.4). The superoxide anion formation was AV (26,6 ± 6,75), IX (65 ± 30,7) and IDO (67 ±12). Analysis in the present study showed a significant increase in superoxide ion in IDO protocol whereas similar mitochondrial potential and membrane integrity were found in both protocols compared to baseline ejaculates. In addition, no alterations in volume and concentration were found in the Protocol IX when compared to the baseline protocol, whereas in the Protocol IDO, the volume were lower and the concentration were higher compared to ejaculates obtained by artificial vagina. We hypothesize that this enhance in superoxide anion is directly regarding to a decreased seminal volume in the IDO protocol. The lower total volume results in reduced seminal plasma, which may imply in lower antioxidants that prevent oxidative stress and production. It probably may be solved by extender addiction in semen as it has besides cryoprotectants and antibiotics, antioxidants in their composition. In conclusion, this alternative method did not modified semen quality being one successful alternative method of semen collection.

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Canine fetal sexing: possibilities and limitations

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Fetal gender by ultrasound has been currently done for bovine and equine where the management decision justifies the investment of the technique and interferes with the outcome (1,2). For cows, the possibility of abortion may overcome the cost of the birth of the undesirable sex and for horses, the value of a sales might change substantially depending on the sex of the foal. In dogs, none of these two reasons are valuable, however, knowing the sex of the puppies prior to birth may impact the strategies of selling and the reservation list. Although the number of studies of fetal sexing in dogs has no clinical impact, there is a growing interest from pet owners to know the sex of the puppies prior to parturition. The principle of fetal sexing is to identify, evaluate the morphology and determine the precise location of the genital tubercle (GT) by ultrasound. In males, the GT migrates cranially around 35 days of gestation to develop into the penis and in females, caudally to form the clitoris (3, 4). Afterwards during pregnancy, it is also possible to visualize other genital parts such as the scrotum and penis. The GT image on ultrasound corresponds to a double hyperechoic line. The important anatomical references on a fetal sonogram that are critical in achieving proper orientation of the fetus, are the head, the beating heart, and the umbilicus (5). The best assessment of the GT is to observe transversally close to the umbilical insertion (male) or to the tail (female) in a cross-sectional (ideal) or frontal view. The aim of this study was to evaluate the efficacy of fetal sexing by ultrasound in dogs. Twenty pregnant bitches from different breeds were examined by ultrasound to determine the sex of the foetus. All the bitches were followed during oestrus to estimate ovulation based on progesterone levels (6 to 10ng/ml). Ultrasonographic examination was performed between days 37 to 42 of gestation. All the females were clipped in the abdominal area to allow a better ultrasound image. In six of the pregnancies with more than 6 fetuses, it was possible to establish the sex of at least three, but not all of them; therefore the confirmation of accuracy at birth was not possible. In the other pregnancies, the success rate of sexing for female was 86,36% (38 in 44 puppies) and for male 78,13% (25 in 32). The overall accuracy was 82,89%, 63 correctly diagnosed on 76 newborns. The difficulty to obtain a good ultrasound image could explain the error. Since, one limitation of the technique, even in a small litter, is to achieve the cross-sectional or frontal view to have a good view of the GT and determine the sex. Another issue is the longer duration of the ultrasound examination with some females not being patient enough to perform a very detailed ultrasonographic evaluation, what could also lead to error. The use of ultrasound to do fetal sexing in the canine species is a reliable technique. The best time to perform the sexing is between days 37 to 42 of gestation. In pregnancies with large numbers of puppies (≥ 6), the technique is less reliable due to the difficulties to identify each puppy and the increased time to perform the examination. In general, a greater level of experience and expertise is necessary for gender determination by ultrasound with the success rate highly associated to the proficiency of the operator.

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