Immunolocalization of progesterone receptors in the canine ovary and their relation to sex steroid hormone concentrations

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The aim of the present study was to describe the normal cellular distribution of progesterone receptors in the canine ovary at different stages of the oestrous cycle. Samples of both ovaries were obtained from 75 healthy adult bitches of various breeds and ages, including five pregnant bitches and three bitches that had just delivered. The presence of progesterone receptors was visualized by immunohistochemistry on paraffin wax sections using a monoclonal antibody. Nuclear staining for progesterone receptors was observed in the surface epithelium, cortical tubules, rete ovarii, follicle cells, thecal cells, luteal cells, granulosa cell cords and ovarian stroma. The staining intensity for progesterone receptors in the follicle cells increased with the stage of follicle development, indicating an intrafollicular role of progesterone in the mechanism of ovulation and luteinization. The stronger staining intensities for progesterone receptors in thecal cells compared with follicle cells may be explained by the fact that thecal

cells mediate some effects of steroid hormones on the follicle cells in secondary and tertiary follicles. Little correlation was found between the expression of progesterone receptors in follicle cells and oestradiol, progesterone or testosterone concentrations. This finding indicates a different regulating mechanism for progesterone receptors in canine ovarian follicles compared with other tissues of the genital tract. During pregnancy all groups of ovarian cells had lower staining intensity scores than during the oestrous cycle, although the sex steroid hormone concentrations in pregnant bitches were similar to those in non-pregnant bitches during the luteal phase of the oestrous cycle. The lower expression of progesterone receptors during pregnancy may be due to higher tissue concentrations of progesterone that are not reflected in the serum because of haemodilution and increased metabolism and clearance during pregnancy.

Introduction

Ovarian steroids regulate ovarian functions; however, it is not clear whether these steroids act directly on ovarian tissues through their steroid receptors and, therefore, are important local regulators of follicle development, or whether they act indirectly via the hypothalamus or the hypophysis (Iwai *et al.*, 1990). Oestrogen receptors and progesterone receptors have been detected in the ovaries of several species using radioligand techniques (Kim and Greenwald, 1987a,b; Vesanen *et al.*, 1991; Vesanen, 1993; Odore *et al.*, 1999), indicating that steroid hormones have various autocrine and paracrine functions in the ovary. In the present study, immunohistochemistry was used to determine the distribution of progesterone receptors in various types of cells in the ovaries of a large group of bitches. To date, the precise localization of progesterone receptors in the canine ovary has not been described. Therefore, the second aim of the present study was to

determine whether changes in the distribution of progesterone receptors occurred throughout different stages of the oestrous cycle and whether the presence of progesterone receptors in different groups of ovarian cells was correlated with the serum concentrations of oestradiol, progesterone and testosterone. Knowledge of the localization and expression of progesterone receptors during the oestrous cycle may lead to a better understanding of hormonal regulation of the ovarian cycle and endocrinological ovarian pathology, such as the development of cystic ovaries.

The present study is part of a global investigation on sex steroid receptors in the different tissues of the genital tract of bitches. In earlier studies, the localization of oestrogen receptors (Vermeirsch *et al.*, 1998, 1999) and progesterone receptors (Vermeirsch *et al.*, 2000a) in the uterus was determined throughout the oestrous cycle and related to the serum concentrations of oestradiol, progesterone and testosterone. In addition, the presence of oestrogen receptors and progesterone receptors in canine pregnant uterus and placenta was determined (Vermeirsch *et al.*, 2000b).

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Materials and Methods

Samples of both ovaries and uterine horns were obtained from 75 healthy adult bitches of various breeds and ages, presented for ovariohysterectomy at the Faculty of Veterinary Medicine of Ghent University and at two veterinary clinics. For each dog, data concerning anamnesis, weight, litters and last pro-oestrus were recorded. The dogs varied in age from 1 to 12 years, and included 22 mongrels, nine Yorkshire terriers, five Maltese, four Labradors, four West Highland white terriers, four Dobermans, three German shepherds, three poodles, two cocker spaniels, two shih tzus, two pinschers, two beagles, one Afghan hound, one bobtail, one boxer, one fox terrier, one French bulldog, one Jack Russell terrier, one keeshond, one Belgian lakenois, one Munsterlander, one bull terrier, one samoyed, one Saint Bernard and one Cairn terrier. The animals were sedated using medetomidine ($40\text{--}50\text{ }\mu\text{g kg}^{-1}$ i.m.) (Domitor; Orion Corporation, Espoo) and subsequently anaesthetized with thiopental (8 mg kg^{-1} i.v.) (Pentothal; Abbot Laboratories, North Chicago, IL). After intubation, anaesthesia was maintained using halothane. Immediately after excision, the tissue samples were fixed for 48 h in 3.5% (v/v) phosphate-buffered formaldehyde (pH 6.7).

