TIMING OF OVULATION USING EOSINOPHILIC INDEX AND PROGESTERONE PROFILE DURING NATURAL AND INDUCED OESTROUS CYCLES IN BITCHES – A RETROSPECTIVE STUDY

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Oestrus was induced in anoestrus bitches (Group A) by administering a sustained release preparation of leuprolide acetate @ 100 µg/kg body weight followed by gonadorelin @ 3 µg/kg body weight on the first day of induced oestrus. All the treated animals evinced proestral bleeding by 4.67±0.21 days and oestrus by 12.67±0.49 days of treatment with a conception rate of 83.3%. Fertile estrus was induced successfully in anoestrus bitches (Group B) by oral administration of diethylstilbestrol @ 0.2mg/kg. body weight consecutively for nine days. Proestrus and oestrus was induced by 6.75±0.48 days and 15.25±0.63 days from the beginning of treatment with a conception rate of 50%. Retrospective studies on timing of ovulation were conducted in natural and induced oestrus using eosinophilic index (EI) and progesterone profile by analyzing samples collected on the first day of treatment, second day of proestrus, first day of oestrus, day of second mating and tenth day of second mating. Ovulation was found to occur 2 to 4 days and 1 to 4 days from the day of oestrus using EI and progesterone profile respectively in Group A and -1 to 7 days and -3 to 3 days from the day of oestrus using EI and progesterone profile respectively in Group B animals. There was good correlation between the predicted days of ovulation as per EI and progesterone profile on first day of oestrus in all the animals. In control animals, good correlation was obtained from the first day of proestrus itself.

Introduction

Interest in dog rearing is increasing greatly and thereby dog breeding is becoming a lucrative venture. Since dogs are monoestrus animals and experience an obligate anoestrus of 2 to 10 months following two months of luteal phase, methods of manipulating canine reproduction are essential for obtaining optimum breeding efficiency. Induction of fertile oestrus in anoestrus bitches is essential in treating infertility conditions and also for maintaining optimum breeding management practices. Cytological and hormonal assessment of ovulation time is essential for the assessment of optimum fertilization period, which is highly essential if artificial insemination using chilled or frozen semen is used. Quality assurance of vaginal cytological studies are highly important since the reduced precision in results may lead to incorrect assessment of the reproductive cycle (Moxon *et al*., 2010) leading to huge economic losses to dog breeders. The main objective of this study is to predict the optimum time of ovulation and thereby breeding in bitches by interpretation of exfoliative vaginal cytology and progesterone profile during natural and induced oestrous cycles.

Materials and methods

Twelve anoestrus bitches of 2 to 5 years old were randomly divided into two equal treatment groups – Group A and Group B. Group A was treated with a sustained release preparation of a GnRH analogue – Leuprolide acetate (Inj. Lupron depot) @ 100µg/ Kg. body weight followed by gonadorelin (Inj. Fertagyl) @ 3µg/ Kg. body weight on the first day of induced oestrus. Animals in Group B were treated with diethylstilbesterol (Tab. Nemestrol) @ 0.2mg/ Kg. body weight orally for nine consecutive days. Six healthy bitches, which showed natural proestrus, formed the control group – Group C.

Exfoliative Vaginal cytological smears and serum samples were collected on the first day of treatment, second day of proestral bleeding, on the first day of induced oestrus, on the day of second mating and tenth day of second mating from all the groups. Vaginal smears were stained using modified Shorr's trichrome stain and evaluated by forming Eosinophilic Index (EI). Expected days of ovulation are calculated by reading off against the EI values of 80%.

$$\text{Eosinophilic Index (EI)} = \frac{\text{Number of keratinised cells} \times 100}{\text{---------}}$$

Number of unkeratinised cells
(Excluding small intermediate and parabasal cells)

Serum samples were analysed for the level of progesterone using Enzymun – Test kit (Boehringer Mannheim). Expected days of ovulation are calculated by reading off against the progesterone level of 4 to 8ng/ml. All the animals were allowed to mate with known fertile males twice during oestrus based on vaginal cytological findings. Conception rate, gestation length and litter size were assessed in all the groups. Retrospective analysis of days of ovulation was calculated in conceived animals using eosinophilic index and progesterone profile based on samples collected on the first day of treatment, second day of proestral bleeding and on the first day of induced oestrus.

Results and Discussion

All animals (6 out of 6) in Group A and 4 out of 6 animals in Group B exhibited proestrus in 4.67±0.21 and 6.75±0.48 days respectively and exhibited oestrus in 12.67±0.49 and 15.25±0.63 days respectively from the first day of treatment. The duration of proestral bleeding was 6.67±0.56, 8.50±0.29 and 8.67±0.42 days and duration of oestrus was 8.0±0.45, 7.75±0.48 and 8.0±0.45 days in Group A, Group B and Group C respectively. A conception rate of 83.3%, 50% and 83.3% with a litter size of 5.6±0.75, 6.0±0.58 and 5.6±1.17 was obtained in Group A, Group B and Group C respectively.

Fertile oestrus could be induced in anoestrus bitches with higher conception rate and normal litter size in Group A animals by administering a slow release preparation of leuprolide acetate @ 100µg/ Kg. body weight followed by gonadorelin on the first day of induced oestrus. This single dose regimen was more effective and convenient as shown by Inaba *et al*., (1998) when compared to other oestrus induction regimens using GnRH where use of continuous infusion or pulsatile infusion pumps were necessary (Vanderlip *et al.*, 1987; Cain *et al.*, 1988, Shille *et al.*, 1989; Cain *et al.*, 1989; Concannon, 1989 and Cain *et al.*, 1990). Oral treatment with diethylstilbestrol in Group B animals was found to be comparatively less effective in inducing fertile oestrus. Even though this regimen was simple and cost effective, result obtained were contrary to that of Bouchard (1992) who obtained 100 per cent conception rate with this regimen. Moreover, Mills and Slatter (1981) reported stilbestrol toxicity by administering 10 mg of diethylstilbestrol daily for five days, manifested by anaemia, thrombocytopenia and severe leukocytosis due to bone marrow suppression.

