Vaginal Cytology and Progesterone Level Correlations during Oestrous Cycle Monitoring in Female Dogs

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Abstract

This study investigated the relationship between vaginal cytological examination and progesterone levels (P4) during a 12.5-month period of cycle monitoring in 16 domestic bitches subjected to oestrus induction treatments. By analysing the correlations between P4 rates and cellular parameters (specifically eosinophilia and basophilia percentage), the study aimed to establish a reliable cellular parameter that can serve as an indicator of low progesterone thresholds. Preliminary monitoring was conducted through bi-weekly visits. At each visit, all animals systematically underwent a vaginal smear stained with the Harris-Schorr trichrome method. For P4 assessment, plasma samples were analysed using a radioimmunoassay method. Cycle control during and after oestrus induction was accompanied by clinical, cytological and hormonal monitoring until the gestational state was confirmed. Chi-square test was applied to study the correlation in a population consisting of 282 smear slides corresponding to the corresponding P4 values. Data showed that at the beginning of proestrus, P4 levels were highly significantly correlated with the percentages of basophilic and eosinophilic cells. Among the cytological criteria evaluated for both cellular percentages, having more than 40% basophilic cells on the vaginal smear proved to be the most reliable with a zero margin of error. Its application indicated that when the percentage of basophilic cells is strictly greater than 40%, P4 values are below 2 ng/mL, and once the percentage of basophilia falls below 40%, the P4 assays become valuable. Thus, in the context of cycle monitoring, this cellular indicator could be particularly useful in avoiding costly and unnecessary progesterone measurements and reducing their frequency at the beginning of proestrus.

Key words: *oestrus monitoring; bitch; vaginal cytology; progesterone*

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Introduction

Accurate monitoring of the oestrous cycle in domestic bitches is crucial for optimising breeding practices and ensuring the health of both the mother and offspring (Jaafar and Al-Mutar, 2024).

Vaginal cytology is an underused, simple diagnostic tool that enhances the ability to diagnose and treat diseases of the reproductive tract, and it has been described as useful in monitoring the stages of reproductive cycle and predicting the optimum mating time in the bitch (Kustritz, 2020; Duran and Duran, 2023). Vaginal cells, under hormonal influence, change periodically during the oestrous cycle (Yahia et al., 2024). At the beginning of the follicular phase, the mucosa only contains a few cell layers. As a result of the divisions of the basal layer cells under the action of oestradiol, this mucosa thickens. Then the superficial layers become keratinized. Under P4 influence, the divisions cease, keratinized cells desquamate, polymorphonuclear cells invade the vaginal lumen and destroy them (Bello et al., 2023). However, an isolated smear is of little interest in monitoring a female dog's heat. Indeed, from one female dog to another, the evolution of the cells can vary and requires observing smear properties over several days (Johnson, 2022).

The use of P4 assay to assess the dates of the LH peak and ovulation is justified because, currently, there is no real indicator of ovulation or the state of oocyte maturation. In addition, P4 is the only hormone that is easily measurable (Tammer et al., 1994). Therefore, the measurement of P4 levels is a reliable indicator of ovulation detection in bitches; however, it is not cost-effective to perform an excessive number of assays during the same heat monitoring. P4 assays are generally used in conjunction with other less expensive methods of assessing the stage of the cycle, such as vaginal smears (Keshav et al., 2023). The relative proportion of different types of vaginal epithelial cells can be used as a marker of the endocrine environment (Lamond and Lang, 2005).

In plasma progesterone assays, it is generally considered that a level between 5 and 10 ng/mL indicates ovulation in the female dog. However, the optimal period of fertility, which is approximately two days after ovulation, occurs at a variable P4 level, making it impossible to set a universally valid reference level, resulting in a very wide range (Hoffmann et al., 1996; Wutthiwitthayaphon et al., 2024). Despite the fact that the measurement of P4 levels is performed in conjunction with the vaginal smear in monitoring heat in bitches and detecting the fertile period, few studies have examined the correlations between different cellular markers of the cycle and progesterone levels (Linde and Karlsson, 1984; Haji et al., 2018). In this context, this study aimed to investigate the correlation between progesterone levels and cellular parameters (specifically eosinophilia and basophilia). More specifically, we aimed to establish a reliable cellular criterion that could serve as an indicator of low progesterone thresholds.

