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Exploring of Ovulation Time in Bitches by Utilizing Cytological Survey and Doppler Ultrasonography

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ABSTRACT

There are challenges when monitoring peri-ovulatory ovarian activity in domestic bitches which only ovulate once or twice a year. There is a difficulty to distinguish between pre-ovulatory follicles and/or corpus luteum. As luteinization of follicles subsists before ovulation, follicles do not collapse after ovulation. Thus, the aim of the present study was to predict the exact day of ovulation. Twenty-seven bitches of mixed breeds were examined daily from the onset of proestrus to five days post ovulation by vaginal smear, real-time B-mode and Doppler ultrasonography. Ultrasound was conducted in both standing and lateral recumbency positions using microconvex and linear probes. Vaginal swabs were stained by DIFF QUICK stain. There was high cornification rate (90 %) of vaginal epithelium and substantial increase in follicular size (0.55±0.02 cm) at ovulation day. There was significant increase in ovarian blood perfusion at Day 0 and 1. Maximum coloring seen two days around ovulation time. We concluded that color Doppler ultrasound performed once daily was more accurate in predicting the preovulatory LH peak than B-mode ultrasound and vaginal cytology.

1. INTRODUCTION

An elaborated knowledge about reproductive physiology of dogs, it is important to support and enhance the prosperity of canine reproductive biotechnology (Uchoa et al., 2012). Reproductive cycle in bitch is unique compared to other domestic animals; with a mean duration about 7 months. Dogs are non-seasonal, mono-estrus animals with spontaneous ovulation and long inter-estrus interval average about 7 months. The estrous cycle consists of five stages; proestrus (9 days), estrus (9 days), metestrus (60 days), diestrus (60 days) and anestrus (120days) (Vermeulen, 2009). Many efforts are exerted to understand the nature of bitch breeding, time of ovulation and standard mating regimes. Thus, there are investigative methods to monitor pre-ovulatory events and optimal mating time such as vulvar tumescence (Nishiyama et al., 2000), plasma hormonal assay (Lévy and Fontbonne, 2007), examination of exfoliated vaginal cells (Hewitt and England, 2000), vaginal endoscope (Moxon et al., 2012) and Doppler ultrasonography (Vermeulen, 2009).

Oocytes of bitch are ovulated in an unripen state that cannot be fertilized until complete maturation at least 48 h after ovulation by rising plasma luteinizing hormone. Oocytes remain viable for 4-5 days within the bitch reproductive tract (England and Concannon, 2002). The appropriate fertilization time in bitch ranges from two to five days after ovulation or after onset of standing estrus with the fertility declines after Day 7 post-ovulation**.** During proestrus, estrogen elevates that causes enlarged and turgor vulva until LH surge then after estrogen diminishes, edema loss with progesterone accretion. There are diversified vaginal epithelium cells in bitch (Vermeulen, 2009). These cells are related to estrous cycle stages for clinical diagnosis. During early proestrus, there is gradual shifting from parabasal, intermediate to superficial cells, neutrophils, background mucus, RBC and bacteria (normal flora). At late proestrus, neutrophils decrease and predominant of superficial cells exist. At estrus, hyperplasia of vaginal epithelium occurs, 90% cornified cells, less mucus, low RBC, clear background and neutrophils are absent. During diestrus, superficial cells diminish, intermediate cells and neutrophils reappear**.** Early proestrus and diestrus are very similar, so one vaginal smear is not adequate for differentiation (Raskin and Meyer, 2001). Ovarian ultrasonography is an auxiliary tool for monitor follicular growth evolution during estrus cycle and defining the time of ovulation by repeat examination. B-Mode ultrasound is a practical and low-cost technique. The ovaries could be found in the area adjacent to the kidney by finding the caudal pole, then scanning in a sagittal plane caudomedial to caudolateral of the pole. A gradual increase in diameter of ovarian structures (follicles) is correlated with the increase of estrogen secretion (Hayer et al., 1993). The accuracy of ultrasonography to evaluate ovulation in bitch is 91.7% (Lévy and Fontbonne, 2007), but Doppler ultrasonography has a complementary information about cyclic changes inside the ovary. It is more accurate tool for identifying pre-ovulatory LH peak than B-mode ultrasonography (Köster et al., 2001).

