EXFOLIATIVE VAGINAL CYTOLOGY – CELLULAR INDICES DURING DIFFERENT STAGES OF NATURAL AND INDUCED OESTROUS CYCLE IN BITCHES

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Exfoliative vaginal cytology indices were calculated in two groups of oestrus induced animals and one group of animals in normal oestrus. Samples were collected on the first day of treatment, second day of proestral bleeding, on the first day of induced oestrus, the day of second mating and tenth day of second mating. Superficial Cell Index (SCI), eosinophilic index (EI) and karyopyknotic cell index (KPI) were calculated. Even though KPI can be used to assess progression of oestrous cycle and ovulation, requirement of good quality stained smears and precise, time consuming evaluation of nuclear details for formulation of KPI limits its use in clinical practice.
Keywords: Bitches, Cellular indices, Exfoliative vaginal cytology, Oestrous cycle.

Reproductive cycle in dog is unique with a prolonged follicular and luteal phase of oestrous cycle. Increased circulating levels of estradiol - 17β for long periods stimulate the growth of vaginal epithelium from a bistratified epithelium during anoestrus to 20 to 30 cell layers at the end of proestrus. This characteristic metastatic change in the vaginal mucosal epithelium is an indirect indicator of oestrogen level and can be used to monitor the progression of proestrus and oestrus in bitches.

Exfoliative vaginal cytology and progesterone profile are commonly employed by clinicians for predicting the optimum mating time in bitches for getting maximum fertility, predicting whelping dates, evaluation of pathological conditions like vaginitis, ovarian and vaginal tumours, subinvolution of placental sites *etc* (Antonov, 2017; Hahn *et al*., 2017). Apart from normal vaginal cytology, compilation and evaluation of vaginal cytology indices are very useful in assessment of aberrant cycles (Lalib *et al.*, 2018) and induced oestrus (Mogheiseh *et al*., 2017). Quality assurance in vaginal cytology is highly important since reduced precision may lead to incorrect assessment of reproductive events leading to huge losses to the dog breeders. Formulation of various cytological indices will greatly reduce errors in cytological studies and increase the precision of results. In this study, characteristic changes of the vaginal mucosa were studied by formulating cytological indices during different stages of natural and induced oestrous cycles in bitches.

Materials and Methods

Animals for this study consisted of twelve healthy anoestrus bitches of two to five years of age with a history of at least one whelping. Anoestrus bitches were selected based on history and confirmed based on exfoliative vaginal cytological studies. Oestrus was induced in six anoestrus bitches (Group A) with a single parenteral administration of a sustained release preparation of 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. Six anoestrus bitches (Group B) were treated with diethylstilbestrol (Nemestrol tab)

@ 0.2mg/Kg. body weight orally for nine consecutive days. Six bitches in natural proestrus (Group C) formed the control. Vaginal cytology smears were collected on the first day of treatment, second day of proestral bleeding, on the first day of induced oestrus, the day of second mating and tenth day of second mating. All the bitches were allowed to mate with proven fertile males twice during oestrus based on vaginal cytology. First mating was recommended when more than 60% of the exfoliated cells became superficial cells and the second mating was recommended when there was a 10 to 20% increase in the number of superficial cells in the vaginal cytology.

The vaginal smears were stained using Wright-Giemsa's stains and by modified Shorr's trichrome method to demonstrate keratinisation of cells. Various cellular indices were compiled for accurate interpretation of vaginal cytology as follows:

$$\textbf{1. Superficial Cell Index (SCI)} = \frac{\text{Number of cells from superficial layer}}{\text{Number of cells from deeper layer}} \times 100$$

$$\text{2. Eosinophilic index (EI)} = \frac{\textit{Number of keratinized cell}}{\text{Number of unkeratinized cells (Excluding small intermediate and parabasal cells)}} \times 100$$

$$\text{3. Karyopyknotic Cell Index (KPI)} = \frac{\textit{No. of superficial cells with pyknotic nuclei}}{\text{No. of superficial cells with vesicular nuclei}} \times 100$$

SCI, EI and KPI values were calculated during different stages of the oestrous cycle in pregnant and non-pregnant animals separately to interpret the vaginal cytology changes. Pregnancy diagnosis was carried out between 28 and 32 days after first mating by trans-abdominal ultrasonography. Conception rate and litter size were also assessed. The results (Mean ± SE) in these studies were analysed between groups using Mann–Whitney non-parametric test.

