



Correlational analysis of anuclear cell index and serum progesterone concentration for predicting the optimum time of breeding in subfertile bitches[#]

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Abstract

Twenty bitches with previous history of subfertility presented for breeding management at University Veterinary Hospital, Kozhikode were selected for the study. The stage of oestrous cycle was determined and breeding scheduled based predominantly on anuclear cell index (ACI) and serum progesterone assay, after giving due weightage to behaviour and clinical signs. First mating was considered as day 0 and the second as day 3. The mean ACI of the bitches under study was 56.4 ± 1.53 per cent and the mean serum progesterone levels was recorded as 2.82 ± 0.16 ng/mL three days prior to breeding (day 3). Since, ACI had highly positive correlation with serum progesterone level (Pearson's correlation coefficient being 0.880) in subfertile bitches three days prior to first breeding, it could be inferred that, when the mean ACI reached more than 55 per cent, serum progesterone levels reach values corresponding to presumably the day of LH surge and by considering both the variables, the day of ovulation could be predicted. Hence, the correlation analysis of ACI and progesterone assay can be considered as reliable method to schedule optimum breeding time in subfertile bitches.

Keywords: Anuclear cell index, serum progesterone, subfertile bitches

Rapid and definitive methods for prediction of ovulation in dogs have a major impact on canine reproductive efficiency. Exfoliative vaginal cytology is a simple and widely accepted diagnostic

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method for breeding management in canines for the prediction of ovulation. Behavioural signs of oestrus do not always correlate changes in peripheral plasma hormones. The surge of plasma luteinising hormone (LH) occurred between three days before to nine days after the onset of oestrus (Concannon and Rendano, 1983). The epithelial cells of anterior vagina have been broadly classified as parabasal, intermediate, superficial and anuclear and further as nine cell types under these four broad categories (Christie *et al.*, 1972). During proestrus, oestrogen levels begin to rise, causing the vaginal epithelium to become hyperplastic, thickened and more cornified. The cornification of the cells increased by around 10 per cent per day until above 80 per cent, during oestrus. When the proportion of cornified cells in the vaginal epithelium appeared above 80%, it was considered as optimal breeding time (Concannon *et al.*, 1989).

Anuclear cell index (ACI) offers advantages over other methods of classification of exfoliative vaginal cells like superficial cell index (SCI) (England, 1992; Simon *et al.*, 1998; Simon, 2001; Simon, 2014). The ACI is calculated by counting superficial cells with small opaque nuclear remnants, superficial epithelial cells with nuclear membrane only and superficial epithelial cells without nucleus. Simon (2014) compared the ACI and SCI and observed higher conception rate in groups mated based on ACI than SCI (84% vs 66%, respectively).

Duration of preovulatory LH surge is comparatively longer in canine and ranged from one to five days compared to other species (de Gier *et al.*, 2006). The progesterone concentration at the time of LH peak was recorded as 2.1 ± 0.7 ng/mL ($n = 16$) and ovulation was contemplated to have occurred 48 h after the preovulatory LH surge (Bouchard *et al.*, 1991). Ververidis *et al.* (2002) reported that the serum progesterone level increased from less than 1.0 ng/mL during anoestrus and early proestrus to greater than 5-6 ng/mL at ovulation. The authors stated that serum progesterone levels ≥ 5 ng/mL indicated ovulation. As per the report from Marseloo *et al.* (2004), progesterone concentration at the time

of ovulation was fairly constant irrespective of breed, which made serum progesterone assays one of the widely accepted and reliable method to determine ovulation in bitches.

Pinpointing ovulation day on the basis of progesterone assay alone require a series of serum progesterone estimation which might not be cost-effective. Reliable correlation of ACI and serum progesterone level will minimise the number of progesterone estimations and thus be helpful for the pet parents of subfertile bitches to schedule breeding with highly pedigreed males. The present study was designed to correlate ACI and serum progesterone level three days prior to first breeding in subfertile bitches so as to predict optimum breeding time in such animals.

