Universidade de Trás-os-Montes e Alto Douro

Molecular markers of the healthy canine endometrial cycle

PhD dissertation in Veterinary Sciences

Celso Alexandre de Sá Santos

Vila Real, 2015

Universidade de Trás-os-Montes e Alto Douro

Molecular markers of the healthy canine endometrial cycle

PhD dissertation in Veterinary Sciences

Celso Alexandre de Sá Santos

Advisor: Professora Doutora Rita Maria Payan Martins Pinto Carreira

Co-advisor: Professor Doutor Dario Joaquim Simões Loureiro dos Santos

Vila Real, 2015

This work was partially sponsored by the Portuguese Science and Technology Foundation (FCT) under the Project PEst-OE/AGR/UI0772/2014.

I declare that the contents of this thesis are my own work and that they have not been presented to any University other than the University of Trás-os-Montes and Alto Douro.

AGRADECIMENTOS

À Universidade de Trás-os-Montes e Alto Douro (UTAD), na pessoa do Professor Doutor Fontainhas Fernandes, seu Magnifico Reitor.

À Professora Doutora Rita Payan-Carreira o meu profundo agradecimento pela orientação científica deste trabalho, pela boa vontade com que aceitou orientar este trabalho, pelo rigor científico que me transmitiu, pelas valiosas correções e sugestões, pela ajuda no planeamento, análise, correções e conselhos que foi dando.

Ao Professor Doutor Dario Santos, gostaria de expressar os meus mais sinceros agradecimentos pela prestimosa colaboração e coordenação, e apoio ao longo do trabalho, pelo importante encorajamento nos momentos mais difíceis, pela disponibilidade e pela amizade com que sempre me distinguiu.

À Professora Doutora Maria dos Anjos, pela ajuda e disponibilidade demonstradas no trabalho realizado.

Agradeço às pessoas que ajudaram de forma mais constante durante a recolha e processamento das amostras.

Na mesma linha, grato a todos os colegas e pessoas do DeBA, e não só, que de alguma forma me deram apoio e incentivaram; por serem muitos não vou citar mais nomes sob pena de esquecer algum.

Aos meus pais por todo o apoio, ajuda e incentivo.

À minha esposa Carla por nada em especial e por tudo em geral.

Às minhas filhas Beatriz e Carolina.

RESUMO

Após a puberdade, o endométrio canino encontra-se sujeito à influência da alternância dos esteróides sexuais que coordenam uma alternância cíclica de eventos que tem como objetivo último permitir a implantação do embrião e levar a gestação a termo. Ao longo do ciclo éstrico o endométrio sofre processos de remodelação intensos, que comportam proliferação, diferenciação, apoptose e regeneração, que são frequentemente agrupados sob a designação geral de "ciclo do endométrio". Estes processos refletem a atividade cíclica de inúmeras moléculas com atuação local, autócrina e parácrina, coordenada através dos recetores para os esteróides sexuais.

Em contraste com o que se observa noutras espécies, ainda existe pouca informação sobre as alterações cíclicas de marcadores moleculares ao longo do ciclo do endométrio em cadela, apesar do seu interesse potencial para a avaliação da fertilidade ou para a compreensão da patogénese de doenças endometriais. Deste modo, acreditamos que o trabalho aqui apresentado possa contribuir para elucidar alguns aspetos da fisiologia do endométrio canino.

O trabalho apresentado neste documento inclui a avaliação das alterações da endopeptidase neutra (NEP)/CD10 no endométrio cíclico normal, assim como no início da gestação. A CD10 é uma endopeptidase neutra transmembranar geralmente utilizada em seres humanos para marcar o estroma endometrial normal. Contudo, esta enzima possui outras funções interessantes, incluindo a regulação da proliferação e diferenciação em muitos sistemas celulares. A imunolocalização de NEP/CD10 no endométrio de cadela revelou alterações cíclicas de todas as camadas do estroma, para além da existência de uma população de células negativas no estroma, subjacentes ao epitélio de superfície, e que partilham algumas caraterísticas morfológicas com as células predeciduais humanas. Por outro lado, a imunorreação observada para NEP/CD10 em amostras de gestação inicial sugere ainda um papel desta molécula na delimitação da invasão do embrião durante o processo de implantação.

Outro aspeto importante na homeostase do endométrio é a integridade, coesão e polaridade que possuem as células epiteliais no útero, a qual é crítica para a fertilidade e a defesa contra infeções. No entanto, o epitélio deve também possuir a plasticidade necessária para participar nos processos cíclicos que ocorrem no endométrio, assim como permitir a interação entre o endométrio e o embrião durante a placentação. A adesão celular entre células epiteliais adjacentes é mantida pelos complexos caderina E/β-catenina. A

identificação, pela técnica de imunohistoquímica, destas duas moléculas no endométrio canino, mostrou que existem variações cíclicas tanto ao nível do epitélio de superfície como glandular, sendo estas menos pronunciadas no epitélio glandular profundo em comparação com o epitélio glandular superficial ou o epitélio de superfície. Encontrou-se uma perda significativa de marcação no diestro inicial, refletindo uma diminuição da adesão intercelular, que poderá favorecer a interação do embrião com os tecidos maternos e a invasão do endométrio durante a implantação. A marcação das amostras utilizadas, que representam as fases de adesão no processo de implantação embrionária (dias 17 a 20), revela uma acentuada redução da marcação membranar no epitélio de superfície materno suportando a hipótese levantada. Além disso, no labirinto, as células deciduais gigantes não apresentaram marcação membranar para a caderina E ou para a β-catenina, enquanto o trofoblasto revela marcação membranar para estas duas moléculas. Por outro lado, verificou-se ainda neste trabalho que a β-catenina mostra variações no padrão de marcação citoplasmática durante o ciclo éstrico, especialmente no estro, em qualquer um dos epitélios analisados, sugerindo a ativação da via de sinalização Wnt/fator de crescimento Wg.

