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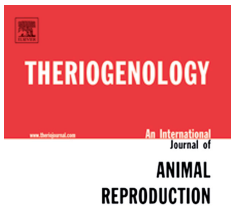

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Expression of 3β-hydroxysteroid dehydrogenase in ovarian and uterine tissue during diestrus and open cervix CEH-pyometra in the bitch

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Abstract

The purpose of this study was to compare the expression of 3β-hydroxystreroid dehydrogenase (3β-HSD) in the uterus and ovary of healthy dogs and those with cystic endometrial hyperplasia/pyometra complex (CEH-pyometra). Eighteen female dogs were included in the study. Eleven bitches with open cervix CEH-pyometra were included in the CEH-pyometra group and 7 diestrus bitches in the control group. For immunostaining a rabbit polyclonal one raised against recombinant human type 2 (adrenal/gonadal) 3β-HSD was used. Progesterone concentrations were not statistically different between the groups. Strongly stained large interstitial cell groups in the ovarian medulla were observed particularly in CEH-pyometra group though these cells in the control group were weakly or moderately stained and existed singly or paired. The expressions of 3β-HSD in luminal epithelium (42.40±22.40 % vs. 18.42±13.15 %, $p<0.05$) and glandular epithelium (32.80±27.05 % vs. 2.94±7.79 %, $p<0.01$) of endometrium were significantly higher in CEH-pyometra group than the control group. The expression of 3β-HSD in corpus luteum was higher (29.38±9.58 % vs 22.94±4.97 %) in CEH-pyometra group than that of control group, although the differences was not significant ($P>0.05$). Similarly, the significant increase in the expression of 3β-HSD in ovarian interstitial cells (33.86±29.44 vs. 1.13±2.97, $p<0.05$) was found in CEH-pyometra group compared to the control group. The study revealed that 3β-HSD expression in the endometrium of canine CEH-pyometra was significantly high.

Keywords: Canine, Cystic endometrial hyperplasia, Pyometra, 3β-hydroxysteroid dehydrogenase

1. Introduction

Canine pyometra is an important disease of intact mature bitches and occurs following estrus [1]. It is thought that there is an association between pyometra and cystic endometrial hyperplasia (CEH) [2]. CEH allows bacterial proliferation in the uterus at the end of estrus and the degenerative process of development of endometrial hyperplasia is linked with formation of pyometra. Because the whole process is mediated by progesterone (P4), it is considered a disease of diestrus [3,4]. Oestrogen and progestagen administration were also linked with development of pyometra [5], whereas pregnancy has a protective effect especially in Rottweiler, Collie and Labrador retriever breeds [6]. There are two forms of pyometra with either an open or a closed cervix. Bitches with open cervix pyometra present with a vaginal discharge while the ones with closed cervix pyometra present without a vaginal discharge [7]. There is also information about ovarian steroid hormonal effects in that estrogen opens the cervical canal and P4 closes [8]. In addition, it has been shown that the presence of pP4 receptors in the uterine cervix is related to the cervical patency [9]. P4 increases secretoric activity of endometrial glands and decreases myometrial contractility therefore causes closure of cervix [10].

Early diagnosis and appropriate treatment of pyometra are required to avoid disastrous consequences such as endotoxemia and specific renal abnormalities as a result of the effects of endotoxins [11]. In addition, presence of systemic inflammatory response syndrome (SIRS) could be detected in canine pyometra that is associated with poorer prognosis [12,13].

Steroid hormones such as P4, mineralocorticoids, androgens and estrogens have a crucial role in the development and growth of most tissues. The biosynthesis of these hormones requires the transformation of delta-5-3β-hydroxysteroids, namely, pregnenolone, 17-hydroxy pregnenolone, dehydroepiandrosterone and androst-5-ene-3β-,17β-diol into 4-ene-3-ketosteroids, P4, 17-hydroxy progesterone, androstenedione and testosterone, respectively.

The membrane-bound 3β-hydroxysteroid dehydrogenase/5-ene-4-ene-isomerase (3β-HSD) catalyzes that conversion [14,15].