Materials and methods

The study was conducted in twenty subfertile bitches aged between three to six years presented for breeding management at University Veterinary Hospital, Kokkalai. Bitches with history of failure of conception after one or more previous breedings were considered as subfertile (Bouchard *et al.*, 1991). The bitches included in the present study belonged to seven different breeds; Rottweiler (25%, $n=5$), Labrador (25%, $n=5$), Dachshund (15%, $n=3$), Dobermann (15%, $n=3$), German Shepherd (10%, $n=2$), Golden Retriever (5%, $n=1$) and St. Bernard (5%, $n=1$). The stage of oestrous cycle was determined and breeding scheduled based predominantly on ACI and serum progesterone assay after giving due weightage to behaviour and clinical signs like vulval softening/compressibility.

Sample collection and examination of slide for exfoliative vaginal cytology

A cotton tipped sterile swab (PW 005, sterile hiculture collecting swab, Himedia laboratories, Mumbai, India), after moistening with sterile saline, was introduced through the vestibule to the caudal vagina through the dorsal commissure of the vulva (Johnston, 1995; Simon, 2014). Care was taken to avoid clitoral fossa and the swab was withdrawn carefully avoiding pressure.

A thin smear was prepared from collected epithelial cells by rolling the cotton tip of the swab along the length of a clean microscopic glass slide which was then air dried.

The prepared smear was then fixed on slide with methanol spray and then air dried. The slides were stained with Field's stain B (Field stain solution B- Eosin Y in phosphate buffer solution, Nice chemicals Pvt. Ltd.) for 20 sec. The stained slide was then rinsed with tap water, air dried and further stained with Field's stain A (Field stain solution A- Methylene blue and azure 1 dissolved in phosphate buffer solution, Nice chemicals Pvt. Ltd.) for 40 sec. The stained slide was then rinsed with tap water and allowed to air dry.

Smears stained by Field's staining were examined microscopically under low power initially and thereafter under a high power (400 X magnification) to establish cell morphology in detail. A count of at least 100 cells was made from a uniform area of the smear to obtain the percentage of cells viewed.

Estimation of serum progesterone

Blood (4 mL) was collected by cephalic venipuncture in the red topped clot activator vacutainer (Ultimate®) for estimation of serum progesterone concentration on Day -3 (in this study, Day 0 was considered as day of first mating). Day -3 was considered as presumably the day of LH surge when ACI reached around 50 per cent by taking in account of the findings of England (1992) and Simon (2014). Sample was kept undisturbed for one hour. Automated chemiluminescent immunoassay (CLIA) by Beckman Access 2 analyser was used to determine serum progesterone level (ng/mL). In cases where the serum progesterone values were below 1.9 ng/mL, a review exfoliative

vaginal cytology and serum progesterone estimation was performed one or two days later depending on the value.

Results and discussion

Anuclear cell index (ACI) on Day -3

The mean ACI of the bitches under study was 56.4 ± 1.53 % (range: 40 to 65%) on Day-3 (Table 1). According to England (1992), the maximum percentage of anuclear cells in bitches with one peak of ACI ranged from 43-94 per cent, while in bitches with two peaks the same ranged from 57-98 per cent at the second peak. Simon (2014) demonstrated that the maximum peak of anuclear cells was between 48 and 96 per cent. However, the mean ACI obtained in the present study was higher than the observations of Anish (2015), where ACI ranged from 10 to 51 on Day -3 (n=5).

