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Abstract of the Doctoral Thesis

"Endometrial expression of cyclooxygenase-2 and prostaglandin E synthase and prostaglandin F synthase in bitches during different phases of the oestrus cycle, with subclinical *endometritis* and at different ages"

Diseases of the uterus in bitches involve important health and reproductive issues with unclear aetiology and pathogenesis despite a substantial number of studies, particularly regarding subclinical pathologies of the *endometrium*. This topic has been investigated in a fragmentary manner and many relations, such as the impact of the individual estrous cycle phases and the age of bitches, have not been entirely elucidated.

This paper provides a comprehensive study of the *endometrium* at different phases of the estrous cycle as well as in animals classified into two age groups: over 5 years of age and below 5 years of age. The investigation was also carried out on animals with a histopathologically diagnosed subclinical *endometritis*.

The study was performed on 48 clinically healthy bitches at different stages of the estrous cycle, without any uterine pathologies as confirmed with ultrasonography and a clinical examination. In the preliminary stage of the study, the stage of the estrous cycle was diagnosed with clinical (vaginal endoscopy), cytological and laboratory (progesterone level) procedures. The animals were then divided into three major experimental groups, according to the diagnosed stage of the oestrus cycle: group 1 (n=16) – *anoestrus*, group 2 (n=16) – *proestrus* + *estrous*, and group 3 (n=16) – *metestrus*. *Ovariohysterectomy* (OVH) was then performed on all animals to collect the sample of the endometrial tissue for a histopathological investigation, a bacteriological examination, cytology (cytobrush) and molecular studies. For the latter study, the expression of mRNA COX-2 (PTGS2), PGFS (PTGFS) and PGES (PTGES) was determined with real-time PCR, and immunolocalization of their protein products in the *endometrium* was carried out with IHC. These molecular analyses included the enzymes involved in prostaglandin metabolism, as they play a key role in inflammation. A histopathological investigation, bacteriological examination and cytology were performed using standard methods. Based on the histopathology results from all bitches in each group, an additional group of animals was distinguished: with subclinical *endometritis* (n=21) and with normal *endometrium* (n=21). The comparison of the above-mentioned expression of the

prostaglandin metabolism enzymes was performed in both these groups. Similarly, the whole study population of animals was divided into two age groups: <5 years of age (n=38) and ≥ 5 years of age (n=10), using the same comparison strategy as above. The results of such investigations were analyzed statistically. The significance of variances in the expression of the selected genes between the treatment groups was determined with a non-parametric Kruskal-Wallis one-way analysis of variance. The post-hoc Mann-Whitney U test was applied to determine the differences between the *anoestrus*, *proestrus/estrous* and *metestrus* groups. The significance of expression of the genes in the *endometritis* group and the regular *endometrium* group (END vs. NE) was determined with an analysis of variance and with the Tukey-Kramer test and Duncan's test. The significance of expression of the genes in the age groups (younger bitches <5 years of age and older bitches >5 years of age) was calculated with a two-sample t-test for equal means (independent samples). Histopathological examination determined the number and percentage of bitches affected by subclinical *endometritis*, CEH and healthy *endometrium* in particular research groups, taking into account the phase of the estrous cycle and the age of the bitches tested (younger bitches <5 vs. older bitches ≥5). Animals with different types of *endometritis* were treated as one group. The results of cytological tests were calculated as the mean ± SEM of the percentages of PMNs in individual groups, taking into account the groups of individual phases of the estrous cycle, the presence of histopathological *endometritis* as well as the age of the tested animals (younger bitches <5 vs. older bitches ≥5).

In both the histopathological and cytological examination, the values were statistically analyzed using the non-parametric Kruskal-Wallis test. The degree of correlation between histopathological and cytological findings was also calculated using the Cohen kapp test.

The molecular studies in the groups classified based on the specific stage of the oestrus cycles demonstrated a varied expression of the investigated factors in these groups. In general, the expression of mRNA PGFS was low and did not differ significantly among the experimental groups (in certain groups I, II, and III P=0.4, in the age groups P=0.943, and for the comparison of END vs. NE P=0.267). However, the anoestrus group was an exception as it demonstrated an slight tendency for PGFS expression.

