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Immunohistochemical detection of progesterone, progesterone receptor, estrogen receptor-α, Vascular Endothelial Growth Factor, Proliferating Cell Nuclear Antigen, Caspase-3, Matrix Metalloproteinase-9 and P-glycoprotein in the canine endometrium

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1. General introduction

1.1. Cyclic changes during the estrous cycle of the bitch

During the estrous cycle of the bitch, the endometrium undergoes significant morphological changes. These changes have been described in previous studies (Mulligan, 1942; Barrau et al., 1975; Spanel-Borowski et al., 1984).

The changes include proliferative and regressive alterations in the surface epithelium, the superficial and deep stromal layers and the endometrial glands, including the basal gland part and the crypts of the endometrium.

A study of Barrau (1975) describes the cyclic changes of the endometrium in those parts and also their relation to the steroid hormones estradiol-17β and progesterone.

In this study two major periods of proliferation are described, with the first one starting at the end of anestrus and lasting until the onset of proestrus (Barrau et. al, 1975).

This period is characterized by growth of the crypts, hypertrophy and hyperplasia of the glandular cells as well as the differentiation of large mucus secreting cells. In accordance with the serosanguineous discharge, which can be observed during this stage of the estrous cycle, extravasation of red blood cells occurs into the upper layers of the endometrium (Barrau et. al, 1975).

During late anestrus and throughout proestrus the level of estradiol-17β is increasing with its peak at the end of this period. This proliferative period therefore correlates with the increase in estradiol-17β concentration. Progesterone concentrations are still low but steadily increasing. Although the estrogen concentration peaks at the end of proestrus, the proliferation comes to a stop a few days after beginning of proestrus and stays in a rather quiescent state until the second major proliferative period occurs (Barrau et. al, 1975).

The second major proliferative change starts in the middle of estrus and terminates around the 16^{th} to 20^{th} day of metestrus, when regressive changes start to appear.

This second period is also characterized by hypertrophy and hyperplasia of the glandular epithelium, as well as proliferation of the basal glands. The glands increase in height and appear coiled at the end of the estrus stage (Barrau et. al, 1975).

In the beginning of metestrus a rapid growth of the glands is seen which marks the peak period of their growth. The basal glands appear highly coiled and branched.

During the stage of estrus the estradiol-17β level starts to decrease and progesterone levels increase. A correlation between the increasing progesterone levels and proliferative changes can be seen (Barrau et. al, 1975).

Following this second period of proliferation, is a period of involution in the non-pregnant bitch. Marked vacuolization is seen in the surface epithelial cells and crypt regions. The endometrium is thinning in this region and debris accumulates in the lumen of the glands. At the end of metestrus the endometrium reaches the appearance of the stage of anestrus which is characterized by debris in the glandular lumen, less coiled basal glands which also occur in a lesser quantity. The epithelial cells appear columnar or cuboidal and the vacuolization disappears (Barrau et. al, 1975).

Another study by Galabova (2003) focused on the morphological changes of the endometrial epithelium during metestrus and anestrus. The findings correspond with the findings of Barrau (1975) and additional immunostaining for the proliferation marker Ki-67 showed that these markers were found in high numbers during early and mid-stage of the metestrus in the surface epithelial cells, the epithelial cells at the base of the crypts and in the gland epithelium of the basal zone (Galabova et al., 2003).

During late metestrus vacuolization was seen which correlates with the time of involution as described by Barrau (1975). It showed that these vacuolated cells accumulate fat which means that these cells are undergoing fatty degeneration. Also the Ki-67 marker was no longer detected during late metestrus in the superficial or crypt epithelium. The glands in the basal zone however, still contained the marker (Galabova et al., 2003).

In early anestrus the nuclei of the vacuolated cells were picnotic due to the large size of the fat vacuoles. These cells were undergoing desquamation and some epithelial sheets could be seen in the lumen of the uterus. Staining for the proliferation marker was observed in the opening of the uterine glands (Galabova et al., 2003).

Progressing to the mid anestrus stage, the fat vacuoles disappeared and the cells had the appearance as described by Barrau (1975). The proliferation marker was seen in the surface epithelium as well as in the glands which shows the regeneration of the epithelium after the desquamation (Galabova et al., 2003).

In late anestrus a remarkable proliferation could be demonstrated with the Ki-67 marker, which correlates with the beginning of the first proliferative period described by Barrau (1975).

1.2. Immunohistochemistry

Immunohistochemistry (IHC) is a very important tool in numerous fields of veterinary medicine. It is widely used for diagnostic as well as research purposes. IHC is useful to understand the pathogenesis of diseases and therefore also essential in therapeutic and prognostic work.

The method is a combination of chemical and immunological reactions based on an antigen-antibody binding principle (Ramos-Vara and Miller, 2014).

In this way antigens can be detected in different types of tissues. By binding specific antibodies to these antigens, complexes are formed and through specific histochemical reactions, these complexes can be detected as colored sections under the light microscope.

In general the IHC examination consists of three steps: Preanalytical, analytical and the postanalytical phase. The first phase includes the proper handling and fixation of the tissue section as well as embedding and sectioning. The most common fixation media that is used is formaldehyde (Ramos-Vara and Miller, 2014).

The analytical phase consists of deparaffinization, antigen retrieval, incubation with primary antibodies, labeling and counterstaining.

The last step is the interpretation of the results and the creation of the IHC report about the findings, which is all done in the postanalytical phase (Ramos-Vara and Miller, 2014).

In this study immunohistochemistry was used to detect progesterone, progesterone receptor (PR), estrogen receptor α (ER), Matrix Metalloproteinase-9 (MMP-9), Vascular Endothelial Growth Factor (VEGF), Caspase-3, Proliferative Cell Nuclear Antigen (PCNA) and P-glycoprotein (Pgp) in the endometrium of the bitch.

2. Literature review

2.1. Progesterone (P4)

In the bitch the steroid hormone progesterone is essential for the preparation of the endometrium to enable nidation of the embryo and further more for the maintenance of pregnancy. It also plays an important role in the growth and development of the mammary glands.

Progesterone is produced by the corpus luteum of the cyclic bitch and throughout the estrous cycle the serum progesterone levels change as followed:

- **Proestrus:** The serum P4 level is lower than 2-4ng/ml
- **Estrus:** P4 levels start to increase around the time of the LH surge, which means it starts to rise before ovulation occurs. At time of ovulation the P4 levels are around 6ng/ml.
- **Metestrus:** The progesterone level stays elevated for a long period of time, regardless if the bitch is pregnant or not. The level ranges from 4 to >10ng/ml. It starts to decline around 35 to 40 days after ovulation and at the end of metestrus levels <1ng/ml can be measured.
- **Anestrus:** During this stage the P4 levels remain at a low level, which means under 1ng/ml.

Progesterone mediates its effect on the uterine tissue through receptors, which can be found in the nucleus of cells.

As described before, progesterone plays an important role in the morphological changes of the endometrium during the estrous cycle, especially during the mid-stage of estrus when proliferative changes appear (Barrau et al., 1975).

2.2. Progesterone receptors (PR)

The progesterone receptors are proteins which are found in the nucleus of cells and are regulated by steroid hormones. Progesterone itself is responsible for different changes within the uterine tissue, including morphological and physiological processes.

The quantitative appearance of PR depends highly on the stage of the estrous cycle (Galabova-Kovacs et al., 2004; Srisuwatanasagul et al., 2006).

