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Cytological, hormonal, and ovarian hemodynamic alteration during the normal oestrus and split heat cycles in bitches

Asmaa A. Abdallah[1*], Elshymaa A. Abdelnaby[2,3], Said Amin[1], Hossam R. El-Sherbiny[2], Ibrahim A. Emam[4], Fatma M. Ibrahim[1]

[1]*Theriogenology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt.*
[2]*Theriogenology Department, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.*
[3]*Department of Clinical Sciences, College of Veterinary Medicine, King Faisal University, Alahsa 31982, Saudi Arabia.*
[4]*Department of Surgery, and Radiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.*

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***Correspondence:**

Corresponding author: Asmaa A. Abdallah
E-mail address: asmaat358@gmail.com

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ABSTRACT

Hormonal levels, particularly progesterone (P4), could be used to predict the day of luteinizing hormone (LH) surge, which is important not only for optimum ovulation but also for the detection of some abnormalities such as split heat. This study aimed to determine cytological, hormonal, and ovarian vascularity changes in normal cyclic and split heat bitches during proestrus and estrous phases. Pluriparous females (n=40) were categorized into two groups as follows: Group A (n=35; with a normal sequence of oestrus cycle) and Group B (n=5; split heat females with a short duration of proestrus). Doppler and hormonal assessments were performed daily from day 0 until day 15. Compared to Group B, both ovarian arteries (OA) Doppler indices decreased in Group A, with the most significant decline occurring between days 7 and 12. While peak velocity (PSV; cm/sec) was elevated in Group A compared to its value in Group B. In Group A, estradiol (E2) levels were higher (P 0.05) than in Group B, with the most significant increase observed between days 7-12. Group A showed an increase in nitric oxide (NO) from days 8 to 12 with no change in split heat bitches ($P<0.05$). P4 levels were slightly elevated ($P<0.05$) until they reached 3.5±0.09 and 8.25±0.22 ng/ml on days 10 and 15, respectively. Nevertheless, bitches showed split heat in Group B, P4 level was low. In Group A, IGF-1 levels were elevated in the proestrus phase until day 9 (155.32±5.26) and in the estrous phase until day 15 (175.56±3.66) compared to other groups. In Group B, the dominance of small intermediate cells and RBCs on all days remained unchanged on all days, in contrast to Group A, where cornified cells began to dominate on day 15. In conclusion, these data suggested that the evaluation of ovarian hemodynamics, in addition to hormonal and cytological assessments, could be critical parameters not only for the optimum ovulation prediction by progesterone assay related to LH but also for the detection of any abnormalities such as split heat or irregular estrous interval.

Introduction

Ovarian cycle in the bitch is connected to the determination of any alterations in the vaginal mucosa in association to hormones (Shah *et al*., 2019). In addition, B-mode ultrasonography could detect the presence of ovarian structures but not provide any valuable results about the genital organ functionality via its vascular perfusion (Günzel-Apel and Dieterich, 1996). According to the histological studies, many changes occur in ovarian vascularization that reflect the functional stage of female gonads (Koster *et al*., 2001), therefore the estimation of Doppler indices and Doppler velocity was very crucial in the detection of organ functionality via ovarian artery only and not related to venous blood flow (Kurjak and Kupesic, 1997) as the venous vascularization not give any important information such as cardiac cycle evaluation that obtained from the well-known examined artery.

