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TUMOUR NECROSIS FACTOR IN THE CANINE ENDOMETRIUM: AN IMMUNOHISTOCHEMICAL STUDY

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Tumour necrosis factor (TNF), a pleiotropic cytokine that regulates cell growth and differentiation as well as the synthesis of other cytokines, has been identified in the uterus of several species describing a cyclic pattern, eventually under ovarian steroid regulation. Information is yet limited on the presence of TNF protein in the canine endometrium during the oestrous cycle and early pregnancy. This study depicts the temporal immunolocalisation of TNF in the bitch endometrium along the oestrous cycle, and changes associated with the early steps of embryo invasion. TNF immunolabelling was found in both the stromal fibroblasts and epithelial components of the canine endometrium in all stages studied. Stromal immunostaining was most intense than that of the epithelia, in all the stages of the oestrous cycle. In addition, a tendency for a decrease in the surface epithelium intensity score was found in early dioestrus. A positive glandular content was only observed in anoestrus and proestrus stages. In early pregnancy (days 13-16), TNF immunolabelling was detected at the embryo-maternal surface, in the syncytium cords and the trophoblast, as well in the endometrial stroma and the basal endometrial glands, but not in the lacunar epithelium. The overall TNF immunoreactivity was higher in early pregnancy samples in comparison to those of the early dioestrus and dioestrus stages, suggesting it plays a role during implantation.

Key words: Tumour necrosis factor; TNF-alpha; canine endometrium; oestrous cycle; early pregnancy; immunohistochemistry; Dog.

Introduction

The mammalian endometrium is a highly dynamic and complex tissue, whose main purpose is to guarantee embryo survival, implantation and the success of pregnancy. In response to stimulation by the sex steroids, the endometrium undergoes cyclic remodelling. This process is ultimately controlled by several autocrine and paracrine factors that include cytokines, interleukins and growth factors, among other molecules (Cavagna and Mantese 2003, van Mourik et al. 2009, Dekel et al. 2010).

Besides epithelial and stromal cells, several immune-related cells, such as macrophages and lymphocytes, can be found in the mammalian endometrium. The recruitment of immune cells into the uterus has been proved to be cycle-dependent, i.e. under ovarian steroid influence (Kaeoket et al. 2001, Gu et al. 2005, Lea and Sandra 2007, Wicherek 2008). It has also been demonstrated that these immune cells participate, together with the endometrial stromal and epithelial cells, in the regulation of the cyclic endometrial remodelling and in embryo implantation (Salamonsen et al. 2002, Kammerer et al. 2004, Kayisli et al. 2004, von Rango 2008).

Tumour necrosis factor (TNF), formerly named as tumour necrosis factor alpha (TNF- α) or cachectin, is a pro-inflammatory cytokine that shows a wide spectrum of activities, frequently in a dual, dose-dependent way (Wang et al. 2003). TNF possesses strong pro-inflammatory and immune-stimulatory actions and is also involved in the control of cell differentiation, proliferation and migration, as well as in tumorigenesis (Wang et al. 2003, Haider and Knofler 2009). When low-levels are expressed, this molecule also participates in tissue remodelling and host responses (Wang et al. 2003).

TNF is expressed and synthesized in human, mouse and cow endometrium (Hunt et al. 1992, De et al. 1993, von Wolff et al. 1999, MacEwan 2002, Haider and Knofler 2009), in a cycle-dependent manner that suggests a regulation by the sex steroid hormones (Hunt et al. 1992, von Wolff et al. 1999, MacEwan, 2002). Although several studies evaluated TNF expression in human endometrium, results remain controversial, which can in part be explained by the use of different methodological procedures (Hunt et al. 1992, De et al. 1993, von Wolff et al. 1999). According to von Wolff and colleagues (1999) progesterone down-regulate TNF expression in the endometrium. However, Hunt et al. (1992) reported an increase in TNF mRNA during the proliferative phases and during mid-to-late secretory phases of the mouse oestrous cycle, while in the early secretory phase its expression declined. In addition, TNF was also found in uterine secretions in the later proliferative and early secretory phases of the menstrual cycle (Hunt et al. 1992).

TNF has also been shown to be expressed by the embryo (Hunt et al. 1992, Ben-Yair et al. 1997, Kawamura et al. 2007) and uterine cells at the implantation site (Hunt et al. 1992). The role of TNF around implantation is not completely understood, and whether TNF affects the embryo viability or not seems to be dependent of the embryonic developmental stage and the amount that is produced locally. *In vitro* matured human embryos secrete TNF until the morula stage, but not at the blastocyst stage (Ben-Yair et al. 1997, Kawamura et al. 2007). In addition, mouse embryos exposed *in vitro* to TNF showed a dose-dependent arrest in growth and development, independently of the embryo stage, along with ultrastructural degeneration (Lalithkumar et al. 2005). However, more recent studies revealed that TNF could be favourable for particular functions of the trophoblast during invasion and may further participate in the control of the invasion process, later in pregnancy (Haider and Knofler 2009).