Materials and Methods

Animal management & study area

The animals included in the study consisted of 16 female dogs of varying breeds (10 German Shepherds, two Belgian Malinois, one Doberman, one Atlas Shepherd, and one Sloughi), aged 4.63 ± 0.53 years and weighing 25.81 ± 1.53 kg (mean ± SE). They were housed in individual kennels at the Canine Training and Breeding Centre of Baïnem (Al-

giers, Algeria), in a system designed to fully comply with sanitary conditions and psychological comfort. They were fed a single daily ration of super-premium quality industrial dry food and had access to water *ad libitum*. For each female, a complete medical-administrative file was available. The medical follow-up since the arrival of each dog at the centre was included in each individual file. It contained the monthly weighing, vaccination dates, pathological history accompanied by the treatments, and breeding career (dates of heat and mating, male(s) used for mating, dates of whelping, and litter size).

Experimental design

The experiment was divided into two phases: a preliminary monitoring period of 4.5 months and an induction period of six months.

Preliminary study

Preliminary monitoring of the 16 females was conducted through bi-weekly visits, which included clinical examination, vaginal cytology, and blood sampling for subsequent progesterone (P4) determination. The dogs were subjected individually to a general clinical examination and then a special examination of the genital tract to detect clinical and behavioural signs of heat.

Vaginal cytology

At each visit, all the animals systematically underwent a vaginal smear (Figure 1). The vaginal exfoliative cytology was obtained as per the technique demonstrated by Feldman and Nelson (1996). Once the vaginal cells were collected, the cotton was immediately rolled onto the end of a microscope slide which was immediately immersed for five minutes in an alcohol-ether mixture for fixation. The slide was then stained according to the Harris-Schorr trichrome method (Schutte, 1967; Corvelyn et al., 2022) using the kit ACCUSTAIN® Harris Hematoxylin Solution (Sigma Aldrich, United Kingdom). In canine oestrous cycle control, the Harris-Shorr technique is the stain of choice. Keratinization of epithelial cells is highlighted: acidophilic cells become orange-red as keratinization progresses. Indeed, this staining makes it possible to identify intracellular keratin precursors that are abundant in epithelial cells during the follicular phase and that stain orange (Neveux, 1999).

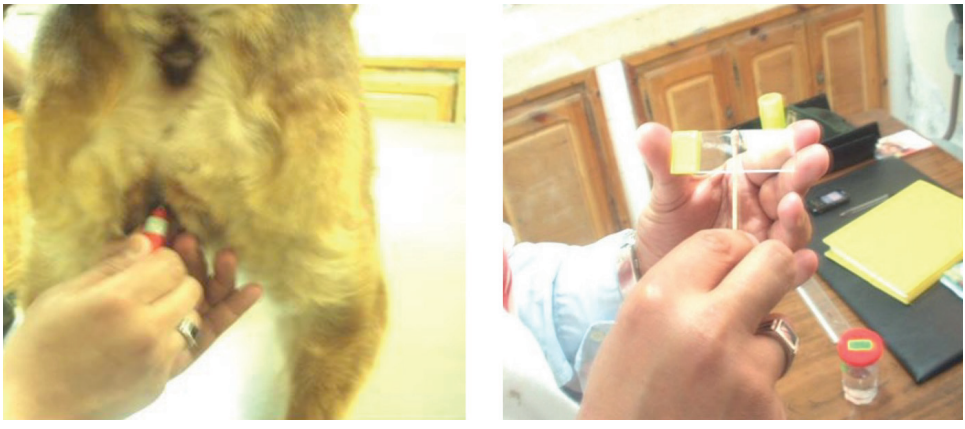


Figure 1. Vaginal smear

Cell counting was performed based on a minimum of 30 epithelial cells. For this, the slides were viewed at a higher magnification (x100 and/or x400). Microphotographs of these slides were captured using a special microscope camera Moticam 350 and professional software Motic Images Plus (Version 2.0, ML Motic China Group Co., Ltd.).

