Original Article

Progesterone Receptors and Proliferation in the Bitch Uterus during Different Stages of the Oestrous Cycle

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Abstract

Immunopresence of the progesterone receptors (PR) and proliferative activity in the bitch uterus were studied during different stages of the oestrous cycle. Uterine samples were collected at 4 different stages of the oestrous cycle; prooestrus, oestrus, dioestrus and anoestrus. Immunohistochemistry was used to evaluate the localization and to semiquantitate PR and uterine proliferation by using monoclonal antibodies to the PR and the proliferative marker, Ki-67 respectively. The results showed that PR and Ki-67 levels were highest during prooestrus and oestrus while they were low at other stages. However, a significant difference was observed only in the surface epithelium for Ki-67 immunostaining. A positive correlation was found between PR positive and Ki-67 positive cells in the surface epithelium. The results from the present study strengthen previous finding that the presence of PR and uterine proliferation differ with oestrous cycle stages and among different uterine compartments; and suggested a possible relationship between the PR and the proliferative marker, Ki-67 in the surface epithelium of the bitch uterus.

Keywords : Progesterone receptor (PR), proliferation, bitch uterus, oestrous cycle.

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บทคัดย่อ

ตัวรับฮอร์โมนโปรเจสเตอโรนและการงอกขยายของมดลูกสุนัขในระยะต่าง ๆ ของวงรอบการเป็นสัด

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การศึกษาถึงการแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนและการงอกขยายของมดลูกสุนัขในระยะต่าง ๆ ของวงรอบการเป็นสัด โดยการเก็บตัวอย่างมดลูกจากสุนัขที่ระยะต่าง ๆ ของวงรอบการเป็นสัดซึ่งได้แก่ ระยะโปรเอสตรัส ระยะเอสตรัส ระยะไดเอสตรัส และ ระยะแอนเอสตรัส และใช้วิธีอิมมูนโนฮิสโตเคมีในการตรวจหาการแสดงออกของตัวรับฮฮร์โมนโปรเจสเตอโรน และการงอกขยายของมดลูกสุนัข พบว่าการแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรน และการงอกขยายของมดลูกมีระดับสูงที่ระยะโปรเอสตรัส และ เอสตรัส และจะลดลงในระยะต่อมา ส่วนการงอกขยายนั้น พบความแตกต่างของดัชนีการงอกขยายเฉพาะในเยื่อบุท่อมดลูกนอกจากนี้ยังพบความสัมพันธ์เชิงบวกระหว่างการแสดงออก ของตัวรับฮอร์โมนโปรเจสเตอโรนกับดัชนีการงอกขยายในเยื่อบุท่อมดลูกอีกด้วย การศึกษาครั้งนี้แสดงให้เห็นว่าการแสดงออก ของตัวรับฮอร์โมนโปรเจสเตอโรนและการงอกขยายมีความแตกต่างกันตามวงรอบการเป็นสัด และแตกต่างกันระหว่างส่วนต่าง ๆ ของมดลูก นอกจากนี้ ยังชี้ให้เห็นว่าการแสดงออกของตัวรับโปรเจสเตอโรนอาจมีส่วนเกี่ยวข้องกับการงอกขยายในส่วนของเซลล์เยื่อบุท่อมดลูกของสุนัข

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Introduction

During the oestrous cycle, morphological and physiological changes in the bitch uterus are under the influence of ovarian steroid hormones which mediate their effects by specific receptors in target cells (Oehler et al., 2000). At oestrus, the presence of steroid receptor including progesterone receptor (PR) in the uterus is greatest while overall reduction is observed during dioestrus (Dhaliwal et al., 1997; Vermeirsch et al., 2000). This reflects the positive effect of estradiol-17β and the negative influence of progesterone on the presence of PR. However, the differences of PR immunolocalization under the influence of these steroid hormones in specific uterine compartments can also be observed. There is the evidence that PR immunostaining is higher in the stromal cells than the epithelial cells which indicates the role of stromal cells in regulation some effects of steroid hormones on the epithelial cells in the bitch uterus (Vermeirsch et al., 2000). Moreover, after treatment with exogenous progesterone, PR score is zero in the glandular epithelium while it is moderate to high in other compartments of the bitch uterus (Dhaliwal et al., 1999). In canine endometrial cell culture, the downregulation effect of PR from

progesterone is less pronounced in the epithelial cell than in the stroma (Galabova-Kovacs et al., 2004). Therefore, these earlier studies suggest that the presence of PR in the bitch uterus was not only under the influences of steroid hormones but also be modified by other factors such as the different cell types, physiological and pathological status of these uterine cells.

