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## A Comparative Study of Steroid Hormone Levels and Oestrogen Receptor alpha (ER $\alpha$ ) between Normal Bitch Uteri and Pyometra during Dioestrus

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*Original Article*

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## **A Comparative Study of Steroid Hormone Levels and Oestrogen Receptor alpha (ER $\alpha$ ) between Normal Bitch Uteri and Pyometra during Dioestrus.**

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### **Abstract**

Ovarian steroid hormones and their receptors play an important role in both physiological as well as pathological changes seen in the uterus. For the further understanding of the mechanism of pyometra, in relation to steroid hormones and their receptors in the bitch, the difference between the expression of the oestrogen receptor alpha (ER $\alpha$ ) between normal and pyometra bitches during dioestrus was investigated. Uterine samples were collected during dioestrus from normal and pyometra bitches. Immunohistochemistry was used to investigate the expression of ER $\alpha$  in the different compartments of the uterus. The results showed that the levels of steroid hormones in the normal and the pyometra group were similar, while ER $\alpha$  staining scores were significantly lower in the epithelia of the pyometra group compared to the normal group. This suggested that the expression of ER in the epithelia was strongly downregulated in pyometra cases compared to normal uteri during dioestrus. The mechanism of this downregulation may be due to more prolonged levels of progesterone and/or the suppression of oestrogen, via ER $\alpha$ , in order to enhance uterine infection. However, any differences of ER $\alpha$  localization in the stroma and the myometrium between normal uteri and pyometra was not obvious.

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**Keywords :** Estrogen receptor alpha (ER $\alpha$ ), pyometra, immunohistochemistry, bitch.

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## บทคัดย่อ

### การศึกษาเปรียบเทียบระดับฮอร์โมน และการแสดงออกตัวรับฮอร์โมนเอสโตรเจนแอลฟา ระหว่างสุนัขที่มีภาวะมดลูกอักเสบแบบมีหนองกับสุนัขปกติในช่วงไดเอสตรัส

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เสตียรอยด์ฮอร์โมนและตัวรับที่จำเพาะของฮอร์โมนนั้น ๆ มีบทบาทสำคัญต่อกลไกการทำงานทั้งปกติและผิดปกติของมดลูก ดังนั้นเพื่อความเข้าใจที่ดียิ่งขึ้นถึงพยาธิกำเนิดของการเกิดภาวะมดลูกอักเสบแบบมีหนอง การศึกษาเปรียบเทียบการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนแอลฟาในสุนัขปกติและสุนัขที่มีภาวะมดลูกอักเสบแบบมีหนองในระยะไดเอสตรัส ตัวอย่างมดลูกสุนัขในระยะไดเอสตรัสถูกนำมาตรวจสอบการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนแอลฟาด้วยวิธีอิมมูโนฮิสโตเคมี ผลการศึกษาพบความแตกต่างอย่างเห็นได้ชัดในเซลล์เยื่อบุมดลูกโดยที่สุนัขที่มีภาวะมดลูกอักเสบแบบมีหนองจะมีคะแนนการแสดงออกของตัวรับเอสโตรเจนแอลฟาน้อยกว่าอย่างมีนัยสำคัญแม้ระดับฮอร์โมนจะไม่แตกต่างกันก็ตาม แสดงให้เห็นว่าตัวรับเอสโตรเจนแอลฟาในมดลูกสุนัขที่มีภาวะมดลูกอักเสบแบบมีหนองถูกระงับการแสดงออกมากกว่าปกติด้วยระดับของโปรเจสเตอโรนที่ไม่แตกต่างกัน ทั้งนี้อาจเนื่องมาจากการที่กลุ่มมดลูกอักเสบแบบมีหนองมีระดับของโปรเจสเตอโรนสูงมาเป็นระยะเวลานานหรือระดับฮอร์โมนเอสโตรเจนถูกกดโดยผ่านทางตัวรับฮอร์โมน ทำให้การติดเชื่อในมดลูกเกิดขึ้นได้ง่าย อย่างไรก็ตามความแตกต่างของการแสดงออกตัวรับเอสโตรเจนแอลฟา ระหว่างสุนัขทั้งสองกลุ่มทดลองในส่วนอื่นๆ ของมดลูกยังไม่เด่นชัด