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Therefore, our study aimed to evaluate the ovulation time in bitches by using the color Doppler ultrasonography, B-mode ultrasonography and vaginal cytology as dependable methods.

2. MATERIAL AND METHODS

2.1. Animals

The present work was carried out on total of 27 bitches; Egyptian Baladi dogs (n= 5) and dogs of mixed breeds (German shepherd, golden retriever, Caucasian, Presa Canario, pug and Rottweiler, n=22) belonged to farms and shelters. Age ranged from 12 months to 4 years with body weight average (20-25 kg) regarding the breed. The study period extended from 2016 to 2020.

2.2. Vaginal cytology

Vaginal smears were taken daily from bitches as soon as they showed signs of proestrus (swollen vulva and bloody discharge) until diestrus. The focus period started from five days before ovulation (Day -5), ovulation day (Day 0) to five days post ovulation (Day +5). Cotton swabs were wetted with one or two drops of sterile saline and inserted in the vagina at the cranio-dorsal part of the vaginal lumen. The cells on the cotton swab were transferred on to a slide and were stained with DIFF QUICK stain according to Jeffcoate and Lindsay (1989). Numbers and percentage of cornified cells were determined by microscopic examination.

2.3. Ultrasound

All ultrasonographic examinations were done by using Sono Site M-Turbo, USA, equipped with 5-10 MHz Linear probe; Esaote My Lab Gamma, Italy, equipped with 4-12 MHz Micro Convex probe and 10-18 MHz Linear probe. All these probes are Auto adapted with multi frequency at Animal Reproductive Research Institute, Al Haram, Giza. Intra-ovarian vascularization was conducted using color-coded Doppler ultrasonography (Duplex-mode). After the ovaries were located, the amount of blood flow within the ovary was determined. The location of blood flow within the ovary was divided into categories; center of the ovary, wall of the ovarian structures as follicles and corpus luteum similar to Vermeulen (2009). There was scoring system for vascular perfusion quantitation, that extended from minimal to maximal scores 1 to 4.

2.4. Statistical analysis

Values were expressed as means ± standard error for all variables. Statistical analysis was carried out by using Kruskal-Wallis test followed by Dunn-Bonferroni Post-Hoc method to determine the significance differences among days. P values ≤ 0.05 were considered highly significant. Statistical analysis was carried out by using SPSS analysis program (IBM SPSS, 2015) (International Business Machines Statistical Package for the Social Sciences).

3. RESULTS

3.1. Vaginal cytology

All bitches (n=27) showed almost the same results of maximum level of cornified cells detected at the day of ovulation (Day 0). Vaginal smear during different stages of estrus cycle is shown at Figure (1-3). At proestrus, neutrophils and a mixture of parabasal, intermediate and superficial epithelial cells were present. The background of the slide showed an abundance of mucus and red blood cells and a variable number of extracellular bacteria, representing the normal flora (Figure 1). At estrus, 90% or greater cornified cells were present (Figure 2). There was less abundance of mucus, red blood cells and a clear background was noted. Neutrophils were absent and bacteria were still present. At the onset of diestrus, cell populations changed abruptly, at least 20% of superficial cells show a dramatic decrease while the number of intermediate cells showed an elevation (Figure 3). The numbers and percent of cornified cells were calculated in (Table 1). Vaginal cytology showed ascension in cornification index at days related to LH surge, 2 days before (Day -2) and after that (Day +2).

