

AN IMMUNOHISTOCHEMICAL STUDY OF THE PROGESTERONE RECEPTOR (PR) EXPRESSION IN PYOMETRA CASES AND NORMAL UTERI AT THE TIME OF DIOESTRUS IN THE BITCH

**Kongkiat Srisuwatanasagul^{1*} Sayamon Sukjumlong¹
Sudson Sirivaidyapong² Sinchai Pianchop¹**

Abstract

Kongkiat Srisuwatanasagul^{1*} Sayamon Sukjumlong¹ Sudson Sirivaidyapong² Sinchai Pianchop¹

AN IMMUNOHISTOCHEMICAL STUDY OF THE PROGESTERONE RECEPTOR (PR) EXPRESSION IN PYOMETRA CASES AND NORMAL UTERI AT THE TIME OF DIOESTRUS IN THE BITCH

The aims of the present study were to investigate the expression of the progesterone receptor (PR) and to compare whether there was any difference between pyometra cases and normal bitch uteri, during dioestrus, by means of immunohistochemistry. The uterine samples were collected from bitches which clients had submitted for spaying and for pyometra treatment. The results showed that a difference was obvious in the connective tissue stroma and myometrium in which a higher expression of PR was observed in the pyometra group. It may be postulated that the difference in PR expression in these uterine compartments may be involved in the pathogenesis of pyometra, which commonly occurs during dioestrus.

Keywords : progesterone receptor, pyometra, immunohistochemistry

¹Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330

²Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330

*Corresponding author

¹ภาควิชากายวิภาคศาสตร์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

²ภาควิชาสูติศาสตร์ เภสัชวิทยา และวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330

*ผู้รับผิดชอบบทความ

บทคัดย่อ

ก้องเกียรติ ศรีสุวรรณสกุล^{1*} ศยามณ สุขจำลอง¹ สุดสรร ศิริไวยพวงศ์² ศิลป์ชัย เพียรชอบ¹

การศึกษาตัวรับฮอร์โมนโปรเจสเตอโรนในมดลูกของสุนัขที่มีภาวะมดลูกอักเสบแบบเป็นหนองเปรียบเทียบกับสุนัขปกติในระยะไดเอสตรัส

การศึกษาจำนวนตัวรับของฮอร์โมนโปรเจสเตอโรนในมดลูกของสุนัขที่มีภาวะมดลูกอักเสบแบบเป็นหนองเปรียบเทียบกับมดลูกสุนัขปกติในระยะไดเอสตรัส (dioestrus) โดยวิธีอิมมูโนฮิสโตเคมี เก็บตัวอย่างมดลูกสุนัขที่ได้รับการทำหมัน หรือผ่าตัดมดลูกเพื่อแก้ไขภาวะมดลูกอักเสบแบบมีหนอง ผลการทดลองพบว่ามีความแตกต่างระหว่างกลุ่มสุนัขที่ป่วยด้วยภาวะมดลูกอักเสบและสุนัขปกติโดยพบจำนวนการแสดงของโปรตีนตัวรับฮอร์โมนโปรเจสเตอโรน (PR) มากกว่าในส่วนเนื้อเยื่อเยื่อเกี่ยวพัน (stroma) และส่วนกล้ามเนื้อมดลูก (myometrium) ของกลุ่มมดลูกอักเสบเป็นหนองเมื่อเปรียบเทียบกับมดลูกของสุนัขปกติ ดังนั้นอาจกล่าวได้ว่าความแตกต่างที่พบในระยะไดเอสตรัส ระหว่างสุนัขสองกลุ่มนี้อาจเกี่ยวข้องกับพยาธิกำเนิดของภาวะมดลูกอักเสบในสุนัขที่พบในระยะไดเอสตรัส