A atividade cíclica normal das células do endométrio, bem como a sua renovação e remodelação, resulta na produção de espécies reativas de oxigénio (ROS), cuja acumulação pode comprometer a homeostase dos tecidos. Assim, as ROS são mantidas sob um controlo estrito por sistemas de defesa antioxidante nos quais se incluem as enzimas antioxidantes. As alterações neste equilíbrio têm sido associadas a infertilidade e ao desenvolvimento de doenças uterinas. Contudo, a informação disponível sobre enzimas antioxidantes e stresse oxidativo no endométrio canino é muito escassa. Num dos estudos apresentados agora, recorrendo à técnica da imunohistoquímica, caracterizou-se a distribuição, no endométrio canino, das células exprimindo duas importantes enzimas antioxidantes, a superóxido dismutase (SOD) e a glutationa-peroxidase (GPx). A marcação para a SOD apresentou variações cíclicas; em particular observou-se um aumento na sua proporção relativa nas fases sob influência da progesterona, em franco contraste com a GPx, que se revelou relativamente constante ao longo do ciclo. Em ambas as enzimas, a imunorreação do estroma foi sempre inferior à do epitélio.

As espécies reativas de oxigénio encontram-se também sob o controlo de outras enzimas antioxidantes, como a catalase (CAT), a glutationa redutase (GSR) e a glutationa-S-transferase (GST). A atividade destas enzimas foi analisada ao longo do ciclo éstrico, sendo completada pela avaliação da peroxidação lipídica, através da medição de espécies reativas ao ácido tiobarbitúrico (TBARS), e ainda da oxidação de proteínas, através da quantificação de tióis (grupos sulfidrilo –SH totais). As enzimas glutationa-dependentes

permaneceram relativamente constantes ao longo do ciclo, em oposição ao observado para a catalase, cuja actividade aumentou do anestro para o diestro, e para a SOD, cuja atividade apresentou um decréscimo do anestro para o diestro. Verificou-se ainda um ligeiro aumento da peroxidação lipídica no proestro, mas não se encontraram indícios de oxidação proteica.

No seu conjunto, as alterações cíclicas verificadas demonstram um eficiente balanço oxidativo, sendo as variações registadas ao longo do ciclo éstrico, associadas às variações hormonais de estrogénio e de progesterona, e a vias de regulação do endométrio, de modo a manter uma adequada homeostase do endométrio.

No global, este trabalho permitiu descrever as alterações na dispersão de algumas das moléculas que contribuem para a homeostase do endométrio nos cães, e também avaliar algumas das alterações que acompanham a interação do endométrio com o embrião, nas fases de gestação inicial, constituindo um ponto de partida para um conhecimento mais aprofundado da fisiologia e homeostasia do endométrio canino

Palavras-chave: Ciclo do endométrio; gestação inicial; endométrio; marcadores moleculares; cão

ABSTRACT

Starting at puberty, the canine endometrium is submitted to a cyclical coordinated sequence of events, in response to the influences of ovarian sex steroids; therefore several regulatory molecules show cyclic changes throughout the estrous cycle with the ultimate goal of allow embryos to implant and the pregnancy to proceed. Along the estrous cycle the endometrial tissue passes for different remodeling processes, encompassing proliferation, differentiation, apoptosis and regeneration that are often named as the “endometrial cycle”. Those processes reflect the activity of several molecules with autocrine and paracrine functions, which are coordinated through sex steroid receptors.

Contrasting to other species, little information still exists concerning the cyclic changes of molecular markers during the canine endometrial cycle, despite their interest for prospecting female dog fertility or to the understand of pathogenesis of canine endometrial diseases. Thereby, we believe that the work presented in here will contribute to highligth some aspects of the physiology of the canine endometrium.

The work presented in this document includes the assessment of the changes of neutral endopeptidase (NEP)/CD10 in the normal cyclic endometrium as well as in early pregnancy in dogs. CD10, a multifunctional transmembrane neutral endopeptidase, is usually used in humans to mark the normal endometrial stroma; however, this enzyme possesses other interesting functions, including the regulation of growth and differentiation in many cellular systems. The immunolocalization of NEP/CD10 in the canine endometrium revealed cyclic changes interesting all stromal layers, and also evidenced the existence of a negative stromal cell population underlying the superficial epithelium, which shared some morphological characteristics with the human predecidual cells. Moreover, the immunoreaction observed for NEP/CD10 in early pregnancy samples further suggests a role for this molecule in the process in limiting canine embryo invasion at implantation.

An important aspect for the endometrial homeostasis respects the integrity, cohesion and polarity of the epithelial barriers in the uterus are critical for fertility and the defence against infection. Nevertheless, these must also present the necessary plasticity to participate in the cyclic processes occurring in the endometrium, as well as to accept the interaction with the embryo at placentation. Adherens junctions between adjacent epithelial cells are maintained by the E-cadherin/β-catenin complexes. These two molecules were immunolocalized in the canine endometrium, in both the superficial and glandular epithelia, displaying cyclic changes during the canine estrous cycle, which was less pronounced in the

deep glandular epithelium. A softening of intercellular adhesion as found during early diestrus should favor embryo-maternal interactions and endometrial invasion during implantation. This hypothesis was supported by the marked reduction in the membrane immunoreaction expressed in the maternal surface epithelia compared to embryonic tissues, as observed in samples at pregnancy day 17 to 20. Moreover, in the labyrinth, giant decidual cells were devoid of membrane labeling for E-cadherin or β-catenin, in contrast to the membrane labeling evidenced in trophoblast cells. In addition, changes in the patterns of β-catenin cytoplasmic immunostaining were found during the cycle in both the surface and the glandular epithelia suggesting the activation of the Wnt/Wg growth factor signaling pathway, particularly in estrus.