The expression of 3β-HSD was confirmed in the human uterine endometrium by Rhee et al especially in the glandular epithelium and decidua [16]. It was detected in nonpregnant mouse endometrium at metestrus [17]. Moreover Ullmann et al [18] demonstrated the presence of 3β-HSD in the ovarian interstitial tissue, the corpus luteum and the granulosa cells of antral and atretic follicles in the South American opossum . Its expression was even demonstrated in Purkinje cells of the cerebellum in canine distemper virus (CDV) infected dogs suggesting its association with demyelination in CDV infection [19]. Concerning the female dog, 3β-HSD expression in corpus luteum during early and late diestrus was presented by Kowalewski et al. [20].

CEH-pyometra is a common disorder in dogs and its pathogenesis is still worth to investigate in detail. Thus in the present study, we examined the expression of 3β-HSD in canine ovarian and uterine tissue during diestrus and open cervix CEH-pyometra complex as well as its relationship with the circulating concentration of P4 in order to light the possible role of enzymatic activity in the uterus. To our best knowledge, this is the first report concerning the expression of 3β-HSD in canine CEH-pyometra complex.

2. Materials and Methods

2.1. Animals

The study was performed in accordance with the principles outlined in Decision no: 2009-12 of Ethical Committee of Animal Research of Turkey. Eighteen privately owned adult female dogs were assigned to the study. Groups consisted of bitches that had not been treated with endogenous progestins or estrogens in the past. All animals were subjected to ovariohysterectomy, either for treatment of CEH-pyometra complex or on request of their owners.

CEH-pyometra group (n=11) included bitches with open cervix CEH-pyometra aged 6.23±0.67 years. The breeds were 6 mongrels, 2 Pekineses, 1 Norfolk Terrier, 1 Doberman Pinscher, 1 Golden Retriever. The diagnosis of CEH-pyometra was based on anamneses, physical, vaginoscopic and ultrasonographic (Falco Vet, Pie Medical Imaging, Maastricht, The Netherlands) examination findings and blood test results.

The control group (n=7) included diestrus bitches aged 2.14±0.32 years and the breeds were 5 mongrels, 1 English Pointer, 1 Dogo Argentino. The ages of the groups differed significantly ($P<0.01$). Since pyometra is considered a diestrus disease, the control group included healthy bitches confirmed to be in diestrus after vaginoscopic, cytologic and ultrasonographic examinations [21]. Vaginal smears were obtained from the anterior vaginal wall with the use of a vaginoscope in order not to be contaminated with vestibulum vaginal material. Afterwards, they were stained using the Papanicolaou technique [22] and evaluated with a light microscope (Leica Microsystems Inc., Illionis, USA).

The blood samples for P4 assesment were taken from the cephalic vein into heparinised tubes before the surgery for hematologic analyses. The leukocyte, lymphocyte and monocyte counts were determined using a haemogram (Abacus Vet Junior, Diatron MI LtD, Budapest, Hungary).

2.2. Progesterone measurement

The plasma was separated after centrifugation at 1550 g for 10 minutes then transferred into labeled micro-centrifuge tubes and stored at -20^{o}C until assayed. P4 concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) method using canine-specific commercial kits (MyBioSource, Inc., San Diego CA, USA). All plasma samples were analyzed twice according to the manufacturer's recommendations. Both intraassay and interassay variabilities for the assay were less than 15%. The ELISA plate was read at 450 nm

on a microplate reader (Digital and Analog Systems, RS 232, Rome, Italy). The concentration of P4 was calculated with reference to a standard curve that was generated by plotting the average O.D. (450 nm) obtained from each standard on the horizontal axis versus the corresponding each standard concentration on the vertical axis. Results were expressed as ng/mL of plasma.

2.3. Sample collection and histopathological examination

Both ovaries and cornu uteri of each dog were fixed in 10% neutral formalin immediately after the surgery, dehydrated through an alcohol series and embedded in paraffin. Tissue sections were cut at a thickness of 5 µm and processed for hematoxylin and eosin staining [23]. Sections were histologically examined to confirm healthy tissue and to verify the presence of CEH-pyometra. Additionally, staging was performed according to the criteria of Dow [24]. Following such verification, sections were processed for immunohistochemistry.