Nowadays, the Importance of dogs as a pet animal is greatly increased, as many owners could read about the bitch cycle and visit the clinic to know more about the pet fertility status, therefore the normal cycle should be reported. As previously known the bitch cycle is divided into prestrous (with an average 9 days), estrous (with an average 9 days with oestrogen dominance), diestrous (with an average 50-70 days with the presence of progesterone), and anestrum (3 to 5 months) (Romagnol, 2014). During prestrous, oestrogen (E2; pg/ml) levels reached the peak 1-3 days prior to the surge levels of luteinizing hormone (LH), with an elevation of follicle stimulating hormone (FSH) at only one day after the surge of LH (Verstegen *et al*., 1993; 1997). Meanwhile, serum levels of P4 slowly increased in prestrous, its levels related to the LH surge that reflected partial luteinization of already histologically visible follicles 6 days ago LH surge. Increased P4, possibly a form of follicular origin and represents an excess of production for E2 synthesis can already start 1-2 weeks before prestrous (Concannon, 1993; 2009). While in infertility problems many alterations could face owners in the form of some physiological abnormalities such as unpredictable ovulation, split heat, silent heat, and long anestrum (Zubair *et al*., 2016). Split heat is a disorder in which the bitch shows estrous behaviour without ovulation in prestrous followed by a few days of anestrum and then a normal estrous cycle. It is usually detected in younger bitches with the first estrous but continuous or frequent split estrous may be due to premature luteolysis (Risvanli and Kalkan, 2016), or follicular development during the last few days of anestrum producing transient elevation of E2 level (Romagnol, 2014). Nitric oxide (NO) is an extraordinary neurotransmitter with a small and lipid-permeable molecule. It considered a paracrine mediator which affects the regulation of oxygen supply and demand, that lead to arterial dilatation/permeability regulation (Abdelnaby *et al*., 2021; Abdelnaby *et al*., 2022; Salama *et al*., 2022), and neurotransmission (El-Sherbiny *et al*., 2022), in addition to the presence of insulin-like growth factor type 1 (IGF-1; ng/mL) that directly lead to a marked increase in the NO production and furthermore affects vasodilatation. Some evidence reported the effect of NO in the regulation of ovulation and luteal function (Abdelnaby *et al*., 2018). The possible applications of the current study are summarized in the estimation of blood flow in the ovarian artery, and cytological and hormonal changes in both normal and abnormal female dogs in order to make a good image with a judgment on the reproductive aspects in this canine species. This study aimed to compare the cytological, hormonal (E2, P4, and IGF-1), and ultrasonographic alterations (B-, color, and spectral modes), in addition to NO changes in bitches during the normal estrous cycle and split heat.

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Materials and methods

Ethical approval

This current work was approved by the Institutional Animal Care and Use Committee (IACUC), at the Faculty of Veterinary Medicine at Cairo University (Approval number: Vet CU 2009 2022506).

Animals and housing

This study was conducted at Cairo University, Faculty of Veterinary Medicine at the Departments of Theriogenology, Small Animal Clinic. The current study was performed on 40 pluriparous females of German shepherd breed as all dogs were owned by our institution. Bitches weighed 40-60 kg, with an average of 35 ± 1 kg, as well as aged 3–4 years, with an average of 3.6±0.4 years. Females were then divided into two main groups, Group A (n=35) had a normal sequence of estrous cycle with normal durations of both prestrous and estrous phases, whereas Group B (n=5) showed an abnormal sequence as split heat with a short duration of both prestrous and estrous with levels of P4 resembles anovulation in addition to all affected bitches enter next proestrous within 3-4 weeks. All animals received pelleted concentrated food and water ad libitum and were housed in cages.

Ultrasound scanning and Doppler analysis

B-mode ultrasonography was performed at prestrous and estrous phases to confirm the ovulation, and this day was referred to as day 0 till day 9 was considered the prestrous phase, while days 10 to 15 were considered the estrous phase (Concannon *et al*., 2009). B-mode ultrasonogram (Hitachi device Aloka F37, 5-12 MHz frequency; brightness= 85%; depth= 2 cm; acoustic power=90%; with the angle of insonation= 60^0 and PRF=3.5 kHz) was performed by convex array probe (Salama *et al*., 2022).

In addition, B-mode ultrasonography was performed to reveal many follicles, as follicles on that day before ovulation are more than one. Color and pulsed wave mode was activated in order to measure the blood flow of OA in bitch in two groups, then the spectral wave was determined via the ultrasound scanner then Doppler parameters were calculated (Figure 1). These parameters include resistance index (RI), pulsatility index (PI), and peak systolic point of velocity (PSV; cm/sec) of the cardiac cycle as previously measured (Abdelnaby *et al*., 2020; Daghash *et al*., 2022; Farghali *et al*., 2022).