One of the most important effects of steroid hormones is the proliferative activity of the reproductive organs (Rider, 2002; Illouz et al., 2003; Bigsby et al., 2004). van Cruchen et al. (2004) studied proliferation of the canine endometrium and found that the proliferation patterns differed between the uterine surface epithelium and the endometrial basal glands. Moreover, a possible correlation between serum progesterone and proliferation of the endometrial basal glands was also observed in that study. In addition, the study by Galabova et al. (2003) shows that higher proliferative rate was found in the epithelia of the bitch uterus at late anoestrus when the level of oestradiol is increasing and the level of progesterone is low. However, only metoestrous and anoestrous stages were included in that earlier study. In vitro, the study of canine endometrial cell culture reveals that high levels of progesterone reduce epithelial proliferation while low progesterone levels, corresponding to late oestrus and early dioestrus supports epithelial proliferation (Galabova-Kovacs et al., 2004). However, in that study, cell signaling between cells types which may have an influence on cellular response to steroid hormones could not be observed as different cell population are studied separately. In addition to steroid hormones, correlations between steroid receptor proteins and proliferative activity in the endometrium have also been reported. In woman, a study by Taylor et al. (2005) showed strong positive correlations between PR and Ki-67 expression, indicating that PR may be a potent regulator of endometrial proliferation. In the mare, the highest hormone receptor expression was correlated with an intense proliferative activity in epithelial cells (Aupperle et al., 2000). However, the data on bitch uterus regarding uterine proliferation pattern during different stages of the oestrous cycle and in different uterine compartments, is still limited.

Knowledge about normal uterine proliferation during the oestrous cycle in relation to steroid hormone receptors may explain some of the pathological conditions found in the uterus, such as cystic endometrial hyperplasia/pyometra complex. Hence, the objectives of the present study were to provide an immunohistochemical evaluation of the progesterone receptors (PR) and of the proliferative marker, Ki-67, and to determine the correlation between PR immunopresence and proliferative activity in normal bitch uterus at different stages of the oestrous cycle.

Materials and Methods

Tissue and blood samples

The present project was in accord with the guidance of the animal ethics committee and was approved by the Research Division of the Faculty of Veterinary Science, Chulalongkorn University. The uteri were obtained by ovariohysterectomy from bitches of various breeds. The uterine samples were classified according to the oestrous stages into 4 groups; prooestrus (n=3), oestrus (n=3), dioestrus (n=3) and anoestrus (n=3). The stages of the oestrous cycle were determined by macroscopic examination of the ovary and the plasma progesterone level, as adapted from van Cruchten et al. (2004), and by vaginal cytology.

Serum steroid hormone measurement

The blood samples were collected from the cephalic veins just before surgery; thereafter the serum was collected and stored at -20 C until assayed. The levels of progesterone were measured using chemiluminescent microparticle immunoassay

(ARCHITECT® Progesterone, Abbott laboratories, USA).

Histological preparation

Uterine samples were collected from the mesometrial side at about the middle of the uterine horns. The samples were fixed in 4% para-formaldehyde for 48-60 hrs prior to histological examination: embedded in paraffin, cut into 5μm thick sections and stained with hematoxylin-eosin (H&E) or stored at room temperature until immunohistochemistry was performed.