Progesterone receptors can be found in the endometrial epithelial cells, glandular ducts and basal glands, endometrial stromal cells as well as in the smooth muscle cells of the myometrium (Vermeirsch et al., 2000).

With immunohistochemical examinations these receptors can be found as brown staining in the nucleus of the cells (Vermeirsch et al., 2000).

Regarding the different stages of the estrous cycle the following is known about the appearance of these receptors: (Vermeirsch et al., 2000;Srisuwatanasagul et al., 2006)

- **Proestrus:** The amount of receptors is high in all cell groups, especially in the basal glands. Higher amounts are found in the stromal cells in comparison to the epithelial cells.
- **Estrus:** Due to the fact that the serum progesterone level slowly increases the amount of PR decreases in comparison to the proestrus stage.

  As the estrus continues the amount of receptors in the epithelial cells increases. A hypothesis that there is a close relationship between the stroma and epithelial cells is used, to explain the phenomenon that the receptors in the epithelial cells increase as the estrus continues. The stromal cells seem to convey the effects of the steroid hormones to the epithelial cells.
- **Early metestrus:** During this stage the concentration of progesterone further increases and the amount of PR decreases accordingly.
- **Late metestrus:** Here different changes can be seen regarding the different cell groups. Whereas in the epithelial cells and the basal glands the amount of PR decreases, the amount in the myometrium and stromal cells increases.

  The decrease of PR in the basal glands may be explained by the fact, that a regression of the basal glands takes place during this time of the estrous cycle.
- **Anestrus:** PR receptors are found in all cells in a higher amount as before, with exception of the basal glands which have the lowest amount in this stage of the estrous cycle.

The highest amount of progesterone receptors were found during proestrus and estrus. This is believed to be due to the high concentration of estradiol-17β, which increases the occurrence of PR. Progesterone itself however, has the opposite effect on the quantity of receptors compared to estradiol. This leads to the fact that the lowest amount of receptors is found during metestrus since this period of the estrous cycle is characterized by a higher progesterone level (Srisuwatanasagul et al., 2006).

However, the absence or presence of progesterone alone is not responsible for the quantity and expression of PR. It is furthermore a combination of both, estradiol and progesterone that is responsible for that (Vermeirsch et al., 2000).

2.3. Estrogen receptor α (ER)

ER are also proteins and as well as the PR found in the cell nucleus. Due to the fact that these ER are also regulated by steroid hormones they differ as the PR in occurrence throughout the estrous cycle of the bitch. Estradiol leads to an increase in the quantity of these receptors and high levels of progesterone are jointly responsible for the decreasing amount of ER (Galabova-Kovacs et al., 2004).

The receptors can be determined microscopically after immunohistochemical staining. ER are stained red and found in the following cells: surface epithelium, glandular epithelium, endometrial stroma cells and smooth muscle cells of the myometrium (Vermeirsch et al., 1999).

In some cases only stromal cells possess ER, although epithelial cells show alterations as well, which shows that the relationship and interaction between stromal and epithelial cells plays and important role here as well, similar as described before for the PR.

Cells that are located closer to the endometrium show higher staining intensity (Vermeirsch et al., 1999).

During the different stages of the estrous cycle the following can be observed in regards to the quantitative distribution of these receptors (Vermeirsch et al., 1999)

- **Proestrus:** During this stage of the cycle the estradiol level is high and therefore the quantity of ER in the stromal and glandular epithelial cells are very high. This stage of the cycle was characterized by the highest number of ER except for the surface epithelium.
- **Estrus:** Since estradiol levels decrease and progesterone levels start to increase the number of receptors decreases accordingly. Stromal cells show the highest decrease.
- **Early metestrus:** During this phase of the cycle the progesterone level further increases which leads to a further decrease in ER, leading to the lowest amount of receptors throughout the cycle. An exception is the surface epithelium cells in which the lowest amount is found during estrus.
- **Late metestrus:** As the progesterone level decreases the amount of receptors start to increase again, with the highest increase rate found in the stromal cells.
- **Anestrus:** Anestrus is characterized by a low serum level of estradiol, progesterone and testosterone. During this stage a high amount of ER can still be found in all cell types. The surface epithelium has the highest amount, even higher than in any other stage of the cycle. An explanation for the high amount of ER during anestrus, although the steroid hormone level is low, could be that even during anestrus sex steroids are

not totally absent and FSH is present to induce follicular growth. The same high amount of ER can be seen in women during menopause.

Summarizing this, the highest amount of ER was found during proestrus and slightly decreased during estrus. The lowest amount of ER was found during early metestrus. During anestrus the quantity was also in a higher range.

In another study, only a weak amount of ER was found in the proestrus stage, including all cell types, except the myometrium, which showed a moderate amount. In the estrus stage the highest amount of ER was found in all cells. The fact that this study showed the highest amount of ER during estrus while other studies showed higher amounts during proestrus can be explained by different definitions of the estrous cycle of the bitch and the difficulty to determine the exact start and end of estrus (Ozyurtlu et al., 2010).

2.4. Matrix Metalloproteinase-9 (MMP9)

Metalloproteinases are zinc-dependent, proteolytic enzymes which degrade and remodel extracellular matrix and play an important role in vascularization (Loukopoulos et al. 2003; Beceriklisoy et al., 2007).

MMP9 belongs to the group of gelatinases, also known as type IV collagenases. They are secreted by different kind of cells as a latent form and need to be activated to gain their proteolytic ability. Activation of MMPs takes place extracellular through other enzymes like other MMPs (Loukopoulos et al., 2003; Beceriklisoy et al., 2007).

MMP-9 plays an important role during changes that take place during endometrial remodeling. A study in humans showed that MMPs play a key role during the degeneration of luminal epithelium but this is not the case in bitches. In bitches they do take part in endometrial breakdown processes, but are rather involved in the uterine remodeling than the degeneration of luminal epithelium (Chu et al., 2002; Loukopoulos et al., 2003).

An increased amount can also be found during inflammatory processes.

A study about the effect of steroid hormones, especially progesterone, on the expression of MMP-2 and MMP-9 during the estrous cycle of the bitch showed, that latent forms were higher at all stages of the cycle in comparison to the activated form (Loukopoulos et al., 2003).

The highest activity of both active and latent forms was seen in metestrus with the latent forms being significantly higher.

The lowest activities were seen in anestrus in case of both forms (Loukopoulos et al., 2003).

In regards to the progesterone level one can say, that there is a positive correlation between the activity of both forms and the plasma progesterone level in the bitch. As the progesterone level increases during metestrus the activity of MMP-9 reaches its maximum.
Since MMPs play an important role during angiogenesis the increased activity can be explained by the fact that this event takes place in the bitch uterus from the stages of estrous till late metestrus.
Hence the highest activities of MMPs are found during the time when the endometrium undergoes morphological changes (Loukopoulos et al., 2003).
Regarding the localization immunohistochemical studies revealed that MMP9 is found in blood vessels, smooth muscle cells and epithelia, uterine crypts and glands of the endometrium. In the placenta they are localized in the deep uterine glands and the epithelium of glandular chambers.
Another study performed to measure the activity of MMP-9 and MMP-2 in the canine uterus before and during placentation, showed that the highest activity in the endometrium is seen during the pre-implantation and placentation period. Therefore preparation of the uterine wall for implantation and placentation is supposed to be another key role of these enzymes (Beceriklisoy et al., 2007; Kanca et al., 2011).