Limited information exists on the expression of TNF in the canine uterus. Previous studies by Schäfer-Somi et al (2008) on cytokine expression by the canine endometrium in early

dioestrus and early pregnancy showed that TNF m-RNA was weakly expressed in dioestrus but was absent from the pre-implantation endometrial samples. These authors also found TNF expression in 10 days-old dog embryos. In pathological conditions, TNF expression was demonstrated not to be up-regulated in uterine samples (Hagman et al. 2009). However, TNF levels in plasma were found to be higher in dogs with clinical pyometra than in animal without pyometra (Fransson et al. 2007). Despite the abovementioned studies, information on the TNF protein expression and localization in the canine endometrium remains sparse. The purpose of this work was to evaluate the TNF protein expression in the dog endometrium throughout the bitch oestrous cycle and in early pregnancy using immunohistochemistry and to determine whether temporal changes occur on the protein immunolocalization during the cycle and in association to early pregnancy events.

2. Material and methods

Animals

A total of 55 mature, clinically healthy bitches (43 mongrel, 5 Portuguese podengo, 2 Boxer, 3 Poodle crossed, 1 Siberian husky and 1 German shepherd), and 8 early pregnant mongrel females (from pregnancy days 13 to 16), submitted to elective ovariohysterectomy were used. Before surgery, a vaginal cytological specimen was obtained. A blood sample was collected by venipuncture from a jugular vein to a controlled vacuum tube (*Serum-gel*, S-Monovette®, Sarstedt, Germany). The serum was stored at -20 °C until analysis. Serum progesterone levels were determined by chemiluminescent immunoassay system (Immulite®, DPC-Diagnostic Products Corp., Los Angeles, CA, USA).

Endometrial samples were collected after expressed consent of the animals' owners, and in respect to the International Guidelines for research involving animals. Excised genital

tracts were fixed in 10% buffered formalin immediately after surgery. A fragment from each uterine horn was collected at its caudal ending, at approximately 1 cm above the uterine body, embedded in paraffin wax, sectioned at 3 µm thickness and routinely stained with haematoxylin and eosin for histological evaluation of the endometrium and staging of the oestrous cycle. Samples were excluded when presenting signs of involution (Jöchle and Andersen 1977, Al-Bassam et al. 1981) or uterine disease, such as metritis or cystic endometrial hyperplasia/pyometra complex.

Oestrous cycle and pregnancy staging

For the non-pregnant bitches, cumulative information provided by vaginal cytology, inspection of the ovaries at OVH, and circulating levels of progesterone was used to stage the oestrous cycle of each animal (Table 1). Vaginal cytology allowed a preliminary staging of the cycle. Afterwards, the stage was further determined by macroscopic and histological examination of the ovaries and uterus (Rehm et al. 2007) and finally the serum progesterone levels were used to confirm the staging of oestrous cycle.

For the pregnant females, cumulative information was gathered from dioestrus-compatible cytology, plus a high progesterone levels and the presence of young corpora lutea in the ovaries and the co-existence of small sized (<3 cm) uterine swellings. Furthermore, the chronology of the pregnancy was determined according to the histological descriptions of canine early pregnancy events provided by Amoroso (1952) and Barrau et al. (1975b), and was established in relation to the first day of dioestrus. Briefly, by day 13 embryo apposition is achieved and the trophoblast grows down and wedges the maternal surface epithelium. Small lacunae are visible. By day 14, the trophoblast continues to spread down, and the syncytium penetrates deeper in the endometrium appearing as strong, linear cords, frequently presenting mitotic figures. On day 16, the crypts at implantation sites are long, tortuous and closely packed, with enlarged lacunae below. The deep endometrial glands start to growth.