Immunohistochemical staining

The uterine samples used for immunohistochemistry were mounted on Polysine™ slides (Menzel-Glaser®, Braunschweig) to prevent the sections from falling off during the procedure. The sections were deparaffinized and pretreated with 0.01M citric acid buffer, pH 6.0 in the microwave in order to enhance the immunohistochemical reaction. The immunohistochemical procedure for progesterone receptor staining was done as described previously by Srisuwatanasagul et al. (2005). The primary antibody used for PR immunostaining in the present study (PR 10A9, Immunotech, Marseille, France) is known to cross-react with the canine PR (Manzel, 1995). For the assessment of uterine proliferation, the same procedure was used but with a monoclonal antibody to the proliferative marker Ki-67 (Clone MM-1, Novocastra Laboratories Ltd, Newcastle, UK) as the primary antibody. For negative controls, some sections were incubated in phosphate buffer saline instead of the primary antibody. All sections were investigated under a light microscope and the stained sections were photographed using Moticam 2000 and Motic Images Plus version 2.0 ML software (Motic China Group Co., Ltd).

Evaluation of the results

All immunostaining evaluation was done under a light microscope at x400. Four different uterine compartments; the surface epithelium, the glandular epithelium, the stroma and the myometrium, were separately evaluated. Each compartment was evaluated for PR immunohistochemical total score as described previously by Vermeirsch et al. (1999). Briefly, the intensity score (I) was ranged from 0 to 3 (0 = negative, 1 = weak, 2= moderate, 3= strong staining) and the proportional score (P) was 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.

For the proliferation marker, the mean percentage of Ki-67 positive cells was presented as a labelling index which was evaluated by classification and number of positive cells per 100 total cells per area, from five random areas of each uterine compartment. In the present study, vascular cells and endothelial cells were not studied in detail.

Statistical analyses

The results of the PR total score from different groups and tissue compartments were compared with a non-parametric method (Kruskal Wallis test, SAS for windows, version 9, Cary, NC, USA). The number of positive Ki-67 cells were analyzed by using one way ANOVA. The correlation between total PR score and Ki-67 labelling index was determined using Spearman rank correlation test. The p values <0.05 were accepted as having significant differences.

Results

The progesterone levels shown in Table 1 were used to indentify stages of oestrous cycle. The

results of PR and Ki-67 immunostaining are summarized in Table 2 and Fig. 2, respectively. The highest PR immunostaining score was significant at prooestrus and oestrus in the stroma and myometrium. The immunostaining scores were lower during dioestrus and anoestrus which were significantly different to the staining scores in the epithelia at oestrus. Though there was some staining in all compartments at dioestrus, and in the surface epithelium at anoestrus, high variations in individual dogs were observed.

Regarding proliferation, the Ki-67 labelling index was highest in the surface epithelium at prooestrus and oestrus, which was significantly different to the other stages. In other compartments, the labelling index did not differ with no proliferating cells found in some compartments and at some stages of the oestrous cycle.

For the result on correlation between PR immunolabelling and proliferation, significant correlation was observed between PR positive and Ki-67 positive cells in the surface epithelium (r =0.69, $p < 0.05$).

Table 1 Mean serum progesterone concentrations in normal bitches during different stages of the oestrous cycle (all values are expressed as means SD).

Group	Progesterone (ng/ml)
Prooestrus	0.56 0.4
Oestrus	1.83 0.6
Dioestrus	21.58 11.0
Anoestrus	0.83 0.8

Table 2 Results of PR-total scores in the different uterine compartments

Stage	Surface epithelium	Glandular epithelium	Stroma	Myometrium
Prooestrus	4.5 0.7^{a,b}	4.75 0.1^{a,b}	7.0 0^{a}	6.5 1.4^{a}
Oestrus	7.8 0.2^{a}	7.33 0.28^{a}	7.16 0.57^{a}	7.33 0.28^{a}
Dioestrus	2.0 3.4^{b}	2.0 3.4^{b,c}	2.16 3.75^{b}	2.0 3.4^{b}
Anoestrus	1.3 2.3^{b}	0 0.0^{c}	0 0.0^{b}	0 0.0^{b}
	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Mean (SD) within the same column followed by the different superscript letters are significantly different ($p < 0.05$).