Progesterone assay

Following the clinical and cytological examination of the female and in order to assess P4, blood samples were taken from the radial or saphenous vein using a vacutainer device on a pre-identified heparinized vacuum tube, centrifuged and stored in Eppendorf tubes at -20°C until analysis. The plasma samples were analysed at the Zootechnics Laboratory of the Draria Nuclear Research Centre (Algiers, Algeria) using a radioimmunoassay method (Kit Immunotech, Beckman Coulter, France).

Oestrus induction

The 16 animals were divided into two experimental groups of eight females and received one of two usual induction treatments (dopamine agonists: bromocriptine and cabergoline). Induction treatment began simultaneously on the same day for both groups. Group A received bromocriptine (Parlodel® 2.5 mg tablets, Novartis) progressively at a dose of 0.05 mg/kg live weight per day orally, while group B received cabergoline orally at a dose of 0.005 mg/kg, equivalent to 0.1 mL per kg live weight of a 15 mL Galastop® product (Galastop® oral solution 15 mL, CEVA Santé Animale; VETEM Sp.A., Italy). The therapeutic approach continued until signs of proestrus appeared or for a maximum of 40 days (Concannon, 1993).

Cycle monitoring during and after induction treatment

Oestrous cycle monitoring during and after induction treatment allowed us to assess the effectiveness of this treatment by triggering oestrus with ovulation, followed by conception and then gestation that resulted in the birth of healthy puppies.

The clinical, cytological and hormonal monitoring accompanied all females in the experiment throughout the study period, however cytology and blood sampling for P4 evaluation were stopped once the gestational state was confirmed. Gestation was clinically monitored until parturition to collect data related to pregnancy and birth rates, as well as litter size. The date of appearance of the female's next proestrus was noted to assess the return to heat after the induced cycle (assessing the duration of the inter-oestrus interval of the cycle following the induced one).

Ethical Statement

All the animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the course of the study. All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA. SDA. 14).

Statistical Analysis

Statistical analysis of the data was carried out using STATISTICA software (Version 10, Stat Soft, France, 2003). Chi-square test (χ^2) was applied to study the relationship (correlation) between cytological indicators (eosinophilia and basophilia cell percentages) and progesterone levels. The significance level was set at 5%.

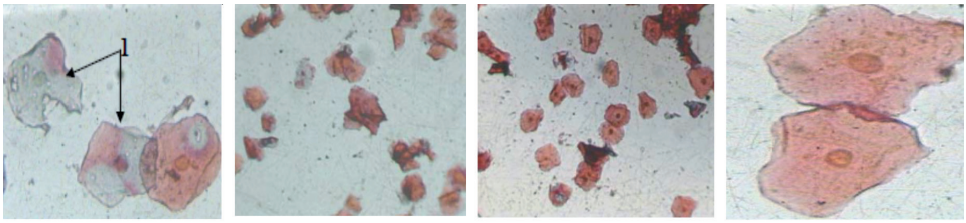
Results and discussion

It is well established in female dogs that the ovulation typically occurs 36–50 h after the luteinizing hormone (LH) peak, which correlates with P4 rates of approximately 2.02±0.18 ng/mL. These concentrations then escalate to a range of 4.00–10.00 ng/mL on the day of ovulation, indicating a significant hormonal shift and the onset of ovulation (Concannon, 1986; Wutthiwitthayaphon et al., 2024).

Correlation Study between Hormonal and Cytological Data

In the current study, the sample consisted of 282 smear slides corresponding to 282 progesterone values. A Chi-square test was applied to this statistical series. However, before proceeding, we established the different cellular classes and progesterone intervals.