Blood sampling and hormonal analysis

Blood samples were collected from the jugular vein using 2-mL syringes and then centrifuged at 2000×g for 10 minutes. Both plasma and serum samples were stored at −18°C for hormonal assay. Estradiol 17β (E2) and progesterone (P4) were assayed in plasma samples using DRG diagnostics (Diagnostic Reagents) using an ELISA kit with a catalogue reference number (eia-4140), with 9.8 pg/mL and 5.44 ng/mL assay sensitivity test for E2 and P4, respectively. At the same time, NO is determined by serum samples using the Griess reagent (Abo El-Maaty and Abdelnaby, 2017; Hahn *et al*., 2017; Hashem *et al*., 2022). Insulin-like growth factor type 1(IGF-1; ng/mL) was measured from plasma samples using a competitive type of ELISA (DRG, Germany, RUO in the USA) (Csapo *et al*., 1973).

Vaginal cytology

Vaginal smears were taken from these bitches once every five days to monitor the development of the vaginal cells. The procedure involved inserting a sterile vaginal swab into the vulva from the top with a clockwise rotatory movement until reaching the anterior vagina. The swab was then placed on the clean glass slide and stained for five minutes with freshly prepared methylene blue 0.1%. Eventually, the slide was inspected with a low-power (X10) and high-power (X400) microscope lens to evaluate the estrous cycle stage and monitor the development of vaginal cells (Faten *et al*., 2018).

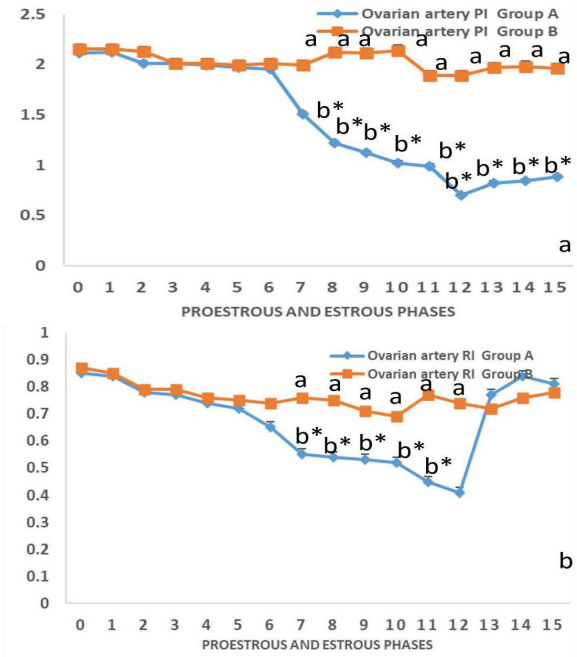

Fig. 1. Alterations in ovarian artery pulsatility index (PI ;a) and resistance index (RI;b) in both groups. Data are presented as mean ± standard error of the mean. a and b values are significantly different at $p < .05$ compared with the control and other group, while * value is significantly different at $p < .05$ between two groups at the same time point.

Statistical analysis

First, all data were analysed using the Kolmogorov–Semenov normality test in SPSS version 20 to confirm data homogeneity. Second, data were expressed as mean ± standard error (SEM) via Student's t-test to determine the significance at each time point between the two groups. Statistical significance was set at $P < 0.05$.

Results

Ovarian artery blood flow

Both OA Doppler indices were measured, as shown in (Figure 2). The PI and RI of the ovarian artery decreased significantly ($P< 0.05$) in Group A compared to Group B, with the most significant decline occurring from day 7 to day 12 during the prestrous and estrous phases, as shown in Figure 2. (Figures 2a and 2b). According to (Figure 2), ovarian artery blood velocity was measured by peak systolic velocity (PSV cm/sec). PSV was significantly ($P < 0.05$) higher in Group A than in Group B, with the greatest increase observed from day 9 to day 12 during the prestrous and estrous phases.