คำสำคัญ : ตัวรับฮอร์โมนโปรเจสเตอโรน, มดลูกอักเสบแบบมีหนอง, อิมมูโนฮิสโตเคมี

Introduction

Pyometra is a pus-filled, infected uterus and the typical time for it to occur is at dioestrus (Noakes et al., 2001; Schoon et al., 1992). Common signs of pyometra are vaginal discharge, polyuria, polydipsia, gastrointestinal signs such as vomiting, and depression. Though the pathogenesis of pyometra was not fully understood, it is assumed to be mainly caused by hormonal disturbances such as stimulation by exogenous progesterone (Nelson and Feldman, 1986; Noakes et al., 2001). Moreover, there is a report that the treatment of pyometra with an antiprogesterin may lead to clinical recovery (Trasch et al., 2003; Zaragoza et al., 2004). On the other hand, earlier studies reported that there is no evidence of high or prolonged increased plasma levels of progesterone, in bitches with pyometra (Chaffaux and Thibier, 1978; Hadley, 1975; Noakes et al., 2001). It may be that the pathogenesis of pyometra is mediated by changes in the expression of progesterone receptors rather than an increase of plasma progesterone levels.

The effects of ovarian, steroid hormones are regulated by their receptor proteins in specific target tissues. The investigation of progesterone receptors may lead to a better understanding of the mechanism of pathological change that occur in the uterus causing pyometra. The aims of the present study were to investigate the expression of progesterone receptors, in different uterine compartments of bitches with pyometra and to compare them with those in normal bitch uteri.

Material and Methods

2.1 Tissue samples

The uterine samples were obtained following ovariohysterectomy, from bitches of many breeds and age. In the pyometra group, the purpose of spaying was because of clinical signs, indicative of pyometra i.e. purulent vaginal discharge, depression, abdominal distention etc. The studies were done on 3 groups : group A, pyometra group (n=6); group B, control group in

dioestrus (n=6) and group C, control group in anoestrus (n=6). In group A, only samples collected from bitches which showed pus in the uterine lumen were included in the experiment. In all groups, the stages of the oestrous cycle were determined by vaginal cytology, as previously described in the earlier studies (Chu et al., 2001; Holst and Phemister, 1975) as well as by macroscopic and microscopic examination of the uterus (Galabova et al., 2003).

2.2 Histological examination

Uterine samples were collected from the mesometrial site near the centre of the uterine horns. They were fixed in 4% paraformaldehyde for 48-60 h and thereafter processed for histological examination: embedded in paraffin, sectioned 4 µm thick and stained with hematoxylin-eosin (H&E) or kept until immunohistochemistry was performed.

2.3 Immunohistochemical staining

The uterine samples used for immunohistochemistry were mounted on 3-aminopropyl-triethoxysilane-coated slides. The sections were deparaffined and pretreated in 0.01M citric acid buffer, pH 6.0 in a microwave (700 watt, Imarflex, MO-7528V) in order to increase the immunohistochemical reaction. Thereafter, they were rinsed in 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 and incubated with normal horse serum for 30 min., at RT. The immunohistochemical detection of PR was performed using mouse monoclonal antibody to PR (1:200, clone 10A9, Immunotech, Hamburg, Germany) added to the sections for 3 h at RT. The negative controls omitted primary antibody in the samples. After primary antibody incubation, all sections were rinsed with PBS in between each step of the staining procedure. The sections were incubated with biotinylated secondary antibody and avidin biotin complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 30 min. at RT,

respectively. Finally to visualise the immunological reaction, 3,3'diaminobenzidine (DAB kit, Vector Laboratories, Inc., Burlingame, CA, USA) was added for 5 min and all the sections were counterstained with hematoxylin in order to distinguish negative reactions from positive ones. All the sections were investigated under a light microscope. Negative controls were done by omitting the primary antibody and normal bitch uteri in oestrus were used as positive controls.