2.4. Immunohistochemistry for 3β-HSD

For immunostaining a rabbit polyconal antibody raised against recombinant human type 2 (adrenal/gonadal) 3β-HSD was used. A universal horseradish peroxidase kit (Zymed Histostain Plus Bulk Kit, San Francisco CA, Cat. No. 85-9043) was used to localize 3β-HSD in the sections. Following routine rehydration and quenching in 3% H_2O_2 in absolute methanol for 10 min, blocking with 5% normal goat serum for 10 min and 1% bovine serum albumin in PBS containing 0.3% triton X 100 for 30 min at room temperature, the tissue sections were incubated with rabbit anti-human type 2 (adrenal/ gonadal) 3β-HSD antibody (1:512 dilution) for an hour at room temperature, incubated with anti-rabbit biotinylated secondary antibody labeled with streptavidin-peroxidase enzyme, reacted with 3-amino-9-ethylcarbazole (AEC) chromogen, counterstained with Mayer's haematoxylin and coverslipped. Between each step of the assay, sections were rinsed three times with tris-buffer (pH 7.4) for 10 min each. Random sections served as negative controls after elimination of

primary or secondary antibody. To double check the endogenous peroxidase background, the primary antibody was omitted with and without the presence of H_2O_2 blocking in random sections. Negative control sections from each animal received identical preparations for immunohistochemical staining, except that primary antibodies were replaced by normal rabbit serum.

The expression of 3β-HSD in luminal and glandular epithelia of uterine endometrium and corpus luteum in the ovarian cortex and intersititial cells in the ovarian medulla were investigated. The percentages of the total area or total cell number of the immunohistochemically 3β-HSD positive cells were assessed with a microscopy image analysis system (Bs200P; BAB Software, Turkey). The distribution of immunoreactive cells was examined with a Nikon Eclipse E-600 microscope. Immunolabelling of 3β-HSD was identified in the cytoplasm of cells. A total of 10 fields were chosen and analysed at X 400 magnification.

2.5. Statistical analysis

All statistical analyses were performed with PASW statistical software (version 11.5, SPSS, Chicago, IL, USA). The normality of features distribution was checked with the Shapiro-Wilk test. Since data were distributed normally, Kolmogorov-Smirnov Z-test was used to assess the differences between groups. Data were expressed as mean±SE. P values of <0.05 were accepted as significant.

3. Results

3.1. Clinical findings

All the bitches in CEH-pyometra group had no fever and vomiting but; inappetence, mucopurulent vulvar discharge, polyuria and polydipsia accompanied by marked

leukocytosis. Cervical patency and uterine discharge in the cranial vagina were observed during vaginoscopic examination. In addition, ultrasonographic examination revealed uterine enlargement characterized by thick uterine walls and anechoic to hypoechoic fluid.

In the control group, none of the bitches had any signs of illness. Ultrasonographic examination indicated normal appearence of uterus. Vaginoscopic appearence of mucosal folds was flattened. Evaluation of vaginal smears displayed intermediate and parabasal cells and abundant neutrophils [21,25]. Based on these findings the bitches were considered to be in diestrus.

The mean blood total leukocyte count in the CEH-pyometra group (35.67±11.60 x 10^3/µL) was outside normal reference range (6 to 17 x 10^3/µL) and greater than in the control group (11.13±0.92 x 10^3/µL). Lymphocytes and monocytes counts were within normal reference range in both groups.

3.2. Histopathological findings

The stages of pyometra (type 1-4) were determined with reference to the description stated by Dow [24] as type 1 (n=1), type 2 (n=4), type 3 (n=3) and type 4 (n=3). Uncomplicated CEH was type 1 with thickening and many cystic irregular elevations on the endometrial surface (Figure 1a). Type 4 was characterized with chronic endometritis. The uterine walls were thickened, endometrium was atrophied and lymphocyte infiltration was present.

3.3. Immunohistochemical findings

The expression of 3β-HSD in luminal and glandular epithelia of uterine endometrium were observed in the tissues both from CEH-pyometra and the control groups (Figure 1b). The expression of 3β-HSD in luminal and glandular epithelia of endometrium and interstitial cells in ovarian medulla were higher in CEH-pyometra group than the control group (Figure 1c, d). Additionally, corpus luteum (Figure 2a) and interstitial cells in both groups (Figure 2b,c,d) were stained. Strongly stained large interstitial cell groups in the ovarian medulla were

observed particularly in CEH-pyometra group (Figure 2c, d) though these cells in the control group were weakly or moderately stained and existed either singly or paired (Figure 2b).