All tissue samples were embedded in paraffin wax, and sections of $5\text{ }\mu\text{m}$ were cut, mounted on 3-aminopropyl-triethoxysilane-coated (Sigma, St Louis, MO) slides and dried overnight at 37°C . Paraffin wax was removed with xylene; the sections were rehydrated and pre-treated in an Antigen Retrieval Citra Solution (BioGenex, San Ramon). The pre-treatment consisted of heating the slides in a microwave oven for 2 min at 700 W and then for 3, 5 and 5 min at 200 W with a 5 min interval between each treatment. After cooling for 30 min at 4°C and rinsing in distilled water, the slides were incubated for 5 min with $100\text{ }\mu\text{l}$ of a 3% (v/v) H_2O_2 solution to block endogenous peroxidase activity. Slides were rinsed in Tris-buffered saline and incubated with $100\text{ }\mu\text{l}$ of 30% (v/v) Tris-buffered saline solution of normal goat serum for 30 min at 25°C to prevent non-specific reactions. All incubations were carried out in a humidified environment. Immunohistochemical detection of progesterone receptors was performed using a monoclonal mouse-anti-human-progesterone receptor antibody (clone 10A9; Medichim, Marseille), which crossreacts with the canine progesterone receptors (Manzel, 1995). Each tissue section was incubated overnight at 4°C with $100\text{ }\mu\text{l}$ of a 1:300 dilution of the concentrated primary antibody in Tris-buffered saline. After rinsing in Tris-buffered saline, the sections were incubated for 30 min at 25°C with $80\text{ }\mu\text{l}$ of a ready-to-use secondary goat-anti-mouse antibody (Envision TM+; DAKO, Glostrup) coupled to dextrane peroxidase. Finally, after rinsing in Tris-buffered saline, $100\text{ }\mu\text{l}$ diaminobenzidine (DAB) chromogen substrate (DAKO) was applied to the sections for 12.5 min. A reaction of DAB with peroxidase produces a brown staining. Mayer's haematoxylin was applied for 5 s as a nuclear counterstain. Positive and negative controls were

included in each staining procedure. The positive controls were human breast tissue and canine uterine tissue known to be positive for progesterone receptors, whereas the same tissue sections incubated with Tris-buffered saline instead of the primary antibody served as negative controls. Additional controls included canine uterine tissue sections incubated without primary and secondary antibody.

Serum samples collected immediately before surgery were stored at -20°C until assayed for determination of the serum concentrations of the sex steroids using a radio-immunoassay. The concentrations of progesterone, oestradiol and testosterone were measured as described by Coryn *et al.* (1981), Henry *et al.* (1987) and Vermeirsch *et al.* (1999, 2000a).

The stage of the oestrous cycle for each dog was determined as described in studies on steroid receptors in the uterus (Vermeirsch *et al.*, 1999, 2000a). The animals were first classified by macroscopic and histological examination of the ovaries and uterus using the classification of Andersen and Simpson (1973). After this preliminary classification, the animals were classified further according to the serum progesterone concentrations (Concannon, 1986; Concannon *et al.*, 1989). Animals with uterine tissue at a proliferative stage and a progesterone concentration $< 1\text{ ng ml}^{-1}$ were classified as being in pro-oestrus. Animals with uterine tissue at a late proliferative to early secretory stage and a progesterone concentration of $1\text{--}10\text{ ng ml}^{-1}$ were classified as being in oestrus. Metoestrus was divided into an early and a late stage for precise evaluation of progesterone receptors during the period in which the corpus luteum is active. Animals with uterine tissue at a secretory stage were classified as being in early metoestrus when progesterone concentrations were $> 10\text{ ng ml}^{-1}$, and in late metoestrus when progesterone concentrations were $< 10\text{ ng ml}^{-1}$. Animals with uterine tissue at an inactive stage were classified as being in anoestrus. The number of animals and mean serum hormone concentrations at each stage are listed (Table 1). Five bitches were pregnant between day 23 and day 42 of gestation. The day of gestation was estimated using the crown-rump length of the fetuses (Evans, 1993; Vermeirsch *et al.*, 2000b), and three bitches had just delivered, that is, they were at 1–3 days post partum.