Estradiol (E2) and nitric oxide (NO) levels alterations

Compared to Group B, plasma levels of oestradiol 17 ß (E2; pg/ml; Figure 3) were significantly ($P < 0.05$) higher in Group A. The greatest increase was determined between day 7 and day 12 during the prestrous and estrous phases. In contrast, the serum levels of nitric oxide (NO; µmol/L) in Group A were significantly ($P < 0.05$) higher than in Group B. The greatest increase was observed between day 8 and day 12 during the prestrous and estrous phases.

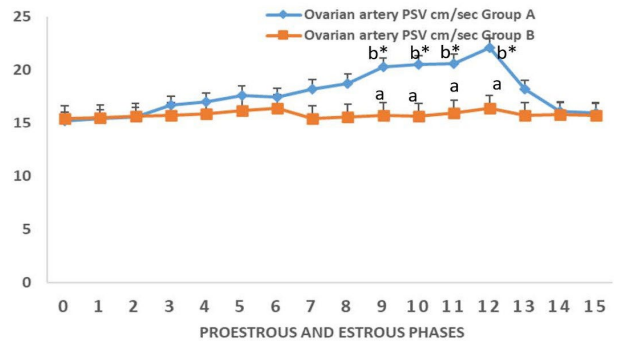

Fig. 2. Alterations in ovarian artery peak systolic velocity (PSV cm/sec) in both groups. Data are presented as mean ± standard error of the mean. a and b values are significantly different at p < .05 compared with the control and other group, while * value is significantly different at p < .05 between two groups at the same time point

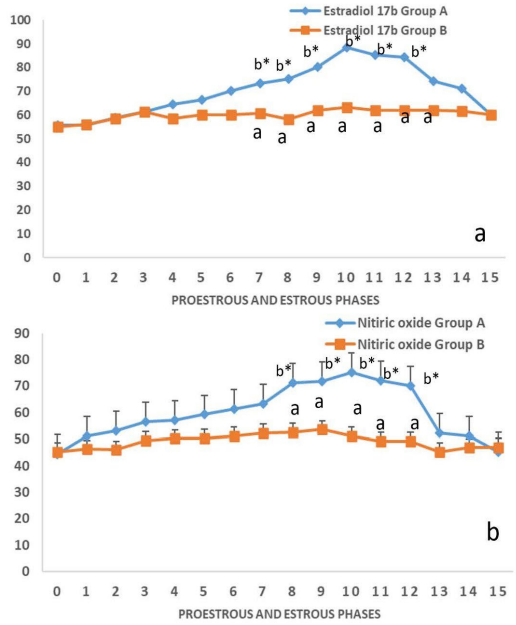

Fig. 3. Alterations in plasma levels of oestradiol 17 b (pg/ml, a) and serum levels of nitric oxide (NO; µmol/L, b) in both groups. Data are presented as mean ± standard error of the mean. a and b values are significantly different at p < .05 compared with the control and other group, while * value is significantly different at p < .05 between two groups at the same time point.

Progesterone (P4), and insulin-like growth factor type -1(IGF-1) levels

By following up and comparing the progesterone hormone profile in the two groups from Day 0 to Day 15 in Group A, it was found that the mean± SE of progesterone concentration was 0.65±0.05 ng/ml on Day 0. Subsequently, it was elevated (P<0.05) slightly until it reached 1.29±0.09 ng/ml on Day 5, and progesterone concentration continued to elevate until reaching 2.88±0.224 ng/ml on Day 9 from the beginning of bleeding, then reached 3.5±0.09 and 8.25±0.22 ng/ml on Day 10 and Day 15, respectively. However, bitches showed split heat in Group B. The concentration of progesterone was low and remained low until Day 15. It started at 0.34±0.09 ng/ml on Day 0 and reached 0.79±0.01 ng/ml on Day 15 (Table 1). IGF-1 was also increased (P<0.05) in the prestrous phases that ended on day 9 (155.32±5.26). In addition, its levels were elevated until day 15 (175.56±3.66) at the oestrous phase in Group A compared to another group (Table 1).