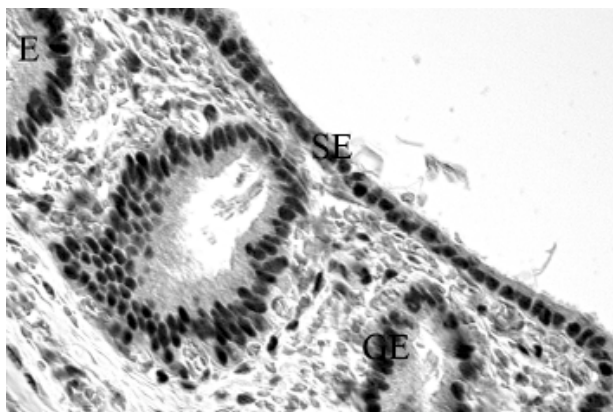
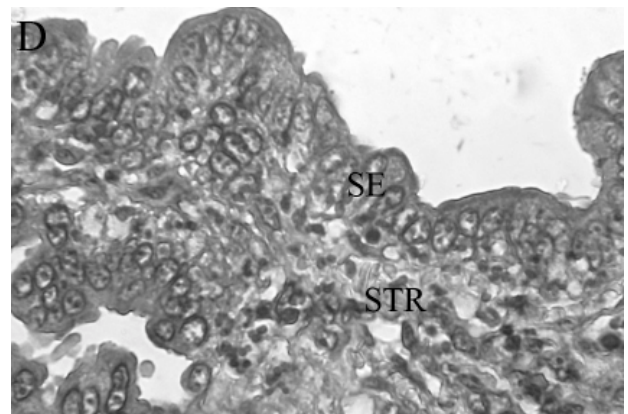
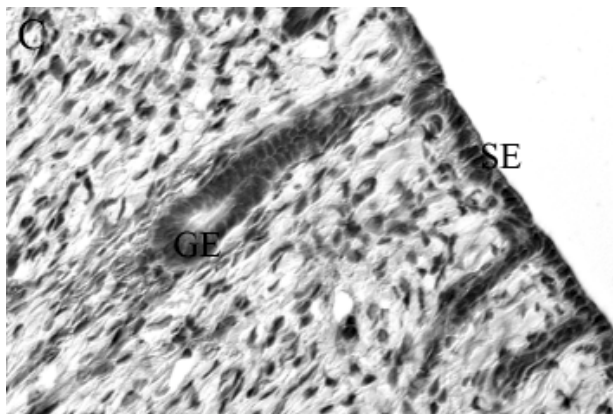
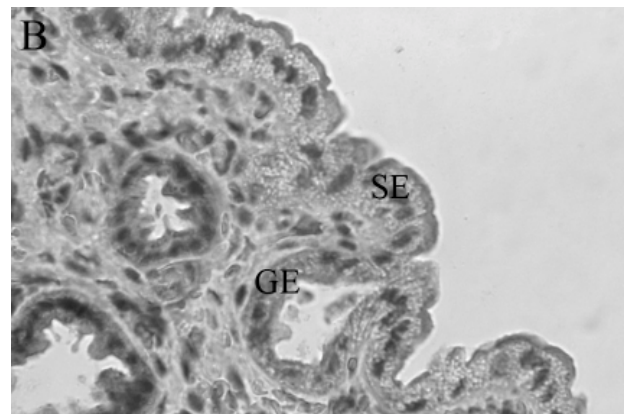
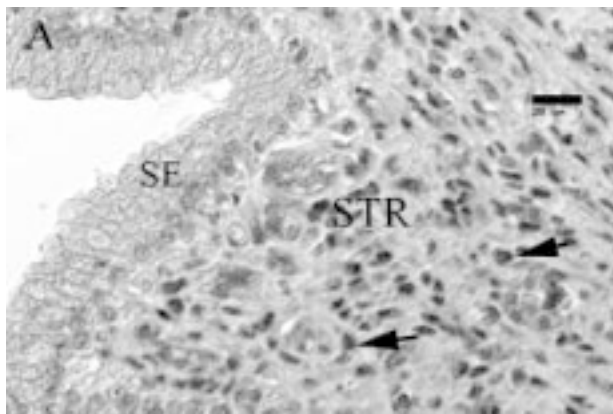
2.4 Evaluation of the results

All the immunostaining evaluation was done under a light microscope at x400. As most of the cells in each compartment (surface epithelium, glandular epithelium, connective tissue and myometrium) were uniformly distributed, only evaluation on staining intensity was done. The results of staining intensity were evaluated semiquantitatively and classified as negative (-), weak (+), moderate (++) and strong staining (+++) (Sukjumlong et al., 2003).