For each dog one tissue section from each ovary was stained for progesterone receptors. The expression of progesterone receptors in the tissue sections was examined three times using a light microscope at $\times 400$ magnification. The microscopist had no knowledge about the animal from which the tissue was obtained. Each group of positively stained cells was given an immunohistochemical total score, which consisted of the sum of an intensity score and a proportional score. The intensity score reflected the intensity of the brown positive staining in the cell nuclei, whereas the proportional score reflected the percentage of cell nuclei that stained positive in the different groups of cells (Table 2). The proportional score was based on all cells of the surface epithelium, rete ovarii and cortical tubules

Table 1. Number of bitches (*n*) at each stage of the oestrous cycle and the corresponding mean serum concentrations of sex steroids

Stage of the oestrous cycle	<i>n</i>	Progesterone (ng ml ⁻¹)	Oestradiol (pg ml ⁻¹)	Testosterone (ng ml ⁻¹)
Pro-oestrus	6	0.44 ± 0.09	19.33 ± 9.29	0.16 ± 0.03
Oestrus	19	3.33 ± 2.49	12.42 ± 8.05	0.15 ± 0.08
Early metoestrus	4	14.79 ± 3.64	8.50 ± 0.71	0.09 ± 0.06
Late metoestrus	8	1.11 ± 0.89	4.67 ± 4.13	0.09 ± 0.05
Anoestrus	30	0.61 ± 0.56	4.26 ± 3.49	0.09 ± 0.04

Values are mean ± SD.

Table 2. Immunohistochemical grading scores

Intensity score	Proportional score
0: no staining	0: no positive nuclei
1: weak staining	1: < 1% positive nuclei
2: moderate staining	2: 1–9% positive nuclei
3: strong staining	3: 10–32% positive nuclei
4: very strong staining	4: 33–65% positive nuclei
	5: > 65% positive nuclei

and on representative areas of the other groups of cells. In these representative areas, up to 100 cells were evaluated. Scores were determined in morphologically normal follicles that were classified into primordial, primary, secondary and tertiary follicles according to the criteria listed by the Nomina Histologica (1994). Note that the term 'follicle cells' includes all cells of a given follicle, except for the oocyte and the cells of the theca interna and externa.

A statistical computer software program (Statistix for Windows; Analytical Software, Tallahassee) was used to perform the statistical tests. The non-parametric Kruskal–Wallis test was applied to determine whether there was any significant difference in staining throughout the oestrous cycle or between the different groups of cells. The Bonferroni test was used to determine in which particular stages or groups of cells the staining scores were significantly different from one another. The Spearman rank correlation test was used for determination of correlations between the immunohistochemical scores and the serum hormone concentrations.

Results

Localization of progesterone receptors

Immunohistochemistry revealed the presence of progesterone receptors, as indicated by brown staining, in the nuclei of different groups of ovarian cells (Figs 1 and 2), namely the surface epithelium, cortical tubules, rete ovarii, follicle cells, thecal cells, luteal cells, granulosa cell cords and in the ovarian stroma. No staining for progesterone receptors was observed in oocytes or blood vessels. Positive staining was not observed in the negative controls.

Variation of progesterone receptor expression in different groups of ovarian cells

Significant differences were observed in staining scores between different groups of cells irrespective of the stage of the oestrous cycle (Fig. 3). In the ovarian follicles the staining intensity for progesterone receptors in the follicle cells increased with the stage of the follicle development. The intensity was significantly lower in the primordial and primary follicles compared with the secondary and tertiary follicles ($P < 0.01$). The proportional scores in the cells of different follicles were similar, except for the primordial follicles in which positive cells were observed less frequently ($P < 0.01$). The total scores increased with the stage of follicle development. Primary follicles were significantly less positive for progesterone receptors than were secondary follicles ($P < 0.05$) and tertiary follicles ($P < 0.01$). Primordial follicles were also significantly less positive than were secondary follicles and tertiary follicles ($P < 0.01$). In luteinizing tertiary follicles, the follicle cells had higher staining intensity and total scores than did other tertiary follicles ($P < 0.01$).

