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Changes in the histomorphology of the canine cervix, ovary and steroid hormone levels through the oestrous cycle

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Abstract

The importance of canine reproduction is steadily increasing and little is known about the canine cervix and ovary so far. The aim of this study is to describe the histomorphology of the canine cervix, ovary and to determine its correlation with the stage of oestrous cycle and to circulating concentrations of progesterone (P4) and estradiol-17beta (E2) and vaginal cytology respectively. Twenty healthy and sexually mature bitches with ages ranging from 2 to 8 years were assigned to the experiment. Ovariohysterectomies were performed according to standard procedures through median laparotomy access. Ovaries and cervix uteri were collected from 20 cross bred bitches at the time of routine ovariohysterectomy. Canine estrous cycles were determined by clinical, macroscopical and histological findings. Histomorphological examinations were performed to evaluate epithelial thickness and epithelial layers of cervix uteri and ovary tissue were significantly influenced by the stage of cycle.The aim of this study is to investigate the histomorphological structure of the cervix and ovary in the cycle phase of non-pregnant bitches. This study showed that the cervix and ovary undergoes cyclic changes during the canine oestrous cycle, in particular in association with circulating P4 and E2 concentration.

Keywords: Histomorphology; ovary; cervix uteri; P4; E2; canine

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Introduction

Reproductive physiology of a dog is different from that of other mammalian species (Feldman and and Nelson, 2000). The importance of canine reproduction is steadily increasing and little is known about the canine cervix and ovary morphology. Histomorphology of the canine cervix and ovary changes with the period of sexual cycles. This situation occurs, depending on the Progesterone (P4) and estradiol-17beta (E2) (Mills et al., 1979; Bouchard et al., 1991; England 1991; Roszel 1992; Wai-Is et al., 1998; Johnston et al., 2001; Chatdarong et al., 2005; Georicke-Pescha, 2010).

The epithelial layer (number of layers, thickness) is significantly influenced by the stage of cycle; significant correlation is found between the epithelial layer, thickness of cervical stroma, follicle number of the ovary and circulating P4 and E2 concentrations. The epithelium of the cervix is dependent on the stage of cycle. E2 and P4 which are related with increase of mean area of glands can be seen as an activation of these cervical glands by P4 and E2 during the cycle (Concannon et al., 1975; Vermeirsch et al., 2002; Goericke-Pescha et al., 2010).

Vaginal cytology is a subjective assessment of epithelial cell morphology using criteria to classify cells into groups to allow determination of the stage of the cycle (Johnston et al., 2001; Schutte 1967a). During anoestrous, the vaginal epithelium of the bitch is only a few cell layers thick and mainly consists of parabasal and small intermediate cells (Post, 1985). The epithelial cells present on the smear, change from initially being mainly small and large intermediate cells with low numbers of parabasal and keratinised cells to large numbers of large intermediate and keratinised cells. Large numbers of erythrocytes are also present in the

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smear but commonly decrease as the bitch progresses towards estrus. As the bitch enters estrus, the percentage of keratinised cells on the vaginal smear increases until peak cornification occurs, coinciding with plasma progesterone concentration reaching the levels after ovulation. At estrus, there are usually no erythrocytes or leukocytes and the smear has little debris compared to smears from proestrus or early diestrus at the start of early diestrus there is an in flux of leukocytes and a large proportion of parabasal and small intermediate cells (Schutte 1967abc; Post 1985).

The aim of this study was to provide detailed information about the histomorphology of the cervix and ovary in non-pregnant bitches and to check for cyclic changes during the canine oestrous cycle in association with circulating sex steroid levels and vaginal smear findings.