2.5. Vascular Endothelial Growth Factor (VEGF)

As the endometrium undergoes changes throughout the estrous cycle one important change is seen in the endometrial blood vessels. Those blood vessels regenerate, including angiogenesis and neovascularization (Sagsöz et al., 2013).
VEGF is a proangiogenic regulator and is an important key element in the development of new capillary blood vessels and forming a microvasculature system.
Three receptors are known to mediate the activity of VEGF. Those receptors are: flt1/fms (VEGFR-1), flk1/KDR (VEGFR-2) and flt4 (VEGFR-3) (Sagsöz et al., 2013).
Besides VEGF an antiangiogenic regulator, the so called vascular endothelial growth inhibitor (VEGI), takes part in the regulation of the angiogenesis.
With immunohistochemistry VEGF can be found in the cytoplasm and membrane of luminal, glandular, stromal and myometrial smooth muscle cells with the highest amount being found in the glandular epithelium. Staining can also be seen in the blood vessels throughout the endometrial layers (Sagsöz et al., 2013).
Hence in comparison to humans and other mammals VEGF can be found throughout the endometrial compartments.

Regarding the stage of the estrous cycle of the bitch, the highest amount can be found during proestrus and estrus stages (Sagsöz et al., 2013).
According to results of a study VEGF helps to form a nutritive environment to enhance the implantation of the embryo by increasing the secretory activity. It may also play a role in the growth and proliferation of epithelial cells (Sagsöz et al., 2013).
Another study about the expression of VEGF and receptors in the early canine pregnancy also showed that there is a higher amount of VEGF during the pre-implantation stage which emphasizes the important role of VEGF during angiogenesis (Schäfer-Somi et al., 2013).

2.6. Caspase-3

Caspase-3 belongs to the effector group of caspase enzymes and is responsible for apoptotic mechanisms taking place in the endometrium. Studies showed that caspase-1 and -3 are connected to each other and an inhibition of either of them will stop apoptosis. Caspases are present in various cell types as inactive forms and therefore require activation. Activation of caspases can happen in different ways, including receptor-ligand mediated mechanism, mitochondrial mechanism or a process involving the endoplasmic reticulum. Apoptotic processes will activate caspase-3, which than leads to changes in the cell such as: ruffling of the cell membrane, condensation and fragmentation (Van Cruchten et al., 2003).
Apoptosis in the canine endometrium is important to maintain a certain number of cells and also plays a key role during implantation and development of the placenta. To detect caspase-3 activity in the endometrium during different stages of the estrous cycle immunohistochemistry can be used. Caspase-3 positive cells stain brown and can be easily seen under the light microscope in the surface epithelium, crypts, stroma and basal glands (Van Cruchten et al., 2003).
Regarding the distribution of caspase-3 during the different stages of the estrous cycle the following can be observed (Van Cruchten et al., 2003):

- **Surface epithelium:** Only a low number of positive cells can be seen throughout the estrous cycle. Higher numbers occur during anestrus compared to early metestrus.
- **Stroma:** An increased amount of active cells is seen in late metestrus and anestrus.
- **Crypts:** Increased amount is found in late metestrus and anestrus.
- **Basal glands:** Increased activity in late metestrus and anestrus.

Low levels of staining throughout the estrous cycle are seen in the surface epithelium and stroma cells. A low level in the surface epithelium does not correspond with findings in studies of other species such as pigs, mice, hamsters and rats.

The high levels in crypts and basal gland cells however coincide with studies in other species. At this stage accumulation of cellular debris in the lumen of the glands and regression of the glands takes place. High levels of caspase-3 activity during this stage suggest that apoptosis is responsible for the regression of the glands (Van Cruchten et al., 2003).
No correlation can be found between the serum progesterone level and the activity of caspase-3 throughout the estrous cycle. Studies in other species such as human, hamsters, mice and rats showed correlation between serum steroid hormone levels and apoptosis but in the canine cycle no such correlation was found. It is to be believed that other factors such as growth factors or cytokines play a more important role regarding the regulation of apoptosis (Van Cruchten et al., 2003).

2.7. Proliferating Cell Nuclear Antigen (PCNA)

The proliferating cell nuclear antigen is a protein which is found in the nucleus of cycling cells. PCNA plays an essential role in the replication and reparation processes of DNA. The detection of this antigen is used in several studies to examine the proliferative activity of certain cells (Lai, 2000).
The immunohistochemical detection of the Ki-67 proliferation associated nuclear antigen for example, was used in several studies to explore the proliferative activity of the endometrium in the bitch as well as in other species throughout the estrous cycle (Salmi, 1998; Gerstenberg et al., 1999; Lai, 2000; Van Cruchten et al., 2003).
A study by Van Cruchten focused on the proliferative activity in the canine endometrium at different stages of the estrous cycle using the detection of the before mentioned Ki-67 with immunohistochemistry.
This study revealed that two major peaks of proliferation can be detected during the cycle: one during proestrus and another one during estrus with different cell groups involved, which show an increase of the proliferation marker Ki-67 (Van Cruchten et al, 2003).
This findings correlate with an earlier study of Barrau in which two peaks of proliferation are described around the same time during the estrous cycle (Barrau et al., 1975).
During the first proliferation peak in the proestrus stage the highest amount of the marker is found in the following parts of the canine endometrium: surface epithelium, stroma, blood vessels and crypts. The amount of Ki-67 in the basal glands is rather low (Van Cruchten et al., 2003).
The second peak during estrus is characterized by a high amount of Ki-67 marker in the basal glands and a rather low amount in the other cell groups (Van Cruchten et al., 2003).

During early metestrus the amount of the proliferative marker decreases further in the surface epithelium, stroma, blood vessels and crypts. The amount in the basal glands stays initially high but starts to decrease in late estrus and all cell types contain a low amount of Ki-67 throughout the anestrus (Van Cruchten et al., 2003).
Regarding this, one can say, that the proliferative activity during proestrus is correlated to the increasing estrogen levels during this period. The surface epithelium, stroma, blood vessels and crypts therefore react to the increased estrogen concentration whereas the basal glands only contain a low amount of the marker. The basal glands rather respond to the increasing concentration of progesterone during estrus which shows in the higher amount of markers during this time (Van Cruchten et al., 2003).

2.8. P-glycoprotein (P-gp)

The P-glycoprotein is known for its role in the multidrug resistance (MDR) in cancer patients. This membrane protein acts like an energy dependent efflux pump, removing all cytotoxic agents from the cell and preventing their intracellular accumulation (Axiotis et al., 1991; Novotna et al., 2004).
Studies in humans as well as in rodents and also canines revealed the distribution of this protein in normal as well as in tumorous tissues (Huang Yang et al., 1989; Axiotis et al., 1991; Ginn, 1996; Novotna et al., 2004; Ceckova-Novotna et al., 2006).
An often used method for detection of P-gp is immunohistochemistry. P-gp is localized mainly in apical cells facing a lumen which shows, that it has a physiological role as a membrane transport protein in normal tissues (Axiotis et al., 1991).
A study by Ginn (1996) on the distribution of P-gp dealt with the distribution of this protein in normal and tumorous canine tissue. The results showed that P-gp can be found consistently in the following normal tissues: liver, adrenal gland, stomach, pancreas, colon, kidney, salivary glands, lung, brain and endothelium (Ginn, 1996).
Normal tissues showing variable staining were: myocardium, mammary gland, apocrine and sebaceous glands, apical margins of the endometrium, luteal cells and developing secondary follicles in the ovary and thyroid gland (Ginn, 1996).
These findings coincide with findings in human studies (Thiebaut et al., 1987). Also the occurrence in malignant as well as benign tumor cells in canines is similar to the distribution in tumorous tissues of humans and therefore P-gp plays an important role in the multidrug resistance in canine cancer patients as well (Ginn, 1996).