Cytological changes

Using vaginal smear to track the progression of vaginal cells and compare the two groups, bitches in Group A cell progression and elevated the percentage of the cornified cells on Day 15, where the cornification index became 95%. However, Group B showed the dominance of small intermediate cells and RBCs from Day 1 until Day 20 without any progress, despite the presence of intermittent bloody vaginal discharge and receptivity to the male dog (Fig. 4).

Table 1. Alterations in progesterone (P4; ng/mL), and insulin like growth factor type 1(IGF-1; ng/mL) levels in both groups during days of proestrous and estrous. Data are presented as mean ±SEM.

Days	P4(ng/mL)		IGF-1(ng/mL)	
	Group A (Normal)	Group B (Split)	Group A (Normal)	Group B (Split)
D0	0.65±0.05^{a}	0.34±0.09^{a}	134.25±6.12^{a}	126.33±6.32^{a}
D1	0.78±0.12^{a}	0.63±0.04^{a}	136.55±6.22^{a}	131.02±2.36^{a}
D2	0.77±0.09^{a}	0.28±0.01^{a}	137.65±6.85^{a}	130.55±3.15^{a}
D3	0.99±0.25^{a}	0.37±0.07^{a}	143.22±2.36^{ab}	129.36±2.33^{a}
D4	1.29±0.09^{a*}	0.69±0.12^{a}	144.25±4.32^{ab}	127.26±2.68^{a}
D 5	1.29±0.12^{a*}	0.69±0.28^{a}	145.55±6.15^{ab}	133.62±4.25^{a}
D6	1.74±0.31^{ab*}	0.56±0.09^{a}	145.62±5.33^{ab}	132.02±6.33^{a}
D7	1.91±0.08^{ab*}	0.67±0.20^{a}	145.85±4.65^{ab}	133.02±.54^{a}
D8	2.01±0.07^{ab*}	0.69±0.13^{a}	152.65±6.94^{ab*}	134.22±2.85^{a}
D9	2.88±0.22^{b*}	0.81±0.15^{a}	155.32±5.26^{b*}	129.52±2.33^{a}
D10	3.5±0.09^{b*}	0.84±0.14^{a}	156.32±6.36^{b*}	131.55±2.74^{a}
D11	4.26±0.14^{b*}	0.87±0.25^{a}	157.32±5.24^{b*}	131.65±6.25^{a}
D12	6.09±0.25^{b*}	0.81±0.12^{a}	161.25±6.44^{b*}	132.66±6.33^{a}
D13	7.05±0.09^{b*}	0.89±0.20^{a}	165.88±5.21^{b*}	128.65±2.58^{a}
D14	7.81±0.44^{b*}	0.93±0.07^{a}	167.25±5.68^{b*}	133.25±6.36^{a}
D15	8.25±0.22^{b*}	0.97±0.01^{a}	175.56±3.66^{b*}	132.36±5.32^{a}

$^{a\text{ and }b}$ values are significantly different at P <0 .05 within two groups compared to day 0, while * value is significantly different at P <0 .05 between two groups at the same time point.

Discussion

The current study is the first to compare the ultrasonographic alterations of ovaries, E2, P4, NO, and IGF-1 concentrations, and vaginal cytology during the normal estrous cycle and split cycle. Numerous studies have demonstrated that ovarian follicles do not change before and after ovulation. Consequently, ovarian vascular perfusion is essential for determining the ovaries' functional status because the follicle remains a non-ovulated structure as they do not collapse (Ginther, 1995). Therefore, using the standard ultrasound Doppler technology to estimate the canine follicular quality and functionality is essential. Ovarian blood flow (OBF) indicates the accurate vascularity and functional status of the genital organs such as ovaries via Doppler ultrasonography which has been previously applied in many other species to determine the changes during prestrous and estrous phases (Evans, 2003; England *et al.*, 2009).