3β-HSD immunopositive interstitial cells were not detected in the ovaries of the control group or there were a few in the medulla of certain ovaries though intensive 3β-HSD immunopositive intersititial cell groups were determined in medullar regions near to cortical border of ovaries of CEH-pyometra group. The immunopositive staining of these cells was more intense than of the luteal cells of corpus luteum. When the primary antibody was omitted and replaced by normal rabbit serum, no staining was observed.

Expression of 3β-HSD in luminal epithelium and glandular epithelium of endometrium, in corpus luteum and in intersititial cells of ovarian medulla were presented in Figure 3. The expressions of 3β-HSD in luminal epithelium (42.40±22.40 % vs. 18.42±13.15 %, $p<0.05$) and glandular epithelium (32.80±27.05 % vs. 2.94±7.79 %, $p<0.01$) of endometrium were significantly higher in CEH-pyometra group than the control group. The expression of 3β-HSD in corpus luteum was detected to be increased (29.38±9.58 % vs 22.94±4.97 %) in CEH-pyometra group than that of control group, although the differences was not significant ($P>0.05$). The expression of 3β-HSD in ovarian interstitial cells (33.86±29.44 vs. 1.13±2.97, $p<0.05$) was significantly higher in CEH-pyometra group than the control group.

3.4. Progesterone concentrations

The average plasma P4 concentration was 8.73±1.65 ng/mL and 5.83±0.72 ng/mL in the study and the control groups, respectively. The difference in plasma P4 concentrations in both groups did not reach statistical significance.

4. Discussion

The domestic dog is known to have a similar P4 profile for pregnant and nonpregnant bitches during diestrus and the corpus luteum is the only source of circulating P4. During the preovulatory LH surge P4 concentration sharply increases more than 2 ng/mL and continue to increase to 15-90 ng/mL to days 30 after LH surge. Afterwards the concentration begins to decline in the following 5 to 6 weeks [21]. It is well known that this P4 dominant period may lead to cystic endometrial hyperplasia and pyometra [2,11]. Although there is much information from studies of the pathophysiology of pyometra, so far the expression of 3β-HSD in uterus and ovary of dogs with CEH-pyometra has not been reported.

Kowalewski et al found that expression of 3β-HSD mRNA in the canine corpus luteum was highest during early diestrus and decreased gradually towards day 65, resembling the circulating P4 profile during diestrus [20]. Rhee et al determined that 3β-HSD was weakly expressed in the glandular epithelium of the proliferative phase and moderately expressed in the secretory phase of the human uterine endometrium [16]. Similarly, 3β-HSD was identified in nonpregnant mouse endometrium [17] and in ovarian interstitial tissue, corpus luteum and granulosa cells of antral and atretic follicles of the South American opossum [18]. Additionally, it has been shown in pregnant cats that 3β-HSD expression in luteal cells peaked during midpregnancy and in the maternal decidual cells of the placenta, the expression was significantly stronger toward the end of pregnancy indicating that the placenta is an additional source of P4 in pregnant cats which is essential for the maintenance of pregnancy [26]. Another study revealed that 3β-HSD expression in the cat placenta elevated clearly in the second half of the pregnancy, again supporting the result that feline placenta is capable of the synthesis of P4 [27]. The results of our study revealed the higher expression of 3β-HSD in luminal and glandular epithelia of uterine endometrium and the cytoplasm of intertitial cells of ovarian medulla in dogs with CEH-pyometra than the control group consisted of healthy diestrus dogs. On the other hand, the expression in corpus luteum of both groups did not show

a significant difference. We therefore put forward the hypothesis that the higher 3β-HSD level in uterus may result in local synthesis of P4 and consequently mucoid secretion of the endometrial glands allows the filling of the uterine lumen thus creating a predisposition to pyometra in the dog. A similar suggestion about the role of 3β-HSD activity in perianal sinus development and possibly in tumorogenesis was made by Stefanow et al in dogs [28]. In that study, a strong immunopositivity of 3β-HSD was observed in the cells beneath the squamous epithelium of the perianal sinus and suggestive of a role in the etiology of squamous cell epithelial tumours and adenocarcinoma [28].