The normal activity of cells, as well as the cyclic cellular turnover or tissue remodeling often produces reactive oxygen species (ROS). Accumulation of these molecules may hamper tissue homeostasis; thereby ROS are maintained under tight control by scavenging system, which includes the antioxidant enzymes. Disturbance of this equilibrium has been associated to infertility and the development of uterine diseases. Still, limited information exists regarding antioxidant enzymes in the canine endometrium. In one experiment, superoxide dismutase (SOD) and glutathione peroxidase (GPx)**,** two important antioxidant enzymes, were localized in the canine endometrium using an immunohistochemistry approach. SOD distribution in canine endometrium showed cyclic variations, the progesterone-associated stages presenting the higher immuno-scores, in which contrasted to a relatively unchanged distribution of GPx1 distribution. In addition, for both the enzymes, the stromal immunoreaction was always lower than that of epithelia.

ROS are controlled not only by SOD and GPx, but also by other antioxidant enzymes such as catalase (CAT), glutathione reductase (GSR) and glutathione-S-transferase (GST). The activity of these enzymes was evaluated throughout the estrous cycle along with lipid peroxidation and protein oxidation, by using thiobarbituric reactive species (TBARS) and total sulphydryls (-SH; thiols) analysis. Glutathione-dependent enzymes remained relatively unchanged during the cycle, contrasting with CAT, presenting increasing activity from anestrus upward, and SOD, which showed a decrease in the activity from anestrus to diestrus. There was only a slight increase in lipid peroxidation in proestrus with no protein oxidation verified once the thiol cell content remained always low. Collectively, cyclic changes registered showed an efficient oxidative balance, with variations along the estrous cycle being associated with steroid hormones fluctuations and with regulatory pathways of the endometrium, in order to maintain a proper endometrial homeostasy.

Overall, the work presented herein allowed to assess the distribution and cyclic variations of particular molecular markers in the canine endometrium, as well as to study the changes they evidence during the interaction of the maternal tissues and the trophoblast during early pregnancy events. Therefore it brought some new information on the physiology and homeostasis of the canine endometrium.

Keywords: Endometrial cycle; early pregnancy; endometrium; molecular markers; dog

CONTENTS

Agradecimentos VII

Resumo IX

Abstract XIII

Index of figures XIX

Index of tables XXIII

Symbols, abbreviations and acronyms XXV

Chapter 1 – General state of the art 1

1. Estrous cycle 1
   1.1. The estrous cycle in the dog 1
   1.2. Gross dynamics of the canine estrous cycle 2
   1.3. Behaviural changes during the canine estrous cycle 3
   1.4. Morphological changes during the canine estrous cycle 4
   1.5. Endocrinology of the canine estrous cycle 7
   1.6. The endometrial cycle in the dog 10
      1.6.1. Sex steroid receptors and the endometrial cycle 11
      1.6.2. Proliferative pattern in the canine endometrial cycle 13
      1.6.3. Angiogenesis pattern during the endometrial cycle 15
      1.6.4 The oxidative stress and the endometrial cycle 16
         1.6.4.1. Reactive oxygen species, cellular antioxidant mechanism and oxidative stress 16
         1.6.4.2. Endometrial cycle and oxidative stress 19

Chapter 2 – Aims 33

Chapter 3 – General description of material and methods 35

Chapter 4 – Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium 45

Chapter 5 – Immunolocalization of E-cadherin and β-catenin in the cyclic and early pregnant canine endometrium 75

Chapter 6 – Distribution of superoxide dismutase 1 and glutathione peroxidase 1 in the cyclic canine endometrium 101

Chapter 7 – Oxidative stress in the canine endometrium during the estrous cycle 129

Chapter 8 – General discussion 151

Chapter 9 – General conclusions 163

INDEX OF GRAPHS AND FIGURES

Chapter 3

Figure 1 35

Drawing of the ovarian-tubal-uterine fragment collected for the study displaying the anatomical points used for measurements.

Chapter 4

Figure 1 54

Western blot for anti-CD10 showing bands corresponding in size to the 100 kDa protein in extracts of canine endometrium.

Figure 2 56

Immunohistochemical expression of NEP/CD10 in normal canine endometrium (counterstained with Gill´s Haematoxylin; bar: 100 µm).

Graph 1 57

Graphic representation of the overall intensity scores for NEP/CD10 in canine endometrium.

Graph 2 58

Graphic representation of the temporal variations in the relative intensity scores for NEP/CD10 in the different endometrial layers.

Graph 3 60

Graphic representation of the relative intensity scores for NEP/CD10 in the pre-attachment period of canine pregnancy (PGr1) and the non-pregnant endometrium in early and full dioestrus.

Graph 4 60

Graphic representation of the relative intensity scores for NEP/CD10 in the attachment period of canine pregnancy (PGr2).

Figure 3 61

Immunohistochemical expression for NEP/CD10 in the early stages of canine pregnancy (PGr2) (counterstained with Gill´s Haematoxylin; bar: 100 µm).

Chapter 5

Graph 1 85

Graphic representation of the endometrial membrane intensity scoring for E-cadherin during the canine estrous cycle**.**

Graph 2 87

Graphic representation of the endometrial membrane intensity scoring for β-catenin throughout the canine estrous cycle.