Another study by Huang-Yang about the interaction of the steroid hormone progesterone and the expression of P-gp in MDR cells and the endometrium of the gravid uterus in mice showed, that progesterone is most likely interacting with P-gp. During the time when progesterone is at its highest level in the pregnant mice (around day 16 of gestation), they observed an increase in the mRNA encoding for P-gp (Huang-Yang, C-P. et al., 1989).

3. Aim of the study

Aim of our study was the demonstration of the expression and distribution of progesterone, progesterone receptor (PR), estrogen receptor α, Matrix Metalloproteinase-9 (MMP9), Vascular endothelial growth factor (VEGF), Caspase-3, Proliferating cell nuclear antigen (PCNA) and P-Glycoprotein (PGP) in the canine endometrium during different stages of the estrous cycle.

4. Materials and methods

Tissue samples

The samples were taken from the uterine wall of bitches which were brought in for ovariectomy or ovariohysterectomy due to medical reasons or requested by the owners. Surgery was performed under general anesthesia. The approximate size of the obtained samples where 2cm x 1cm. They were excised from the apical part of the uterus horn or dissected from the removed uterus.

Data, including ID-code, date of sampling, stage of the estrous cycle and possible uterine diseases were recorded for each patient. Since a lot of samples were taken from dogs originating from shelters and taking part in a spaying campaign, it was difficult to evaluate the stage of the cycle in some individuals. Arrangement of animals in estrous stages was based on anamnesis/clinical signs or serum progesterone concentration collected before anesthesia. The bitches were of various breeds and age, with most of them being mongrel dogs.

Processing of samples

After obtaining the samples they were placed in small containers containing 8% neutral buffered formaldehyde for 24 hours.

During the next 5 days the samples were washed daily with a phosphate buffered saline solution. After this washing process they were placed and preserved in flasks containing 75% alcohol.

Immunohistochemistry

After fixation the tissues were further dehydrated, cleared and embedded in paraffin wax. 4 μm thick slices were collected from the paraffin blocks and placed onto glass slides. The next steps included deparaffinization and rehydration. For this the slides were placed in a xylene bath followed by ethanol treatment. For the antigen retrieval the microwave oven heating method was used. The slides were heated in citrate buffer (pH 6.0, 10 nM) for 10 minutes at 750 W and two periods of 10 minutes at 300 W. After cooling at room temperature for approximately 10 minutes the slides were washed with buffered saline.

3% hydrogen peroxide in PBS and 2% milk powder diluted in PBS were used to block endogenous peroxidase activity and eliminate non-specific background.

Specific primary monoclonal antibodies were used and the slides incubated overnight at 4°C. The further steps included rinsing with PBS, incubation with the En Vision system, application of substrate and chromogen and finally counterstaining with heamatoxylin (Thuróczy et al., 2007).

Evaluation

The prepared slides where examined for each marker under a light microscope. It was evaluated whether the sample contained the marker (+) or not (-). If it contained the marker it was further classified into three different categories:

- + (low positive): contains only a small amount of marker and is found only in a small amount of individual cells.
- ++ (medium positive): contains a medium amount of the examined marker and is found in more than one part of the sample.
- +++ (high positive): contains a large amount of the marker and is found in almost all parts of the tissue.

5. Results

ID Number	P-gp	MMP9	Estrogen receptor	Serum P4	Stage of cycle	Diseases
231774	++	+++		0,71	Juvenile	
232803	++			0,73	Anestrus	
232804	+			4,48	Late metestrus	
232805	-			17,62	Early metestrus	
232875	++			1,34	Late metestrus/anestrus	Neoplasm mammae
232876	-			13,69	Early metestrus	
232916	++	++	++		Metestrus	
232918	-	+	-			
232919	+	-	++		Late metestrus	
233045	-	++	++	1,9	Late metestrus/anestrus	Neoplasm mammae
233057	-	-	+			
233122	+	++	++	1,88	Late metestrus/anestrus	
233123	-	++	-	0,45	Anestrus	
233160	-	++	+++	0,81	Anestrus	
233166	-	-	-			
233248	-	++	++	0,83	Juvenile	
233420	+	++	-	0,94	Anestrus	
233433	-	-	-	0,69	Infantilis, praepubertalis	
234395	++	++	++	1,03	Proestrus	Prolapsus vaginae
B-5067		++				
Beagle 55			++	6,34	Metestrus (pregnant)	

Table 1: Expression of P-gp, MMP-9 and estrogen receptor-α in the canine endometrium at different stages of the estrous cycle.

ID Number	Progesterone	Progesterone receptor	Serum P4	Stage of cycle	Diseases
231774			0,71	Juvenile	
232803			0,73	Anestrus	
232804	+++		4,48	Late metestrus	
232805			17,62	Early metestrus	
232875			1,34	Late metestrus/anestrus	Neoplasm mammae
232876			13,69	Early metestrus	
232916	-	++		Metestrus	
232918	+	-			
232919	-	-		Late metestrus	
233045	++	++	1,9	Late metestrus/anestrus	Neoplasm mammae
233057	-	-			
233122	+++	-	1,88	Late metestrus/anestrus	
233123	-	++	0,45	Anestrus	
233160	-		0,81	Anestrus	
233166	-	++			
233248	-	-	0,83	Juvenile	
233420	-	-	0,94	Anestrus	
233433	-	-	0,69	Infantilis, praepubertalis	
234395	-	++	1,03	Proestrus	Prolapsus vaginae
B-5067		+++			
Beagle 55			6,34	Metestrus (pregnant)	

Table 2: Expression of progesterone and progesterone receptor in the canine endometrium at different stages of the estrous cycle.

ID Number	VEGF	PCNA	Caspase	Serum P4	Stage of cycle	Diseases
231774			-	0,71	Juvenile	
232803				0,73	Anestrus	
232804				4,48	Late metestrus	
232805				17,62	Early metestrus	
232875				1,34	Late metestrus/anestrus	Neoplasm mammae
232876				13,69	Early metestrus	
232916	++	+++	+		Metestrus	
232918	-	++	+			
232919	-	-			Late metestrus	
233045	+	+++	-	1,9	Late metestrus/anestrus	Neoplasm mammae
233057	+	+	-			
233122	-	-	++	1,88	Late metestrus/anestrus	
233123	++	++	-	0,45	Anestrus	
233160	-	+++	-	0,81	Anestrus	
233166	-	++	-			
233248	+++	++	+	0,83	Juvenile	
233420	+	++	-	0,94	Anestrus	
233433	-	-	-	0,69	Infantilis, praepubertalis	
234395	+++	+++	-	1,03	Proestrus	Prolapsus vaginae
B-5067						
Beagle 55				6,34	Metestrus (pregnant)	

Table 3: Expression of Vascular Endothelial Growth Factor, Proliferating Cell Nuclear Antigen and Caspase-3 in the canine endometrium at different stages of the estrous cycle.