The intensity, proportional and total scores of granulosa cell cords, which are thought to originate from atretic secondary follicles, were not significantly different from those of secondary follicles. However, compared with the ovarian stroma, the granulosa cell cords had a lower staining intensity and a higher proportional score ($P < 0.01$).

The other epithelial cells in the ovary, namely, the surface epithelium, cortical tubules and rete ovarii, had different staining intensity scores ($P < 0.01$). The surface epithelium had the lowest staining intensity and the cortical tubules had the highest staining intensity. In the cortical tubules and rete ovarii, nearly all cells stained with the same intensity, whereas the surface epithelium contained negative cells more frequently ($P < 0.01$). These differences were reflected in the total scores. The cortical tubules had higher total scores than did the rete ovarii ($P < 0.05$), whereas the total score of the surface epithelium was lower than that of both other groups of cells ($P < 0.01$).

Cells of the corpora lutea stained with a low intensity, which was intermediate between that of the primary and secondary follicles, and was significantly lower than that of the tertiary follicles ($P < 0.01$). Positive staining was observed less frequently in luteal cells than in follicle cells

Fig. 1. Immunohistochemical staining for progesterone receptors in different groups of canine ovarian cells. Arrows indicate strong to moderate positive staining; black arrowheads indicate weak positive staining; white arrowheads indicate negative staining. (a,b) Progesterone receptors were expressed in the surface epithelium (se), cortical tubules (ct), stromal cells (s), follicle cells of primordial (p) and primary (I) follicles. (c) The rete ovarii also expressed progesterone receptors. (d) In the secondary follicles no positive staining for progesterone receptors

($P < 0.01$) and luteal cells had a lower total score ($P < 0.01$; except for the primordial follicles). In old corpora lutea that were generally observed in anoestrus and pro-oestrus, a higher staining intensity was observed than in the other corpora lutea ($P < 0.01$).

The staining scores of the ovarian stroma and the theca externa of tertiary follicles were lower than those of the theca interna of tertiary follicles and the theca layer of secondary follicles ($P < 0.01$). The thecal interna and externa of luteinizing tertiary follicles had lower staining scores compared with other tertiary follicles ($P < 0.01$; proportional score of the theca externa $P < 0.05$). In large antral follicles in particular, the expression of progesterone receptors in the theca externa was not distributed evenly, as the theca externa cells located at the side of the ovarian surface stained positive, whereas those located at the medullary side stained negative (for a comparison, see Fig. 2a,b). Positive staining of stromal cells occurred less frequently in the tunica albuginea than it did in the other stromal cells, but the intensity of the staining was similar.

Cyclic variations of expression of progesterone receptors

Cyclic changes in the staining intensity for progesterone receptors were observed throughout the oestrous cycle (Table 3; Figs 4–6). In groups of cells with significant cyclic changes, the staining intensity for progesterone receptors decreased from pro-oestrus to oestrus, except for the stroma. In the thecal cells of tertiary follicles, granulosa cells of secondary follicles and granulosa cell cords, the staining intensity was lower during oestrus than in any other stage of the oestrous cycle. In the corpus luteum and cortical tubules, the staining intensity was lowest during early metoestrus. The ovarian stroma was an exception as in early metoestrus staining for progesterone receptors was at the strongest intensity.

The proportional scores showed no cyclic variations, except for the corpus luteum and the theca externa of tertiary follicles, in which changes were significant ($P < 0.05$). In the corpus luteum, more cells stained positive during pro-oestrus and anoestrus than during any other stage of the oestrous cycle. The theca externa cells had lowest proportional scores in oestrus and highest scores in late metoestrus.

Cyclic variations in the total scores reflected those in the intensity scores and were only significant for the corpus luteum, the thecal cells of the tertiary follicle and the cortical tubules ($P < 0.01$).