Results and discussion

The results are shown in Table 1 under the specific uterine compartments, which were, surface epithelium, glandular epithelium, connective tissue and myometrium. Positive cells, with brown staining in the nuclei and no cytoplasmic staining, were observed in any of the samples examined. Cells of the vessels such as endothelial cells were all negative, and they were not evaluated. In the negative controls (Fig D), no specific staining was found in any uterine cells, while most of the cells stained positively for the positive controls (Fig E).

The results showed that the monoclonal antibody used in the study (PR clone 10A9) was able to stain progesterone receptors in the paraformaldehyde fixed, paraffin embedded uterine tissues from the bitches. It was reported in earlier studies that pyometra is frequently found at dioestrus (Noakes et al., 2001; Schoon et al., 1992). Moreover, pyometra can be induced experimentally by the inoculation of *Escherichia coli* suspension (De Bosschere et al., 2002a; Nomura and Funahashi, 1999). Therefore,



Immunohistochemical staining of progesterone receptors in the bitch uterus :

A, pyometra group: B, control group at dioestrus; C, control group at anoestrus; D, negative control and E, positive control

SE: surface epithelium, STR: stroma and GE: glandular epithelium. The arrow in figure 1A shows a strong positive cell in the stroma and the bar in figure A represents a distance of 20 μ m

in the present study normal uterine samples at dioestrus were used so as to compare them with pyometra samples.

In group A (pyometra group), prominent staining was found in the connective tissue, while the staining intensity in the epithelia (both surface and glandular epithelia) was weak or negative. The staining intensity in the myometrium was moderate (Fig A). In group B (controls in dioestrus), most of the epithelial cells (surface and glandular epithelia) were negative with a few cells showing weak intensity in the surface epithelium. Most of the cells in the connective tissue compartments were weakly stained (Fig B). In group C (controls in anoestrus), the staining intensity was stronger in the surface epithelium than in the other compartments (Fig C).

Earlier studies in bitches have reported that progesterone receptors (PR) varied during the different stages of the oestrous cycle and also in different uterine cells (Dhaliwal, et al., 1997). The present study confirmed that variation was observed, not only between groups but also between different tissue compartments. However, in a study by Dhaliwal et al. (1997), some positive staining in the glandular epithelium during dioestrus was observed, whereas no positive staining could be found in the glandular epithelium in the present study during dioestrus. The discrepancy between these two studies could be because the study by Dhaliwal et al. (1997) was done on frozen tissues, while this present study was done on paraformaldehyde fixed, paraffin embedded tissues, where immunoreactivity may be lower than in frozen tissues.

In the pyometra group, staining intensity was found to be prominently strong in the connective tissue and only moderate in the myometrium, while negative or weak staining was observed in the other compartments i.e. the epithelia. In the control group at dioestrus (group B), positive staining was negligible in most uterine compartments except in the connective tissue stroma, in which weak staining was observed. These results showed the difference in the expression of PR between normal uteri and pyometra, at dioestrus, with stronger staining

observed in the connective tissue and myometrium of the pyometra group.

There is an earlier study suggesting that stromal cells can mediate some of the effects of steroid hormones on epithelial cells e.g. epithelial proliferation (Vermeirsch et al., 2000). In the present study, it appeared that PR in stromal cells remained positive in all the groups examined compared to the PR in the epithelia, therefore, some of the regulatory mechanisms in the epithelia may be mediated by the PR in the stroma as PR was absent from the epithelia. These mechanisms may also be involved in the development of pyometra, in which proliferation might be strongly induced, as a higher expression of PR was found in the stroma compared to the epithelia in pyometra group.

When comparing the results of the present study with earlier studies by De Bosschere (2003), in which pyometra was induced by the intraluminal inoculation, of a ligated uterine horn, with an *Escherichia coli* suspension, a similar pattern was observed with a higher expression of PR in most uterine compartments in the pyometra group. This indicated a similar mechanism regarding PR expression in both experimental pyometra in the earlier study and natural pyometra in the present study. It would seem that the pathogenesis of natural pyometra may involve an interaction between bacteria and hormones, as was shown in the experimental pyometra (De Bosschere et al., 2003).