Materials and Methods

Animals

Twenty healthy and sexually mature bitches with ages ranging from 2 to 8 years were assigned to the experiment. The group included 20 cross bred bitches which were admitted to Small Animal Clinic of Firat University for ovariohysterectomy. Ovariohysterectomies were performed according to standard procedures through median laparotomy access. Before surgery, animals were sedated with the normal pre-anesthetic protocol. Ten min. later following intramuscular atropine sulfate (0.02-0.04 mg/kg, sc.) injection xylazine hydrochlorid (2 mg/kg, im.) injection were performed. Finally, ketamine hydrochlorid (2-4 mg/kg, iv.) were injected intravenously. The stage of estrus cycle was determined according to Concannon et al. (1975) on the basis of the cumulative information provided by the vaginal cytology, sex hormone determinations, and visual inspection of the ovarian surface for information on the ovarian dominant structures. The cycle stages were defined as explained by (Goericke-Pescha et al., 2010) on the basis of the hormone levels. Proestrus was characterized by high E2 and low P4 concentrations, estrus by high E2 and increasing P4, early diestrus by falling E2 and high P4 levels, late diestrus by low E2 and decreasing P4 and anestrus by low E2 and P4. In the group of bitches used in this study, five bitches were in proestrus, six bitches were in estrus, six bitches were in diestrus (early diestrus 3, late diestrus 3), and three bitches were in anestrus. Ovaries and cervix uteri of 20 cross bred bitches were collected at the time of routine ovariohysterectomy. Cervix and ovaries were conserved for further examinations.

Blood sampling and hormone determination

Blood samples were collected from Vena cephalica antebrachii into anti coagulant vacutainer tubes which includes EDTA and centrifuged at 3000g for 5 min. Plasma was separated and then stored at -20^{o}C until hormonal analyses. The plasma P4 and E2 levels were measured by the ELISA method using P4 and E2 kits (E2 ELISA Kit CSBE-06846c, PROG Kit CSB E07285c, 96 Well kit, Scientific Research Center Cusabio Biotech Co. Ltd. Newark China) according to the kit manufacturer's instructions.

Histological evaluation

For histology, tissue samples were fixed for 24h in 10% neutral phosphate buffered formaline, washed in phosphate buffered saline (PBS), and subsequently dehydrated in a graded ethanol series as previously described (Mutembei, 2005). Then tissue samples were embedded in a paraffin substitute (Histo-Comp, Vogel, Giessen). Sections 4-5 µm thickness were cut with microtome and were stained with haemotoxylin-eosin. Sections were examined with a light microscopy (Olympus BX-51) and photograps were taken. The number of layers and thickness of the superficial epithelium, were evaluated in the cervix uteri.

Vaginal cytology

A collection cotton swab (9–10 mm large), previously dipped in a 0.9% NaCl solution, was gently placed into the vagina and rotated. After rolling on a slide, the smear was stained following the Giemsa technique. The aspect of the surface of the slide, apart from cells, known as the smear background, was observed under a magnification X 100. Hunderd epithelial cells were examined and classified as basal, parabasal, intermediate, superficial, or anucleated superficial cells (Schutte 1967a; Johnston 2001).

Statistical analyses

All values were presented as mean ± SEM. P value less or equal to 0.05 was considered statistically significant. Data were analyzed by one-way analysis of variance (ANOVA) and post-hoc Tukey HSD test using SPSS/PC computer program (version 13.0) (2011) to determine the differences among all groups in the whole parameters.

Results

Regarding gynaecological status as determined on the basis of the vaginal cytology, sex hormone determinations, and the visual inspection of the ovarian surface, five bitches were in proestrus, six bitches were in estrus, three in early diestrus, three in late diestrus, and three in anestrus. Canine oestrus cycle was determined by clinical, macroscopical and histological findings. Epithelial thickness (µm), number of epithelial layers, Progesterone (P4) and estradiol-17beta (E2) levels and visual ovarium findings for each case were

presented in Table 1. Epithelial thickness and epithelial layers of cervix uteri were significantly influenced by the stage of cycle. The numbers of epithelial layers were higher in estrus stage compared to all others. In addition, epithelial thickness increased in estrus and proestrus stage, however it decreased in diestrus and anestrus. Steroid hormone levels were influenced by the stage of cycle. Proestrus was characterized by circulating high E2 and low P4 concentrations, estrus by circulating high E2 and increasing P4, diestrus by circulating low E2 and high P4 levels, and anestrus by low E2 and P4.