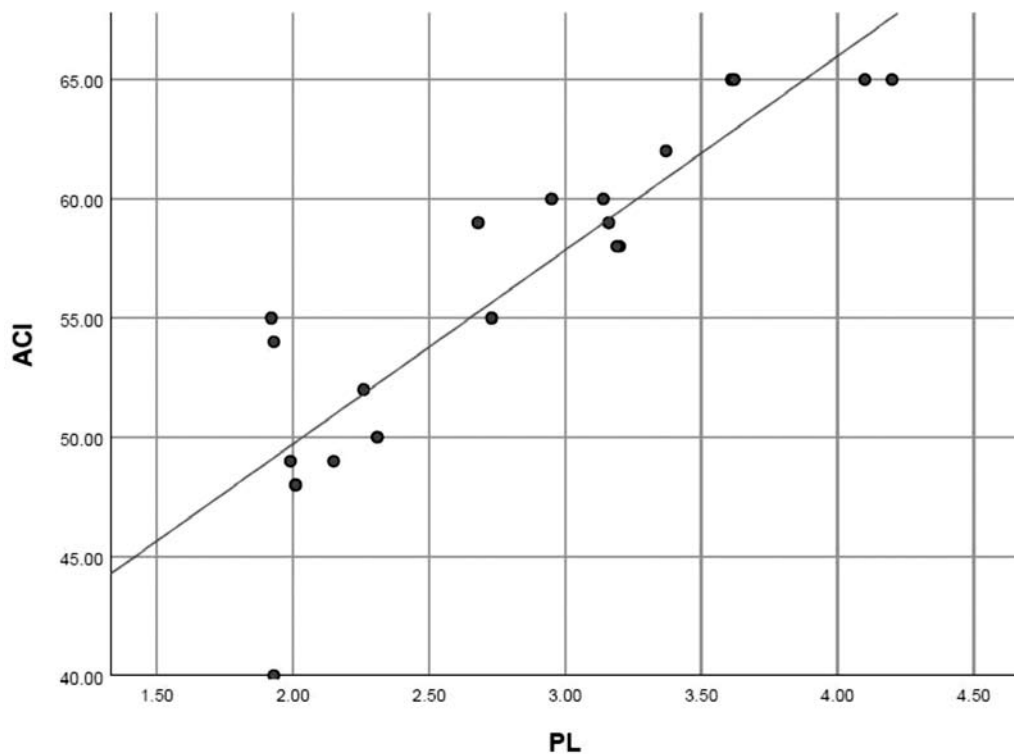
In the present study bitches were mated on Days 0 and +3 and the mating time was in accordance with the findings of England (1992) where the mean interval between calculated day of LH peak and anuclear cell peak was 1.9 ± 2.1 days, ranged from -2 to +6 days for the bitches with one anuclear cell peak. Similarly, Simon (2014) also reported that the mean interval between the calculated day of LH and the peak of anuclear cells was 3.6 ± 2.1 days.

Serum progesterone level (ng/mL) on Day -3

The mean serum progesterone level of the bitches under study was 2.82 ± 0.16 ng/mL (1.93 to 4.1 ng/mL) on Day-3 (Table 1). This was in accordance with Concannon *et al.* (1977), who reported that the plasma progesterone levels reached 1.0-1.9 and 2.0-2.9 ng/mL at 3 and 2 days prior to ovulation.

Table 1. Mean value of anuclear cell index and serum progesterone level three days prior to breeding in twenty subfertile bitches

Variable	Mean	Range
ACI (%)	56.4 ± 1.53	40 – 65
Serum progesterone level (ng/mL)	2.82 ± 0.16	1.93 to 4.1



ACI; anuclear cell index, PL; serum progesterone level in ng/mL

Fig.1. Scatterplot of anuclear cell index and serum progesterone assay three days prior to first breeding- Pearson's correlation= 0.880

Correlation between anuclear cell index and serum progesterone level on day 3

The Pearson's correlation coefficient for these variables (ACI and serum progesterone concentration) was 0.880 (Table 2). In the present study, ACI had significant ($p < 0.01$) correlation with serum progesterone concentration.

In the present study, ACI was having highly significant ($p < 0.01$) correlation with serum progesterone concentration. The Pearson's correlation coefficient for these variables was 0.880 (Fig.1; Table 2). Since there is a positive correlation between these variables, serum progesterone level is elevated with increase in ACI. The mean ACI on Day -3 in the present study was recorded as 56.4 ± 1.53 and the mean progesterone level was 2.82 ± 0.16 ng/mL. By taking in account of the findings of England (1992) and Simon (2014),

Table 2. Correlation between anuclear cell index and serum progesterone level three days prior to breeding in twenty subfertile bitches

ACI	Progesterone level	
	Pearson's correlation	0.880
	Sig. (2-tailed)	0.000**
	N	20

**Correlation is significant at the $p < 0.01$ (2 tailed)

Day -3 was considered as presumably the day of LH surge when ACI reached around 50 per cent.