Expression of progesterone receptors during pregnancy and post partum

During pregnancy all groups of cells had lower staining scores for progesterone receptors than during the oestrous cycle. Except for the rete ovarii and the cortical tubules, all

progesterone receptor-positive groups of cells stained with a significantly lower intensity ($P < 0.05$ for the granulosa layer of the secondary follicle, theca externa of the tertiary follicle, stroma, epithelium and granulosa cell cords; $P < 0.01$ for the other follicle cells, the theca interna cells and the corpora lutea). The proportional score was also lower during pregnancy than during the oestrous cycle ($P < 0.01$; epithelium $P < 0.05$), but this difference was not significant for the rete ovarii, cortical tubules and theca interna and externa of the tertiary follicle. As a consequence, the total scores were also lower in pregnant bitches ($P < 0.01$; $P < 0.05$ for the theca externa of the tertiary follicle and the rete ovarii; not significant for the cortical tubules).

During postpartum anoestrus the staining scores for progesterone receptors in the different groups of ovarian cells were similar to those during the anoestrous stage of the oestrous cycle. No significant differences were found.

Relationships between steroid hormone concentrations and expression of progesterone receptors

The serum progesterone concentration was negatively correlated with: (i) the staining intensity scores of the cortical tubules ($P < 0.01$), the theca interna of the tertiary follicle, the corpus luteum and the granulosa cells of the secondary follicle ($P < 0.05$); (ii) the proportional scores of the corpus luteum and the theca externa of the tertiary follicle; and (iii) the total scores of the cortical tubules, the corpus luteum and the theca externa of the tertiary follicle ($P < 0.05$). In contrast, the progesterone concentration was positively correlated with the staining intensity score of the ovarian stroma ($P < 0.05$).

The oestradiol concentration was positively correlated with: (i) the intensity scores of the surface epithelium, stroma ($P < 0.01$) and cortical tubules ($P < 0.05$); (ii) the proportional scores of the surface epithelium ($P < 0.05$); and (iii) the total scores of the surface epithelium ($P < 0.01$) and stroma ($P < 0.05$). In contrast, the oestradiol concentration was negatively correlated with the proportional and total scores of the theca interna of tertiary follicles ($P < 0.01$).

The testosterone concentration was negatively correlated with: (i) all staining scores of the theca interna and externa of tertiary follicles ($P < 0.01$); (ii) the proportional score of the rete ovarii ($P < 0.01$); and (iii) the total score of the primordial follicles ($P < 0.05$). In contrast, the testosterone concentration was positively correlated with all staining scores of the corpus luteum ($P < 0.01$).

Discussion

Immunohistochemistry revealed a species-specific expression of progesterone receptors in the ovary and localized progesterone receptors in the surface epithelium (Hild-Petito

was observed in the oocyte (o), but weak staining was observed in the follicle cells (f) and moderate staining in the thecal cells (t). Some stromal cells (s) were positive. Scale bars represent 32 μm .

Fig. 2. Immunohistochemical staining for progesterone receptors in canine tertiary follicles and corpora lutea. Arrows indicate moderate positive staining; black arrowheads indicate weak positive staining; white arrowheads indicate negative staining. (a) In the follicular wall of large antral follicles at the side of the ovarian surface, weak positive staining was observed in the follicle cells (f) and moderate staining in cells of the theca externa (te) and the theca interna (ti). (b) In the follicular wall of large antral follicles at the medullary side, weak positive staining

et al., 1988; Korte and Isola, 1988; Press and Greene, 1988; Slomczynska *et al.*, 2000), follicle cells (Hild-Petito *et al.*, 1988; Korte and Isola, 1988; Iwai *et al.*, 1990; Revelli *et al.*, 1996; Slomczynska *et al.*, 2000), thecal cells (Hild-Petito *et al.*, 1988; Korte and Isola, 1988; Iwai *et al.*, 1990; Suzuki *et al.*, 1994; Revelli *et al.*, 1996; Slomczynska *et al.*, 2000), stromal cells (Hild-Petito *et al.*, 1988; Korte and Isola, 1988; Press and Greene, 1988; Iwai *et al.*, 1990; Suzuki *et al.*, 1994; Revelli *et al.*, 1996) and luteal cells (Hild-Petito *et al.*, 1988; Korte and Isola, 1988; Press and Greene, 1988; Iwai *et al.*, 1990; Suzuki *et al.*, 1994; Revelli *et al.*, 1996; Duffy *et al.*, 1997; Sasano and Suzuki, 1997; Slomczynska *et al.*, 2000). Furthermore, progesterone receptor mRNA has been detected in the surface epithelium, in follicle cells (Chandrasekhar *et al.*, 1994) and in luteal cells (Chandrasekhar *et al.*, 1994; Misao *et al.*, 1998). In the present study in dogs, progesterone receptors were observed in all these groups of cells and in the granulosa cell cords, rete ovarii and cortical tubules, indicating that progesterone has a direct influence on ovarian tissue. The absence of progesterone receptor expression in oocytes and blood vessels has also been noted in the human ovary (Revelli *et al.*, 1996).

