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Periovulatory time in the bitch: What's new to know? Comparison between ovarian histology and clinical features

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ABSTRACT

The ability to recognize specific events happening in the ovaries during periovulatory time allows optimal management of canine reproduction. The objective of this study was to assess the efficacy of vaginal cytology and blood progesterone (P4) assay to identify accurately the changes occurring at the ovarian structures, mainly during the fertile period. Tertiary follicles, *corpora hemorrhagica* (CHs) and *corpora lutea* (CLs) from forty healthy bitches undergoing ovariohysterectomy were evaluated by histo-morphometry based on their aspect, number and size. The tertiary follicles distribution (small, medium and large) was statistically different ($P < 0.002$) among all the stages of the reproductive cycle, except for small follicles (<2 mm), which were always observed from proestrus to anestrus. Very large follicles (>4 mm) were predominant ($P = 0.008$) around ovulation when P4 mean level was 6.1 ± 1.7 ng/mL. The early postovulatory estrous period was characterized by CHs ($P < 0.002$) and P4 level of 16.7 ± 5.9 ng/mL. The end of the fertile period – start of diestrus – coincided with the development of CLs ($P = 0.001$) associated with a P4 mean level of 73.9 ± 9.9 ng/mL. The small ($P < 0.001$) and medium ($P < 0.05$) follicle diameters were positively correlated with the bitch size. The number of follicles larger than 4 mm was significantly lower in bitches younger than 4 years ($P < 0.02$). This study provides insight into some critical steps in the canine reproductive processes in the periovulatory phase and the end of the fertile period, essential to plan breeding programs.

1. Introduction

Canine breeding is increasingly professional and paying close attention to the reproductive physiology. The incorrect identification of the fertile period results in apparent hypo-/infertility in the bitch, especially in cases of single mating/insemination, chilled-frozen or low quality semen. Changes occurring at the ovary level during the periovulatory period and the transition to the diestrus phase constitute the main determinants of the reproductive success in the bitch. Different techniques for monitoring periovulatory events, such as vaginoscopy, behavioural cues and ovarian ultrasound, have been reported (Johnston et al., 2001; Bergeron et al., 2013). Currently, the most reliable method applied to routine breeding management is based on vaginal cytology together with blood progesterone concentration measurement (Fontbonne, 2010). Preovulatory blood progesterone concentration provides an indirect estimate of the ovulation and of the LH surge, which is associated in the canine species with a

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granulosa cells preluteinisation that occur several days before ovulation (Concannon, 2009).

Although behavioural and hormonal aspects are the obvious effects of the ovarian modifications happening throughout the sexual cycle, little is known about the precise quali-quantitative features of the follicular population and luteal structures and their influence on the hormonal profile during the fertile period in the bitch. Therefore, the accurate correlation between the ovarian morphology and hormonal aspects might add novelty, providing specific information relative to crucial events in canine reproduction. This study provides a histologic and histometric survey of the gonads of healthy bitches throughout all the stages of the reproductive cycle, mainly the periovulatory period. Number, structure and dimension of the ovarian structures (follicles, *corpora hemorrhagica* and *corpora lutea*) were evaluated and compared to the vaginal cytology and blood progesterone outcomes.

2. Materials and methods

2.1. Animals

Gonads were obtained from forty healthy cycling bitches (*Canis lupus familiaris*) attending the Reproduction Unit of the Faculty of Veterinary Medicine, Milan (Italy) for routine spaying. Only bitches whose reproductive cycle was monitored before spaying were used in this study. The bitches belonged to different breeds (small sized breeds: <10 kg, $N = 13$; medium sized breeds: 10–30 kg, $N = 18$; large breeds: >30 kg, $N = 9$) and ages (<4 years, $N = 25$; 4–9 years, $N = 10$; >9 years, $N = 5$). The bitches' average weight was 18.1 ± 11.6 kg (from 2.5 to 41.5 kg); average age was 4.1 ± 3.6 years (from 6 months to 13 years). All animals underwent an accurate anamnestic and clinical assessment. Only bitches clinically healthy at the time of the first screening were included in the study. Ovariohysterectomy was performed at specified times depending on the stage of the reproductive cycle, using a standard technique through median laparotomy access in supine bitches (Howe, 2006).