Figure 1 88

Immunohistochemical expression of E-cadherin and β-catenin in the normal canine endometrium throughout the estrous cycle (bar: 100μm).

Graph 3 89

Graphic representation of the endometrial cytoplasmic intensity scoring for β-catenin during the canine estrous cycle.

Graph 4 90

Graphic representation of the cytoplasmic pattern of immunoreaction for β-catenin throughout the canine estrous cycle.

Figure 2 92

Immunohistochemical expression of E-cadherin and β-catenin in early pregnant (days 17 to 20) endometrium (bar: 50μm).

Chapter 6

Figure 1 110

Total scoring grades for SOD1 (images A to C) and GPx1 (images D to F) molecular expression and examples of the subcellular dislocation of the immunoreaction in the canine endometrium. The immunoreaction was counterstained with Gill´s Haematoxylin. Scale bar = 300μm.

Graph 1 113

Graphic representation of the intensity scores for SOD1 in the different endometrial structures during the canine estrous cycle.

Graph 2 115

Prevalence of nuclear staining (upper) and the apical reinforcement (bottom) patterns in SOD1 in the different endometrial epithelia throughout the canine estrous cycle.

Graph 3 117

Graphic representation of the intensity scores for GPx1 in the different endometrial structures during the canine estrous cycle.

Graph 4 118

Graphic representation of the expression of apical reinforcement pattern in GPx1 immunolabelling in the different endometrial epithelia during the canine estrous cycle.

Chapter 7

Figure 1 139

Box-plot graph for Superoxide dismutase (SOD) activity in the different stages of estrous cycle of the bitch.

Figure 2 140

Box-plot graph for Catalase (CAT) activity in different stages of estrous cycle of the bitch.

Figure 3 140

Box-plot graph for Glutathione Peroxidase (GPx) activity in different stages of estrous cycle of the bitch.

Figure 4 141

Box-plot graph for Glutathione reductase (GSR) activity in different stages of estrus cycle of the bitch.

Figure 5 142

Box-plot graph for Glutathione-S-transferase (GST) activity in different stages of estrous cycle of the bitch.

Figure 6 142

Box-plot graph for TBARS levels in different stages of estrous cycle of the bitch.

Figure 7 143

Box-plot graph for total sulfhydryls (-SH) levels in different stages of estrous cycle of the bitch.

INDEX OF TABLES

Chapter 3

Table 1 37

Summary of the distribution of the number of samples used in each chapter according to the cycle stage or status (Cyclic *vs.* Early pregnancy).

Table 2 38

Criteria used to the estrous cycle staging of the canine endometrium.

Table 3 41

Primary antibodies used for immunohistochemistry

Chapter 4

Table 1 55

NEP/CD10 immunoreactivity scores in the endometrial samples of female dogs throughout the stages of the estrous cycle and in pre-attachment PGr1.

Table 2 55

NEP/CD10 immunoreactivity scores in the implantation area in samples from canine pregnancy days 16 to 23 (attachment period, PGr2).

Chapter 5

Table 1 85

E-cadherin and β-catenin membrane immunoreactivity scores in the canine endometrium throughout the stages of the estrous cycle

Table 2 86

Cytoplasmic intensity and pattern of immunoreaction against β-catenin in the cyclic canine endometrium.

Table 3 91

E-Cadherin and β-Catenin membrane immunoreactivity scores in early pregnant canine endometrium (pregnancy days 17 to 20).

Chapter 6

Table 1 109

Criteria for grading SOD1 and GPx1 Immunoreactivity.

Table 2 112

Immunoreactivity scores for SOD1 – the intensity of immunolabeling and the percentage of labeled cells – in canine endometrial samples throughout the stages of the estrous cycle.

Table 3 116

Immunoreactivity scores for GPx1 – the intensity of immunolabeling, the percentage of labeled cells and the number of cases displaying apical reinforcement – in canine endometrial samples throughout the stages of the estrous cycle.

Chapter 7

Table 1 138

Basic statistics descriptive for endometrial oxidative stress enzymes and thiols and lipid oxidation (TBARS) in female dogs throughout the estrous cycle.

SYMBOLS, ABBREVIATIONS AND ACRONYMS

AN	Anestrus
Bcl-2	B-cell lymphoma 2
BHT	Butylated hydroxytoluene
CAT	Catalase
CD10	Cluster of differentiation 10
CDNB	1-chloro-2,4-dinitrobenzene
CH	Corpus hemorrhagicum
CL	Corpus luteum; *pl.*: corpora lutea
Cu	Copper
DbS	Deep basal stromal layer
DGE	Deep glandular epithelia
DI	Diestrus
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
E	Estrus
eDI	Early diestrus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptors
FSH	Follicle-stimulating hormone
GE	Endometrial glandular epithelium
GnRH	Gonadotropin-releasing hormone
GPx	Glutathione peroxidase
GSR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
IgG	Immunoglobulin G
Ki-67	Ki-67 antigen
LamS	Lamellar stroma
LE	Luminal epithelium
LH	Luteinizing hormone
LHAP	Laboratório de Histologia e AnatomiaPatológica

mM	Millimolar
Mn	Manganese
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NBT	Nitroblue tetrazolium
NEP	Neutral endopeptidase
O_2^-	Superoxide radical anion
OH^-	Hydroxyl radical
OVH	Ovariohysterectomy
P_4	Progesterone
P450	Cytochrome P450
PAF	Platelet activating factor
PAS	Periodic acid schiff
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PE	Proestrus
PF	Primary follicle
PGr	Pregnancy
PR	Progesterone receptors
ROS	Reactive oxygen species
SE	Superficial epithelium
SF	Secondary follicle
SGE	Superficial glandular epithelia
SOD	Superoxide dismutase
SSL	Sub-surface layer
Str	Stroma
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TC	Trophoblast
TF	Tertiary follicle
TGF	Transforming growth factor
TGF-β1	Transforming growth factor beta1
TNF	Tumour necrosis factor
UpS	Upper stromal layer
VEGF	Vascular endothelial growth factor
Zn	Zinc

Chapter 1 – General state of the art

1. Estrous cycle

The estrous cycle is a coordinated sequence of events which is observed after puberty, determined by ovarian sex steroid produced within a proper sequence, and accordingly to the species. In general, there is an alternating dominance of estrogen to progesterone, and again to estrogen, with or without interleaving a pause phase with a reduction in the sex steroids secretion depending on the species concerned, which is reflected in the morphology and function of the reproductive organs.