Immunohistochemistry (IHC)

IHC analysis was performed using a streptavidin-biotin-peroxidase complex method and the UltraVision Detection System® (Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) on formalin-fixed, paraffin-embedded tissue sections. Three µm thick sections, placed in silan-coated slides, were submitted to routine deparaffinization and rehydration in graded alcohol. Antigen retrieval was performed by using a pressure cooker for 2 min, with slides immersed in citrate buffer (pH = 6). For quenching endogenous peroxidases the sections were immersed in 3% hydrogen peroxide for 30 min. Non-specific binding of primary antibodies was blocked using a polyvalent blocking serum (Ultra V Block®, Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA) for 5 minutes. A specific mouse monoclonal primary antibody raised against full length recombinant canine TNF molecule (sc-80386; Santa Cruz Biotechnology, Inc., Europe, Heidelberg, Germany) was used at a 1:50 dilution in PBS. After an overnight incubation with the primary antibody, at 4°C, in a humid chamber, tissue sections were incubated with Biotinylated Goat Polyvalent Plus® antibody (Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA), followed by incubation with Streptavidin-peroxidase Plus® (Thermo Fisher Scientific, LabVision Corporation, Fremont, CA, USA). The 3,3'-diaminobenzidine (DAB) was used as chromogen. The sections were then counterstained with Mayer's Haematoxylin, dehydrated and mounted for evaluation by light microscopy. Samples of canine ovaries with mature corpus luteum (Engel et al. 2005) were submitted to the same procedure and used as positive control. Uterine vessels included in the tissue sections were also utilized as individual positive controls. Endometrial specimens incubated with mouse IgG (sc-2025; Santa Cruz Biotechnology, Inc., Europe, Heidelberg, Germany) and with PBS were used as negative controls. In neither negative control was TNF-immunoreactivity observed.

Immunohistochemical scoring

A blind assessment of the degree of staining was performed with a NIKON Eclipse 80i (Nikon Instruments Europe, BV) photomicroscope. Digital images were captured using a Nikon Digital Sight DF-Si1 camera and a NIS-Elements imaging software (version F2.30). Positivity was indicated by the presence of a distinct brownish to gold labelling.

The distribution of TNF immunoexpression was studied independently to the stroma and the epithelial elements of the endometrium, and the later were further distinguished as surface epithelium (SE) and glandular epithelium (GE). In early pregnancy samples, the trophoblast, the syncytium cords, the endometrial stroma and the glandular epithelium were individually scored. The evidence of immunostaining was recorded and its intensity was scored as negative (0), weak (1), moderate (2) or strong (3).

Statistical analysis

Statistical comparisons were performed by using the IBM SPSS Statistics Base 17.0 statistical software for Windows®. Statistical analysis of the differences in the intensity of immunoexpression for TNF between the stages of the oestrous cycle and the cell type were performed using the chi-square and Fisher exact tests. A *P* value ≤ 0.05 was regarded as statistically significant.

3. Results

Immunohistochemical staining indicating that TNF was present in the canine uterus was found in all samples studied (Table 2 and 3). TNF immunolabelling was expressed in both the uterine stroma and the endometrial epithelial cells in samples from cyclic females (Figure 1), and in different maternal endometrial structures and embryonic trophoblast. Strong to moderate intensity of immunolabelling was found in mature canine corpora

lutea, which were used as positive control for the technique. The corpora lutea showed a slight variation of the intensity of immunostaining within the cells of the same individual structure, ranging from strong to moderate intensity of immunolabelling (Figure 1).

Immunoreactive-TNF was present in the endometrial stroma in all stages of the oestrous cycle, with an intensity of immunolabelling varying from moderate to weak (Table 2). The staining intensity of TNF in the endometrial stroma appeared to be rather high in anoestrus and prooestrus (Figure 1; Graph1), whilst it tended to decrease in oestrus and was significantly lower in early dioestrus and dioestrus ($P < 0.001$; Fisher = 19.488) (Figure 1; Graph1). Within the stroma, besides positive fibroblast cells, strong immunolabelling on the vessel endothelia (which was used as internal positive control) and on resident immune cells of the endometrium was also detected in all samples.

TNF-immunolabeling in the surface epithelium of the canine endometrium showed little variation during the oestrous cycle (Table 2; Graph1); a weak intensity of immunolabelling predominated for the majority of samples and for all the cycles stages (Figure 1; Graph1). Occasionally, absence of immunoreaction for this molecule was found in oestrus (1:10) and early dioestrus (3:12) samples. However, no statistical differences were found for TNF-immunoexpression in the surface epithelium between cycle stages ($P = 0.090$; Fisher = 5.556).

Immunoreactivity for TNF was also found in the glandular epithelium. No visual differences were found between the superficial and deep endometrial glands, and consequently they were scored jointly. Glandular epithelial cells showed a weak cytoplasmic immunostaining during all stages of the cycle, and in some samples absence of TNF-immunoreactivity was detected (Graph1). For the glandular epithelium, although no statistical differences were found between stages of the oestrous cycle ($P = 0.359$;

Fisher = 4.129), a relative increase in the number of samples where TNF-immunoexpression was absent at the glandular epithelium was observed in early dioestrus (6:12) (Graph1; Figure 1).