**คำสำคัญ:** ตัวรับฮอร์โมนเอสโตรเจนแอลฟา, มดลูกอักเสบเป็นหนอง, อิมมูโนฮิสโตเคมี, สุนัขเพศเมีย

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## Introduction

Pyometra is a most frequent condition in the reproductive system of bitches, but the mechanism of this pathological condition is not fully understood (Nelson and Feldman, 1986; Noakes et al., 2001). It has been suggested that an excessive quantity of progesterone or an oversensitivity of the uterus to progesterone can cause this pathological status in bitches (Sokolowski and Zimbelman, 1974; Verweridis et al., 2004). Moreover, pyometra usually occurred during the period of dioestrus as high levels of progesterone during that stage promote endometrial growth and glandular activity, while suppressing

myometrial activity (Nelson and Feldman, 1986). Oestrogen may also be a regulator in this mechanism, because high levels of oestrogen during prooestrus and oestrus can increase uterine sensitivity to progesterone in the following stages (Buhi et al., 1992; Chen et al., 2001). Moreover, exogenous oestrogen-like substances such as zearalenone can lead to apoptotic changes and hyperplasia of uterine cells (Obremski et al., 2003; Gajecka et al., 2004). From our earlier study, the differences of progesterone receptor (PR) immunoreactivities between normal and pyometra uteri, as well as in

different compartments of the uterus, may be involved in the pathogenesis of pyometra (Srisuwatanasagul et al., 2005). The difference in ER $\alpha$  between normal and pyometra uteri may also explain some part of this complicated mechanism of pyometra.

Sex steroid hormones and their receptors play an important role in both physiological as well as pathological changes in the uterus (Nelson and Kelly, 1976). A study on the expression of steroid hormone receptors is important to predict the function or malfunction of hormones on the target organs. From our previous study in bitches on the expression of PR, the prominent difference between pyometra and normal uteri was found in the connective tissue. For further understanding of the expression of reproductive steroid hormone receptors in pyometra bitches, the present study has investigated the differences in the expression of the ER $\alpha$  between normal and naturally affected pyometra bitches at dioestrus.

## Materials and Methods

### *Tissue and blood samples*

The uteri were obtained by ovariohysterectomy from bitches regardless of the breed. The uterine samples were classified into 2 groups, a normal uterine group during dioestrus (n=6) and a pyometra group (n=6). In the pyometra group, the purpose of spaying was because of clinical signs indicated pyometra i.e. depression, abdominal distention, mucopurulent vaginal discharge etc., and samples were only collected from bitches which showed a mucopurulent discharge from the uterine lumen. The stage of dioestrus was determined by vaginal cytology and macroscopic examination of the ovary, as well as the plasma level of progesterone (Holst and Phemister, 1974). The uterus at oestrus was used as a positive control and a negative control was obtained by omitting primary antibody.

### *Serum steroid hormone measurement*

The blood samples were collected from the cephalic vein just before surgery, and the serum obtained was stored at -20°C, until being assayed. The level of oestradiol 17 $\beta$  and progesterone was measured by the competitive immunoassay method (Immulite®, Los Angeles, CA, USA). The intra-assay coefficient of variation was 2.56 % and 2.07 % for oestradiol 17 $\beta$  and progesterone respectively. The lower limit of detection was 20 pg/ml for oestradiol 17 $\beta$  and 0.2 ng/ml for progesterone.

### *Histological examination*

Uterine samples were collected from the mesometrial side at about the middle of the uterine horns. They were fixed in 4% paraformaldehyde for 48-60 h and thereafter processed for histological examination, embedded in paraffin, sectioned 5  $\mu$ m thick and stained with hematoxylin-eosin (H&E) or kept until immunohistochemistry was performed.

### *Immunohistochemical staining*

The uterine samples used for immunohistochemistry were mounted on Polysine slides (Menzel-Glaser®, Braunschweig, Germany) to prevent the sections from falling during the procedure. The sections were deparaffined and pretreated in 0.01M citric acid buffer, pH 6.0 in a microwave in order to increase the immunohistochemical reaction. Thereafter, they were rinsed in phosphate buffered saline (PBS) and incubated in 3% hydrogen peroxide in methanol, to block endogenous peroxidase, for 10 min at room temperature (RT). After this step, all incubations were done in a humidified chamber at RT. After blocking endogenous peroxidase, the sections were rinsed in PBS, and incubated with normal horse serum for 30 min. Then, immunohistochemical detection of ER $\alpha$  was performed by adding mouse monoclonal antibody to ER $\alpha$  (1:75) (ER $\alpha$  1D5, Dako Cytomation Glostrup,