Proestrus

During proestrus, primordial, primary, multiple secondary antral follicles and tertiary follicles were seen in the ovarian cortex (Fig. 1a). Four or five antral follicles were present in both ovaries. Large ovarian antral follicles with liquor folliculi were present in the ovaries. There were also shrunken corpora lutea (corpora albicans) from the previous cycle. Ovarian medulla was edematous and with congestion (Fig. 1b). Cervical epithelial thickness was higher than in diestrus and anestrus

Cervical stroma showed an edematous expansion at proestrus, and in transversal sections, the lumen presented as a X in shape (Fig. 1c). Cervical basal glands and epithelium proliferated, and epithelial invaginations into the stroma formed crypts. Propria was hyperemic and hemorrhagic (Fig. 1d). In proestrus, the epithelial thickness tends to increase from the cranial to the caudal (vaginal) part of the cervix

Estrus

There were multiple tertiary follicles in the ovaries at estrus. These follicles lines by stratified layers of elongated granulosa cells protruding into the antral space that became increasingly noticeable (Fig 2a). Granulosa cells stated along a basement membrane and were separated from the vascularized theca (Fig. 2b&c).

During estrus, cervical stroma was edematous and hyperemic. In addition, hemorrhages in some areas were seen. Basal glands became proliferate and the lumen of cervix was dilated (Fig. 2d).

Diestrus

During diestrus, histological sections of the ovaries composed of luteal cells in the corporal lutea. The cytoplasm of the cells generally appeared homogenously granular and eosinophil (Fig. 3a). In addition, small antral follicles were observed filled with inflammatory cells, erythrocytes and eosinophil fluid (Fig. 3b).

Cervical endometrium became thick and cellularity. Basal glands generally had a coiled appearance and there was eosinophil fluid in the lumen of the glands. Stroma was composed of dense collagen (Fig. 3c).

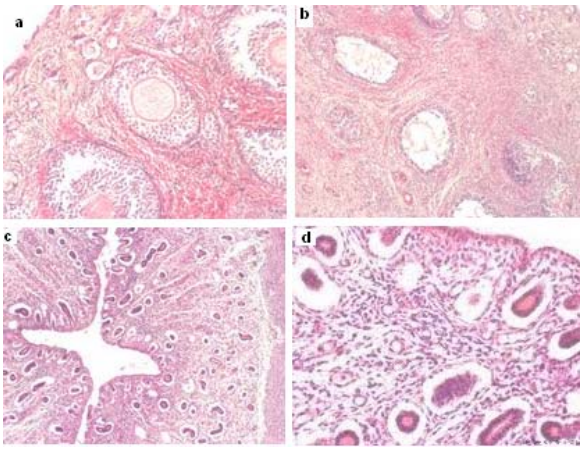

Fig.1.a: Ovarium, proestrus, cortex with primordial, primer and multiple secondary antral follicles. H&E, 20x. b: Large ovarian antral follicles with liquor folliculi are present in the ovaries during proestrus. H&E, 10x. c: Canine cervix, the lumen presented as a X in shape H&E, 10x. d: Cervical basal glands and epithelium proliferated, and epithelial invaginations into the stroma formed crypts. H&E, 40x.