The increase in OBF velocity during the early estrous phase (PSV; cm/sec) may be directly related to the significant decrease in both Doppler indices (RI and PI) in this study, which may be due to the inverse relationship between Doppler indices and velocities (Daghash *et al.*, 2022). Similarly, it was determined that intra-ovarian vascularization increased during the follicular phase during prestrous and the early luteal phase but decreased during the late luteal phase (Terazonoa *et al.*, 2012).

The significant elevation in both estradiol (E2; pg/ml) and (NO; µmol/L) in the early estrous phase in a group with normal cyclic activity and the no change effect observed in another group with split heat could be related to the vasodilation effect of both E2 and NO (Byers *et al.*, 2005). This leads to increased ovarian artery (OA) blood flow pattern and the declination of the vascular wall resistance with pulsatility with the aid of LH. Hence, it helps the arterial dilatation to elevate the flow to preovulatory follicles that directly enhance the follicular quality and increase the nutrient and oxygen delivery (Hellberg *et al.*, 1991), as these nutrients are vital in the ovulatory process. In accordance with our finding, a similar study reported an elevation of OBF at the early estrous phase in normal cyclic bitches (Koster *et al.*, 2001). A similar study reported the use of color Doppler as a method to detect preovulatory follicle vascularity and functionality via hormonal changes (Jurczak and Janowski, 2018). While in split heat, there was a deficiency in the normal progesterone rise, and the process of ovulation, and ovulation did not occur, as this may be related to chronic premature luteolysis with a problem in the LH hormone, which is responsible for angiogenesis and ovulation (Hoffmann *et al.*, 2004;

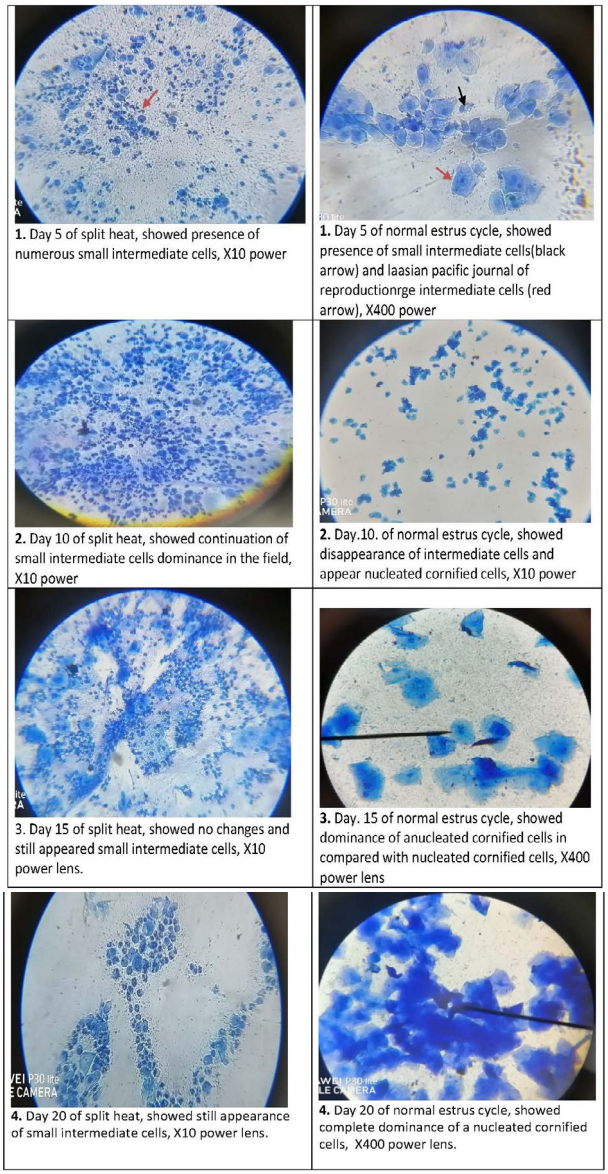

Fig. 4. Image for cytological changes in split heat and normal estrous cycle on Days 5,10, 15, 20 from the beginning of bloody vaginal discharge, showed static appearance of small intermediate cells from Day 5 to day 20 at Fig. A1, 2, 3, 4. But on another side in fig. B1, 2, 3, 4 appeared the progress of vaginal cells from small intermediate cells and large intermediate cells to cornified cells.