Moreover, samples from anoestrus and prooestrus also presented moderate to strong immunoreactivity to TNF in the glandular secretions, which was absent from the glands at other stages of the oestrous cycle ($P < 0.001$; Fisher = 23.470). Strong immunopositivity was also observed in the tail of spermatozoa adhering to the SE or in the glandular lumen in 3 of the oestrus samples (Figure 1).

Studied individually, the oestrous cycle stages presented a significant influence on TNF-immunoexpression by stromal fibroblasts ($P < 0.001$; Fisher = 19.488) and the glandular secretions ($P < 0.001$; Fisher = 23.470), but not for the epithelial cells on the surface and glandular epithelia (respectively $P = 0.090$, Fisher = 5.556 and $P = 0.395$, Fisher = 4.129). However, when the intensity of the TNF immunolabelling was evaluated together in all studied endometrial structures, a significance influence of the canine stage of oestrous cycle was found ($P < 0.001$; Fisher = 48,879).

TNF-immunolabelling was detected in all early pregnancy samples studied (Table 3; Graph 2). Between canine pregnancy days 13 to 16, in response to the embryonic activity, the adluminal endometrial compartment displays important architectural changes that impair a direct comparison to equivalent structures in the non-pregnant, early dioestrus endometrium. Consequently, TNF-immunoreactivity was evaluated at the level of the trophoblast, the embryo-maternal interface (as the SE was no longer recognised as an independent structure), the syncytium cords, the endometrial stroma and the glandular epithelium (Table 3; Graph 2). Although more compact than the more basal endometrial stroma, the peri-lacunar stroma displayed the same intensity of immunolabelling and they

were scored together. Comparative evaluation between early pregnancy samples and the early dioestrus and dioestrus non-pregnant samples were only established for the stroma and the glandular epithelium.

A weak to moderate TNF immunolabelling was found in early pregnancy endometrial stroma (Graph 2; Figure 2). No statistical differences were found between the intensity scores of early dioestrus, dioestrus and early pregnancy samples ($P=0.202$; Fisher = 3,703), even though a slight increase of the intensity of the immunolabelling was found in the later. Strong TNF immunoreactivity was evidenced by immune-related cells and small vessels in either the superficial or the basal stroma.

The embryo trophoblast showed a moderate intensity of immunolabelling that seemed to increase in areas of adherence compared to the non-adherence areas (Figure 2). In addition, a moderate intensity of immunolabelling was observed at the embryo-maternal interface, where a surface epithelium is no longer recognisable. Also the syncytium cells showed a prevalence of weak intensity of immunolabelling. No TNF immunolabelling was observed in lacunar epithelial cells, neither was there a positive glandular content ever seen. In pregnancy days 13 to 16, only the deep endometrial glands are comparable to similar structures in non-pregnant endometrial samples, and in early pregnancy the absence of TNF immunoexpression in GE was observed. The marked decrease in the intensity of the glandular epithelium immunostaining registered in early pregnancy samples was found to be significantly different from those observed in early dioestrus and dioestrus ($P=0.003$; Fisher = 11,717).

4. Discussion

TNF is a multifunctional, pleiotropic pro-inflammatory cytokine that may exert beneficial functions in cell growth and proliferation and in tissue remodelling (Wang et al. 2003, Haider and Knofler 2009).

Studies in different species showed that TNF is expressed in the endometrium not only by immune cells, but also by epithelial and stromal cells (Hunt et al. 1992, Tabibzadeh et al. 1995, von Wolff et al. 1999, Fumuso et al. 2003, Sakumoto et al. 2009). Although the different studies on human endometrium failed to reach consensus about phase-related changes, it is generally accepted that TNF is expressed in a cyclic pattern that suggests its regulation by the ovarian steroids (Hunt et al. 1992, Tabibzadeh et al. 1995, von Wolff et al. 1999).

In dogs, limited information exists on TNF expression in the endometrium. Studies by Schäfer-Somi et al. (2008) on gene expression of several cytokines in the canine pre-implantation uterus and embryo showed TNF expression in low levels in the dioestrus endometrium. Although the authors fail to detect the expression of m-RNA for this molecule in the pre-implantation uterus, it was present in the 10 day-old dog embryos.

In the present study we investigate the temporal pattern of TNF immunoexpression in the canine endometrium during the oestrous cycle and also at pregnancy days 13-16, by using a primary antibody specific for the canine TNF molecule. Here we demonstrate that TNF protein is present in the canine endometrium during all stages of the oestrous cycle and also in maternal endometrium and in the embryo trophoblast at days 13 to 16 of pregnancy.