The estrous cycle can be divided into different stages, although the proposed classification may not always be unanimous or cannot be indifferently applied to all animal species. Generally the estrous cycle encompasses the following phases: anestrus, proestrus, estrus, and metestrus (diestrus).

1.1. The estrous cycle in the dog

Similar to other domestic species, dogs reach puberty at about 55-65% of adult weight in case of males. Reproductive activity in females starts a little later, when the female reaches 75 to 80% of the adult weight [1]. In practical terms, due the wide range of possible sizes/breeds for this species, contrasting to other non-seasonal domestic species, age at puberty translates into a large temporal variation [1, 2], which is about 6 to 7 months for dwarf and small breeds, 8 months for medium to large size dogs, but may be 12 to 18 months for giant sized breeds. Age at puberty is dependant from the breed size (genetic factor) but the nutritional level and the social environment may also influence it. Thus, the occurrence of the first estrus seems to be a function of reaching adult body size, an adequate physical condition and social synchrony [1-3]

The dog estrous cycle presents several features that distinguish it from other domestic species, on what concerns the pattern and regularity of the estrous activity. First, dogs are not seasonal, and yet they only experience a period of estrus during the breeding season, and thereby it is classified as a monoestric non-seasonal species [1, 3, 4]. In dogs, ovulation and luteal function are spontaneous events [5].

Another important difference for this species is that they present a considerably longer cycle than most domestic species [5]. Moreover, there is also a large variation concerning the length of its various stages, according to the breed or even with the genetic line within each breed [1, 5]. Though, the length of the canine luteal stage is relatively constant, and the progesterone synthesis by the canine corpus luteum lasts for similar period whether or not it is associated with pregnancy [1, 3]. Each estrous cycle is separated by a period of variable duration, the anestrus [5]. Inter-estrus intervals range from 4 to 13 months, averaging, in most breeds, 6 months. The interval between estrous cycles in the bitch depends mostly on the length of the anestrus phase which may vary from 2 to 10 months [1, 5, 6]. Despite the individual variation in the cycle length, it is important to remember that the interestrous interval, whatever the duration, is regular for each female.

Contrasting to most domestic species, ovulation in the bitch is preceded by an increase in circulating levels of progesterone arising from the pre-ovulatory luteinization of granulosa cells. Another important difference is that, in dogs, ovulation results in the release of a primary oocyte; meiosis is resumed in the oviduct in a process that takes 2 to 3 days to achieve the maturation necessary to the oocyte fertilization [1, 7-9].

1.2. Gross dynamics of the canine estrous cycle

After puberty, the canine ovaries enter a cycle of regular alternance between a follicular stage, which encompasses the proestrus and initial estrus, and a luteal stage (starting in early estrus and prolonged to diestrus), that are separated from those of the following cycle by a quiescent period named as anestrus [1].

As said, the length of canine estrous cycle is rather variable; the two stages that most contribute for this variability is the heat period (proestrus/estrus) and anestrus. Diestrus is a rather constant stage, of about 60 days (if considering its end when progesterone levels decline below 1 $ng.mL^{-1}$), whether pregnancy occurs or not. In contrast, the heat may last for 7 – 27 days (with an average of 9 days each) and anestrus can vary between 80 and 240 days [1, 3, 4].

In dogs, due to early luteinization of pre-ovulatory follicles, follicular and luteal stages seem to overlap as blood progesterone levels raise above 2 $ng.mL^{-1}$ prior to ovulation [10].

Changes issuing from the structures developing in the ovaries (follicles or corpora lutea) result in an alternation of the dominance of major sex steroids (estrogens and

progesterone, respectively), which determine a number of changes in the steroid-target organs. These translate into changes on the female behavior, vulvar and vaginal appearance, vaginal cytology, uterine tone, progesterone concentrations, among others, that allow to determine the stage of the estrous cycle during the female clinical evaluation [3, 4].

Proestrus is quite variable. It may last for 1-3 weeks, but in the average cycle its length is 9 days [4, 11]. The beginning of proestrus stage is determined by the appearance of a bloody vaginal discharge [4, 12], that may vary in intensity on individual basis, and of an increasing edematous enlargement of the vulva [11]. The anestrus is a period of relative ovarian quiescence [4], where circulating progesterone is maintained below 1 $ng.mL^{-1}$. Its length is the major determinant of the individual length of the interestrous interval. It would correspond to the interval between parturition and the onset of new cycle, incorporating the lactation. Its length ranges between 2 months to 11 months, for an average of 3 months. It is an important stage for female fertility.