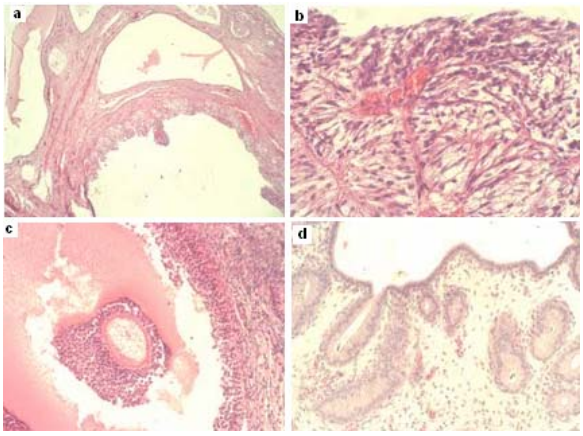

Fig.2.a: Ovarium, early estrus. Large tertiary follicles in ovarian cortex. H&E, 4x . b:Early Estrus, ovary, Tertiary follicle. Tall granulosa cells with slightly vacuolated cytoplasm pushed into folds by budding theca interna (arrow). H&E, 20x. c: Large tertiary follicle in ovarium. H&E, 40x. d: During estrus, cervical stroma and basal glands became proliferate H&E, 20x.

Early and Late Anestrus

There were primordial and primary follicles in the ovarial cortex at early anestrus (Fig. 4a). Regressing corpora lutea grows long to show irregular lobulation (Fig. 4b) and luteal cells contained cytoplasmic vacuoles. At this period, cervical propria had a few atrophic gland, and macrophage infiltration, and yellow pigment deposition were also observed. Cervical stroma

Table 1: Cyclic changes of circulating progesterone (P4) and estradiol-17 Beta (E2) concentrations, and cervical tissues epithelial thickness (µm) and number of epithelial layers throughout the canine estrous cycle

Stage of Estrous Cycle	Epithelial thickness (µm)	Number of epithelial layers	P4 (ng/ml)	E2(pg/ml)	Ovarium visual inspection
Proestrus	16.34±0.26[a]	1.60±0.10[a]	0.748±0.04	19.493±0.72	pinpoint-sized follicles
Estrus	36.08±1.24[b]	3.30±0.15[b]	4.176±0.28	14.162±0.39	More than one follicle (6-9)
Diestrus	13.69±0.49[a]	1.93±0.12[a]	9.923±0.89	6.916±0.14	Luteal structures like clusters of grapes (9-10)
Anestrus	10.23±0.30[c]	1.59±0.10[a]	1.076±0.16	2.27±0.34	Don't have any structure

Means bearing different superscripts within same columns were significantly different (P<0.05)

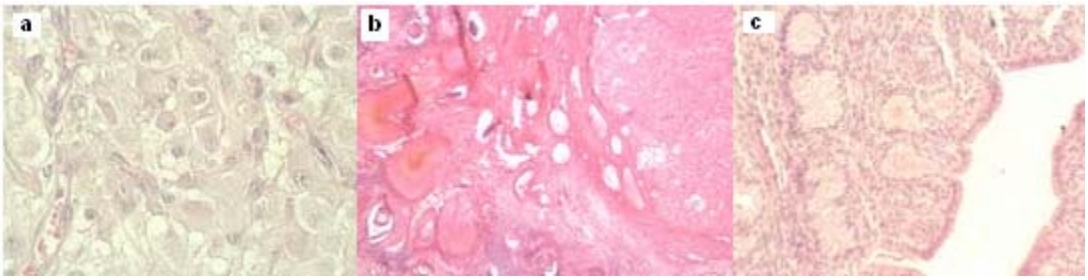

Fig.3.a: Ovary, diestrus, corpus luteum. Luteal cells became increasingly vacualated. H&E, 40x. b: Ovary, diestrus,H&E, 4x. c: Diestrus, cervix, cervical basal glands generally had a coiled appearance and there was eozinophilic fluid in the lumen of the glands H&E, 20x.

was completely atrophic and had dense cellularity at late anestrus (Fig. 4c&d).

Discussion

The histological structure of the cervix is according to the details previously described in dogs (Johnston et al., 2001; Chatdarong et al., 2005; Georicke-Pescha et al., 2010). But only a little part of the histological structure of ovarium is known. To increase the knowledge about the histomorphology of the canine cervix and ovary; the stage of oestrous cycle, concentrations of circulating progesterone (P4) and estradiol-17beta (E2), and vaginal smear findings were examined to determine correlations between these parameters respectively.