As it has been reported for human and mice (Hunt et al. 1992, Roby and Hunt 1994, von Wolff et al. 1999), in the cyclic canine endometrium TNF immunoexpression was localized in both the stromal and the epithelial cells, in addition to the immune-related cells and the endothelia of endometrial vessels. Although Sakumoto and colleagues (2009) did not

observe TNF protein expression in bovine endometrial fibroblasts, also they found TNF expression in the endometrial epithelia.

Interestingly, TNF immunolabelling was also found in the midpiece of canine spermatozoa; further studies are needed to highlight its putative function in either the female reproductive tract or on spermatozoa.

In all species studied so far, cyclic variations have been found in TNF gene and protein expression throughout the endometrial cycle. In the study presented here, although cyclic variations were detected in the protein immunolabelling in canine endometrium, overall TNF immunoreaction remained in low to moderate levels throughout the oestrous cycle. This finding is partially corroborated by the work of Schäfer-Somi and colleagues (2008), who report that TNF expression is maintained in low-levels in canine dioestrus endometrium, although they did not evaluate its expression at any other stage of oestrous cycle. In cows, Sakumoto et al. (2009) evidenced an increased expression in the overall TNF at the follicular phase and at late luteal phase in relation to the low level of expression found in the early luteal phase. The results from their study show some similarities with the observations presented here for the cyclic canine endometrium. Also previous work in humans and mice demonstrated that both TNF protein and gene are expressed in the endometrium in low to moderate levels (Hunt et al. 1992, Roby and Hunt 1994, von Wolff et al. 1999).

In the present study, some fluctuations in the intensity of TNF immunoexpression were observed during the oestrus cycle in the different components of the dog endometrium. Both the stromal and epithelial cells presented lower intensity scores in the secretory stages of the cycle, particularly at early dioestrus in relation to stages not-associated to progesterone, in particular at anoestrus and prooestrus. Although in humans no apparent differences were detected in TNF protein expression by stromal cells during the menstrual cycle (Hunt et al. 1992, von Wolff et al. 1999), in the mouse, Roby and Hunt (1994)

detected differences in the protein and gene transcripts between the secretory and proliferative stages of the cycle.

In humans and rodents, published reports do not differentiate between the superficial and the glandular epithelium when TNF protein expression is evaluated (Hunt et al. 1992, Roby and Hunt 1994, von Wolff et al. 1999). However, in the dog, the superficial and the glandular epithelium play different roles in early embryo-maternal interactions, and consequently they might show different features concerning cytokine expression.

The apparent difference in the pattern of TNF expression detected between observations in the dog and woman endometrium, in particular concerning the highest immunoexpression in early proliferative stage (prooestrus) and the lowest immunoexpression in early dioestrus found in dogs could be explained by the species-specific particularities, which in turn could be associated to known TNF functions.

TNF has been associated with tissue remodelling and renewal (Wang et al. 2003), and its levels are increased previous to intense remodelling of the woman endometrium at menstruation (Hunt et al. 1992, von Wolff et al. 1999). The study presented here revealed an increased intensity of immunolabelling of this molecule during anoestrus, particularly in the stroma. According to previous studies, during anoestrus in the canine endometrium degeneration of the SE cells occurs (Chu et al. 2006), along with a rather low proliferative activity in all cell types (Van Cruchten et al. 2004) and an increase in the matrix metalloproteinases expression (Chu et al. 2002). We hypothesize that TNF may be involved in the endometrial remodelling that occurs during anoestrus in dogs (Barrau et al 1975a), which has been proven to be crucial to bitch fertility. In contrast to women, in which the circulating levels oestrogens start to increase in early proliferative stage, in the bitch a small raise in blood oestrogens occurs during the last third of anoestrus, and rapidly increase during prooestrus (Jeffcoate, 1993; Concannon, 2009). This could explain why in prooestrus overall TNF expression was increased, in both the stromal and epithelial components. Oestrogens have been shown to up-regulate TNF expression in

the uterus (Hunt et al. 1992, Hunt 1993), which in turn stimulates the endometrial proliferative activity (Hunt, 1993). Moreover, TNF has also been associated with an increased permeability of the endothelial linings and with oedema (Tabibzadeh et al. 1999). In prooestrus in the canine endometrium all these three main events associated to TNF co-exist: epithelial cell proliferation (Van Cruchten et al. 2004), oedema, and diapedesis (Tabibzadeh et al. 1999).

In oestrus, pre-ovulatory luteinisation of the dog ovarian follicles is observed. This stage is characterized by gradual decreasing levels of oestrogens and increasing levels of progesterone. When high levels of oestrogens are reached, the TNF expression by endometrial epithelial cells is inhibited (Hong et al. 2004, Grant-Tschudy and Wira 2005), an event that was proposed to be mediated by a putative soluble factor produced by stromal cells (Grant-Tschudy and Wira 2005). In the study presented here, a decrease in the stromal TNF immunoeexpression in oestrus was observed, possibly reflecting these changes in ovarian steroids.