Results and Discussion

All animals in Group A and four out of six animals in Group B evinced proestral response in 4.67±0.21 and 6.75±0.48 days, respectively. The duration of proestrus, oestrus, conception rate and litter size in different groups are shown in Table 1. Fertile oestrus could be induced in bitches by using GnRH, gonadotropins as also mentioned by Jaafar and Al-Mutar, 2024, synthetic oestrogens and prolactin antagonists as also reported by Ohtaki *et al.*, 2020.

Superficial cell index (SCI):

Superficial cells and large intermediate cells were taken as cells from superficial layer and small intermediate cells and parabasal cells were taken as cells from the deeper layers of the vaginal epithelium for calculating this index. Superficial cell index (SCI) in all the group of animals are presented in Table 2 (Pregnant animals) and 3 (Non-pregnant animals). In treatment groups, SCI increased rapidly to high values during follicular phase and then declined rapidly during metoestrus which are in consistence with the findings of Arlt (2018). Statistical analysis revealed significant difference in SCI in pregnant animals between treatment groups on the first day of treatment, between Group A and Group C on the first day of oestrus and on the day of second mating. There was significant increase in SCI values from the day of treatment to early oestrus and significant decrease from late oestrus to metoestrus in all animals. This progression in SCI values is due to the metastatic changes in the vaginal mucosal epithelium in response to increased levels of oestrogen in circulation, but the peak values were found fluctuating

between animals. Therefore SCI can be effectively used for monitoring the response to hormonal oestrus induction and to assess the progression of oestrus, but could not be reliable in predicting ovulation in bitches.

Eosinophilic index (EI):

Modified Shorr's trichrome staining method was very effective in distinguishing the keratinised and non-keratinised cells in the smear. Eosinophilic index (EI) in all the group of animals are presented in Table 4 (Pregnant animals) and 5 (Non-pregnant animals). EI values increased gradually in both treated and control animals during proestrus and oestrus and dropped to pre-treatment values during metoestrus. Peak values ranged from 63 to 93%. Retrospective studies revealed that ovulation and mating occurred during EI peak in all the animals. Therefore EI can be effectively utilised for monitoring ovulation and thereby timing of mating in bitches. Statistical analysis revealed significant difference in EI in pregnant 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.

Kariopyknotic index (KPI):

KPI values during various stages of the oestrous cycle in all the group of animals are presented in Table 6 (Pregnant animals) and 7 (Non-pregnant animals). It increased gradually and reached peak during oestrus and decreased to lower values during metoestrus. Statistical analysis revealed significant difference in KPI in pregnant animals between treatment groups and control group on the second day of proestrus and on the day of second mating. KPI values remained significantly higher during early metoestrus in Group B animals which might be due to an extended effect of diethylstilbestrol therapy. Even though KPI can be used to assess progression of oestrous cycle and ovulation in bitches, good quality stained smears and precise, time consuming evaluation of nuclear details of cells in smears were required for formulation of KPI values. Gupta *et al.* (2022) observed high fertilityin Chippiparai bitches when natural mating was allowed at oestrus with a KPI value of 80% or more.

Table 1. Oestrus response, conception rate and litter size in treated and control groups

Group (n=6)	No. of animals responded	Onset of proestrus (Days)	Duration of proestrus (Days)	Duration of oestrus (Days)	Conception rate (%)	Litter size
A	6	4.67±0.21^{a}	6.67±0.56^{a}	8.00±0.45^{a}	83.3^{a}	5.6±0.75^{a}
B	4	6.75±0.48^{b}	8.50±0.29^{b}	7.75±0.48^{a}	50.0^{b}	6.0±0.58^{a}
C	NA	NA	8.67±0.42^{b}	8.00±0.45^{a}	83.3^{a}	5.6±1.17^{a}