In the control groups, differences could be observed with a lower presence of PR at dioestrus compared with anoestrus. A downregulation of PR in epithelia by progesterone was reported in earlier studies i.e. it was shown that progesterone treatment can reduce the number of progesterone receptors in uterine cell cultures (Galabova-Kovacs et al., 2004) and also in reproductive tissues (De Bosschere et al., 2002^b; Dhaliwal et al., 1999^b). Therefore, the low expression of PR at dioestrus in the present study may be the result of an expected higher level of progesterone at that period of the cycle as is shown in other studies (Dhaliwal et al., 1999a; Galabova-Kovacs et al., 2004). In the present study, this downregulation

mechanism was absent in the pyometra group, as a high expression of PR was found in the stroma and myometrium during dioestrus. The loss of a down regulation mechanism on PR by progesterone may cause the onset of a pathological state leading to pyometra in the bitch uterus.

At anoestrus, prominent staining was found in the surface epithelium, even though the levels of steroid hormones; oestrogen and progesterone, were supposed to be low, as was shown in an earlier study (Chu et al., 2002). This indicated that the level of plasma steroid hormones may not be the only regulator for the expression of PR. Instead, some other factors such as the stage of the oestrous cycle, the physiology of different uterine compartments (Galabova-Kovacs et al., 2004) and the pathological status of the bitch uterus, could also be involved.

When comparing the tissue compartments, different staining patterns was observed in the two types

of epithelia (surface and glandular epithelia), in the control group at anoestrus (group C), and it could be suggested that the surface epithelium and the glandular epithelium response differently, at the same stage of the oestrous cycle and that these two have different functions or roles in the reproductive physiology of the bitch uterus.

In conclusion, the differences in PR expression found between the pyometra and the control groups, indicate that the different immunostaining patterns of PR in the stroma and myometrium, may at least, be involved in the pathogenesis of pyometra of bitches during dioestrus. Moreover, it is speculated that the expected down-regulation mechanism by progesterone at dioestrus, in these compartments, was lost in the pyometra group. Further information such as the presence of PR, as well as the levels of steroid hormones, at different stages of the oestrous cycle are needed to confirm this finding.

Table 1. The Staining intensity of progesterone receptors in the bitch uteri with pyometra and in control groups.

| Uterine compartments | Staining intensity | | |
|----------------------|--------------------|---------|---------|
| | Group A | Group B | Group C |
| Surface epithelium | - | -/+ | +++ |
| Glandular epithelium | -/+ | - | - |
| Connective tissue | +++ | + | + |
| Myometrium | ++ | - | -/+ |

- negative; + weak, ++ moderate, +++ strong, +/- negative to weak staining

Group A : Pyometra group in dioestrus

Group B : Control group in dioestrus

Group C : Control group in anoestrus

Acknowledgement

This study was supported by The Veterinary Research Fund of the Faculty of Veterinary Science, Chulalongkorn University. The authors would like to thank Dr. Sukanya Maneein for helping to collect samples collections and Mrs. Jantima Intarapanya for her technical support on immunohistochemistry.