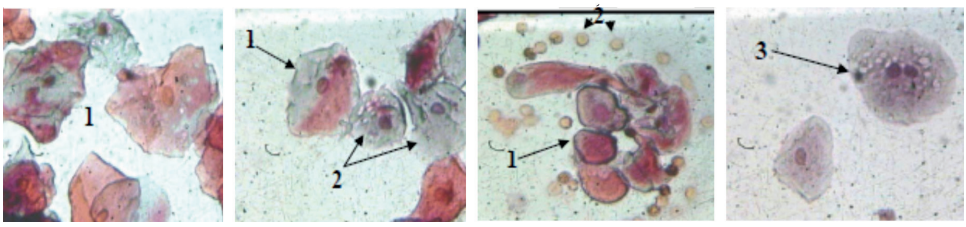
Cellular classes were determined based on the staining affinity of vaginal cells, and since our smears were stained using the Harris-Schorr trichrome method, we defined three cellular classes: basophilic cells, including parabasal and intermediate cells; polychromatophilic cells; eosinophilic or acidophilic cells, including fully and partially keratinized cells. From these three cellular classes, we determined the respective cell percentages (Figure 2).



Proestrus (1: Large intermediate polychromatophilic vaginal cells (X400)

Late Proestrus (Partially keratinized superficial cells (x100 left, x400 right)

Oestrus (1: Superficial keratinized cells (eosinophils). 2: Clusters of superficial keratinized cells. 3: Superficial completely keratinized "anuclear" cells. 4: Spermatozoid, X400)



Metoestrus (1: Reappearance of large polychromatophilic intermediate cells. 2: Reappearance of basophilic intermediates. 3: Metoestral cells.)

Figure 2. Vaginal cytology according to the oestrus cycle

Table 1. Progesterone rate Intervals

Progesterone Level Range (ng/mL)	*n* of samples	Relative Frequency (%)
[0.00 – 0.03]	37	13.12
[0.03 – 0.09]	55	19.50
[0.09 – 0.5]	45	15.96
[0.5 – 2.00]	32	11.35
[2.00 – 4.00]	23	8.16
[4.00 – 10.00]	25	8.87
[10 - 20]	34	12.06
> 20	31	10.99
TOTAL	**282**	**100**

Table 2. Percentages of the Three Cellular Classes Based on Progesterone Levels

Progesterone level ranges (ng/mL)	*n* of samples	% basophils	% olychromato-philic cells	% eosinophils
< 0.03	37	70.56	21.30	7.97
[0.03 – 0.09]	55	39.60	40.87	19.47
[0.09 – 0.5]	45	27.43	26.66	45.90
[0.5 – 2.00]	32	9.75	39.14	51.11
[2.00 – 4.00]	23	6.68	27.10	66.22
[4.00 – 10.00]	25	0.09	1.65	98.26
[10.00 – 20.00]	34	5.25	61.66	73.09
>20	31	28.28	46.69	24.97

Regarding progesterone levels, based on the raw data and for statistical analysis purposes, we performed a flat sorting and created a frequency table by grouping the P4 values into eight intervals (Table 1).

Evolution of the Three Cellular Classes Based on Progesterone Levels

The evolution of the percentage of the different cells based on P4 levels for the 282 smear slides studied is shown in Table 2.

These findings were in agreement with previous studies that reported the microscopic appearance of vaginal cells during the oestrus cycle under major steroid influence (oestrogens and mainly progesterone) in bitches. During proestrus, high oestradiol levels (while P4 rates are low) during the maturation of ovarian follicles causes proliferation of the vaginal epithelium and diapedesis of red blood cells through uterine capillaries. These two processes explain the presence of keratinizing

epithelial cells and erythrocytes in vaginal smears (Baker and Lumsden, 2001). At the beginning of proestrus, the parabasal cells disappear in favour of the intermediate basophilic cells and acidophilic coloration begins to appear, indicating the beginning of keratinization. However, parabasal cells can persist in large numbers for the first two days before disappearing. At the end of proestrus, only superficial acidophilic cells are observed, with angular contours and pyknotic or even anucleate. During oestrus, the vaginal smear is very rich in cells and shows a clean background. The cells are mainly superficial, entirely keratinized, anucleate and grouped in clusters. Acidophilia is greater than 60% or even 80 or 90%. Approximately six days after ovulation, and eight days after the LH surge, the majority of female dogs will have a metoestrus smear. The onset of oestrus corresponds to the oestrus-metoestrus transition (Neveux, 1996). The smear shows keratinized cells, intermediate cells that reappear, as well as grouped parabasal cells and polymorphonuclear cells. Towards the end of metoestrus, the smear is dominated by basophilic parabasal and intermediate cells (Malandain and Fontbonne, 2006; Haji et al., 2018).