The presence of progesterone receptors in granulosa cell cords in the canine ovary is consistent with the presence of progesterone receptors in the granulosa cell layer of secondary follicles from which the granulosa cell cords originate (Anderson and Simpson, 1973). The rete ovarii has a secretory capability in several species (Wenzel and Odend'hal, 1985), and it may have a role in the control of meiosis and be the source of follicle cells (Wenzel and Odend'hal, 1985). The reasons for its progesterone receptivity and its precise function have not been determined. Progesterone receptors were also expressed in the cortical tubules, which are invaginations of the surface epithelium that are common in adult ovaries of some species (Mossman and Duke, 1973; Mülling *et al.*, 1998), including dogs (Anderson and Simpson, 1973). It has been suggested that these cortical tubules are a source of oocytes (Mossman and Duke, 1973), interstitial cells (Mülling *et al.*, 1998) or follicle cells (Pineda, 1989), or that they are the remains of the fetal germinal strands (Ellenberger, 1911). Alternatively, it is possible that the cortical tubules result from entrapment of ovarian surface epithelium within the ovarian stroma after ovulation, forming an ovarian inclusion cyst, as observed in human ovaries (Resta *et al.*, 1987). This suggestion is more likely as the cortical tubules become more prominent as the animal advances in age (Anderson and Simpson, 1973) and show similar cyclic changes in the expression of progesterone receptors as the surface epithelium.

In the canine ovary, the staining for progesterone receptors increases with the development of the follicles. In primates, follicles that developed beyond the primary stage

Table 3. Significance of cyclic changes in staining intensity for progesterone receptors in the canine ovary

Sample	Significance
Follicle cells of primordial follicles	Not significant
Follicle cells of primary follicles	
Follicle cells of tertiary follicles	
Theca layer of secondary follicles	
Rete ovarii	
Surface epithelium	$P < 0.05$
Follicle cells of the secondary follicles	
Granulosa cell cords	
Corpus luteum	
Ovarian stroma	
Theca externa cells of the tertiary follicles	$P < 0.01$
Theca interna cells of the tertiary follicles	
Cortical tubules	

no longer expressed progesterone receptors until after the surge of LH, when the luteinizing preovulatory follicles again stained positive for progesterone receptors (Hild-Petito *et al.*, 1988). Revelli *et al.* (1996) observed weak to moderate staining for progesterone receptors in human follicle cells. Other studies observed no staining for progesterone receptors in human preantral follicles (Iwai *et al.*, 1990; Suzuki *et al.*, 1994), whereas cells of the dominant follicle showed marked positive staining at the time of ovulation (Iwai *et al.*, 1990). In all species examined to date, luteinizing follicle cells expressed progesterone receptors during the short interval between the preovulatory gonadotrophin surge and ovulation (Park and Mayo, 1991; Revelli *et al.*, 1996; Slomczynska *et al.*, 2000). In dogs, cells of antral follicles and luteinizing follicles stained with the strongest intensity, especially during oestrus when ovulations occur. This finding indicates that progesterone has an important intrafollicular role in modulating follicular growth and granulosa cell functions, as in other species. However, the exact role of progesterone has not been established (Hsueh *et al.*, 1984). The expression of progesterone receptors in preovulatory follicles may be critical for successful ovulation (Pinter *et al.*, 1996; Revelli *et al.*, 1996; Duffy *et al.*, 1997) and luteinization (Revelli *et al.*, 1996; Duffy *et al.*, 1997), and the maintenance of luteal structure and function (Duffy *et al.*, 1997). This notion is supported by the fact that female progesterone receptor $-/-$ homozygote mice are infertile because no differentiation of granulosa cells to luteinized cells occurs in their ovaries and no ovulations occur (Revelli *et al.*, 1996).