Thus, it could be concluded that the first mating or insemination can be done at the second peak of anuclear cells or two days after a single peak followed by a second mating two or three days later, so as to achieve maximum breeding efficiency in subfertile canines. Hence, the recommended mating days in the present

study (Days 0 and +3), which was calculated based on ACI and serum progesterone concentration on Day -3, fall within the optimum breeding time.

According to Bouchard *et al.* (1991), progesterone concentrations were 2.1 ± 0.7 ng/mL ($n=16$) at the time of the LH peak and an increase in progesterone level was rapid after LH surge (2.1 ng/mL/day). Ovulation was contemplated to have occurred 48h after the preovulatory LH surge. Hence, the results of this study were in accordance with the findings of Bouchard *et al.* (1991) and Romagnoli (2014) who reported the serum progesterone level of 2 to 3 ng/mL on the day of LH surge. The progesterone concentration recorded in the present study is in agreement with Kutzler *et al.* (2003) where they reported the value at the time of LH peak as 2.2 ± 0.18 ng/mL while the values are slightly higher than the findings of England and Concannon (2002) who reported 2.0 ng/mL of serum progesterone at the time of LH surge. However, bitches exhibited higher values compared to the observations of Anish (2015) where the mean value ranged from 0.73 to 2.32 ng/mL with a mean of 1.32 ± 0.29 ng/mL and the author suggested that the lower values observed might be due to the stressful environment and displacement from their familiar surroundings that resulted in disturbed neuroendocrine pathway and possibly due to ultrasonography performed on a daily basis continuously for two to three weeks from day 1 of pro-oestrus. Kunanusont *et al.* (2021) reported that the serum progesterone concentrations of 2.0 ng/mL indicating the day of the LH surge and 4-10 ng/mL on the ovulation day. As per Rota *et al.* (2016), serum progesterone concentrations in the different phases of oestrous cycle were statistically different with values; anoestrus; 0.38 ± 0.14 ng/ml, proestrus; 1.04 ± 0.67 ng/ml and oestrus; 6.8 ± 7.26 ng/ml which was in accordance with this study.

The stage of oestrous cycle was determined and breeding was scheduled based predominantly on ACI and serum progesterone assay after giving due weightage to vulval compressibility/softening and receptivity/interest shown by male/female. Vulval softening

usually happened about one day after LH surge or during the same period; and ovulation occurred over a period of 38 to 48 h following LH surge (Concannon *et al.*, 1989). England and Concannon (2002) opined, the onset of standing oestrus and LH surge happened at about same period. According to the same authors, three or four days after the onset of LH surge would be considered as the optimum time for breeding in bitches. As per the observations of Bouchard *et al.* (1991), increase in progesterone level was rapid after LH surge (2.1 ng/mL/day). In the present study, first mating was considered as Day 0 and the second as Day +3. Johnston (1995) observed that best conception rate and litter size was attained in female dogs when mating or artificially inseminating with fresh or chilled semen two to three days post ovulation or with frozen semen three to five days post ovulation.

Conclusion

The mean ACI (56.4 ± 1.53) of the subfertile bitches under the study showed significant positive correlation ($p < 0.01$: Pearson's correlation coefficient for the variables: 0.880) with the mean serum progesterone level (2.82 ± 0.16 ng/mL), three days prior to first breeding. When serum progesterone estimations are made based on the peak of ACI the number of progesterone estimations can be reduced. According to the present study, combined use of progesterone estimation and determination of ACI can be considered as reliable methods to schedule the optimum breeding or fertile period in subfertile bitches.

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Conflict of interest

The authors report no conflict of interest.

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