Denmark) to the sections overnight. The negative controls were obtained by omitting the primary antibody in the procedure. After the primary antibody incubation, all sections were rinsed with PBS 3 times, before being incubated for 30 min with biotinylated anti-mouse antibody, followed by a 30 min incubation with avidin-biotin complex. Finally, to visualize the immunoreactivity, 3,3'-diaminobenzidine (DAB kit, Vector Laboratories, Inc., Burlingame, CA, USA) was added for 5 min. All the sections were subsequently counterstained with hematoxylin, in order to distinguish negative reactions from positive reactions. The results were investigated under a light microscope.

#### ***Evaluation of the results***

All the immunostaining evaluation was carried out under a light microscope at x400 by the same observer who was unaware of the sample identity. Four different uterine compartments, the surface epithelium, the glandular epithelium, the stroma and the myometrium, were evaluated separately. Each compartment was evaluated by giving it an immunohistochemical total score, as described before by Vermeirsch et al. (1999). Briefly, the intensity score (I) ranged from 0 to 3 (0 = negative, 1 = weak, 2 = moderate, 3 = strong staining) and the proportion score (P) varied from 0 to 5 (0 = no positive cells, 1 = <1% positive cells, 2 = 1-9% positive nuclei; 3 = 10-32% positive nuclei, 4 = 33- 65% positive nuclei and 5 = >65% positive nuclei). The total score was calculated by adding I + P.

#### ***Statistical analyses***

The results of the steroid hormone levels were analysed by using a student *t*-test and the results of total ER $\alpha$  scores from the different groups were compared using a non-parametric method (Kruskal Wallis test, SAS for windows, version 9, Cary, NC, USA). *P* values < 0.05 were accepted as significantly

different.

## **Results**

### ***Clinical finding***

In the normal group, the uteri had a normal appearance while in the pyometra group, the uterine lumens contained mucopurulent discharge. Microscopically, uterine samples from the normal group showed no cystic glands in the endometrium, with neither an enlargement nor an inflammatory response. In the pyometra group, all uterine horns were enlarged with cystic endometrial glands and the uterine lumens were filled with cell debris and inflammatory cells. The stroma was also infiltrated with abundant inflammatory cells.

### ***Hormone levels***

The results of the steroid hormone levels are shown in Table 1. No significant differences in the oestradiol 17 $\beta$  or the progesterone levels between normal and pyometra uteri were found.

### ***ER $\alpha$ immunolocalization***

The immunostaining scores are summarized in Table 2 (Figs. 1C-1F). In the control group during dioestrus (Figs. 1C and 1E), staining was prominent in the surface epithelium and the endometrial glands, while low staining scores were observed in the stroma and myometrium. In the pyometra group (Figs. 1D and 1F), the staining scores were significantly lower in most compartments compared to the normal group. Moreover, no staining was observed in the inflammatory cells of the uterine stroma nor in the uterine vessels. Positive controls showed strong staining, as well as a high proportion of immunostaining cells in all compartments, while no specific staining was found in the negative control as shown in Figs. 1A and 1B, respectively.

**Table 1** Mean serum sex steroid hormone concentrations in normal dog and dog with pyometra (all values are expressed as means  $\pm$  S.D.).

Group	Oestrodiol 17 $\beta$ (pg/ml)	Progesterone (ng/ml)
Normal	35.76 $\pm$ 27.7	14.3 $\pm$ 10.8
Pyometra	32.26 $\pm$ 6.6	5.8 $\pm$ 5.4
Overall significance	NS	NS

NS = not significant

**Table 2** Results of ER-total scores for the different uterine compartments.

Group	Surface epithelium	Glandular epithelium	Stroma	Myometrium
Normal uteri	4.25 $\pm$ 2.3 <sup>a</sup>	6.00 $\pm$ 1.1 <sup>a</sup>	0.80 $\pm$ 2.0	2.00 $\pm$ 3.16
Pyometra	0 $\pm$ 0 <sup>b</sup>	2.91 $\pm$ 12.33 <sup>b</sup>	0.67 $\pm$ 1.63	2.16 $\pm$ 2.40
Overall significance	p < 0.01	p < 0.05	NS	NS

Mean ( $\pm$  S.D.) within the same column followed by the different superscript letters are significantly different (p < 0.05).