A significant decrease in the intensity of immunolabeling for TNF was found in the endometrial stroma in early dioestrus and dioestrus. The decreased TNF immunolabelling found in the epithelial cells of the canine endometrium in early dioestrus is comparable to that described by Sakumoto et al (2009) in the cow endometrium. Also, the low level TNF immunoeexpression found in early dioestrus in the canine endometrial epithelia could be associated to the fact that in dogs the embryo enters the uterus by dioestrus day 9 (Barrau et al. 1975b) as young blastocyst, a stage that has been proved to be more sensitive to negative effects exerted by TNF (Kawamura et al. 2007).

In the study presented here the intensity of TNF immunolabelling in the SE remained relatively constant along the cycle, which could be associated to the fact that, in the dog endometrium, degeneration of the SE, but not apoptosis, is observed. The work of Chu and colleagues (2006) showed that in the bitch endometrium apoptosis is involved in the regression of the glandular epithelium, but not in degeneration of the surface epithelium.

TNF expression has also been detected in early embryos and in the early pregnant endometrium in humans and rodents (Chen et al. 1991, Vince et al. 1992, De et al. 1993, Jerzak and Bischof 2002, Kawamura et al. 2007). TNF m-RNA transcripts have also been detected in canine 10 days embryos but not in the endometrium (Schäfer-Somi et al. 2008). The study presented here evidenced the presence of TNF protein immunolabelling in both embryonic and maternal structures. In pregnancy days 13-16, TNF immunoexpression was detected in the trophoblast, in the syncytium cords, at embryo-maternal interface and also in endometrial stroma and basal glandular epithelium. No differences were found in the intensity of immunolabeling between pregnancy days 13-16 endometrial stroma and early dioestrus or dioestrus stoma. However, a significant decrease in the intensity scores was found in the glandular epithelium at early pregnancy in relation to those observed in early dioestrus and dioestrus.

The presence of TNF in early stages of the pregnancy have also been reported in human and mice (De et al. 1993, Chen et al. 1991, Ben-Yair et al. 1997), and it has been proposed that this molecule could be associated with regulation of apoptosis and embryo invasiveness during early implantation, either directly (Jerzak and Bischof 2002) or through other molecules (Leisser et al. 2006). Chen and collaborators (1991) detected TNF expression in the first-trimester human placenta. They reported that the strongest immunostaining was observed in the syncytiotrophoblast when compared to the cytotrophoblast, the villous stroma or the decidual cells. Their results have been later supported by the work of Ben-Yair et al. (1997) in human and mouse. In the study presented herein, the canine trophoblast showed the highest intensity of immunolabelling, along with cells of the syncytium cords, in relation to other epithelial-like cells at the fetomaternal interface.

In the dog, we speculate that the intensity of immunoexpression for TNF found in maternal and embryonic structures could be associated with both the proliferation and invasiveness

of the trophoblast, the remodelling of the maternal endometrium and the differentiation of new structures associated with decidua formation. It has been shown that TNF stimulates tissue proliferation (Spaczynski et al. 1999, Cohen et al. 2006), angiogenesis (Fajardo et al. 1992) and that it favours cell invasion through the release of matrix metalloproteinases (MMPs) (Han et al. 2001, Cohen et al. 2006). In a recent work, Beceriklisoy and colleagues (2007) demonstrated that in the non pregnant endometrium MMP2 is only detected in the endothelia and smooth muscles of blood vessels and in myometrium, whilst in the pregnant endometrium this MMP was additionally found in the trophoblastic cells and in fetal capillaries. Moreover, MMP9 was found, in the non-pregnant uteri, in all the epithelial cell types, while in the pregnant endometrium this collagenase was located in the DGE and the lacunar epithelial cells. Previous works have associated TNF with the regulation of MMP secretion in either the endometrium (Braundmeier and Nowak 2006) or in tumour progression (Han et al. 2001).

However, further studies on TNF and its receptors expression in the canine pregnant and non-pregnant endometrium are required to clarify the functions of this molecule, and the possible pathways involved, as TNF may mediate tissue homeostasis through diverse and sometimes opposite, time-specific actions (Wang et al. 2003).

To conclude, this work provided information on the space and temporal TNF immunolabelling in the canine endometrium. It documented the existence of an overall tendency for lower intensity of this protein immunolabelling during the secretory stages of the cycle, whilst a marked increase in stromal immunostaining was detected in anoestrus and dioestrus. During early pregnancy, when implantational invasion begins, TNF immunolabelling was detected in the trophoblast, in the syncytium cords and in endometrial stroma, but not in the lacunar epithelium nor in the glandular epithelium, suggesting a role during implantation in the domestic dog.