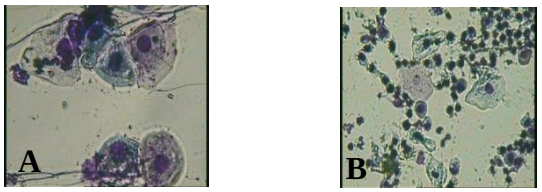

Figure (1): Photomicrograph of a vaginal smear collected during proestrus. A: Parabasal cells have large round stippled nucleus that is surrounded by round basophilic cytoplasm. They are uniform in size and shape. B: Intermediate cells vary in size but are usually two times the size of parabasal cells. The nucleus is smaller than those of the parabasal cells; they have large amounts of keratinized cytoplasm. Their borders are round to irregular and folded.

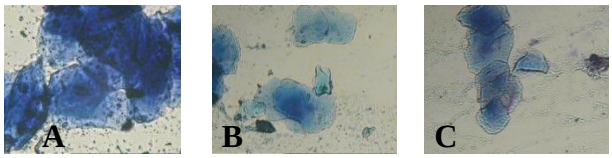

Figure (2): Photomicrograph of the vaginal cells during estrus phase. A & B &C: Superficial cells with small, round, pyknotic nuclei. Their cytoplasm is abundant, keratinized. Cell margins are angular with folded edges. Sometimes these cells lack a nucleus and are than called a nuclear cell.

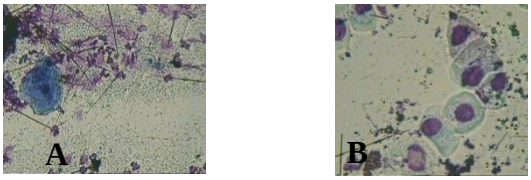

Figure (3): Photomicrograph of the vaginal cells during diestrus phase. A-Superficial cells show a dramatic decrease. B-Intermediate cells show marked increase.

Table 1: Proportion of cornified cells relative to the expected ovulation time.

Time (Days)	Cornification index
-5	1.28 ± 0.11^{a}
-4	1.48 ± 0.10^{a}
-3	1.72 ± 0.11^{a}
-2	2.48 ± 0.13^{a}
-1	2.56 ± 0.13^{a}
0	2.80 ± 0.08^{b}
1	2.48 ± 0.12^{c}
2	2.16 ± 0.12^{d}
3	1.80 ± 0.10^{d}
4	1.44 ± 0.10^{d}
5	1.36 ± 0.18^{d}

Day 0 is the expected ovulation time based on ultrasound examination and/or progesterone level. Values (Mean ± SEM) with different superscripts (^{a,b,c}) significantly differed at $p < 0.0001$ (ANOVA test).

3.2. Ultrasound

3.2.1. B-Mode ultrasound

Follicles started to appear five days before ovulation as a round anechoic structure filled with clear anechoic fluid on both right and left ovaries, over the evaluated days (Figure 4). The largest follicle was noticed at Day -1 estimated as (0.54 ± 0.09 cm) and the thickness of wall increased about 1 mm as shown in Table (2).

The process of ovulation was completed within 24hr in all animals but some follicles might undergo total collapse in less time, follicles reached maximum diameter at D-1(0.55 cm). On ovulation day (D0), the ultrasonographic

characteristics were different among animals; as some follicles undergone complete collapse remaining no structures on ovary. Some cases undergone partial collapse leaving an un-ovulated follicle as an anechoic structure (cystic structure) which remained after ovulation for 3-5 days (D3-D5). They were oval in shape, in other cases hypoechoic structure was detected in the day of ovulation which was irregular in shape as in Figure (5). An echoic free fluid (unovulated follicles) was detected in some cases in the ovarian bursa few hours after ovulation and remained up to three days, this fluid released from ruptured follicles.

At the day after ovulation (D+1), the blood accumulated in a young corpus haemorrhagicum which was very similar to the preovulatory follicle so it was harsh to differentiate between them by B-mode thus color Doppler helped us at this time. Corpus luteum appeared as a hypoechoic structure with or without anechoic cavity and remained for three to five days.