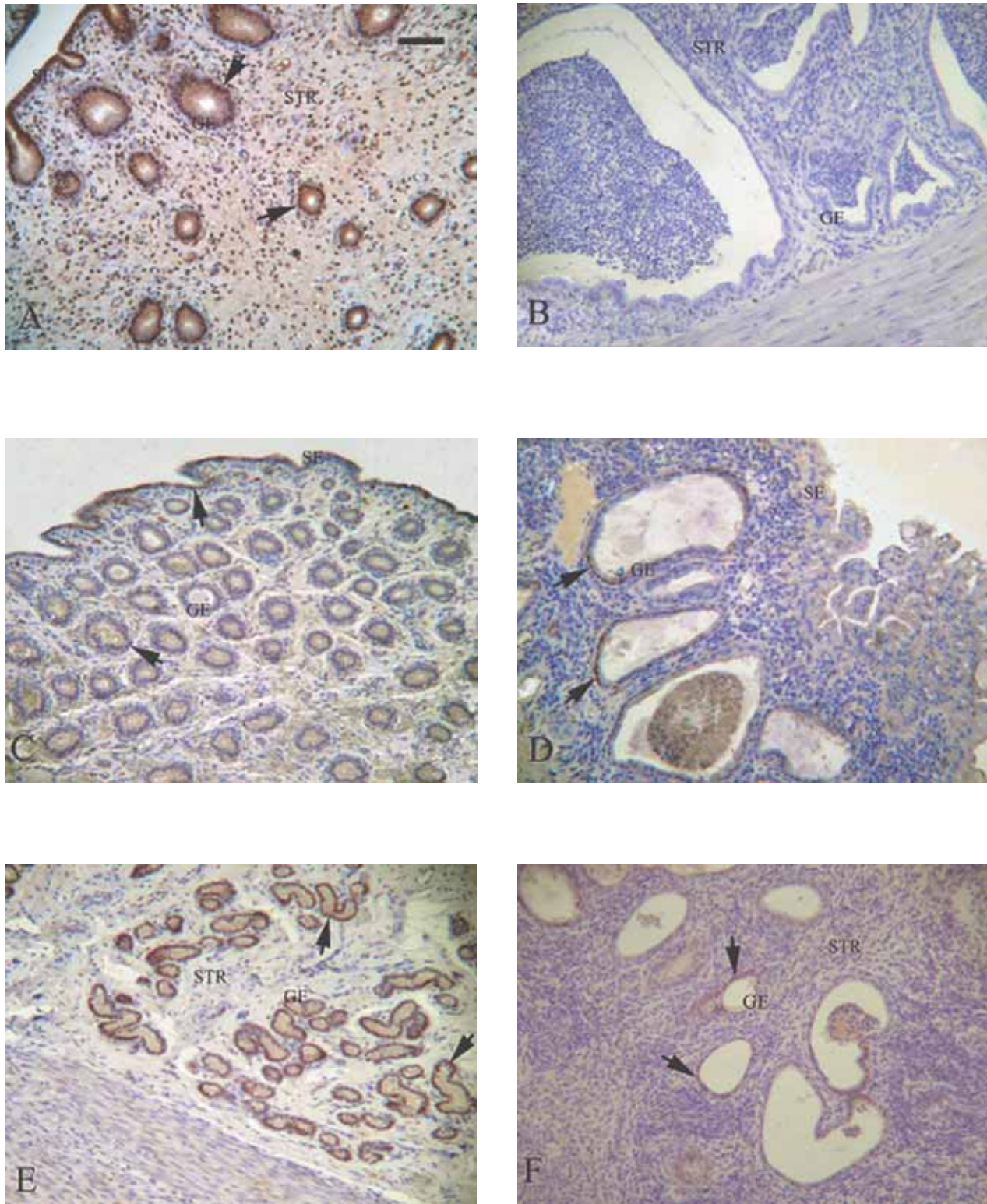
NS = not significant

### Discussion

In the present study, the serum levels of oestradiol 17 $\beta$  and progesterone at dioestrus were similar in normal and pyometra bitches. This was in agreement with earlier studies (Hadley, 1975; Austad et al., 1979) and supported the concept that circulatory progesterone levels may not be directly involved in the pathogenesis of pyometra (Chaffaux and Thibier, 1978). Regarding the expression of ER $\alpha$ , the present study showed different immunolocalization of ER $\alpha$  among the different uterine compartments, as well as between normal and pyometra uteri. At oestrus, the positive control sample showed higher immunostaining intensity and proportion in all compartments of the uterus, compared to both groups during dioestrus. Moreover, in the normal group at dioestrus, immunostaining scores in the epithelia were higher than in the stroma and the myometrium. This is simply explained by the increasing plasma level of progesterone during dioestrus which resulted in a

downregulation of ER $\alpha$  in the uterus. This downregulation effect from progesterone on the expression of ER $\alpha$  was compartment-specific (Dhaliwal et al., 1997). Moreover, it indicated that the expression of ER $\alpha$  was still needed in the epithelia during dioestrus, but was less important in other compartments such as the myometrium or the stroma.