Depending on the increased number of developing follicles, close to ovulation and corpora lutea in ovary increased estrogen and progestrone levels. Depending on the situation are caused changes in histomorhology of cervix and vaginal structure (Bouchard et al., 1991; Concannon et al., 1975; Vermeirsch et al., 2002). Our study showed that, the period of estrus in dogs with extensive superficial-anuclear cells were found in vaginal cytological examination. During estrus the histologicial structure of cervix uteri was changed, especially epithelial thickness and the number of epithelial layer were increased.

Apart from this study, previous investigators have shown that (Wai-Is et al., 1998; Vermeirch et al., 2002; Ortega-Pacheco et al., 2007; Groppetti et al., 2010; Georicke-Pescha et al., 2010) epithelial layer thickness

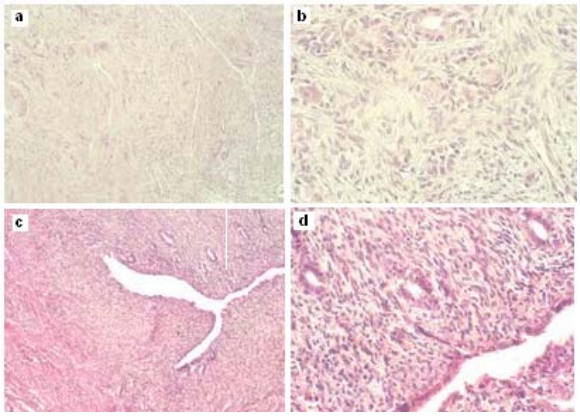

Fig.4.a: Ovary, anestrus. There were primordial and primary follicles in the ovarial cortex at anestrus H&E, 10x. b: Ovary, anestrus. H&E, 40x c: Anestrus, cervical stroma was completely atrophic and had dense cellularity. H&E, 10x. d: Anestrus, cervix, basophilic endometrial stroma. H&E, 40x.

and the number of epithelial varied according to the periods of sexual cycle phases, as shown histologically. Follicular structures have been observed during anestrus in ovary. Accordingly, this period has decreased the number of epithelial layer and the epithelial layer thickness. As a result of increased levels of progesterone during estrus in dogs, histological changes were observed in ovary and vaginal smear. Reducing the intensity of superficial cells in vaginal smear at diestrus period was explained by decreased estrogen levels. Also during this period, increased number of corpus luteum was observed histologically.

During anestrus vaginal smear findings, histology of the cervix uteri and ovaries and hormone levels were also consistent particularly the increase in the number of parabasal cells and, decreased, or not visible other cell types. Depending on the number of rising tertiary follicles in ovary were increased E2-level and anuclear cells of the vaginal cytology. This situation was identified for estrus period.

During proestrus, progesterone levels was below 1 ng/ml. (Vermeirsch et al., 2002) Antral follicles were histologically monitoring in ovary. Vaginal smear findings in proestrus were different from other stages. High amount of erythrocytes and non-cornified cells and less anuclear cells which were connected to changes in E2 levels were seen.

Investigators reported that progesterone level was very high during diestrus (Bouchard et al., 1991; England, 1991; Vermeirsch et al., 2002). High levels of P4 was indicated the presence of luteal cells in ovaries. In our study showed that thickness of epithelium and the number of epithelial layer were decresased but luteal cells and tissues were seen as the greatest concentration in diestrus period. As a result of investigations elevation of progesterone was increased the number of parabasal and intermediate cells.

Our study showed that relationship between E2 and P4 were influenced by the thickness of epithelium and the number of epithelial layer, the relationship between E2 and P4 were different according to sexual cycle. This study provided detailed information about the histological changes of the ovary in non pregnant bitches and showed that the ovary undergoes cyclic changes during the canine oestrous cycle, in particular in association with circulating progesterone concentrations, vaginal smear results and visual examination of the ovary also supported this position.

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