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Research Article

Evaluation of Vaginal Smears, and Progesterone and Relaxin Levels in Pregnant, and Overt and Covert Pseudopregnant Bitches

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Abstract: Progesterone and relaxin levels, vaginal smears, and physical differences were evaluated in pregnant, overt pseudopregnant, and covert pseudopregnant bitches. The study included 30 bitches of various breeds, aged 3-12 years and 20 ± 2 kg in body weight, that were divided into 3 groups of 10 animals each. Bitches in group 1 were in the last trimester of pregnancy. Group 2 contained overt pseudopregnant bitches and group 3 consisted of covert pseudopregnant and late dioestrus (between 40 and 60 days of dioestrus) bitches without any signs of overt pseudopregnancy. Clinical, ultrasonographic, and vaginal smear examinations were performed, and cyclic stage or pregnancy was diagnosed. Qualitative relaxin and quantitative progesterone levels were assayed in blood samples.

The evaluation of the vaginal smears of groups 2 and 3 revealed that basal, parabasal, and intermediate epithelial cells were present in greater quantity than superficial cells. The results of the vaginal smears of groups 2 and 3 were statistically similar. Moreover, the difference between group 1 and the other groups was statistically significant. While mean progesterone values of groups 2 and 3 were similar, the progesterone level of group 1 was statistically higher than that of groups 2 and 3 ($P < 0.01$). Qualitative relaxin test results (ReproCHEK®) were positive in the pregnant bitches and negative in the covert and overt pseudopregnant bitches.

Taking into account the higher plasma progesterone concentration in bitches after the 40th day of pregnancy, progesterone level can be used to detect pregnancy; however, concentrations of serum progesterone in pregnant bitches after the 40th day of pregnancy are of minor clinical importance in the diagnosis of pregnancy. It has been shown that relaxin levels are present in the blood of pregnant bitches. Relaxin was not seen in the blood of overt or covert pseudopregnant bitches.

Key Words: Bitch, progesterone, relaxin, vaginal smear

Gebe, Klinik ve Klinik Olmayan Hayali Gebe Evresindeki Köpeklerde Vaginal Smear, Progesteron ve Relaksin Hormonu Düzeylerinin Değerlendirilmesi

Özet: Bu çalışmada, köpeklerde gebelik, klinik ve klinik olmayan hayali gebelik olgularında fiziki değişiklikler, progesteron ve relaksin hormonu düzeyleri araştırıldı. Çalışmada 3-12 yaşlı ve 20 ± 2 kg ağırlığında, toplam 30 adet dişi köpek kullanıldı. Her grupta 10 dişi köpek değerlendirildi. Grup 1; gebeliğin son dönemindeki gebe köpekler, Grup 2 klinik hayali gebe ve Grup 3; diöstrusta (40-60 günler arası) hayali gebelik belirtisi göstermeyen dişi köpeklerden oluşturuldu. Klinik ve ultrasonografik muayenelerden sonra vaginal smear yapılarak gebelik ve siklik dönemleri belirlendi. Toplanan kan örneklerinde nitel relaksin ve nicel progesteron hormonu düzeyleri belirlendi.

Grup 2 ve Grup 3'teki köpeklerin epitel hücrelerinin değerlendirilmesinde bazal, parabazal ve intermediyer hücrelerin süperfisiyel hücrelerden baskın olduğu belirlendi. Vaginal smear bulgularına göre Grup 2 ve Grup 3 arasında istatistiksel olarak benzerlik bulundu, buna ilaveten Grup 1 ve diğer gruplar arasında istatistiksel bir fark vardı.

Grup 2 ve Grup 3'teki köpeklerde ortalama progesteron seviyesi benzer iken, Grup 1' deki progesteron seviyesi, Grup 2 ve Grup 3'e göre istatistiksel olarak daha yüksekti ($P < 0,01$).

ReproCHEK relaksin kiti ile yapılan değerlendirmelerde, gebe köpeklerde sonuçlar nitel olarak pozitif bulundu. Klinik ve klinik olmayan hayali gebe köpeklerden alınan tüm kan örneklerin sonuçları ise negatif olarak saptandı.

Gebe köpeklerde 40. günden sonra yüksek progesteron konsantrasyonu dikkate alınarak progesteron seviyesi ile gebelik teşhisi yapılabilir. Ancak, gebe köpeklerde 40. günden sonra progesteron konsantrasyonunun gebelik teşhisi açısından önemi düşüktür. Relaksin seviyesinin incelenmesi sonucu relaksinin yalnızca gebe köpeklerin kanında tespit edildiği görülmektedir. Gebe köpeklerde relaksin seviyesinde tespit edilen artış, klinik ve klinik olmayan hayali gebe köpeklerde görülmemektedir.