The results of the immunohistochemical examination regarding the earlier mentioned markers are shown in Table 1, 2 and 3.
Further on the different markers will be evaluated separately and pictures will illustrate the findings. For some of the samples the serum progesterone level was unknown and the anamnesis or clinical examination inconclusive. Therefore these samples could not be related to a certain stage in the estrous cycle.

Progesterone (P4)

Progesterone was examined in 14 samples with two being high positive, one being medium and low positive respectively and 10 showing no signs of progesterone (Table 2).
The high and medium positive samples were collected from bitches being either in late metestrus or anestrus.
Five of the negative samples were found in dogs being in metestrus or anestrus as well.

Progesterone receptor (PR)

13 samples were checked for the presence of PR. One sample was high positive, five medium positive and in seven slides no marker was seen (Table 2).
Unfortunately the high positive sample could not be matched to a certain stage of the estrous cycle, but the medium positive samples showed that in three of these samples the bitches were in late metestrus or anestrus. One medium positive sample was observed in a bitch being in proestrus.
Regarding the negative samples three were observed in bitches being in late metestrus or anestrus and two in juvenile dogs.
As for the distribution in the uterine tissue, the majority of PR were found in the endometrial basal glands (Figure 1, 2, 3). Staining was also seen in the endometrial stromal cells, endometrial epithelium and myometrium, but to a lesser extent.

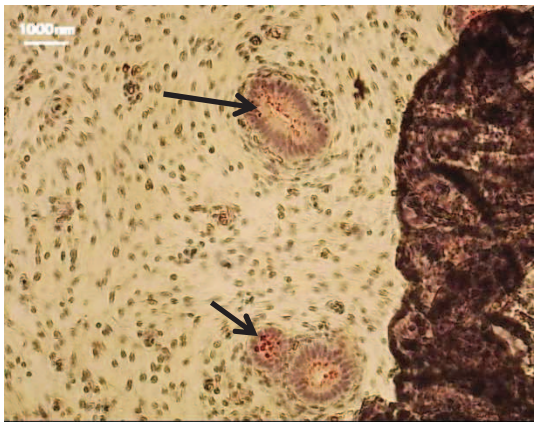

Figure 1: Progesterone receptors in endometrial glands from a bitch in metestrus

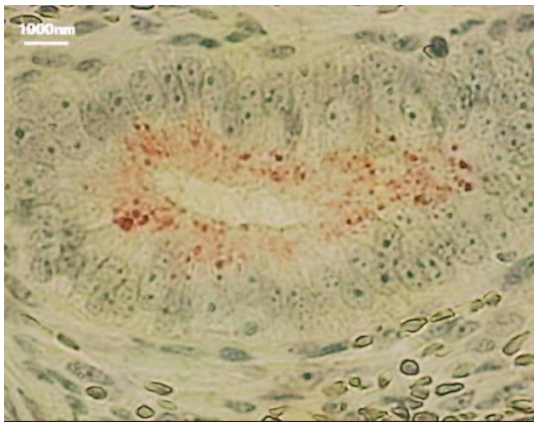

Figure 2: Progesterone receptors (brown staining) in endometrial glands from a bitch in late metestrus/anestrus, higher magnification

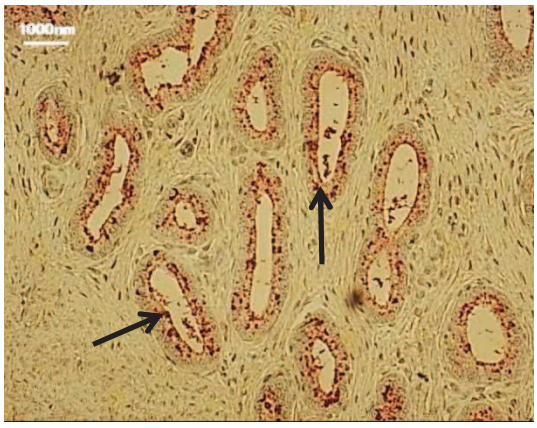

Figure 3: Progesterone receptors in endometrial glands in a sample from a bitch in proestrus

Estrogen receptor (ER)

14 samples were checked for the presence of the estrogen receptor α. One of the samples showed high positivity, seven were medium positive, one low positive and in five samples the marker was absent. (Table 1)

Regarding the location, the receptors could be found mainly in the glandular epithelium but also in the surface epithelium, stromal cells as well as in the myometrium (Figure 4, 5, 6).

In relation to the stage of the estrous cycle the highest amount of ER could be seen during anestrus and late metestrus. The sample showing high positivity was a sample taken from a bitch in anestrus and five samples out of the medium positive samples were taken from bitches in late metestrus. One medium high sample was seen in proestrus and one in a juvenile dog. The negative samples were observed in two bitches in anestrous and one in an infantile/praepubertal dog.

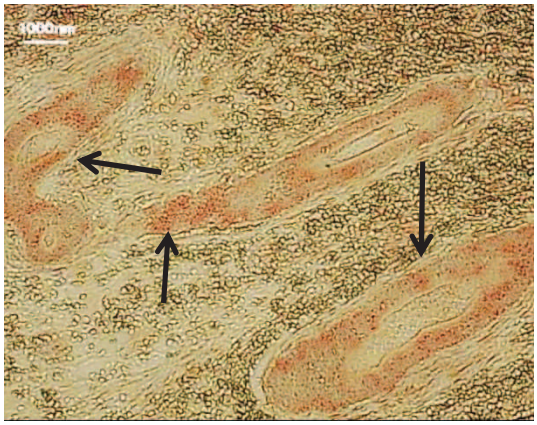

Figure 4: Estrogen receptor-α in a sample from a bitch in late metestrus/anestrus

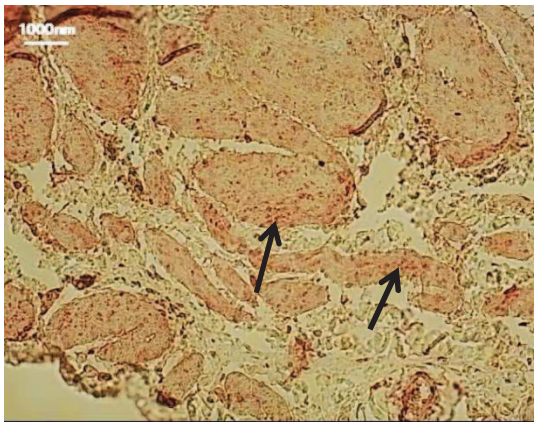

Figure 5: Estrogen receptor-α in myometrial cells in a sample from a bitch in anestrus

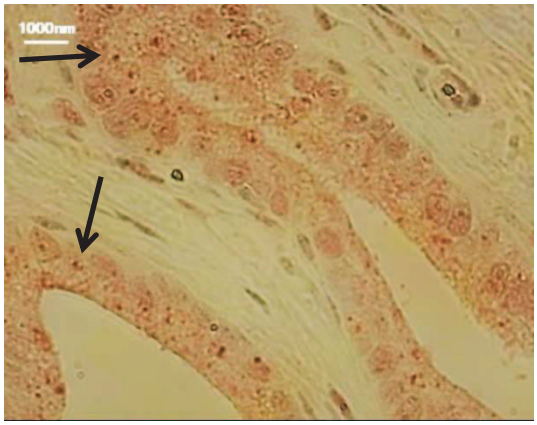

Figure 6: Estrogen receptor-α in glandular ducts in a sample from a bitch in proestrus, higher magnification

Matrix Metalloproteinase-9 (MMP-9)

The MMP-9 marker was examined in 15 samples, with only one sample being high positive. Nine of the samples were medium and one low positive. In four samples no staining could be seen (Table 1).