The presence of progesterone receptors in corpora lutea indicates that ovarian steroids are important for the

was observed in the follicle cells (f) and moderate staining in the cells of the theca interna (ti), whereas most theca externa cells (te) were negatively stained. (c) In luteinizing tertiary follicles, thecal cells (t) were weakly positive and follicle cells (f) were moderately positive. (d) Cells of the granulosa cell cords (gcc) and the corpus luteum (l) were weakly positive for progesterone receptors. Scale bars represent 32 μ m.

Fig. 3. Mean ($\pm$ SEM) staining scores for progesterone receptors in different groups of ovarian cells, irrespective of the stage of the oestrous cycle, in histological sections of both ovaries of 75 bitches. (a) Mean intensity score: 0 = no staining; 4 = very strong staining. (b) Mean proportional score: 0 = no positive nuclei; 5 = > 65% positive nuclei. (c) Total score: sum of intensity score and proportional score.

regulation of the corpus luteum (Revelli *et al.*, 1996). Progesterone acts locally to regulate the proliferation and development of luteinized granulosa and thecal cells (Sasano and Suzuki, 1997). In primates, expression of progesterone receptors in corpora lutea is greatest during the mid-luteal stage (Hild-Petito *et al.*, 1988; Iwai *et al.*, 1990; Suzuki *et al.*, 1994; Revelli *et al.*, 1996; Sasano and

Suzuki, 1997; Misao *et al.*, 1998). In general, the expression of progesterone receptors in the canine corpora lutea was low compared with other groups of cells, as in rabbits (Korte and Isola, 1988), and was negatively correlated with the progesterone concentrations, which is in contrast to findings in monkeys (Hild-Petito *et al.*, 1988) and pigs (Slomczynska *et al.*, 2000). This low expression of

Fig. 4. Mean ($\pm$ SEM) staining intensity scores (0 = no staining; 4 = strong intensity staining) for progesterone receptors in different stages of follicular development (■: primordial follicle; ▨: primary follicle; ▩: secondary follicle; □: tertiary follicle) and in corpora lutea (□) in 75 bitches grouped by the stage of the oestrous cycle.

Fig. 5. Mean ($\pm$ SEM) staining intensity scores (0 = no staining; 4 = very strong staining) for progesterone receptors in the ovarian epithelia (■: surface epithelium; ▨: cortical tubules; ▩: rete ovarii) and in granulosa cell cords (□) in 75 bitches grouped by the stage of the oestrous cycle.

Fig. 6. Mean ($\pm$ SEM) staining intensity scores (0 = no staining; 4 = very strong staining) for progesterone receptors in ovarian stromal cells (■: theca follicularis; ▨: theca interna; ▩: theca externa; □: ovarian stroma) in 75 bitches grouped by the stage of the oestrous cycle.

progesterone receptors may be due to a negative effect of progesterone on production of progesterone receptors. However, it is possible that fewer progesterone receptors are required in the corpus luteum to enable progesterone to act, as a high progesterone concentration is present in this tissue. Higher staining scores were observed in old corpora lutea in bitches at anoestrus and pro-oestrus, which could be the consequence of a decrease in progesterone

production. According to Iwai *et al.* (1990), the weaker staining for progesterone receptors in the human corpus luteum of pregnancy compared with that in the periodical corpus luteum may be associated with the downregulation of progesterone receptors by endogenous progesterone and/or with the degeneration of luteal cells. Similarly, in dogs, a weaker staining for progesterone receptors was observed in the corpora lutea during pregnancy. However,