1.3. Behavioral changes during the canine estrous cycle

Stages of the canine estrous cycle may be defined by sexual behavior and physical signs, such as vulvar swelling and vaginal bleeding that drive the attention of male counterpart. However, except for proestrus and estrus, also commonly named as heat, all other stages of the cycle are silent from the point of view of sexual behavior. Diestrus and anestrus are characterized by the female rejection of the male. Also, the animals are less inclined to explore the environment and to interact with other animals, although the environment seems to modulate the hormonal effect on animal behavior; unless for animals under free ranging conditions, females turn out to be more protective, barking for a longer time and reducing some static behaviors, such as sitting [13].

The proestrus onsets with the first notorious change in the bitch behavior, which in turn triggers the male sexual behavior and courtship [7]. The vulvar swelling and a bloody vulvar discharge are the major signs; pheromones present in the discharge drives the male attention. Another behavior change includes an increased restlessness, urine marking, and increased roaming; a tendency for disobedience is also commonly found [14]. Males demonstrate their interest but most often bitches are unreceptive to the male [4]. Proestrus ends with the onset of the receptive behavior; true estrus behavior starts close to luteinizing hormone (LH) surge and consists in the acceptance of the mounting [1]. During estrus, the female is pro-active in the search for the partner and increases the increased male-seeking

behavior; she expresses posturally inviting behaviors, such as presenting the hind limbs, deviation of the tail and exposure of the vulva to the male, and lordosis, inviting the male to approach and indicating its availability for breeding [1, 10, 15]. This is determined by the fall in estrogens and the increasing progesterone levels, although its full expression is dependant of the priming of the rising estrogen occurring during proestrus. The vulvar discharge becomes less intense, more watery and pink-tan in estrus. Estrus ends when the female refuses the male for two consecutive days [10, 15-18].

Although this may be considered the most practical and accurate method of determining estrus in dogs, in fact, in dogs, the behavioral signs of male acceptance used in many species for estrus detection and identification of the fertile period are not specific and may vary individually; therefore they are, for themselves, of few reliability to identify the estrus stage [19, 20]. To surmount this pitfall, vaginal cytology and progesterone are currently used for staging the follicular stage and identify the moment for ovulation, thereby delimitating the fertile period of the bitch [3, 8, 21-23].

1.4. Morphological changes during the canine estrous cycle

Prepubertal ovary shows a smooth surface. It is only around two to three months prior to puberty onset, at an average of 5 to 6 months of age for the medium sized bitches, that small antral follicles are first seen, which most often suffer atresia. But eventually a cohort of follicles are rescued to maintain its development and to produce the necessary amount of estrogens to start the first proestrus [24].

In mature females in anestrus, regressing corpus luteum may be found in the ovaries; they are located deeper towards the medulla, and do not project at the ovarian surface. Under the microscope, regressed corpora lutea show an irregular lobulation, increased macrophage infiltration and deposition of a yellow pigment (lipofuscin); the luteal cells contain large cytoplasmic vacuoles. As corpus luteum regresses the ovarian cortex becomes more apparent; in the last third of anestrus, numerous primary and secondary follicles may be observed in active growth, or in regression. Remains of corpus luteum may persist for several months and be clearly visible during the next estrous cycle [4].

During anestrus, the non-diseased uterus is small and presents a reduced cross-shaped lumen [5]; during this stage, under basal estrogen and progesterone levels, the length and diameter of the uterine glands are at its lowest point; both the glandular and the surface epithelia is cuboidal or low columnar [25]. Occasionally, the glandular lumen

presents a small amount of PAS-positive material and debris, in particular in postpartum females [26].

Accordingly, the vaginal epithelium is low; in vaginal cytology small intermediate and parabasal cells predominate; neutrophils and mucus may be found as well as bacteria that are sporadically observed in the smear [4].

Proestrus is characterized by the presence of antral follicles of moderate diameter on both ovaries, which are surrounded by multiple layers of granulosa cells. There may also be found atretic corpus luteum formed by vacuolated cells containing pigments that derive from the previous cycle [4, 5]. At the beginning of proestrus, the dominant pre-ovulatory follicles have 2 to 3 mm diameter and fairly protrude above the surface of the ovary. They are grossly distinct, semi-opaque, showing well-vascularized walls. Follicles then increase to 5 to 8 mm and at late proestrus, several days before the LH peak, follicles start to show histological signs of luteinization over some areas of the follicular wall; follicular growth increase after LH peak and they reach over 9 to 12 mm at ovulation [3].

During proestrus, the uterus is tense and slightly increased in diameter, due to estrogen-associated edema, with a lumen resembling a cross [4, 5]. The endometrium presents marked interstitial oedema and extravasated erythrocytes infiltrate the stroma, in particular on the upper layers; endometrial epithelia increase in height [26]. Endometrial basal glands and surface epithelium proliferate, the later forming surface invaginations into the stroma, named crypts. Increased capillary hyperaemia is associated with extravasation of erythrocytes into the lumen, which contribute to the bloody vaginal discharge [4, 5]. The proliferation of endometrial surface epithelium and crypts, vasculature and stroma correlates with increasing levels of estradiol, while proliferation of the endometrial basal glands is correlated with increased levels of progesterone [4, 27]. The uterine lumen fills with PAS positive mucinous secretion. The myometrium is thicker compared with that observed in anestrus [5].

During proestrus, under the effect of increasing levels of estrogens, the vaginal epithelium progressively proliferates and increases the number of layers, increasing its thickness, and becoming keratinized close to the moment of LH surge. Consequently, as proestrus elapse, the analysis of vaginal cytology shows a gradual shift from intermediate and parabasal cells to superficial cells, with karvopyknotic nuclei, and folded cytoplasm are observed; the erythrocytes are present in large numbers at the beginning of the stage and neutrophils are commonly observed, but decrease gradually by the end of proestrus. Large numbers of bacteria are also often present [3, 4].