Manifold studies proved that the expression of 3β-HSD was not only in the ovaries but also in other organs [16,18,19,20,28]. Even the epithelial cells in the human lacrimal gland, cornea and conjunctiva express 3β-HSD mRNA [29]. This multicentric distribution was identified with the role of the enzyme in the intracrine formation of sex steroids in peripheral tissues [15]. According to the physiological mechanism of intracrinology, sex steroids are made in target tissues and exert their action locally without release in the circulation [30]. This could explain why in our study plasma P4 concentrations of both groups were not statistically different. Accordingly, it is known that plasma P4 concentrations in the bitch with pyometra are not different from the concentration in healthy bitches [31]. In addition, plasma P4 concentrations in our study were not found to be parallel with 3β-HSD expression in ovarian and uterine tissue.

The intracrine formation of sex steroids are known to have a role in the aetiology of breast cancer in women after cessation of ovarian estrogen secretion at menopause [32]. There could be a similar link between local production of P4 and pyometra formation. Thus, in the present study, the presence of intensive 3β-HSD immunopositivity both in the interstitial cells of the ovary and the epithelium of endometrium in CEH-pyometra group might be the influence of increasing age. This deduction might explain why the incidence of pyometra increases with

age [4,11]. The effects of P4 in canine uterus, such as endometrial proliferation, glandular secretions, decrease in myometrial activity are indicated to be cumulative and might be more powerful with each estrus cycle [7], this additionally shows the impact of age on the pathogenesis of pyometra.

Sadasivam et al [33] detected that treatment with bacterial lipopolysaccharide, a component of the cell wall of gram negative bacteria, resulted in the increase of the mRNA expression of 3β-HSD and 17β-HSD in the brain of rats but in contrast significant decrease in the testis at 24 h and 48 h following the treatment. These differences in the enzyme activity in different tissues was thought to be a result of impaired antioxidant defenses [33]. In our study, the infection in the uterine tissue might have affected the expression level of the enzyme. However, one dog in CEH-pyometra group was determined to be type 1 as histologically which means uterine tissue contains only thickening and many cystic irregular elevations on the endometrial surface and even this dog had also strong expressions in the endometrium and this finding of that single dog caused to suspect that the level of expression could be independent of infection. Therefore, the effect of the infection in the canine uterus on 3β-HSD expression needs to be investigated in future studies. On the other hand, the fact that upregulation of the expression of 3β-HSD reflects the production of steroid hormones made us thought that intracrine synthesis of P4 in canine endometrium during CEH-pyometra might be probable.

In conclusion, the present study revealed that the immunopositivity of 3β-HSD in luminal and glandular epithelia of endometrium and interstitial cells in ovarian medulla were higher in dogs with CEH-pyometra than the control dogs. Though P4 concentration in uterine tissue has not been determined in the study, the results made us thought that the dog uterus may have an ability to synthesize P4. However, 3β-HSD has a role for the biosynthesis of the other steroid hormones. Thus, the importance or aetiological effect of intracrine synthesis requires detailed

295 future studies including other steroidogenic enzymes and further results might be helpful to
296 create more effective treatment plans for CEH-pyometra in the dog.