Kowaleweski *et al*., 2011), along with other vital hormones (E2 and NO). Therefore, bitch with split heat suffer from no change in hormonal levels as well as in Doppler velocities, but when split heat comes in, young bitches usually recover without any intrusion (Grundy *et al*., 2002).

In a typical estrous cycle, P4 levels progressed from Day 1 to Day 15. The LH surge was found to be concurrent with the initial noticeable increase in circulating P4, making the evaluation of circulating P4 level the most common method for determining the timing of ovulation (Concannon and Patrick, 2011). The P4 increases during prestrous from basal levels to pre-LH surge levels, and then it elevates quickly around pre-LH surge levels and luteinization of follicles, peaking at 4 to 10 ng/ml during ovulation (Davidson, 2006). The progesterone levels in Group A increased from 0.65 ng/ml on day 1 to 8.25 ng/ml on day 15 after the onset of prestrous, as supported by previous research. However, P4 levels in the bitches in Group B with split heat did not rise above 1 ng/ml. Additionally, split estrous in dogs will prevent ovulation from occurring, but without the characteristic progesterone rise, this ailment usually resolves on its own (Grundy *et al*., 2002), continuous or frequent split estrous is indicative of hypothyroidism or chronic premature luteolysis.

In a typical estrous cycle, P4 levels progressed from d1 to d15. The LH surge is discovered to be concurrent with the initial noticeable increase in circulating P4, making the evaluation of circulating P4 level the most used method for determining the timing of ovulation (Concannon and Patrick, 2011). The P4 increases during prestrous from basal levels to pre-LH surge levels, and then it climbs quickly around pre-LH surge levels and luteinization of follicles, peaking at 4 to 10 ng/ml during ovulation (Davidson, 2006). These earlier investigations supported our findings in group A, which showed that progesterone levels increased from 0.65 ng/ml on day 1 to 8.25 ng/ml on day 15 after the onset of prestrous. However, P4 levels in the bitches in group B with split heat did not rise above 1 ng/ml.

In veterinary clinics, vaginal cytology is the quickest and easiest approach since it is simple to perform and needs less equipment. Rapidly available results make it a useful adjunct to gynaecological examination for distinguishing the stage of the cycle and diagnosing infectious, inflammatory, and tumorous diseases in the bitches. This study, which tracked the typical estrous cycle of Group A using vaginal cytology, discovered the presence of cornified cells 7 days after a bloody vaginal discharge. At day 15, these cornified cells still increased above 90%. According to a study, breeding should continue at the time when more than 80% of epithelial cells are cornified since the elevated cornification index is crucial for establishing the best time to do so (Feldman and Welson, 1987). This finding is supported by a study that tracked the development of vaginal cells in a split heat estrous cycle in fetuses of Group A, which revealed the emergence of tiny intermediate cells, RBCs, a small number of parabasal cells, and a complete lack of cornified cells. According to Koster *et al*. (2001), split heat has an abnormally brief time of prestrous followed by a failure of ovulation, which is caused by low levels of E2, and LH is accompanied by serum P4 pattern of anovulation. IGF-1 directly contributes to the endometrial binding sites leading to the increased production of NO, which affects the vasodilatation mechanism (Schini-kerth, 1999; Galderisi *et al*., 2002), and increases the blood flow in the posters expressed by prestrous bleeding and together in estrous to increase the blood flow toward the ovaries in order to perform the optimum ovulation with the highest quality future oocytes (Zhong and Zhou, 2013).

Conclusion

Assessment of ovarian vascularization allowed the accurate detection of hormonal alterations and estimating ovulation to those with split heats. Doppler ultrasound requires professional experience detecting very small blood vessels such as OA, but it is very accurate and repeatable when combined with blood sampling for hormonal detection and vaginal cytology.

Conflict of interest

The authors declare no conflict of interest.

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