References

- Chaffaux, S. and Thibier, M. 1978. Peripheral plasma concentrations of progesterone in the bitch with pyometra. *Ann. Rech. Vet.* 9(3):587-592.
- Chu, P. Y., Lee, C. S., Moore, P. F., and Wright, P. J. 2001. Oestrogen and progesterone treated ovariectomized bitches: a model for the study of uterine function. *J. Reprod. Fertil. Suppl.* 57:45-54.
- Chu, P. Y., Wright, P. J., and Lee, C. S. 2002. Apoptosis of endometrial cells in the bitch. *Reprod. Fertil. Dev.* 14(5-6):297-305.
- De Bosschere, H., Ducatelle, R., and Tshamala, M. 2002^a. Is mechanically induced cystic endometrial hyperplasia (CEH) a suitable model for study of spontaneously occurring CEH in the uterus of the bitch? *Reprod. Domest. Anim.* 37(3):152-157.
- De Bosschere, H., Ducatelle, R., and Tshamala, M. 2003. Uterine oestrogen and progesterone receptor expression in experimental pyometra in the bitch. *J. Comp. Pathol.* 128(2-3):99-106.
- De Bosschere, H., Ducatelle, R., Vermeirsch, H., Simoens, P., and Coryn, M. 2002b. Estrogen-alpha and progesterone receptor expression in cystic endometrial hyperplasia and pyometra in the bitch. *Anim. Reprod. Sci.* 70(3-4):251-259.
- Dhaliwal, G. K., England, G. C., and Noakes, D. E. 1997. Immunocytochemical localization of oestrogen and progesterone receptors in the uterus of the normal bitch during oestrus and metoestrus. *J. Reprod. Fertil. Suppl.* 51:167-176.
- Dhaliwal, G. K., England, G. C., and Noakes, D. E. 1999^a. The influence of exogenous steroid hormones on steroid receptors, uterine histological structure and the bacterial flora of the normal bitch. *Anim. Reprod. Sci.* 56(3-4):259-277.
- Dhaliwal, G. K., England, G. C., and Noakes, D. E. 1999^b. Oestrogen and progesterone receptors in the uterine wall of bitches with cystic endometrial hyperplasia/pyometra. *Vet. Rec.* 145(16):455-457.
- Galabova, G., Egerbacher, M., Aurich, J. E., Leitner, M., and Walter, I. 2003. Morphological changes of the endometrial epithelium in the bitch during metoestrus and anoestrus. *Reprod. Domest. Anim.* 38(5):415-420.
- Galabova-Kovacs, G., Walter, I., Aurich, C., and Aurich, J. E. 2004. Steroid receptors in canine endometrial cells can be regulated by estrogen and progesterone under in vitro conditions. *Theriogenology.* 61(5):963-976.
- Hadley, J. C. 1975. Unconjugated oestrogen and progesterone concentrations in the blood of bitches with false pregnancy and pyometra. *Vet. Rec.* 96(25):545-547.
- Holst, P. A. and Phemister, R. D. 1975. Temporal sequence of events in the estrous cycle of the bitch. *Am. J. Vet. Res.* 36(5):705-706.
- Nelson, R. W. and Feldman, E. C. 1986. Pyometra. *Vet. Clin. North Am. Small Anim. Pract.* 16(3):561-576.
- Noakes, D. E., Dhaliwal, G. K., and England, G. C. 2001. Cystic endometrial hyperplasia/pyometra in dogs: a review of the causes and pathogenesis. *J. Reprod. Fertil. Suppl.* 57:395-406.
- Nomura, K. and Funahashi, H. 1999. Histological characteristics of canine deciduoma induced by intrauterine inoculation of *E. coli* suspension. *J. Vet. Med. Sci.* 61(4):433-438.
- Schoon, H. A., Schoon, D., and Nolte, I. 1992. [The pathogenesis of the "endometritis-pyometra complex" in the female dog]. *Zentralbl. Veterinarmed. A.* 39(1):43-56.

- Sukjumlong, S., Kaeoket, K., Dalin, A. M., and Persson, E. 2003. Immunohistochemical studies on oestrogen receptor alpha (ER alpha) and the proliferative marker Ki-67 in the sow uterus at different stages of the oestrous cycle. *Reprod. Domest. Anim.* 38(1): 5-12.
- Trasch, K., Wehrend, A., and Bostedt, H. 2003. Follow-up examinations of bitches after conservative treatment of pyometra with the antigestagen aglepristone. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* 50(7):375-379.
- Vermeirsch, H., Simoens, P., and Lauwers, H. 2000. Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth. *Anat. Rec.* 260(1):42-50.
- Zaragoza, C., Barrera, R., Centeno, F., Tapia, J. A., and Mane, M. C. 2004. Canine pyometra: a study of the urinary proteins by SDS-PAGE and Western blot. *Theriogenology*. 61(7-8):1259-1272.11.