In the pyometra group, positive staining was low in most compartments, with no staining of the surface epithelium, suggesting that the inactive appearance was in response to steroid hormones (De Bosschere et al., 2003). The results showed that the downregulation effect from progesterone during dioestrus was stronger in pyometra cases compared to that seen in the normal uteri. If ER $\alpha$  in the epithelia regulated some of the reproductive mechanisms during dioestrus, it suggested that those mechanisms were absent in pyometra cases. Additionally, it was shown that the genital tract was more resistant or



**Figure 1** Immunohistochemical staining of the bitch uteri. A, normal uterus at oestrus as a positive control; B, negative control from a pyometra bitch; C and E, normal uterus at dioestrus; D and F pyometra bitch at dioestrus; E and F show endometrial glands and cystic endometrial glands in the deep layer of the uterus respectively. SE = surface epithelium; GE = glandular epithelium, STR = stroma. The arrows showed positive staining cells and the bar in Figure 1A represents a distance of 50  $\mu$ m.

less susceptible to infection under oestrogen domination (Rowson et al., 1953; Roth et al., 1983; Ramadan et al., 1997; Sugiura et al., 2004) and that the susceptibility to uterine infection increased when progesterone concentrations increased (Lewis, 2003). The mechanism of pyometra may be initiated by high levels of progesterone causing a downregulation of ER $\alpha$ , which consequently caused desensitization of oestrogenic effects leading to higher susceptibility to uterine infection.

It was suggested by earlier studies that oestrogen mediated proliferation in the epithelia, both directly and indirectly, via ER $\alpha$  (Yamashita et al., 1990; Cooke et al., 1997; Felix and Farahmand, 1997; Bigsby et al., 2004; Helguero et al., 2005). Our present study, however, showed the different results in the endometrial glands, where the lower ER $\alpha$  immunostaining scores were found in the epithelia of pyometra uteri compared to those in normal uteri. Moreover, an earlier study in experimentally induced pyometra showed ER $\alpha$  positive staining only in normal endometrial glands (De Bosschere et al., 2003), while the present study showed stainings in both the normal and the cystic endometrial glands, though the staining score was lower in cystic glands. These results supported the findings in human endometrium, that ER $\alpha$  was likely to be lower in hyperplastic compared to normal proliferative endometrium (Thornton and Wells, 1987; Papadimitriou et al., 1992; Bircan et al., 2005). Therefore, lower ER $\alpha$  may be involved in the formation of cystic hyperplasia of the uterine glands in pyometra cases, as seen in the present study.

In the myometrium, uterine contractions have been shown to increase during oestrus (Langendijk et al., 2002) and ER $\alpha$  is believed to mediate the oestrogenic influence that stimulates myometrial contraction (Sukjumlong et al., 2004). As a result, a low level of ER $\alpha$  in the myometrium at dioestrus will reduce the myometrial activity, which may

exacerbate a condition of pyometra, as expulsion of uterine pus can not be done.

From a previous study on PR in bitches, it was shown that the downregulation of PR in the uterine stroma was altered in pyometra cases, compared to normal bitches (Srisuwatanasagul et al., 2005). On the other hand, ER $\alpha$  was strongly downregulated in the epithelia in the present study. From these results, it is suggested that PR and ER $\alpha$  play different roles in the pathogenesis of the pyometra.

In summary, the expression of ER $\alpha$  in the epithelia during dioestrus was strongly downregulated in pyometra cases compared to that in normal uteri, although the serum levels of the steroid hormones were similar. The mechanism of this strong downregulation may be due to a more prolonged high level of progesterone and/or the suppression of oestrogen via ER $\alpha$ , which enhance uterine infection in the pyometra group. However, the difference in the ER $\alpha$  localization in the stroma and the myometrium between normal and pyometra uteri was not obvious.

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