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Captions:

Table captions

Table 1 – Parameters used for staging the canine oestrous cycle.

Table 2 – TNF-immunoreactivity scores in the canine non-pregnant endometrial samples throughout the stages of the oestrous cycle.

Table 3 – TNF-immunoreactivity scores in the samples from canine pregnancy days 13 to 16.

Graph captions:

Graph 1 – Graphic representation of the relative intensity scores for TNF in the different structures of the non-pregnant endometrium during the canine oestrous cycle.

Graph 2 – Graphic representation of the intensity scores for TNF in the early pregnant and the non-pregnant endometrium progesterone-associated stages.

Figures captions:

Figure 1 – Immunohistochemical expression of TNF in normal canine endometrium (bar: 100 µm). (A) In anoestrus and prooestrus, a prevalence of moderate intensity of immunostaining, along to a strong to moderate intensity of immunolabelling in the glandular content was observed. (B) During oestrus, a reduction in the stromal intensity of immunolabelling and the absence of glandular content positive to TNF was found. (C) In early dioestrus and dioestrus, a weak immunostaining was detected in the GE and in stromal fibroblasts. (D) In the oestrus a tendency to a decline in the intensity of

immunostaining was observed in the surface and glandular epithelium, and in some samples, a strong intensity of TNF immunolabelling was also found in the spermatozoa midpiece. (E) In early dioestrus, a reduction in the intensity of immunolabelling for this molecule was found in both the surface and the glandular epithelia. (F) The intensity scores increased in the endometrial epithelia in dioestrus, in particular in the SE. (G) Strong to moderate intensity of immunolabelling was found in the cells of mature canine corpora lutea (H) Positive immunostaining was found in the endothelium of the endometrial vessels.

Figure 2 – Immunohistochemical expression of TNF in canine endometrium at pregnancy days 13-16 (bar: 100 μm). (A) In the embryonic trophoblast. (B) At the embryo-maternal interface. (C) In the syncytium cords. (D) In the basal glands and stroma.

Table 1 – Parameters used for staging the canine oestrous cycle.

	N	VAGINAL CYTOLOGY	OVARY	BLOOD PROGESTERONE
ANOESTRUS	12	> 90% of the cells are parabasal or intermediate	Smooth ovarian surface, without visible structures (longitudinal cuts may show regressed corpora lutea)	Baseline [< 2ng/ml]
PROOESTRUS	10	Presence of erythrocytes and an increasing percentage of superficial and intermediate cells	Large antral follicles, 2-3 mm in diameter are clearly visible in the ovarian cortex	Below 2 ng/ml until the LH surge
OESTRUS	10	> 90% of superficial cells and only very few erythrocytes	Presence of large, luteinized follicles 5-8 mm in diameter with signs of collapse after ovulation	Above 2 ng/ml
EARLY DIOESTRUS [first 20 days]	12	Sharp decrease in superficial cells, while intermediate and	Dark carmine corpora lutea remained cavitory	>16ng/ml and rising
DIOESTRUS	11	parabasal cells, along with neutrophils, are the major cell types visualised	Corpora lutea were carmine and compact	Progesterone levels remaining high

Table 2 – TNF-immunoreactivity scores in the canine non-pregnant endometrial samples throughout the stages of the oestrous cycle.

	Scores	Anoestrus (n=12)	Prooestrus (n=10)	Oestrus (n=10)	Early Dioestrus (n=12)	Dioestrus (n=11)
Stroma	neg	0	0	0	0	0
	1	3	3	7	11	10
	2	9	7	3	1	1
	3	0	0	0	0	0
Surface epithelium	neg	0	0	0	3	0
	1	12	10	10	9	11
	2	0	0	0	0	0
	3	0	0	0	0	0
Glandular epithelia	neg	2	2	3	6	2
	1	10	8	7	6	9
	2	0	0	0	0	0
	3	0	0	0	0	0
Glandular content	neg	4	4	10	12	10
	1	0	0	0	0	1
	2	3	4	0	0	0
	3	5	2	0	0	0

Table 3 – TNF-immunoreactivity scores in the samples from canine pregnancy days 13 to 16.