Anahtar Sözcükler: Dişi köpek, progesteron, relaksin, vaginal smear

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Introduction

Reproductive activity in the bitch differs from the polycyclic pattern of other species in that there are no frequent, recurring periods of heat. The oestrus cycle of the bitch consists of 4 stages: prooestrus, oestrus, dioestrus, and anoestrus (1). After a fertile mating, pregnancy occurs. If bitches are not mated or mated with a sterile male, oestrus is followed by dioestrus, which occurs about 6-12 weeks after oestrus. These differences are investigated by physical and hormonal changes (2-5).

Serum progesterone levels begin to rise until late prooestrus, reaching > 8 ng/ml at the time of ovulation. This increase in serum progesterone continues after ovulation and reaches a peak of 15-90 ng/ml by 15-30 days after the luteinizing hormone (LH) surge. Following this, serum progesterone concentrations begin a gradual decline that continues for 5 to 6 weeks (4). Dioestrus is considered to last for as long as blood levels of progesterone are > 1.0 ng/ml (6). Concannon (7) reported that progesterone profiles were similar for pregnant, non-mated, and ovariohysterectomised bitches during dioestrus.

Unlike concentrations of serum progesterone, among pregnant and non-mated bitches, concentrations of serum immunoreactive relaxin differ between pregnant and non-pregnant dogs (4). Serum immunoreactive relaxin concentrations are < 0.25 ng/ml in dioestrus bitches that are not pregnant. Mean serum immunoreactive relaxin concentrations increase to maximum levels (> 3.0 ng/ml) at about week 6 to 7 of gestation in pregnant bitches (4).

It was initially postulated that pseudopregnancy was caused either by an overproduction of progesterone, or abnormal persistence of the corpus luteum. Later work demonstrated that prolactin concentrations increase in non-pregnant bitches at a time similar to that in pregnant bitches. This suggested that the clinical signs of pseudopregnancy were related to increased concentrations of prolactin in the late luteal phase (8). Following the cessation of progestin therapy, more rapid decline of progesterone levels, hyperprolactinemia associated with pituitary microadenomas, and psychogenic or reflexive hyperprolactinemia occurring in response to stimulation by surrogate neonates or other visual, physical, or social stimulation, may be causes of clinical pseudopregnancy (9,10).

The vaginal epithelium is one of several target tissues for ovarian hormones. Characteristic changes in exfoliated vaginal epithelial cells occur as a result of changing levels of oestrogens. Changes in the percentage of epithelial cells in vaginal smears can be used to monitor the oestrus cycle in bitches (4).

The aim of this study was to measure progesterone and relaxin levels, and characteristic changes in exfoliated vaginal epithelial cells in an effort to contribute to the knowledge about the physiological changes in pregnant, and overt and covert pseudopregnant bitches.

Materials and Methods

The study included 30 bitches aged 3-12 years and 20 ± 2 kg in body weight. Bitches (n = 30) were allocated to the following groups: group 1 (n = 10): bitches whose pregnancies were detected with ultrasonographic examination (PieMedical 100 Falco) and were in the last trimester of pregnancy; group 2 (n = 10): overt pseudopregnant bitches that had signs of overt pseudopregnancy; group 3 (n = 10): covert pseudopregnant and late dioestrus (between 40 and 60 days of dioestrus) bitches without any signs of overt pseudopregnancy.

The bitches were brought to the clinic, where they were physically examined. Overt pseudopregnant bitches had signs such as weight gain, restlessness, decreased activity, mothering behaviour, nesting, mammary enlargement, and milk secretion. Ultrasonographic examination was performed to rule out pregnancy. While creating the groups, these criteria were taken into account.

Inguinal mammary glands were measured longitudinally and transversally with a calliper compass. In addition, secretion of mammary glands was recorded. Quantitative measurements of mammary gland size were compared between the groups.

Vaginal smear samples were taken from all bitches and stained according to the Papanicolaou staining procedure (11). The percentages of vaginal cells (basal, parabasal, intermediate, and superficial) in vaginal smear samples were classified in order to determine reproductive stage.

Blood samples were collected from all bitches, in both dry tubes containing no additive and in heparinised tubes.

Then the collected blood samples were centrifuged (3000 rpm/15 min) and serum samples were stored at –20 °C until the hormone assay. Before evaluation, serum samples were stored at 4 °C for 24 h and then for 1 h at laboratory temperature. Progesterone levels were determined by radioimmunoassay (RIA) (12).

Qualitative relaxin levels were determined with an enzyme-linked immunosorbent assay (ELISA) for canine relaxin (ReproCHEK®, Synbiotics, Europe Corp., France) in blood. This assay measures relaxin concentration in serum and whole blood samples, and the presence of significant amounts of relaxin is indicative of pregnancy.