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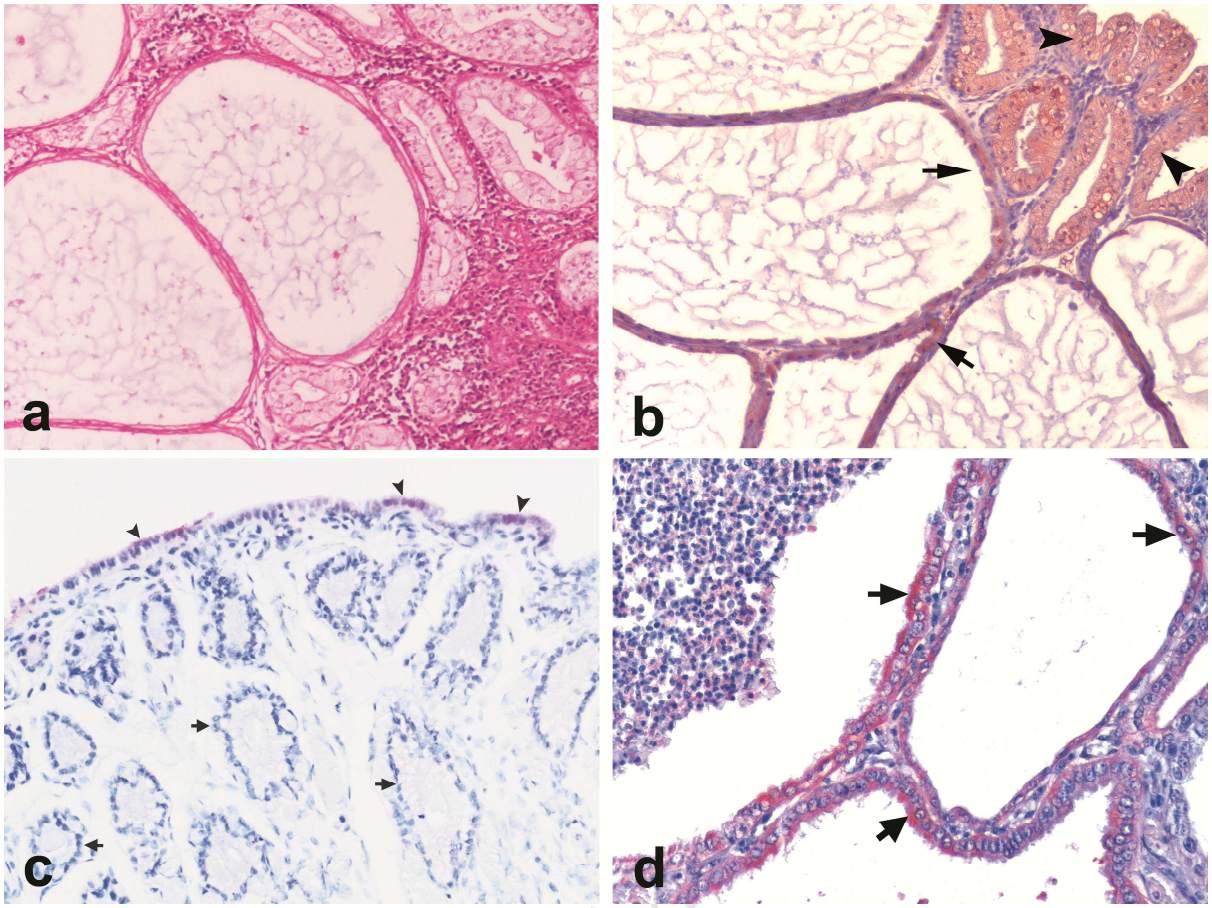
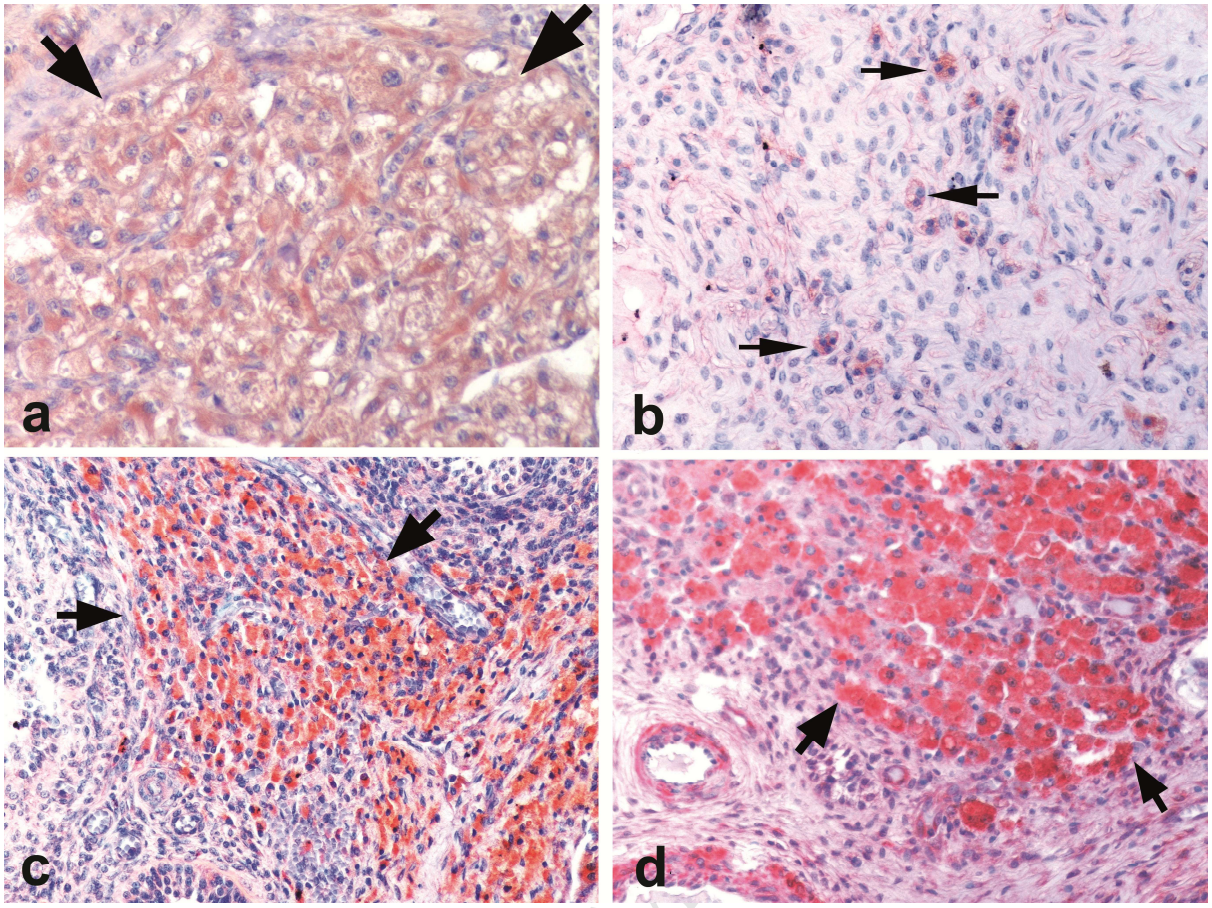
Figure Legends