Figures having different superscripts in a column differ significantly (P<0.05)

Table 2. SCI during different stages of oestrous cycle in pregnant animals (Mean±SE)

Group	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	10th day of second mating
A (n=5)	25.12±4.18^{a}	132.11±19.39^{a}	1361.59±482.85^{a}	1051.54±191.75^{a}	34.43±4.11^{a}
B (n=3)	15.99±2.15^{b}	162.74±17.10^{a}	730.39±117.46^{ab}	1682.54±461.67^{a}^{b}	37.98±2.08^{a}

C (n=5)	---	147.26±13.14[a]	786.55±102.18[b]	1109.81±63.64[b]	37.77±5.51[a]

Figures having different superscripts in a column differ significantly (P<0.05)

Table 3. SCI during different stages of oestrous cycle in non-pregnant animals

Group n=1	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	10th day of second mating
A	13.79	89.48	430.22	526.57	51.94
B	13.87	222.23	523.83	1167.30	47.50
C	-	118.87	480.72	725.08	27.90

Table 4. EI during different stages of oestrous cycle in pregnant animals (Mean±SE)

Group	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	10th day of second mating
A (n=5)	19.40±1.41[a]	48.59±1.86[a]	71.28±3.37[a]	83.68±1.30[a]	22.82±4.36[a]
B (n=3)	18.82±1.36[a]	60.49±2.93[ab]	85.67±2.30[ab]	91.48±2.25[ab]	24.19±0.41[a]
C (n=5)	-	63.15±1.44[b]	85.81±1.35[b]	89.38±1.05[b]	21.62±1.71[a]

Figures having different superscripts in a column differ significantly (P<0.05)

Table 5. EI during different stages of oestrous cycle in non-pregnant animals

Group n=1	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	10th day of second mating
A	14.42	41.18	67.33	72.82	43.27
B	26.67	61.39	64.15	63.11	27.45
C	-	63.37	86.27	92.16	21.78

Table 6. KPI during different stages of oestrous cycle in pregnant animals (Mean±SE)

Group	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	10th day of second mating
A (n=5)	21.03±2.21[a]	70.79±2.57[a]	79.90±0.99[a]	94.65±1.01[a]	39.66±4.21[a]
B (n=3)	23.09±3.35[a]	75.62±2.08[a]	85.35±1.49[a]	95.23±2.76[a]	50.96±2.46[b]
C (n=5)	-	58.34±2.15[b]	81.97±1.10[a]	89.63±1.67[b]	28.88±1.26[a]

Figures having different superscripts in a column differ significantly (P<0.05)

Table 7. KPI during different stages of oestrous cycle in non-pregnant animals

Group n=1	First day of treatment	Second day of proestrus	First day of oestrus	Day of second mating	10th day of second mating
A	35.19	66.02	81.73	92.93	52.88
B	20.19	79.25	87.62	98.02	56.48
C	-	61.54	74.51	85.58	19.80

Summary

Superficial Cell Index (SCI), Eosinophic Index (EI) and Kariopyknotic index (KPI) were formulated and evaluated during different stages of natural and induced oestrous cycles in bitches. SCI was found to

be effective in monitoring the response to hormonal oestrus induction therapy and to assess the progression of oestrus in bitches. Ovulation can be predicted using EI and KPI, but the staining procedure was lengthy for formulating EI and good quality stained smears with precise, time consuming evaluation of nuclear details of cells in smears were required for formulation of KPI values.

Several researchers like Meghasree *et al*., 2019 also compared the efficacy of different methods for ovulation timing in bitches like us. Skliarov *et al.* 2022 also opined that none of the diagnostic techniques for ovulation timing in dogs is absolutely reliable; therefore use of several diagnostic modalities may be combined to get a most accurate result as we have done. Most of them used a combination of different methods or repeated use of vaginal cytology to identify a time to start progesterone monitoring in serum for timing of mating or insemination. Like us Reckers *et al.* 2022, also developed a tutorial as a flow chart for the accurate identification of different types of vaginal cells, which made the evaluation more objective in nature for a reliable and accurate prediction of events.

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