Results were evaluated comparatively in all groups. The statistical calculations of serum progesterone and mammary gland size were made with one-way ANOVA. The differences in vaginal smear rates were analyzed statistically using the Kruskal-Wallis test.

Results

Among the 30 bitches, 10 (group 1) were in the last trimester of pregnancy according to the ultrasonographic examination, whereas 20 bitches (groups 2 and 3) were not pregnant. Foetal heart beats and ossification were detected with ultrasonography in all pregnant bitches.

The results of vaginal smears were evaluated microscopically and mean results with standard error are shown in Table 1.

In addition, leukocyte levels detected in vaginal smear samples were higher in the non-pregnant bitches than in the pregnant bitches.

Serum progesterone levels of all the groups are shown in Table 2. Serum progesterone levels were similar in groups 2 and 3. In contrast, these values in group 1 were statistically higher than in groups 2 and 3 ($P < 0.01$). Qualitative relaxin test results were positive in group 1 and negative in groups 2 and 3.

Behavioural changes and mammary secretion were recorded only in overt pseudopregnant bitches. Quantitative measurement results of mammary glands and mean values are shown in Table 3. Measurements of mammary glands were similar in groups 1 and 2; however, these measurements were significantly lower in group 3.

Discussion

In this study, differential diagnoses were performed in all groups, taking into account such parameters as serum progesterone, blood relaxin, vaginal smear, and mammary enlargement.

Burke (13) and Concannon (14) reported that basal, parabasal, intermediate, and superficial cells, and neutrophils were common in early dioestrus between 7 and 11 days after LH surge. The onset of cytological dioestrus was defined as the first day a decrease occurred in the number of superficial cells, and the decrease was often greater than 50%. Parabasal, basal, and intermediate cells are dominant cell types present in vaginal smears from late dioestrus bitches (4). Johnston et al. (4) and Jones et al. (15) stated that concentration of oestradiol-17β was low in pregnant bitches. Oestradiol-17β begins to rise significantly from mid-gestation to the 50th day of gestation and reaches 13.8 ±

Table 1. Vaginal smear results in the study groups.

Groups	Basal (%) (X ± SE)	Parabasal (%) (X ± SE)	Intermediate (%) (X ± SE)	Superficial (%) (X ± SE)
Group 1 (n: 10)	$3.60^{a} \pm 2.10$	$17.60^{a} \pm 2.75$	$56.00^{a} \pm 4.77$	$22.80^{a} \pm 5.13$
Group 2 (n: 10)	$30.90^{b} \pm 5.98$	$29.80^{b} \pm 2.06$	$30.50^{b} \pm 4.08$	$8.20^{b} \pm 1.87$
Group 3 (n: 10)	$30.00^{b} \pm 1.91$	$32.40^{b} \pm 1.63$	$29.20^{b} \pm 1.20$	$8.80^{b} \pm 1.77$
P	***	***	***	**

[a-b] Difference is statistically significant in groups with different letters in the same column (**P < 0.01, ***P < 0.001).
X = mean SE = standard error

Table 2. Minimum, maximum, and mean serum progesterone concentrations in the study groups.

Groups (n: 10)	Serum progesterone concentration (ng/ml)		
	Minimum	Maximum	Mean and standard deviation (X ± SD)
Group 1	4.38	32.28	17.05[a] ± 10.05
Group 2	1.18	11.31	5.07[b] ± 3.00
Group 3	0.56	26.54	9.66[b] ± 8.92

[a, b]: Difference is statistically significant in groups with different letters in the same column ($P < 0.01$).

Table 3. Quantitative measurements of mammary glands.

Groups (n: 10)	Mammary dimension (cm)				Mean and standard deviation (X ± SD)	
	Minimum		Maximum			
	longitudinal	transversal	longitudinal	transversal	longitudinal (cm)	transversal (cm)
Group 1	2.8	2.5	8.8	6.6	4.92 ± 1.81	4.19 ± 1.32
Group 2	2.3	1.6	8.4	5.3	4.86 ± 1.86	3.50 ± 1.28
Group 3	1.2	1.0	4.2	3.5	2.59 ± 1.15	2.23 ± 0.74

Results of measurements were significantly different between pregnant/overt pseudopregnant and covert pseudopregnant bitches ($P < 0.001$). X = mean, SD = standard deviation.