The only high positive sample was from a bitch being juvenile. The majority of the medium positive samples were from bitches being in metestrus or anestrus. One medium positive sample was found in a dog in proestrus and one in a juvenile dog.

The negative samples were found in a bitch being in late metestrus and one being infantile.

Regarding the location of the MMP-9 the majority of positive cells were seen in glandular cells (Figure 7, 8).

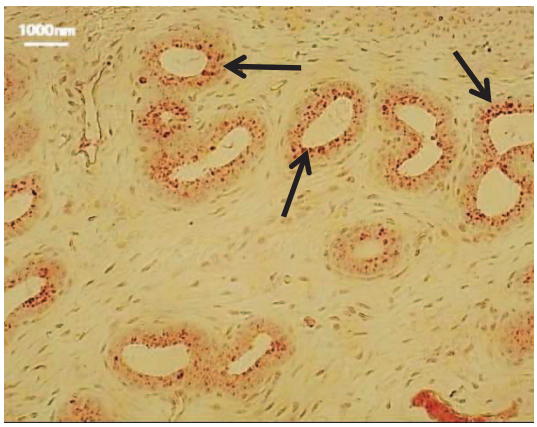

Figure 7: MMP-9 positive glandular cells in the canine endometrium from a bitch in proestrus

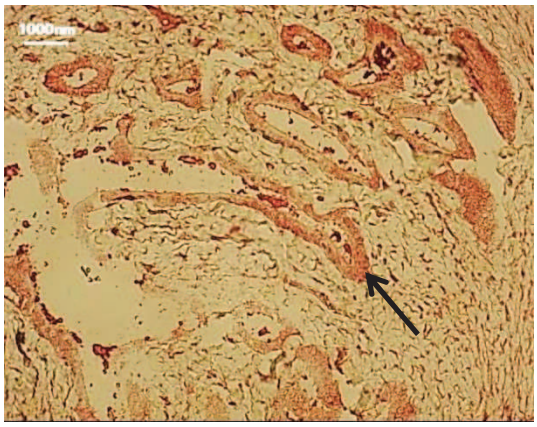

Figure 8: MMP-9 positive cells in the endometrium of a bitch in late metestrus/anestrus

Vascular Endothelial Growth Factor (VEGF)

This marker was examined in 13 slides. Two showed high positive, two medium positive, three low positive staining. In six samples no staining was seen (Table 3).

The two high positive samples were from a juvenile dog and one being in proestrus. The medium and low positive samples were mainly from dogs being in late metestrus or anestrus. Out of the six negative samples, three were from dogs being in late metestrus or anestrus and one from an infantile dog.

As for the distribution, positive cells were found in glandular (Figure 9) as well as in stromal and myometrial cells (Figure 10).

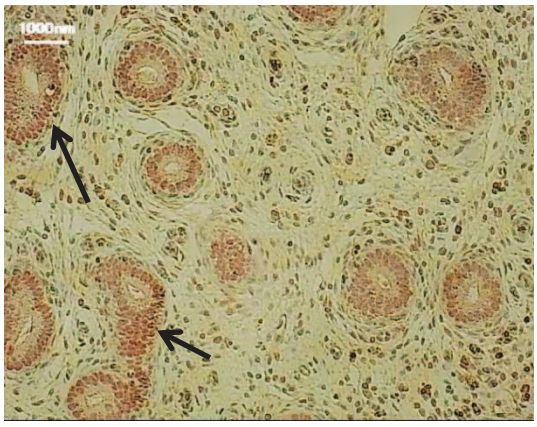

Figure 9: VEGF positive glandular cells in the canine endometrium from a bitch in metestrus

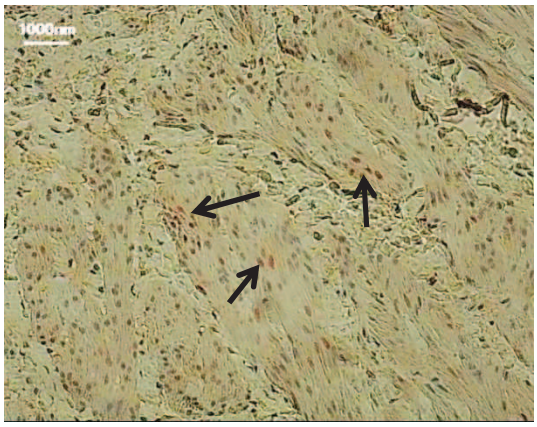

Figure 10: VEGF positive stromal and myometrial cells in the canine endometrium

Caspase-3

Caspase was examined in 14 slides. None of the samples were high positive. Only one sample was medium positive, three low positive and the 10 others showed no evidence of the marker (Table 3).

The medium positive sample was seen in a bitch in late metestrus or anestrus and the three low positive samples in a dog in metestrus stage and one in a juvenile dog.

Regarding the distribution, positive cells were found in basal glands (Figure 11, 12) as well as in stromal cells (Figure 13).

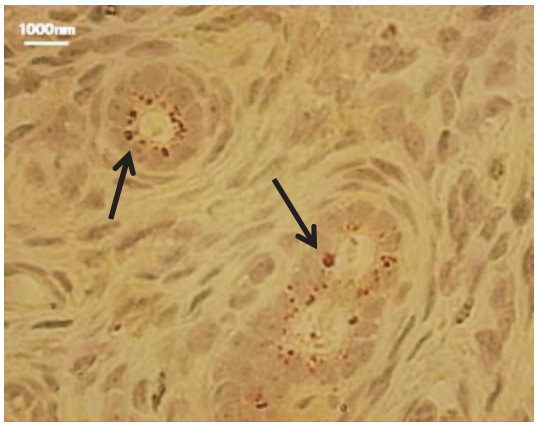

Figure 11: Caspase-3 positive glandular cells in the canine endometrium from a bitch in late metestrus/anestrus

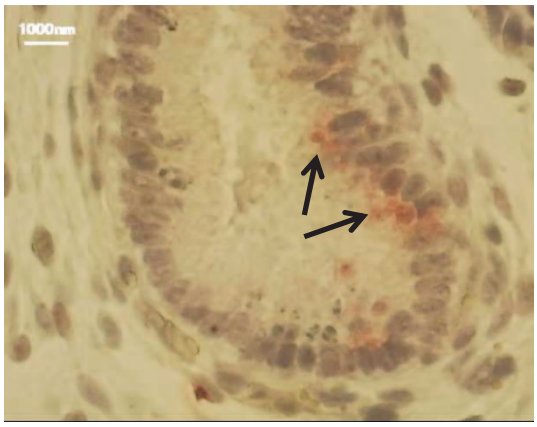

Figure 12: Caspase-3 positive cells from a bitch in metestrus, higher magnification

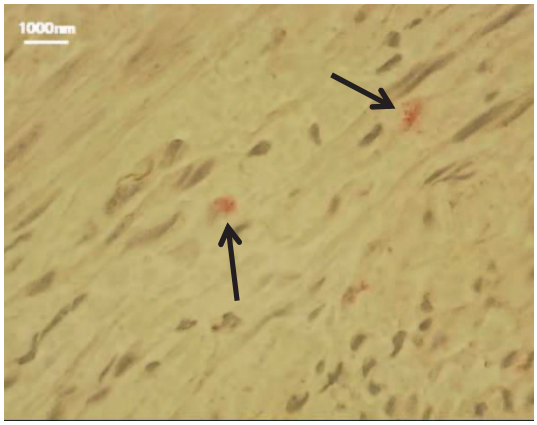

Figure 13: Caspase-3 positive stromal cells in the canine endometrium from a juvenile bitch

Proliferative Cell Nuclear Antigen (PCNA)

13 slides were examined for the occurrence of PCNA. Four showed high positive, five medium positive, one low positive and three negative staining (Table 3).