Estrus starts close to LH surge. It includes the final developmental phase of follicular development but, in contrast to other domestic species, in dogs is associated to increasing progesterone levels associated to the pre-ovulatory luteinization [4]. In the ovaries, large cyst-like structures – the dominant follicles – are visible; in cross-section these show thickened follicular wall, which increases wall rigidity. Microscopically, during early estrus, each ovary section may present 4 to 6 large tertiary follicles lined by stratified layers of elongated granulosa cells, which protrude in the antrum area, reaching from 3 to 8 mm in diameter. Granulosa and theca cells form folds into the antrum that become increasingly noticeable. In the bitch, luteinization of granulosa cells precedes ovulation. The follicular luteinization, in dogs, occurs during and immediately after the LH peak. Consequently, closer to ovulation, follicles are surrounded by thick layers of rounded luteal cells [4, 5]. Histologically there may be observed a dramatic loss of morphological integrity of granulosa cells and an ingrowth of cells morphologically similar to the internal theca cells, suggesting that theca cells can contribute largely to the formation of corpus luteum [3]. In dogs, ovulation occurs about 48 to 60 hours after the LH peak, after a period of 2 to 3 days of granulosa luteinization [3, 21]; due to the follicular wall luteinization, in opposition to the observed in other domestic species, follicles do not collapse after ovulation [28].

At the onset of estrus, the uterus is tense. The endometrium is still oedematous, though the edema decreases after ovulation. Microscopically, a burst of growth occurs in the endometrium, increasing the height of the glands: the proliferation of crypts is more pronounced and the basal glandular elements start to coil. By the end of estrus, the basal glands are marked increased in coiling and the crypts appear appreciably elongated and start to coil. The size of epithelial cells increases only slightly throughout estrus [26], the stromal content in collagen raises [4, 5].

The loss of estrogen impregnation early in estrus, which will induce the decrease in the number of epithelial cell layers, takes some time to translate into the vaginal cytology features. Thus the estrus vaginal cytology is characterized by the predominance of pyknotic or enucleated keratinized cells, as most bitches will undergo full cornification. Smears are visually more clear and clean, as seldom erythrocytes or neutrophils are normally present [4].

The diestrus is characterized by the presence of functional ovarian corpus luteum [10, 29], which lasts close to 2 months [4]. Thereafter, luteolysis results in a decrease of progesterone levels below 1 $ng.mL^{-1}$, and technically anestrus begins, though progesterone may remain between 1 and 2 $ng.mL^{-1}$ for an additional period of 30-45 days [19, 30-32]. During diestrus, the ovaries are larger and seem lobulated, mainly during the first 45 days, each lobule corresponding to a corpus luteum. Cross sectioning of the corpora lutea shows

that for the first days of diestrus they remain cavitary; some authors named this period as early diestrus, and matched it with the pre-implantation period of the canine pregnancy [33, 34]. These large antral corpora lutea present closely packed, prismatic luteal cells. By the end of diestrus, when luteolysis begins, luteal cells have rarefied to vacuolated cytoplasm [5], due to increased lipid deposition [4].

The uterus in diestrus is thickened, due to the increased thickening of the endometrium and myometrium. At the onset of diestrus the uterine glands grow rapidly, mostly the basal glands, which become highly coiled and branched. No further growth of the glandular compartment is observed unless the bitch becomes pregnant [26]. The epithelial cells are high columnar [5, 25] until the progesterone levels decrease in association with initial regression of corpora lutea [4]; thereafter, epithelial cells show a finely vacuolated aspect and return to the size characteristic of anestrus [5]. In parallel it is noticed a loss of stroma collagen. The endometrial collapse initially results in enlargement of endometrial lumen [4].

In diestrus, the vaginal epithelium regresses to its normal non-estrogenic primed thickness. The reappearance of round cells and neutrophils usually corresponds to the beginning of diestrus; cellular debris may also be observed. There are no clear cytological signs when the diestrus ends and anestrus begins; the division can only be defined by plasma progesterone levels (≤ 1 $ng.mL^{-1}$) [4].

1.5. Endocrinology of the canine estrous cycle

As for other mammals, the ovarian cyclic activity is regulated by the main central hypothalamic-hypophyseal axis, under the influence of environmental and internal cues. These influences allow the organism to integrate nutritional and social information with those from the surrounding environment to boost the reproductive success of the female, and thus that of the species. These influences are exerted through several neurotransmitters (such as leptin, opioids, galanin or gamma-aminobutyric acid) and hormones (adrenocorticotropic hormone and cortisol, melatonin, thyroid or metabolic hormones), which modulate the gonadotropin-releasing hormone (GnRH) secretion [35]. GnRH is the hormone that coordinates the neuro-gonadal axis (or hypothalamic-gonadal axis) through the secretion of follicle-stimulating hormone (FSH) and LH. Grossly, FSH stimulates the ovarian follicular development while LH triggers ovulation and promotes luteogenesis [1]. In carnivores, the follicular development is associated to increasing levels of estrogens that control by negative

feedback loop FSH secretion. Progesterone produced by the corpora lutea modulates LH secretion, impairing LH surge during the luteal phases of the cycle [1, 3, 35]. This is a very simplistic way to present the complex mechanism that characterizes the cyclic changes escorting the estrous cycles of carnivores. A myriad of small molecules are involved in the regulation of the physiological processes that warrants an adequate follicular development in the ovaries or the uterine function, as it will be discussed later within this manuscript.