12 pg/ml. Then, serum concentration of oestradiol-17β begins to decline, which continues until the time of parturition (15). In the present study, basal, parabasal, and intermediate epithelial cells were dominant in the vaginal smears of covert and overt pseudopregnant bitches. The intermediate cell rate (56%) and superficial cell rate (22.8%) were more dominant in the pregnant bitches than in the overt and covert pseudopregnant bitches. Feldman and Nelson (16) reported that the presence of superficial-intermediate cells was similar to that of superficial cells because of an insufficient effect of oestrogen on the vaginal wall. A major proportion of the pregnant bitches in this study were in late pregnancy; therefore, some superficial cells in the vaginal smears of the pregnant bitches were probably thought to be superficial-intermediate cells.

Serum progesterone is at peak concentration between 15 and 30 days of the cycle. Then, concentration of progesterone begins to decline and remains 4-16 ng/ml (7). Serum progesterone concentration, like in pregnancy, reaches 15-80 ng/ml 15-25 days after LH surge and is greater than 1.0 ng/ml 60 days after the LH surge in dioestrus bitches (17,18). Progesterone levels may decline abruptly in pseudopregnant bitches. It is suggested, based on indirect evidence, that pseudopregnancy may occur as a result of increased concentrations of prolactin being induced more rapidly than the normal decline of progesterone levels in the late luteal phase (10). Gobello et al. (19) reported that serum progesterone concentrations decreased rapidly in bitches with a history of pseudopregnancy that showed signs of overt pseudopregnancy. Baştan et al. (20) reported that mean serum progesterone concentration was 17.4 ± 1.2 ng/ml during dioestrus. In the present study, serum progesterone levels were 17.05 ± 10.05 ng/ml, 5.07 ± 3.0 ng/ml, and 9.66 ± 8.92 ng/ml in the pregnant, and overt and covert pseudopregnant bitches, respectively. Although some authors have reported a pregnancy-

specific increase in progesterone level after implantation and placental development, the difference is not great enough to provide a diagnostic test to determine pregnancy (4). However, in the present study, serum progesterone concentrations in pregnant bitches were higher and statistically more significant ($P < 0.01$) than those in overt and covert pseudopregnant bitches. In addition, serum progesterone concentrations were similar in overt and covert pseudopregnant bitches. Schaefers-Okkens (21) reported that in the pregnant bitch there were often secondary increases in circulating progesterone concentrations between the 25th and 40th day of gestation that may reflect a pregnancy-specific mechanism that results from additional stimulation of progesterone production. However, concentration of serum progesterone in pregnant bitches after the 40th day of gestation has minor clinical importance for pregnancy diagnosis.

Immunoreactive relaxin is detected in pregnant bitches between 3 and 4 weeks of gestation, after the LH surge, reaching peak levels 2 to 3 weeks before parturition (6). It is usually detected between 21 and 26 days of pregnancy (22). Serum relaxin concentration is not detected in pseudopregnant bitches, or in foetal resorption and abortion cases (23). Relaxin measurement might provide useful information about whether or not a pregnancy had been established, or if foetal death and resorption have occurred (24). In this study, qualitative relaxin test results were positive only in the pregnant bitches and were negative in overt and covert pseudopregnant bitches. It was thought that these results were normal because the highest concentrations of relaxin are in the canine placenta.

Mammary gland development is stimulated by prolonged exposure to serum progesterone, and the stimulation of lactogenes and galactopoiesis is regulated by the presence of serum prolactin (4). Development of mammary glands may cause mammary oedema and lactation. Pregnant bitches usually lactate near the time of parturition, and lactation can also occur in non-pregnant bitches near the end of dioestrus (18). Although bitches were selected with regard to similar body weight in the present study, evaluation of mammary gland development was not sufficiently objective due to differences in breed, age, and number of parturitions. Longitudinal and transversal measurements of mammary glands in covert pseudopregnant bitches were not as statistically significant as those in pregnant and overt pseudopregnant bitches in the same stage. Furthermore, measurements of mammary glands of the covert pseudopregnant bitches were smaller than those of pregnant and overt pseudopregnant bitches ($P < 0.001$).

In conclusion, relaxin, progesterone, vaginal cytology, and mammary enlargement size were compared among the groups in this study. Relaxin test results were positive only in the pregnant bitches. Mammary gland development and mammary secretion were observed in the pregnant bitches and overt pseudopregnant ones. According to vaginal smear results, there were more superficial cells in the pregnant bitches than in the overt and covert pseudopregnant bitches. Serum progesterone concentrations were significantly higher in the pregnant bitches than in overt and covert pseudopregnant bitches. Taking into account the higher serum progesterone concentration after the 40th day of gestation in the pregnant bitches, further studies may be carried out using only progesterone measurements in pregnancy diagnosis (between 40 and 60 days of gestation) in bitches. Although the difference in progesterone levels was not great enough to provide a diagnostic test to determine pregnancy, there was apparently a difference in progesterone levels between pregnant and non-pregnant bitches.

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