Discussion

This present study investigated the immunolocalization and levels of PR and proliferation by mean of the Ki-67 labelling index, at different stages of the oestrous cycle and in different compartments of the canine uterus. It was shown that the highest PR immunostaining score was significantly found at oestrus when the plasma level of oestrogen was supposed to be high. This finding is in agreement with other studies on the uterus of bitches (Dhaliwal et al., 1997; Vermeirsch et al., 2000) and of other species, such as cows (Robinson et al., 2001); mares (Aupperle et al., 2000; Hartt et al., 2005) and sows (Sukjumlong et al., 2005). At dioestrus, when the level of progesterone increased, the immunostaining score was significantly lower compared to those at oestrus in all compartments and also at prooestrus in the myometrium and stroma. This suggests and supports the findings in other studies, that oestrogen upregulates progesterone receptors while progesterone itself downregulates the progesterone receptors in reproductive organs (Donnay et al., 1995; Xiao and Goff, 1999; Hartt et al., 2005).

Regarding the different uterine compartments, the PR immunostaining pattern tended to be similar with high scores during prooestrus and oestrus and lower at the later stages. However, at prooestrus, the PR score was higher in the stroma compared to the epithelia, but at the following stage, oestrus, the PR score increased in the epithelia and the PR levels were similar at this stage in all compartments. Therefore, we speculate that the presence of PR in the epithelia may be partly mediated by PR in the stroma or that the upregulation of PR by oestradiol is slower in the epithelia than in the other compartments.

For the uterine stroma in sows, it has been shown that PR was present at all stages of the oestrous cycle, which indicates that stromal PR could mediate certain reproductive functions while it is absent from other compartments (Sukjumlong et al., 2005). However, this is different from the bitch uterus in the present study that PR in the uterine stroma followed the same pattern as in the other compartments. Therefore, the pattern of PR immunolabelling, is likely to be species-specific. Moreover, the absence of PR at certain stages may be required for some reproductive events such as onset of differentiated functions by the uterine gland, as suggested by Spencer et al. (2004).

In the canine myometrium, the upregulation of PR during prooestrus and oestrus as found in the present study results from the high levels of oestrogen which prepares for PR-mediated progesterone regulation in the later stages of the oestrous cycle, as suggested in sows (Sukjumlong et al., 2005). High levels of progesterone have been shown to provide a quiescent uterus with low myometrial activity (Porter and Watts, 1986) and low PR expression (Bouchard, 1999) and therefore, the lower PR score in the bitch at dioestrus and anoestrus compared to prooestrus and oestrus, may represent lower myometrial activities.

For canine uterine proliferation, a significantly higher Ki-67 labelling index was found only in the surface epithelium at prooestrus and oestrus when compared to other stages. This is in agreement with an earlier study on the bitch uterus showing that proliferation in the surface epithelium peaked during prooestrus (van Cruchten et al., 2004). During dioestrus, a lower proliferative activity was observed in all uterine compartments which may be due to downregulation by the increasing levels of progesterone during that stage, as suggested for rodents (Das, 1972; Clark, 1973). However, during anoestrus, uterine proliferation was also low in all compartments in spite of a low level of serum progesterone. Therefore, it indicates that plasma levels of progesterone may not be the main regulator of uterine proliferation. This differs to the study by

van Cruchten et al. (2004) who suggested that the proliferation of endometrial basal gland was likely to be regulated by progestin. In addition, results from the present study also showed a positive correlation between PR immunopresence and Ki-67 labelling index in the surface epithelium. These results suggest a common regulator of the PR and the proliferation in the surface epithelium. Moreover, as endometrial cell proliferation and the presence of steroid receptors, including PR, have been shown to be upregulated by oestradiol (Galabova-Kovacs et al., 2004), the high proliferation in the surface epithelium at prooestrus and oestrus as well as the high immunoscore of PR around oestrus in all tissue compartments are also likely to be mediated by oestradiol. A relation between PR and proliferation was also indicated by Taylor et al. (2005), but in different uterine compartment suggesting that PR may be a potent regulator of human endometrial gland proliferation, since PR and Ki-67 expression show strong positive correlations in the glandular epithelium. However, the mechanism of a potential physiological relationship between PR and proliferation in the canine uterus requires further studies.