Table 2: The changes in follicular diameter during pre-ovulatory period.

Time (Days) of estrus cycle	Follicle diameter (cm)
-5	0.32±0.02[a]
-4	0.37±0.01[a]
-3	0.42±0.02[b]
-2	0.50±0.02[c]
-1	0.55±0.02[d]
0	0.49±0.02[d]

Day 0 is the expected ovulation time based on ultrasound examination. Values (Mean ± SEM) with different superscripts ([a,b,c]) significantly differed at p< 0.0001 (ANOVA test).

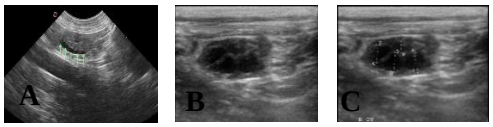

Figure (4): Trans-abdominal ultrasonogram of a bitch showing an ovary at day -5, day-4 before ovulation. A: Ovary at Day -5: Growing follicles appeared as multiple, anechoic structures within the ovarian stroma. B &C: Ovary at Day -4: Follicles increase in size as anechoic fluid filled structure.

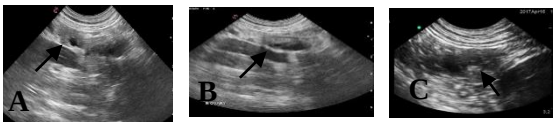

Figure (5): Trans-abdominal ultrasonogram of a bitch showing an ovary at and after ovulation with different ovarian changes. A: Anechoic free fluid at ovarian bursa. B: Remaining unovulated follicle. C: The ovarian bursa surrounding the ovary appeared as a hyperechoic 'halo'.

3.2.2. Doppler ultrasonography

Color-coded blood flow was detectable in most ovaries (D -2, 0, +2). As the follicular phase progressed, the number, extent and intensity of intra-ovarian color pixels increased gradually (Figure 6). The increase in pixel density often occurred around the LH peak. In most dogs, maximal coloring was seen two days after the LH surge. At Day 0, the highest vascular perfusion reached to peak, then gradually decreased after that (Table 3). By using a scoring system for subjective quantitation of vascular perfusion, the colored density varied from minimal to maximal scores 1 to 4, as group 1: 0 -10% (none to almost none), group 2: 11- 20% (low), group 3: 20- 40% (medium) and group 4: 40% and more (high).

Blood flow before the LH surge was only detectable in a small area at the base or center of the ovary. Subsequently blood flow was detectable in the walls of the ovaries and/or in the area involving the corpus luteum (Figure 7). Blood flow was noticed at the base or center of the ovary during early stages of follicular development. At Day-2, blood flow started to increase in the vicinity of follicular wall and then at ovulation (Day 0) the color pixel increased in follicular wall at the center of the ovary. After ovulation, blood perfusion increased with high color flow mapping as in Table (3), blood flow was present at the area of CL, then following Day 5 color flow gradually decreased and became at the center until it completely disappeared.

Table 3: Mean area of ovarian blood flow (blood perfusion density) in days related to LH surge.

Time (Days)	Doppler
-5	1.32±0.11
-4	1.52±0.13
-3	2.00±0.14
-2	2.56±0.12
-1	2.64±0.13
0	3.64±0.11
1	3.48±0.14
2	3.36±0.15
3	2.60±0.21
4	2.04±0.21
5	1.80±0.19

Day 0 is the expected ovulation time based on ultrasound examination. Values (Mean ± SEM) with different superscripts significantly differed at p< 0.0001 (ANOVA test).