Three out of the high positive samples were from bitches being in late metestrus or anestrus and one sample from a dog in proestrus. Two out of the medium high samples were in anestrous stage as well and one from a juvenile dog. Regarding the negative samples two out of three were from bitches in late metestrus and one from an infantile dog.

Positive cells were found in glandular cells (Figure 14, 15, 16) as well as in stromal cells (Figure 16) and in the surface epithelium.

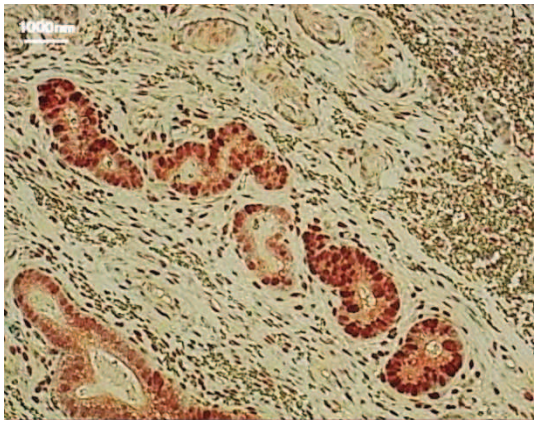

Figure 14: PCNA positive glandular cells (brown staining) in the canine endometrium from a bitch in late metestrus/anestrus

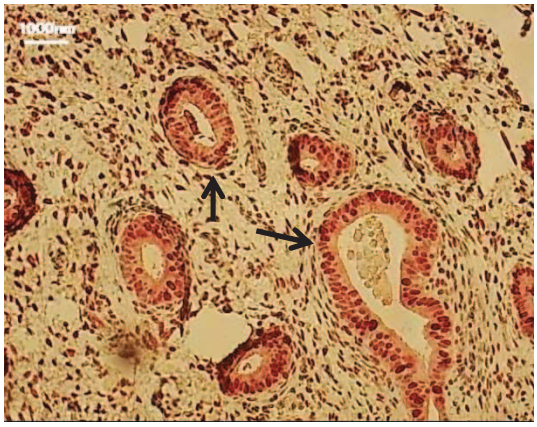

Figure 15: PCNA positive glandular cells in the canine endometrium from a bitch in metestrus

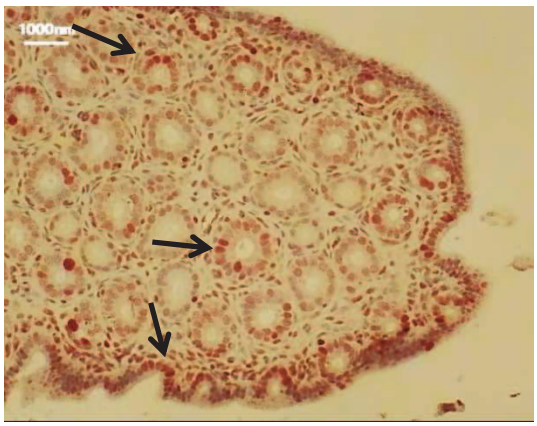

Figure 16: PCNA positive stromal and glandular cells in the canine endometrium from a bitch in proestrus

P-glycoprotein (P-gp)

The P-glycoprotein marker was examined in 19 samples with five of them being medium positive, four low positive and 10 showing no staining for P-gp (Table 1).

In relation to the estrous cycle three of the medium positive samples were seen in late metestrus and anestrus stage. One medium positive sample was observed in proestrus and one in a juvenile dog.

As well as the medium positive samples, the low positive samples were also seen in bitches being in either late metestrus or anestrus.

Although most of the positive samples were found in metestrus or anestrus some negative samples could be seen during these stages as well. Three samples were found negative during these stages of the cycle, as well as during early metestrus and in juvenile bitches.

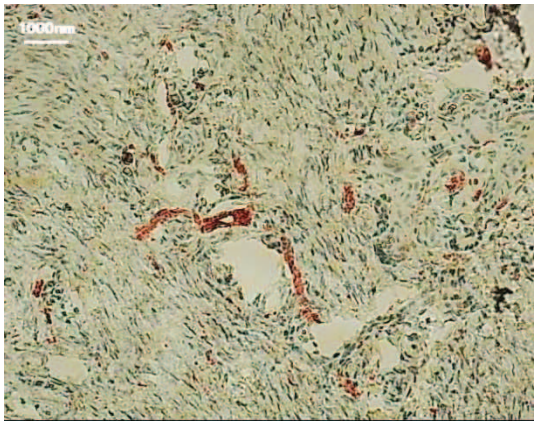

Figure 17: P-gp positive stromal cells (brown staining) in the canine endometrium from a juvenile bitch

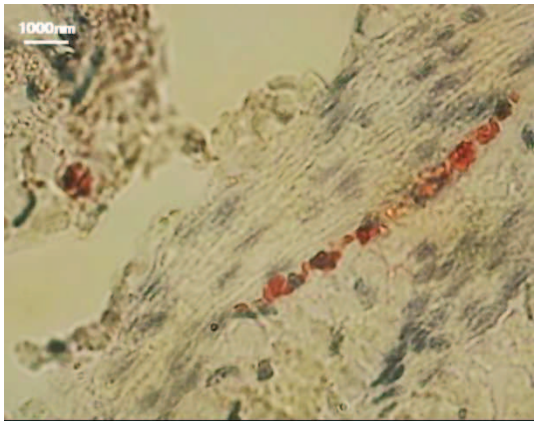

Figure 18: P-gp positive stromal cells (brown staining) in the canine endometrium from a bitch in late metestrus/anestrus

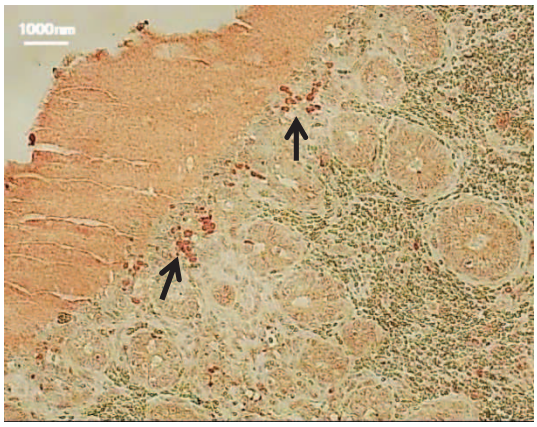

Figure 19: P-gp positive stromal cells in the canine endometrium from a bitch in late metestrus/anestrus

6. Discussion

Progesterone

The results show, that 10 out of 14 examined samples showed no positivity for progesterone. Most of these samples were from bitches being in late metestrus or anestrus as well as one sample from a bitch in proestrus and two from juvenile dogs.

As described before, the serum progesterone level starts to decrease around day 35-40 after ovulation, than reaches a low value around the end of metestrus and stays at this level throughout anestrus.