The eosinophilic index (EI) values and progesterone profile values of conceived animals were tabulated in Table 1 and 2.

Table 1. Eosinophilic Index (EI) during different stages of the oestrous cycle in conceived animals (Mean ± SE)

Animal	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	Tenth day of second mating
Group A	19.40±1.41	48.59±1.86	71.28±3.37	83.68±1.30	22.82±4.36
Group B	18.82±1.36	60.49±2.93	85.67±2.30	91.48±2.25	24.19±0.41
Group C	---	63.15±1.44	85.81±1.35	89.38±1.05	21.62±1.71

Table 2. Serum Progesterone profile (ng/ml) during different stages of the oestrous cycle in conceived animals (Mean ± SE)

Animal	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	Tenth day of second mating
Group A	0.72±0.33	0.28±0.08	3.8±1.07	15.12±3.73	17.92±1.15
Group B	0.47±0.27	0.2	5.53±0.66	13.67±2.84	15.87±0.27
Group C	---	0.36±0.12	3.56±0.50	8.12±0.36	17.8±0.58

Modified Shorr's trichrome staining method was very effective in distinguishing the keratinised and non-keratinised cells in the smear. EI values increased gradually in both treated and control animals during proestrus and estrus and dropped to pre-treatment values

during metestrum. Peak values ranged from 63 to 93% as against 56 to 100% reported by Schutte (1967). Wright (1991) studied the ovulation time in Labrador bitches and found that ovulation occurred from four days before to seven days after the maximum EI was reached. Retrospective studies revealed that ovulation and mating occurred during the period of EI peak in all the animals. Therefore EI can be effectively utilised for monitoring ovulation and thereby timing of mating in bitches as reported by Mestre *et al.*, (1990) and Wright, (1991). Statistical analysis revealed significant difference in EI in conceived animals between Group A and Group C on the second day of proestrus, on the first day of oestrus and on the day of second mating. In GnRH analogue treated animals, the EI values and EI peak values were found to be lower during different periods of follicular phase of oestrous cycle when compared to control and diethylstilbestrol treated animals. Even though, EI can be used for predicting the time of ovulation in bitches, the staining procedures are time consuming which limits its routine use in clinical practice.

Serum progesterone levels increased gradually during oestrus and drastically after ovulation. Concannon (1986) concluded that ovulation occurs in bitches when the serum progesterone level reaches 4 to 8 ng/ml. Retrospective studies conducted in conceived animals revealed that ovulation occurred during these levels of progesterone in dogs. Therefore progesterone profile can be effectively used for timing of mating in canines for optimizing fertility. Progesterone levels were significantly higher in treated animals when compared to control animals during late oestrum, which may be due to enhanced luteinisation as observed by Inaba *et al*. (1998) in oestrus induction trials using slow release formulation of GnRH analogue.

Predicted days of ovulation based on EI values and serum progesterone levels are shown in Table 3. Ovulation was found to occur in oestrus induced animals earlier than that in control animals which was probably due to shortened proestrus in treated animals. But in animals where oestrus was induced using diethylstilbestrol, the ovulatory period was inconsistent (over a period of 6 days) which necessitates more frequent assessment of progesterone profile for timing of ovulation. In all animals, the frequency of blood sampling for serum progesterone estimation has to be increased (at 2 to 3 days interval) for more precise timing of ovulation (Concannon, 1986).

Table 3. Predicted days of ovulation based on EI values and serum progesterone level

Animal	Predicted days of ovulation					
	From first day of treatment		From first day of proestrus		From first day of oestrus	
	Using EI values	Using P_4 profile	Using EI values	Using P_4 profile	Using EI values	Using P_4 profile
Group A	13 to 15	13 to 16	8 to 10	8 to 11	2 to 4	1 to 4
Group B	13 to 21	13 to 19	6 to 14	6 to 11	-1 to 7	-3 to 3
Group C	---	---	6 to 13	10 to 14	-2 to 6	0 to 4

The correlation in prediction of ovulation days between these two techniques (EI and progesterone profile) are shown in Table 4.

Table 4. Correlation on prediction of ovulation using EI and progesterone levels

Animal	Days of sampling		
	From first day of treatment	From first day of proestrus	From first day of oestrus
Group A	0.98	-0.63	0.75
Group B	0.50	0.00	0.87
Group C	---	0.92	0.85

There is good correlation between the predicted days of ovulation as per EI and progesterone profile on first day of oestrus in all the animals. In control group good correlation obtained from the first day of proestrus itself. This clearly shows that ovulation time is variable in oestrus induced bitches and prediction of ovulation time is possible only by the onset of oestrus. But good prediction of ovulation time is possible in control animals from the first day of proestrus itself.

Since dogs are multiple ovulators, ovulations occurs over a duration of 2 to 3 days and the fertile life of spermatozoa in the female genital tract is 7 to 10 days which favour the process of fertilization. But precise timing of ovulation is necessary in animals with oestral aberrations, oestrus induced animals and when chilled/ frozen preserved semen is used for artificial insemination where the life span of spermatozoa are compromised. For more precise timing of ovulation, cytological and / or serum samples for progesterone estimation has to be collected optimally at an interval of 3 days from the first day of oestrus.

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