However, significant differences were demonstrated for mRNA COX-2 and PGES. The expression of these factors was statistically significantly higher, especially in the anoestrus group (for COX-2 P=0.01; for PGES P=0.017). Furthermore, an enhanced and statistically significant expression level of both factors was recorded for the bitches in the *endometritis* group as compared to the animals with a normal *endometrium* (for COX-2 P=0.001; PGES P=0.002). Such a difference was not demonstrated in the groups classified by the age of the

bitches (COX-2: P=0.437; for PGES: P=0.718). It is difficult to explain and clarify this part of the study. It appears that the mRNA COX-2 and PGES expression in the bitches affected with *endometritis* can be linked to mild inflammation, though this response is unusual since the PGFS stimulation, which is discussed with pyometra, was missing. The enhanced secretion of these factors during anoestrus may derive from histological endometrial regeneration, which takes place in this phase of the estrous cycle. In contrast, low expression of PGFS and a lack of differences between the experimental groups suggest that this prostaglandin metabolic path demonstrates low activity in the *endometrium* in bitches at all. It is consistent with the known phenomenon that the uterus in the bitch does not produce luteolytic PGF2α during the estrous cycle.

The immunohistochemical investigations demonstrated that the surface epithelium and glandular epithelium of the *endometrium* and the blood vessels were the main locations for the agents investigated with real-time PCR. The intensity of colour reactions was substantially consistent with the above-discussed phases and groups of the animals with mRNA expression.

The histopathological examinations demonstrated that in many bitches, despite the absence of clinical symptoms and gross lesions, the *endometrium* was affected with pathologies. Subclinical *endometritis* was the predominant type of these pathologies, with acute and chronic histopathological presentations. This pathology was diagnosed in 33-60% of the bitches, depending on the phase of the estrous cycle, although a statistically significant correlation with the cycle phase was not found (P>0.05). Another detected pathology was CEH, yet its occurrence was much lower (6-25%). In contrast, the study found a strong impact of the age on the occurrence of these pathologies in the bitches. In the animals below 5 years of age, these conditions were significantly less common (39%), than in the older bitches. In the latter group, the animals with a normal *endometrium* were absent.

The cytological examinations performed to determine the percentage of PMNs in the endometrial swabs demonstrated that their presence in the *endometrium* during the individual phases of the estrous cycle was generally low: <2%. The phase of the estrous cycle and the age of the animals were not shown to have any impact on the average percentage of PMNs (in the certain groups I, II, and III P=0.056; in the age groups: P=0.97). This observation is a major drawback for practical cytological diagnostics. A statistically higher percentage of PMNs with a statistically significant difference was found between the bitches with *endometritis* and without *endometritis* (2.05 % vs. 0.96%), although the correlation between the results of histopathology (the reference method) and cytology was generally low (P=0.511). These results

are difficult to consider, but it seems that they are insufficient to make a recommendation on cytology as a practical tool for diagnosing the *endometritis* in bitches.

In summary, based on the presented study, the following conclusions are drawn:

a. the expression of mRNA COX-2 and PGES in the canine *endometrium* during the *estrous* cycle is detected mainly in anoestrus and in animals affected with subclinical *endometritis*. This phenomenon was not recorded in the other phases of the ovarian cycle or in the bitches in the different age groups.
b. the surface epithelium of the uterine lumen and the glandular epithelium, as well as the blood vessels, were the main locations for the protein products of mRNA *COX-2*, *PGFS*, and *PGES*.
c. it seems that the expression of PGE metabolic path is the main phenomenon in the *endometrium* during the physiological estrous cycles in the bitch and the clinically healthy uterus in canine females, whereas the expression of PGF2α is less active.
d. a high percentage of the uterine pathologies with the predominance of histopathological *endometritis* (35-60%) and a significantly lower occurrence of CEH (6-25%) were found in the clinically healthy bitches and with the grossly regular *endometrium* during the physiological estrous cycle. With age, the percentage of the bitches with these conditions increases, in contrast the phase of the estrous cycle did not impact this phenomenon.
e. a cytological investigation of the *endometrium* with the cytobrush does not allow for a precise examination of the endometrial condition. A low degree of correlation between the result of histopathology and the percentage of PMNs in the cytological endometrial swabs was demonstrated, despite the statistically significant difference between the percentage of PMNs in the bitches with *endometritis* and the animals with a normal *endometrium*.