Only bitches having initial plasma progesterone concentration <2 ng/mL were included in the study. Based on the stage of the reproductive cycle (defined as described below) bitches where categorized into 7 groups: proestrus (P; $N = 6$), estrus before ovulation (E1; $N = 6$), periovulatory estrus (E2; $N = 5$), estrus after ovulation (E3; $N = 5$), early diestrus (D1; $N = 7$), middle-late diestrus (D2; $N = 6$), anestrus (A; $N = 5$).

2.2. Methods for reproductive cycle monitoring

The reproductive cycle was monitored by vaginal cytology and plasma progesterone (P4) measurement, starting from proestrus. The optimal time for surgery was chosen on the basis of subsequent monitoring of these two parameters. Vaginal cells were collected from the anterior portion of vagina using a sterile metal spatula after vulvar labia spreading. The spatula was inserted at dorsal commissure of the vulva, avoiding clitoral fossa then advanced at 45° angle and gently rubbed on vaginal vault. Cells from spatula were gently transferred to cytological slides, air-fixed for haematoxylin and eosin staining (Hemaquick; Tektron, Bornheim, Germany) and examined under an Olympus (Olympus, Tokyo, Japan) BX51 photomicroscope at 100×, 200× and 400× magnifications. Eosinophilic index (EI) was also calculated (England, 2013) from proestrus to early diestrus as:

EI = number of anuclear cells/total number of epithelial cells, examined at 100× magnification.

For progesterone analysis blood samples were collected from the cephalic vein into heparinized tubes. Plasma progesterone concentration was determined using a quantitative test based on ELFA technique (Enzyme Linked Fluorescent Assay; MiniVidas®, BioMérieux, France). The assay combines an enzyme immunoassay competition method with a final fluorescent detection (Brugger et al., 2011).

2.3. Definition of the stages of the reproductive cycle

On the basis of the available literature, the stages of the reproductive cycle, including the intermediate ones, were classified by vaginal cytology and blood progesterone outcomes as follows:

- Proestrus (P) was defined as the period between the first day of appearance of vulvar bloody discharge and just before the LH surge, that is blood progesterone concentration values below 2 ng/mL associated to a corresponding vaginal cytology characterized by the presence of erythrocytes and a mixture of parabasal, intermediate and superficial cells together with neutrophils and bacteria (Concannon and Rendano, 1983; Wright, 1990; Simpson et al., 1998; Johnston et al., 2001; Kutzler et al., 2003; Michel et al., 2011).
- Estrus started at the LH surge, which was identified by P4 values above 2 ng/mL (Wright, 1990; Johnston et al., 2001; Kutzler et al., 2003; Michel et al., 2011). Due to the ovulation occurring at blood progesterone concentration between 4 and 10 ng/mL (Wright, 1990; Johnston et al., 2001; Michel et al., 2011), preovulatory (E1) and periovulatory (E2) estrus stages were categorized from 2 to 4 ng/mL and from 4 to 10 ng/mL blood progesterone concentration values, respectively. Postovulatory estrus stage (E3) was characterized by progesterone concentrations above 10 ng/mL. Superficial cells are the predominant epithelial cell type present in vaginal smears during estrus, together with a decreasing number of erythrocytes and usually absent neutrophils (Johnston et al., 2001).
- Early diestrus (D1) was defined as the period between the first day of appearance of cytological diestrus until the ninth day after (Groppetti et al., 2010). The abrupt change in relative number of epithelial cells, mainly superficial *versus* parabasal/intermediate cells, marks the onset of cytologic diestrus (Johnston et al., 2001). After this time and for a further 50 days middle-late diestrus was considered (D2) (Groppetti et al., 2010).
- Anestrus (A) was characterized by basal values of blood progesterone concentration (<2 ng/mL) and corresponding vaginal cytology characterized by parabasal and small intermediate cells (Johnston et al., 2001; Okkens and