In conclusion, the present study strengthens previous findings showing that the presence of PR and uterine proliferation differed with stages of the oestrous cycle as well as among different uterine compartments. It also suggests a possible relationship between immunopresence of the PR and the proliferative marker Ki-67, in the surface epithelium. Further investigations are required to understand the mechanisms of uterine proliferation in the bitch uterus under the influence of steroid hormones via their specific receptors.

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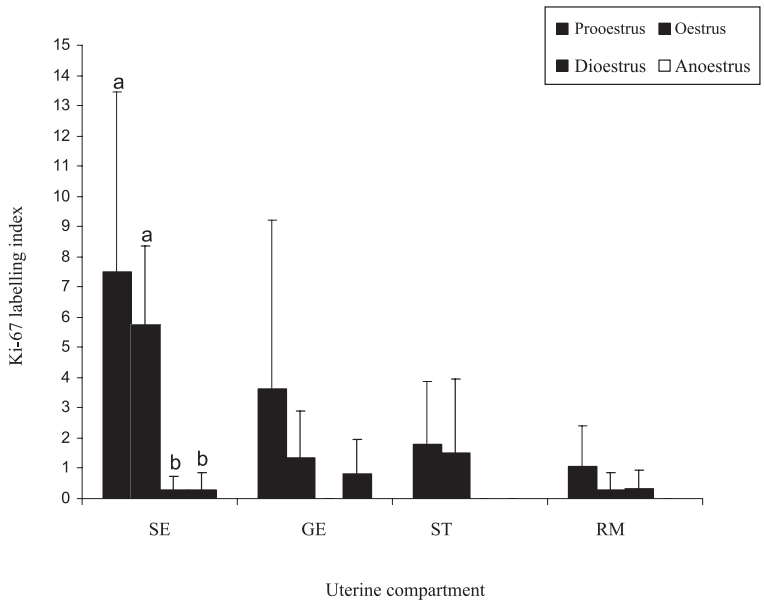

Figure 1 Ki-67 labelling index at different stages of the oestrous cycle and in different uterine compartments presented as bars (mean SD). The uterine compartments are defined as SE = surface epithelium; GE = Glandular epithelium; STR = Stroma and M = Myometrium. Bars marked by different letters in the same uterine compartment category are significantly different ($p < 0.05$).

PR **Ki-67**

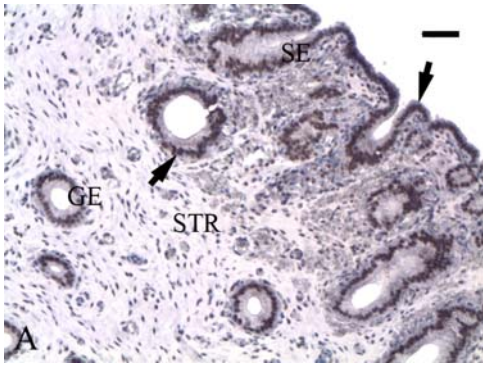

Figure 2 Immunohistochemical staining of the progesterone receptor (PR) in bitch uteri. A and C, PR immunostaining at oestrus; B and D, Ki-67 immunostaining at prooestrus; E and F, PR and Ki-67 immunostaining at anoestrus respectively; G and H, negative controls. SE = surface epithelium; GE = glandular epithelium, STR = stroma and M= myometrium. The arrows show positive staining cells and the bar in Fig. 2A represents a distance of 50 μm.

References

Aupperle, H., Ozgen, S. H. A., Schoon, D., Hoppen, H. O., Sieme, H. and Tannapfel, A. 2000. Cyclical endometrial steroid hormone receptor expression and proliferation intensity in the mare. Equine Vet. J. 32 (3): 228-232.

Bigsby, R. M., Caperell-Grant, A., Berry, N., Nephew, K. and Lubahn, D. 2004. Estrogen induces a systemic growth factor through an estrogen receptor-alpha-dependent mechanism. Biol. Reprod. 70 (1): 178-183.

Bouchard, P. 1999. Progesterone and the progesterone receptor. J. Reprod. Med. 44 (2 Suppl): 153-157.