Percentage of basophilic cells according to progesterone levels

Data revealed that when progesterone dropped below 0.03 ng/mL, the percentage of basophilic cells was 70.56%. Then it decreased to 0.09%, corresponding to a progesterone interval of 4.00–10.00 ng/mL. From this point, the curve took a new upward trend, and the percentage of cells increased to 5.25% when the P4 level was in the interval 10.00 – 20.00 ng/mL, and then 28.28% at P4 levels >20 ng/mL. In reality, the percentage of basophilia includes two types of vaginal cells: parabasal and basophilic intermediate cells. When studied separately, these two cell types produced almost the same curve profile. Here, a correlation was seen between the decline of the curve and rising P4 concentrations, which changed direction from a value of 10 ng/mL. This correlation will be further analysed later.

Polychromatophilic cells

Data showed a relatively constant percentage, oscillating between 20% and 40% during the first part of the cycle, up to the P4 interval of 2.00 – 4.00 ng/mL. Between 4 and 10 ng/mL P4, the proportion of polychromatophilic cells decreased significantly, reaching a value of 1.65%, after which the curve raised again to 61.66% in the interval 10.00 – 20.00 ng/mL and 46.69% in the interval of > 20 ng/mL. This profile indicated that this cellular class served as a reservoir of cells undergoing keratinization throughout proestrus. This trend will become clearer in the comparative curve of the three cellular classes.

Eosinophilic cells

Our results showed that when P4 levels were below 0.03 ng/ml, the eosinophilia proportion was 7.97%. This then gradually increased, forming a plateau ranging from 45.90% to 51.11% between 0.5 and 2 ng/mL P4. This plateau was followed by rise in cell numbers to 66.22% between 2–4 ng/mL P4, with a sharp peak of 98.26% between 4–10 ng/mL P4. Beyond this value, the curve started to decrease, reaching a percentage of 24.97% once P4 levels exceeded 20 ng/mL.

We noticed that the evolution of the three cellular classes based on P4 levels changed the direction of correlation once P4 rates reached 10 ng/mL. This suggests that cellular parameters lost their significance beyond 10 ng/mL. To analyse this situation, we will focus solely on the values below 10 ng/mL P4, which corresponded to 217 smears, represent-

ing 76.95% of the 282 studied smears.

Figure 3 clearly illustrates the inverse correlations between P4 levels and the percentages of eosinophilic and basophilic cells. The profile of the polychromatophilic cell percentage curve differed, indicating constancy in the percentage as P4 levels raised from basal to the 2–4 ng/mL interval. Beyond this, the curve decreased in parallel with the rise of eosinophilic cells, forming a near-perfect symmetry between the two curves.

According to the chi-square test, there is a highly significant correlation between progesteronemia and the different intervals of basophil and eosinophil percentages ($P<0.001$). Basophilia seems to have an even stronger correlation with progesteronemia than the percentage of eosinophilia. However, proving this correlation alone is not sufficient to use the basophil percentage as an indicator of the progesteronemia threshold. To achieve this, it is necessary to correlate specific progesteronemia thresholds with certain cytological criteria.

Progesterone levels based on cytological criteria

Linde and Karlsson (1984) were the first to investigate the relationship between vaginal keratinization changes and P4 and oestradiol levels. However, their approach differed from this study and unfortunately did not allow for direct comparison. In our results, we were unable to identify a clear minor peak in keratinization in the 3 to 4 days preceding the main keratinization peak of oestrus, as reported in their study. In France, as in this current study, Professor Alain Fontbonne focused on establishing cytological criteria to estimate progesteronemia at the beginning of proestrus in a retrospective study on data from an entire year (2003) (Luc, 2005).