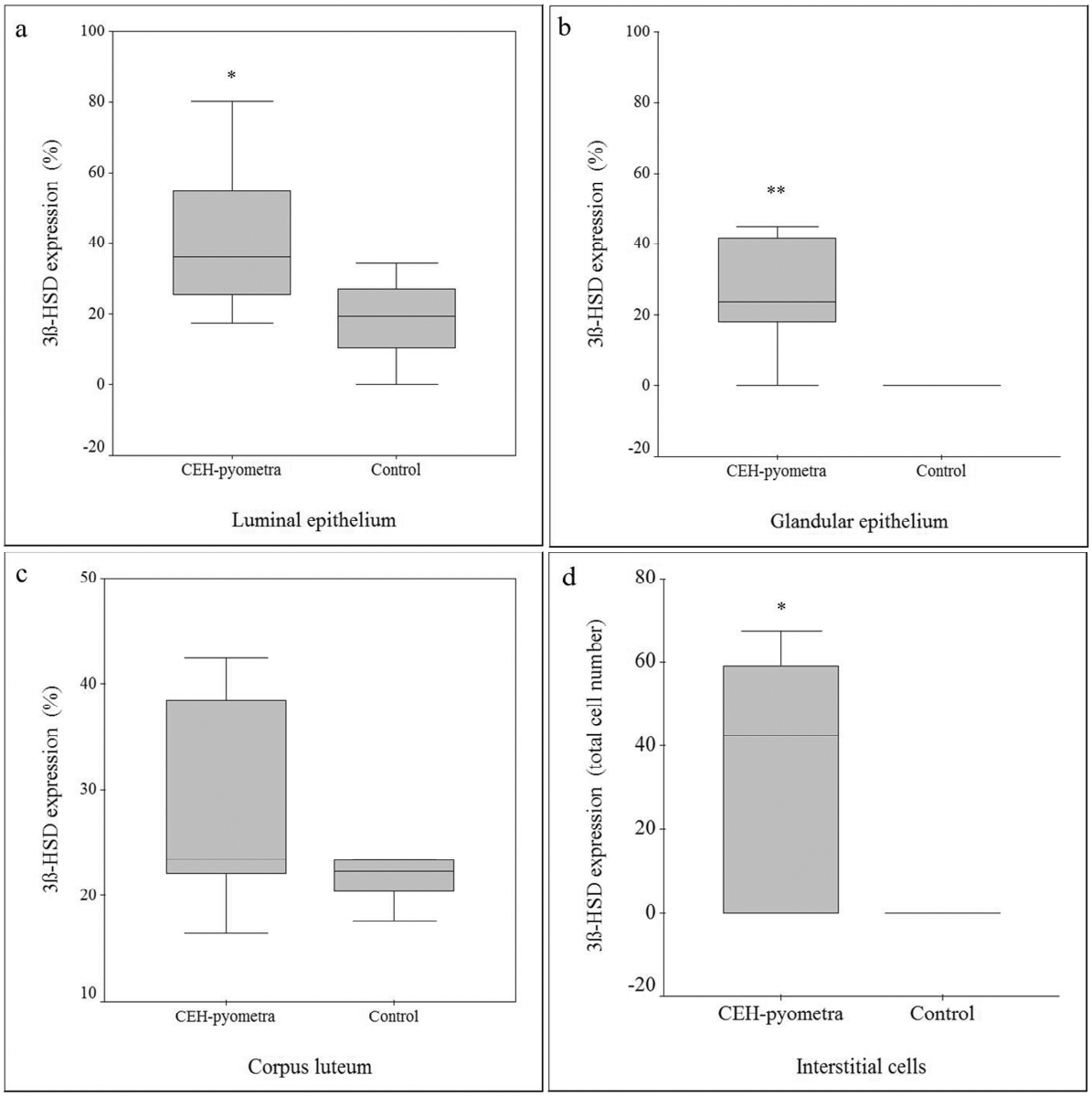
Figure 1. Endometrium of the control bitch and endometrium with CEH-pyometra. (a) Cystic endometrial hyperplasia in the bitch. (b) Immunopositivity of 3β-HSD in luminal epithelium (arrow heads) and glands (arrows) of endometrium with cystic endometrial hyperplasia. (c) Immunopositive staining of 3β-HSD in luminal epithelium (arrow heads) and immunonegative staining of glands (arrows) in endometrium of the control bitch. (d) Immunopositivity of 3β-HSD in glands (arrows) of endometrium with CEH-pyometra. Haemotoxylin and eosine, a; x10; Streptavidin peroxidase counterstained with haemotoxylin, b; 10x; c, d; 40x.