Scores	Stroma	Trophoblast	Cells at the embryo-maternal interface	Syncytium cords	Glandular epithelium
neg	0	0	0	0	7
1	5	1	4	7	1
2	3	7	4	1	0
3	0	0	0	0	0

Graph 1

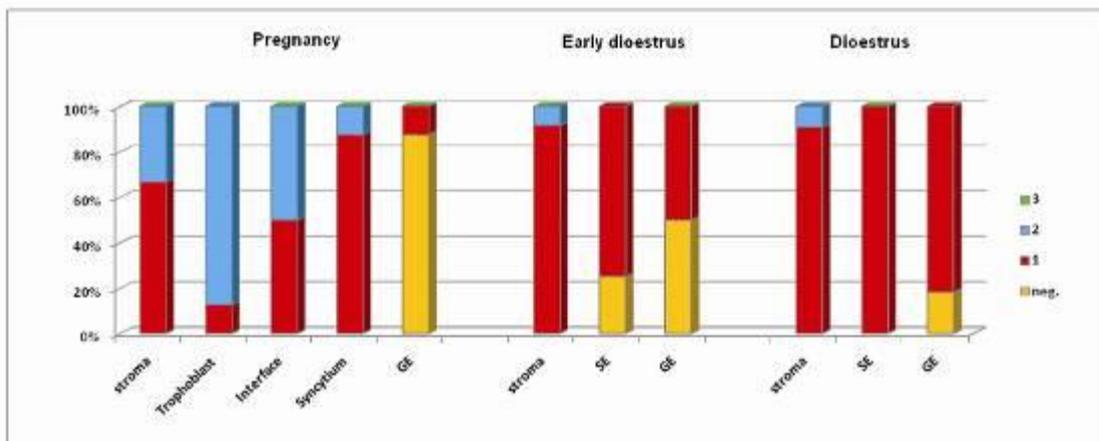


Figure 1

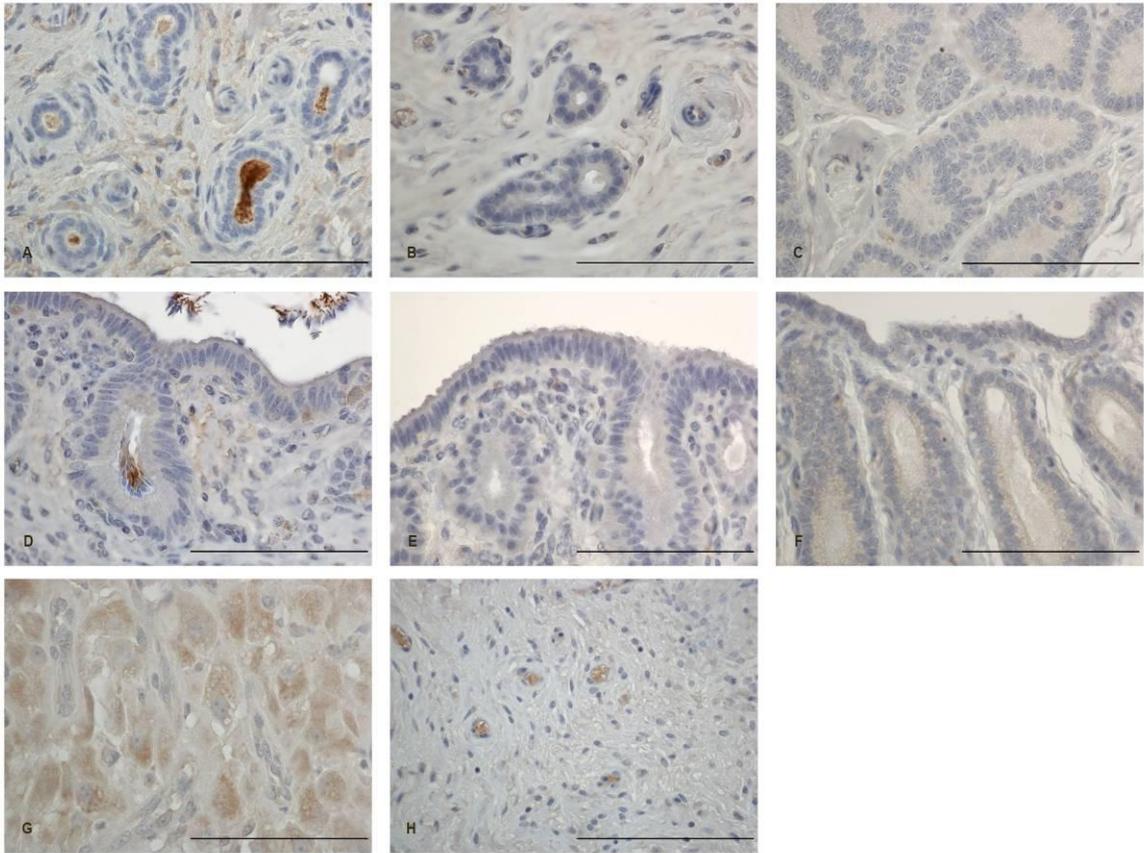


Figure 2