For the following percentage calculations, we only considered the smears where progesteronemia did not exceed 10 ng/mL (217 of 282 smears). This is the level at which the correlation between cellular percentages and progesteronemia changed direction.

Furthermore, this value represented the maximum threshold of the progesteronemia interval that corresponds to the incidence of ovulation. Beyond this value, cellular parameters lost their diagnostic value. For example, in our data, there were progesteronemia values at the end of oestrus and the beginning of metestrus associated with high basophilia rates, which may skew the calculation.

In this approach, several cytological criteria were defined (>50% and >40% basophilia; <10%, <25%, and <33% eosinophilia) which were correlated with three progesteronemia thresholds (<1 ng/mL, <1.5 ng/mL, and <2 ng/mL). The objective was to compare these criteria to identify

Table 3. Cytological criteria and progesteronemia threshold values

Criteria		*n* of samples	P4 < 1 ng/mL	P4 < 1.5 ng/mL	P4 < 2 ng/mL
% eosinophil cells	<33%	107	91.59%	94.39%	95.33%
	<25%	96	92.71%	95.83%	95.83%
	<10%	78	96.15%	97.44%	97.44%
% basophil cells	>50%	59	100%	100%	100%
	>40%	67	98.51%	100%	100%

the one with the highest precision and the smallest margin of error (Table 3).

The three eosinophilic cell percentage criteria studied all demonstrated intolerable margins of error. The most restrictive criterion (*i.e.*, less than 10% eosinophilic cells) presented a margin of error of 2.66% for the progesteronemia threshold P4< 2 ng/mL and 3.75% for the threshold P4<1 ng/mL. Respectively, 2 of 78 smears meeting the criterion corresponded to progesteronemia levels exceeding 2 ng/ml, and 3 of 78 smears corresponded to progesteronemia levels exceeding 1 ng/mL. The least restrictive criterion (*i.e.*, less than 33% eosinophilic cells) presented a margin of error of 4.67% for the progesteronemia threshold P4< 2 ng/mL, meaning that 5 of 107 smears corresponded to progesteronemia levels exceeding 2 ng/mL. It also showed a margin of error of 7.29% for the threshold P4< 1 ng/mL, meaning that 9 of 107 smears corresponded to progesteronemia levels exceeding 1 ng/mL. The percentage of eosinophilic cells did not appear to provide reliable or usable criteria as indicators of a progesteronemia threshold.

Conversely, the criterion of finding more than 40% basophilic cells on a vaginal smear offered maximum precision, with a zero margin of error for the progesteronemia thresholds P4 < 2 ng/mL and P4< 1.5 ng/mL, and a margin of error of only 1.49% for the most restrictive threshold P4<1 ng/mL. These results indicated that of the 67 smears meeting the cytological criterion of more than 40% basophilia, only one smear corresponded to a progesteronemia level above 1 ng/mL. This smear, with a basophilia percentage of 45.98%, corresponded to a P4 level of 1.01 ng/mL. The difference between this progesteronemia value and the threshold value P4 < 1 ng/mL was minimal, just 0.01 ng/ml. This criterion (more than 40% basophilic cells) could be considered a reliable indicator of the progesteronemia threshold P4 level < 1 ng/mL. This implies that if, on a vaginal smear, 4 of 10 cells or 12 of 30 cells are basophilic, measuring progesterone levels would be unnecessary, as the level would be below 1 ng/mL.

Regarding the evolution of progesterone levels in relation to cytological criteria (more than 40% basophilic cells), our results align perfectly with those reported previously (Luc, 2005). In contrast, this author reported results of 93.41%, 94.74%, and 98.68% with margins of error of 6.59%, 5.26%, and 1.32%, respectively, for the thresholds P4< 1 ng/mL, P4 < 1.5 ng/mL, P4< 2 ng/ml. This discrepancy may be explained by the difference in sample size.