Figure 2. Expression of 3β-HSD in ovary of the control bitch and ovary with CEH-pyometra. (a) Immunopositivity of 3β-HSD (arrows) in luteal cells of corpus luteum in the ovary of bitch with CEH-pyometra (arrows). (b) A few 3β-HSD immunopositive interstitial cells located in ovarian medulla of the control bitch. (c) Large cell groups of 3β-HSD immunopositive interstitial cells located in ovarian medulla of bitch with CEH-pyometra (arrows). (d) Large cell groups of 3β-HSD immunopositive interstitial cells located in ovarian medulla of bitch with CEH-pyometra (arrows). Streptavidin peroxidase counterstained with haemotoxylin. a,b,c,d; 20x.

Figure 3. Expression of 3β-HSD in luminal epithelium (a), glandular epithelium (b) of endometrium, in corpus luteum (c) and intersititial cells of ovarian medulla (d) of the CEH-pyometra and the control group. In each box, the central mark represents the median, the edges of the box represent the 25^{th} and 75^{th} percentiles, and the whiskers are the most extreme data points not considered outliers. **P<0.01, *P<0.05.






a
3ß-HSD expression (%)
100
80
60
40
20
0
-20
*
CEH-pyometra
Control
Luminal epithelium
b
3ß-HSD expression (%)
100
80
60
40
20
0
-20
**
CEH-pyometra
Control
Glandular epithelium
c
3ß-HSD expression (%)
50
40
30
20
10
CEH-pyometra
Control
Corpus luteum
d
3ß-HSD expression (total cell number)
80
60
40
20
0
-20
*
CEH-pyometra
Control
Interstitial cells

Highlights

The expression of 3β-HSD in the uterus and ovary of healthy dogs and those with CEH-pyometra was investigated.

The immunopositivity of 3β-HSD in luminal and glandular epithelia of endometrium and interstitial cells in ovarian medulla were higher in dogs with CEH-pyometra than the healthy dogs in diestrus .

Strongly stained large interstitial cell groups in the ovarian medulla were observed particularly in the dogs with CEH-pyometra.

There was no significant relationship between plasma P4 concentration and 3β-HSD expression in ovarian and uterine tissue of the dogs.