The high amount of negative samples found during these stages of the estrous cycle, therefore correspond with the decreasing serum progesterone level.

Progesterone receptor

The results show that six out of 13 samples were found positive for the PR (Table 2). According to previous studies the highest amount of PR can be found during proestrus and estrus, whereas the amount of receptors varies in metestrus and anestrus according to different locations within the uterine tissue. Three out of the positive samples were found in samples from bitches being in either metestrus or anestrous; one was from a bitch in proestrus. Three out of the negative samples were also found in samples being from late metestrus and anestrous. Previous studies already revealed that during metestrus a high number of PR can be found in the stromal and myometrial cells, whereas the number in the basal glands decreases (Vermeisch et al., 2000). This might explain why some samples in metestrus or anestrus were found to be negative, whereas others showed a medium positive staining because the slides did not contain all parts of the uterine tissue all the time.

Too little amount of samples from bitches in proestrus were collected, to confirm the fact that the highest number of PR can be found during this stage. One sample from a bitch in proestrus however did show a medium positive result.

Estrogen receptor

Our results show, that nine out of 14 samples were positive for the estrogen receptor. The majority of the positive samples were from bitches in metestrus or anestrus (Table 1). These findings coincide with the findings from previous studies (Vermeisch et al., 1999). As earlier described, the number of estrogen receptors increases during late metestrus and stays at a high level throughout anestrus and the following proestrus. Previous studies also

revealed that the highest number of ER was found either in proestrus or estrus (Vermeisch et al., 1999; Ozyurtlu et al., 2010).
Unfortunately the number of samples from bitches in proestrus was low; therefore it was not enough to confirm these results. One sample from a bitch in proestrus did however show a medium amount of estrogen receptors.
According to these results one can say that as the measured progesterone levels decrease, the number of estrogen receptors start to increase.

Matrix Metalloproteinase-9

A study by Loukopoulos (2003) on the expression of MMP-9 in the canine endometrium and their relation to the steroid hormone progesterone revealed, that the highest quantity of MMP-9 is usually found during metestrus when the serum progesterone levels are high. In our study 11 out of 15 samples showed positivity for MMP-9. Three out of these 11 positive samples were from bitches in metestrus, which coincides with the findings of Loukopoulos (2003). Another three positive samples were found in bitches being in anestrous which does not coincide with the findings of the other study, since according to Loukopoulus (2003) the amount of MMP-9 decreases together with the progesterone levels. A direct correlation between the serum progesterone levels and the expression of MMP-9 could not be seen in our results.

Vascular Endothelial Growth Factor

As an important proangiogenic regulator, VEGF plays a key role in the angiogenesis of the endometrium of the bitch. A study by Sagsöz (2013) revealed that the highest amount of VEGF can be usually seen during the proestrus and estrus stages. Since in our study no samples of dogs in estrus and only one sample from a dog in proestrus was collected it is difficult to prove the findings described by Sagsöz (2013).
In our study seven out of 13 samples showed positivity for VEGF. One sample, taken from a bitch in proestrus, showed high positivity, which therefore coincides with the findings of Sagsöz (2013). Another highly positive sample was from a juvenile dog, which can be related to the fact that VEGF plays a key role during angiogenesis. Another four positive samples were from bitches being either in metestrus or anestrus in which no positivity was expected.

Caspase-3

As described earlier, Caspase-3 is an enzyme found in an inactive form in various cells and once activated it plays an important role during apoptotic processes in the endometrium. According to previous studies the highest amount of cells showing positivity for Caspase-3 can be usually found during late metestrus and anestrus (Van Cruchten et al., 2003). In our study 14 samples were examined for the activity of Caspase-3 and only four of them showed positivity. Although a lot of our samples were from dogs being in either late metestrus or anestrus only two showed positivity. A lot of samples from dogs in metestrus or anestrus showed no sign of caspase-3 activity, so the findings did not coincide totally with the findings in the study of Van Cruchten (2003).

A correlation between the serum progesterone levels and the activity of caspase-3 could not be observed in our study, which coincides with the results described in the study by Van Cruchten (2003).

Proliferative Cell Nuclear Antigen (PCNA)

Previous studies showed that there are two major proliferative peaks during the estrous cycle of the bitch in which a high amount of the PCNA marker can be found in the endometrium (Barrau et al., 1975; Van Cruchten et al, 2003).

These two peaks are found during proestrus and during the middle of estrus. Since our samples were taken mainly from bitches in metestrus and anestrus, it is difficult to confirm these results. One sample was taken from a bitch in proestrus and this sample did show a high positive result for the PCNA marker. But among the other 10 positive samples were also samples that were taken from bitches in metestrus and/or anestrus were found and they showed some positivity for PCNA as well as samples taken from juvenile dogs.

P-glycoprotein

Regarding the occurrence of P-glycoprotein in the canine endometrium nine out of 19 samples were positive for P-gp and in 10 samples no marker could be found. Seven positive samples were from bitches being either in metestrus or anestrus. The same applies to the negative samples were the majority was also found in either metestrus or anestrus stages (Table 1).

As described before, studies about the presence of P-gp in the endometrium revealed that this tissue usually shows a variable staining (Ginn, 1996).

The fact that a study in gravid mice showed, that progesterone might have an influence on the expression of P-gp in endometrial cells cannot be transferred to dogs according to our study.

Whereas in gravid mice it seems that there is a positive correlation between the high progesterone level and the expression of P-gp in endometrial cells this cannot be seen in dogs according to these results. It seems that the progesterone level has no influence on the expression of P-gp in canine endometrial cells.

7. Summary

In this study immunohistochemistry was used to detect progesterone, progesterone receptor, estrogen receptor-α, Vascular Endothelial Growth Factor, Proliferating Cell Nuclear Antigen, Matrix Metalloproteinase-9, Caspase-3 and P-glycoprotein in the canine endometrium. Furthermore serum progesterone levels were measured and anamnesis as well as clinical examination was used to relate the immunohistochemical findings to the stages of the bitch estrous cycle.

Parts of the results in this study coincided whereas other parts differed from findings described in previous studies.

The results regarding the estrogen receptor-α for example, coincided in great parts with the results in a study done by Vermeisch (1999) and the highest quantity of receptors was found during the late metestrus, anestrus as well as during proestrus stages.

As for the progesterone receptor a high amount of positive cells was found during late metestrus, anestrus and proestrus. But also negative samples were found during the same time of the estrous cycle, so the findings coincided only in parts with the results of a study done by Vermeisch (2000).

A high amount of Caspase-3 positive cells was expected to be seen during late metestrus and anestrus but, although a high amount of samples was taken from bitches in these stages, only a few showed positivity which therefor does not correlate with previous studies. On the other hand, the fact that no correlation between the serum progesterone levels and the expression of caspase-3 was found did coincide with a study by Van Cruchten (2003).

Regarding P-gp, our study showed that a study done on mice, in which a positive correlation between the serum progesterone levels and the expression of P-gp exists, can most likely not be transferred to dogs, since in our study no correlation was found.

Due to the fact, that our samples were mainly originating from bitches being in either metestrus or anestrus and also from juvenile bitches, in some of the markers like PCNA or VEGF, it is hard to say that the results of our study coincide with the findings of previous studies. It is advisable to repeat the study with more bitches, concentrating on those that are in proestrus and estrus and evaluate the results accordingly.

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