For the eosinophilia-related cytological criteria, our results are in agreement to those found by Luc (2005), who reported 93.13%, 92.23%, and 90.17% with margins of error of 6.87%, 7.77%, and 9.83%, respectively. In both studies, these margins of error are unacceptable, and the three eosinophilia-related cytological criteria (<10%, <25%, and <33%) appear unreliable. Even the most restrictive criterion could not be considered a good indicator of progesteronemia levels below 2 ng/mL.

The correlation studies between cytological and hormonal data indicate a significant relationship at the onset of proestrus, particularly between progesterone levels and the percentages of basophilic and eosinophilic cells. While the percentage of eosinophilic cells is a straightforward criterion to apply since it requires only counting fully acidophilic cells, it suffers from a significant margin of error and is thus considered unreliable for indicating a progesteronemia threshold. On the other hand, the percentage of basophilic cells is also easy to analyse as it does not require any cell typing according to morphology. Moreover, it is more reliable

than the eosinophilia rate. Among the cytological parameters evaluated, the presence of more than 40% basophilic cells in a vaginal smear emerged as the most reliable indicator, demonstrating a zero margin of error. This finding suggests that when the percentage of basophilic cells exceeds 40%, there is a high likelihood that progesterone levels are below 2 ng/mL, making progesterone assays unnecessary. Conversely, a basophilia percentage below 40% requires hormonal measurements. Thus, this criterion has the potential to enhance the utility of cytological examinations at the onset of proestrus, allowing for the avoidance of costly and unnecessary P4 assays. This approach offers significant financial advantages for facilities engaged in the medical monitoring of dog breeding through vaginal cytology and P4 testing.

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Korelacija vaginalne citologije i razine progesterona tijekom nadziranja ciklusa estrusa u kuja

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Ova studija istražila je odnos između pregleda vaginalne citologije (VCE) i razina progesterona (P4) tijekom razdoblja od dvanaest i pol mjeseci nadziranja ciklusa u šesnaest domaćih kuja podvrgnutih tretmanima indukcije estrusa. Analizirajući korelaciju između P4 stopa i celularnih parametara (posebno postotka eozinofila i bazofila), ovaj rad imao je za cilj utvrditi pouzdani celularni parametar koji može poslužiti kao indikator niskih pragova progesterona. Preliminarni nadzor 16 kuja proveden je putem dvotjednih posjeta. Kod svake posjete, sve životinje su sistematski podvrgavane vaginalnom brisu i Harris-Schorr trikrom metodi za naknadno bojanje stakalca. Za P4 procjenu, uzorci (plazma) su analizirani uporabom metode radioimunotesta. Kontrola ciklusa tijekom i nakon tretiranja indukcije estrusa popraćena je kliničkom, citološkom i hormonalnom nadzoru dok nije potvrđena gestacija. Primijenjen je Hi-kvadrat test za istraživanje korelacija u populaciji koja se sastojala od 282 stakalca briseva koji su odgovarali 282 P4 vrijednosti. Podatci su pokazali da su na početku proestrusa, P4 razine bile vrlo značajno korelirane (P<0,001) s postotcima bazofilnih i eozinofilnih stanica. Među procijenjenim citološkim kriterijima za oba celularna postotka, više od 40 % bazofilnih stanica na vaginalnom brisu (> 40 % bazofila) dokazano je kao najpouzdanije s nultom marginom greške. Njegova primjena pokazala je da kada je postotak bazofilnih stanica bio strogo veći od 40 %, P4 vrijednosti su ispod 2 ng/mL (što znači da je P4 mjerenje nepotrebno), a kada ovaj postotak bazofila padne ispod 40 %, P4 testovi postaju vrijedni. Stoga, u kontekstu praćenja ciklusa, ovaj celularni indikator mogao bi biti posebno koristan za izbjegavanje skupih i nepotrebnim mjerenja progesterona i smanjenje njihove učestalosti na početku proestrusa.

Ključne riječi: *nadzor estrusa, kuja, vaginalna citologija, progesteron*