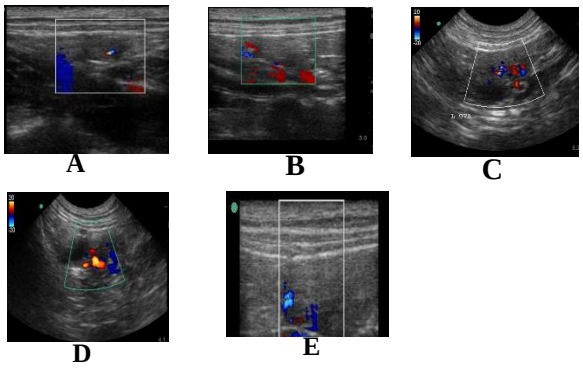

Figure (6): Ovarian parenchyma with Color-Doppler ultrasonographic images showing blood flow increase within follicular phase progression and after that (around ovulation time). A: Day -5 with low blood flow at ovarian parenchyma, with no apparent follicles. B: Day-4 with increase in blood flow and apparent follicles with average size 0.4cm with blood perfusion in wall. C: Day0 (ovulation day) with marked increase in blood supply in base of ovary. D: Day2 with blood supply at the wall of CL. E: D5 after ovulation with formation of CL and blood flow at CL wall.

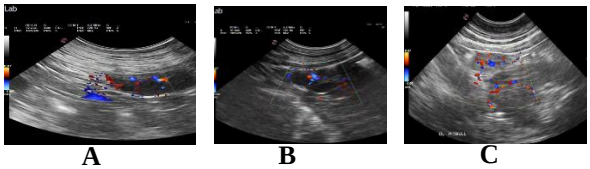

Figure (7): Doppler ultrasonographic image showed different blood flow in the base and center of the follicles and in the wall of CL of ovary. A: Blood flow at follicular wall (early stage of proestrus). B: Blood flow at the center/base of ovary (ovulation time). C: Blood flow at the wall of CL.

4. DISCUSSION

It is clarified that canine reproduction physiology is diverge than other animal species. There is a relation between onset of vulvar bleeding at pro-estrus and the ovulation time. The day of ovulation is not linked to estrous cycle duration. The ovulation time occur at 11 days after onset of vulvar bleeding (Hori et al., 2012). In the current work, we could predict ovulation day by using B-Mode ultrasonography and vaginal cytology. The ovarian follicles at five days before ovulation and CL after ovulation by 5 days could be estimated. Largest follicles were existed at Day-1 and Day 0 (0.55 and 0.49 cm respectively). Also, Mansour et al. (2020) recorded that the average size of pre-ovulatory follicle was 5.8 mm. These values were similar to those reported by Fontbonne (2008) and Le Fresne and Juillet (2010) as pre-ovulatory size was 5.9 to 5.3 mm, respectively. Thus, maximum numbers of follicles and/or corpus luteum was

related to the day of LH surge (Day -1, 0, 1) (Köster et al., 2001).

The most common cause of conception failure is an opportune breeding time (Johnston et al., 1994). Microscopic examination of vaginal epithelial cells is a simple method to keep an eye on the stages of estrous cycle. In the current study, there were mixtures of neutrophils, parabasal cells, intermediate and superficial cells at proestrus. At estrus, 90% of cells were cornified, and no neutrophils were observed. At diestrus, superficial cells wane and intermediate cells boosted. In a similar manner, at estrus, the percentage of superficial and squamous cells was above 80 % (Manda et al., 2004). Breeding can occur at two to three days interval until diestrus begins, as recognized by the appearance of parabasal cells and neutrophils. Likewise, England and Concannon (2002) and Vermeulen (2009) found that vaginal cytology from craniodorsal part of vaginal lumen showed 90% cornification at estrus or at the day of LH surge and 4-5 days after until reach to 80-100 %. Leucocytes were absent in smear 10 days before and 10 days after LH surge. It could be concluded that color doppler was a promising noninvasive diagnostic mean particularly when combined with vaginal cytology for prediction of ovulation day in bitch.

5. CONCLUSION

In conclusion, we can determine the exact ovulation and mating time in bitch with help of reliable useful methods as vaginal cytology and color Doppler ultrasonography.

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