in dogs, the lifespans of corpora lutea during the luteal stage of the oestrous cycle and during pregnancy are nearly similar, and the concomitant increase in serum progesterone concentrations is as high and prolonged in non-pregnant as in pregnant bitches (Concannon, 1986). However, Guderath *et al.* (1998) reported pregnancy-specific increases in faecal concentrations of progesterone, oestradiol and testosterone in dogs. This finding indicates that the corpora lutea of pregnant bitches synthesize and secrete more steroids than those in non-pregnant bitches, but this increase in steroids is not reflected in the serum because of increased haemodilution, metabolism and clearance during pregnancy. Thus, in dogs, increased tissue concentrations of progesterone may be the reason for significantly weaker staining for progesterone receptors in nearly all groups of ovarian cells during pregnancy. In pregnant compared with non-pregnant bitches there is also a marked increase in the concentration of FSH (Reimers *et al.*, 1978). This finding raises the question of whether these increased FSH concentrations may participate in the decrease in progesterone receptor expression in the canine ovary, as progesterone receptor expression does not occur in rabbit granulosa cells treated with progesterone after FSH treatment (Iwai *et al.*, 1991).

In the canine ovary, the expression of progesterone receptors in thecal cells of secondary follicles and theca interna cells of tertiary follicles was more prominent than that in follicle cells and was correlated with at least two different sex steroid hormones, in contrast to follicle cells. Furthermore, theca interna cells, which are more closely related to follicle cells than theca externa cells, showed stronger staining for progesterone receptors, as observed in pigs (Slomczynska *et al.*, 2000). This finding indicates that progesterone exerts its influence on follicle cells also via thecal and stromal cells, particularly via the theca cell layer lying closest to the follicle cells. This could also explain why positive staining was observed less frequently in stromal cells of the tunica albuginea, which is not as closely related to the follicles. Similar indications for stromal-epithelial interactions were found in the canine endometrium (Vermeirsch *et al.*, 1998, 1999, 2000a). The possibility of a paracrine action of ovarian steroids on follicles and corpora lutea, mediated through stromal steroid receptors and steroid-linked local factors, has also been suggested in humans (Revelli *et al.*, 1996). There are several examples of interaction between thecal and follicle cells. The biosynthesis of oestrogens in follicle cells requires interaction with neighbouring thecal cells that provide steroid precursors (Hsueh *et al.*, 1984; Sasano and Suzuki, 1997). Early follicular growth may be regulated by progesterone via stromal and thecal cells (Hild-Petito *et al.*, 1988). The thecal cells may secrete factors that promote granulosa cell growth or differentiation (Hsueh *et al.*, 1984). Furthermore, progesterone via progesterone receptors in thecal cells might facilitate rapid growth of preovulatory follicles by remodelling of the extracellular matrix (Yoshimura and Bahr, 1991). Progesterone is also thought to

be important in the mechanism of LH-induced follicular rupture, but it remains to be determined where this progesterone action occurs. Just before ovulation, localized processes of digestion and dissociation of the collagen fibres result in an opening in the follicular wall and the formation of the stigma and follicular rupture (Morioka *et al.*, 1989). In the present study, progesterone receptors in the theca externa of large preovulatory follicles were more prominent at the side of the ovarian surface compared with the medullary side. If these progesterone receptors play a role in follicular rupture it could explain why stigma formation and rupture always occur at the apex of the follicle, although weakening of the follicular wall occurs throughout the entire wall (Morioka *et al.*, 1989). However, an explanation is required as to how this difference in progesterone expression can occur.

The serum concentrations of oestradiol, progesterone and testosterone showed few correlations with the expression of progesterone receptors in the ovarian follicles, although oestrogen is considered to be the primary regulator of progesterone receptor expression in many tissues (Katzenellenbogen, 1980; Hild-Petito *et al.*, 1988; Revelli *et al.*, 1996). There are indications that expression of progesterone receptors in the ovary is not under direct oestrogenic control, although this does not exclude a indirect role of oestrogen in ovarian progesterone receptor expression (Pinter *et al.*, 1996). In follicle cells in particular, the expression of progesterone receptors is regulated by the action of LH together with gonadotrophin in humans and primates (Iwai *et al.*, 1991; Duffy *et al.*, 1996). Thus, there is an important contrast between progesterone receptor induction in the ovarian follicles, which is regulated by gonadotrophins via membrane receptors, and receptor induction in other well known progesterone target tissues, such as the uterus, which is regulated by oestrogens via nuclear receptors (Pinter *et al.*, 1996). This contrast in progesterone receptor induction mechanisms has been observed in several species and the present study indicates that it is also present in dogs.

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