Clark, B. F. 1973. The effect of oestrogen and progesterone on uterine cell division and epithelial morphology in spayed-hypophy sectomized rats. J. Endocrinol. 56 (2): 341-342.

Das, R. M. 1972. The time-course of the mitotic response to oestrogen in the epithelia and stroma of the mouse uterus. J. Endocrinol. 55 (1): 203-204.

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. 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.

Donnay, I., Wouters-Ballman, P., Devleeschouwer, N., Leclercq, G. and Verstegen, J. 1995. Changes in oestrogen, progesterone and epidermal growth factor receptor concentrations and affinities during the oestrous cycle in the normal mammary gland and uterus of dogs. Vet. Res. Commun. 19 (2): 101-113.

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.

Hartt, L. S., Carling, S. J., Joyce, M. M., Johnson, G. A., Vanderwall, D. K. and Ott, T. L. 2005. Temporal and spatial associations of oestrogen receptor alpha and progesterone receptor in the endometrium of cyclic and early pregnant mares. Reproduction. 130 (2): 241-250.

Illouz, S., Dales, J. P., Sferlazzo, K., Garcia, S., Carpentier-Meunier, S., Boubli, L., Lavaut, M. N. and Charpin, C. 2003. Effects of progestins of human proliferative endometrium: an in vitro model of potential clinical relevance. Int. J. Mol. Med. 12 (4): 517-523.

Manzel, O. 1995. Immunohistochemische Darstellung der Ostrogen- und Progesteronerezeptoren in Gewebeschnitten des Hundes. PhD dissertation, University of Hannover. Hannover, Germany.

Oehler, M. K., Rees, M. C. and Bicknell, R. 2000. Steroids and the endometrium. Curr. Med. Chem. 7 (5): 543-560.

Porter, D. G. and Watts, A. D. 1986. Relaxin and progesterone are myometrial inhibitors in the ovariectomized non-pregnant mini-pig. J. Reprod. Fertil. 76(1): 205-213.

Rider, V. 2002. Progesterone and the control of uterine cell proliferation and differentiation. Front. Biosci. 7: d1545-1555.

Robinson, R. S., Mann, G. E., Lamming, G. E. and Wathes, D. C. 2001. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle

and early pregnancy in cows. Reproduction. 122 (6): 965-979.

Spencer, T. E., Johnson, G. A., Burghardt, R. C. and Bazer, F. W. 2004. Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biol. Reprod. 71 (1): 2-10.

Srisuwatanasagul, K., Sukjumlong, S., Sirivaidyapong, S. and Pienchop, S. 2005. Immunohis-tochemical study of Progesterone receptor (PR) expression between pyometra and normal uterus at dioestrus in the bitch. Thai J. Vet. Med. 35 (2): 57-64.

Sukjumlong, S., Dalin, A. M., Sahlin, L. and Persson, E. 2005. Immunohistochemical studies on the progesterone receptor (PR) in the sow uterus during the oestrous cycle and in inseminated sows at oestrus and early pregnancy. Reproduction. 129 (3): 349-359.

Taylor, A. H., Guzail, M., Wahab, M., Thompson, J. R. and Al-Azzawi, F. 2005. Quantitative histomorphometric analysis of gonadal steroid receptor distribution in the normal human endometrium through the menstrual cycle. Histochem. Cell Biol. 123 (4-5): 463-474.

van Cruchten, S., van den Broeck, W., D'Haeseleer, M. and Simoens, P. 2004. Proliferation patterns in the canine endometrium during the estrous cycle. Theriogenology. 62 (3-4): 631-641.

Vermeirsch, H., Simoens, P., Hellemans, A., Coryn, M. and Lauwers, H. 2000. Immunohis-tochemical detection of progesterone receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology. 53 (3):773-788.

Vermeirsch, H., Simoens, P., Lauwers, H. and Coryn, M. 1999. Immunohistochemical detection of estrogen receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology. 51 (4):729-743.

Xiao, C. W. and Goff, A. K. 1999. Hormonal regulation of oestrogen and progesterone receptors in cultured bovine endometrial cells. J. Reprod. Fertil. 115 (1): 101-109.