In prepubertal females and in late anestrus mature females, small antral follicles are first observed around 5 to 6 months old, in medium sized bitches, which often suffer atresia. But eventually a cohort of follicles is rescue to maintain its development and produce the necessary amount of estrogens to start the first proestrus. This event is associated to a pre-proestrus increase in the GnRH pulsatility, which in turn increases the frequency of pulsatile LH release. Yet, FSH secretion is maintained continuous elevated and slow increases are described in late prepubertal or late anestrus stages. These are parallel with low levels of estrogen secretion [3, 36, 37].

In mature females, anestrus starts when circulating progesterone levels falls below 1 $ng.mL^{-1}$, the transition from diestrus being gradual [3, 27]. Progesterone further decreases into values below 1 $ng.mL^{-1}$ for an additional period of almost 20-30 days after the onset of anestrus [4]. In anestrus, overall serum LH levels remain low (≤ 1 $ng.mL^{-1}$), with occasional LH pulses ranging from 2 to 25 $ng.mL^{-1}$ typically detected at 4 to 24 hours intervals. Basal FSH levels become progressively higher during anestrus without any signs of induced activity by estrogens [3].

In middle and late anestrus, FSH levels increase to near or equal the pre-ovulatory peak levels, reaching, in average, 40% of the peak concentration. In the week before proestrus onset, LH pulses maintain its relative large magnitude, but their intervals decrease to 60 to 90 minutes; therefore, the average LH value rises to 3 $ng.mL^{-1}$ or even more, and they are distinctly higher than the preceding anestrus pulses [3, 36]. There is a concomitant modest increase in mean FSH levels although less pronounced than those of LH (20 to 50% *vs.* 300 to 600% above the anestrus levels, respectively for FSH and LH) which can trigger the beginning of proestrus [3].

Prior to the onset of proestrus, FSH concentration, and at a lesser extent those of LH, rise but soon decrease when plasma estradiol increases at the beginning of proestrus. The estradiol produced by granulosa cells of growing follicles is responsible for the clinical signs of proestrus. At early proestrus, plasma estradiol slowly rises from baseline levels of approximately 26 ± 4 $pg.mL^{-1}$, increasing until day 3 (43 ± 4 $pg.mL^{-1}$) and then increases rapidly, reaching a peak of 62 ± 4 $pg.mL^{-1}$ and then decreases before the beginning of estrus

[4, 12]. The occurrence of maximum plasma level of estrogen (46-113 $ng.mL^{-1}$) at the end of proestrus, preceding the LH peak in dogs, suggests that, as in other species, an increase in plasma estrogen is the major factor for the mechanism that triggers the pre-ovulatory LH release [3, 12]. The middle and late follicular phases of proestrus are potentially autonomous or semi-autonomous once intra-follicular estrogen remains folliculo-trophic with a follicle fate of either atresia or ovulation, as the ability to increase endogenous estrogen secretion becomes limited [3]. During proestrus, LH levels become progressively lower and its pulses less detectable or undetectable, due to the negative estrogen feedback [3, 12]. At the end of proestrus, just after the estradiol peak and during rising progesterone levels, there is a sudden increase in LH and FSH [4, 38]. LH levels rise to peak levels of 4 to 40 $ng.mL^{-1}$ (average of 8 to 15 $ng.mL^{-1}$) during the preovulatory surge. The LH surge involves a rise for 12 to 36 hours and a subsequent decrease of 12 to 24 hours, and elevated FSH typically results in a peak half to one day after the LH peak and a decline phase to baseline levels one or two days beyond the LH decrease [3, 12]. This LH and FSH peak can occur from 1 day before to 3 days after the beginning of behavioral estrus, due to individual variations [3, 4, 12]. The pre-ovulatory surge in plasmatic LH in the bitch lasts 18-48 hours [12].

Plasma progesterone levels slowly increase mainly in late proestrus, from baseline values of 0.2 to 0.4 $ng.mL^{-1}$ to values up to 0.6 to 0.8 $ng.mL^{-1}$ close to the LH surge, partially reflecting the luteinization of the growing follicles. Follicles synthesize not only estrogen but also progesterone well before ovulation and full luteinization [3, 4, 38]. Progesterone rises sharply and rapidly, concomitant with the acute onset of the LH surge, so that the first sharp rise in progesterone can't be separated from the initial rise in LH [3]. The increase in progesterone levels in late proestrus is thought to play a key role in sexual behavior changes of the bitch, with its eventual acceptance to the male mating at the end of proestrus [4, 38].

The blood estrogen levels, which peaked before the end of proestrus, decrease rapidly during estrus, reaching baseline values of 18±3 $pg.mL^{-1}$, below those of the beginning of proestrus, 5 days after the LH peak [4, 10, 39]. Coincident with the estrogen peak, the LH surge occurs [10, 36]. The LH surge and the onset of estrus occur from 1 to 3 days after the estradiol peak and half to 1 day after the first detectable sharp increase in preovulatory progesterone to above 0.9 $ng.mL^{-1}$. The initial increase in plasma progesterone may reflect the preovulatory LH-induced luteinization [10]. Progesterone levels rise from low levels of about 0.6 $ng.mL^{-1}$ at the end of proestrus and early estrus, increasing to higher levels about 6 days after the LH surge [10, 29], when the transition of granulosa cells into luteal cells is complete. Ovulation occurs at the beginning of estrus, about two days after the LH/FSH peak when serum levels are already declining to baseline levels during the rest of the estrus [4,

40, 41]. Plasma progesterone reaches peak concentration of approximately 20 $ng.mL^{-1}$ about 10 days after the LH surge and remains high for 25 to 30 days, already in the diestrus stage [4, 10]. Its origin is exclusively from the corpus luteum and is dependent on the secretion of either LH or prolactin [3]. Plasma levels of progesterone decrease smoothly from day 30 onwards, but the sharp decrease in the progesterone levels is commonly noticed after day 45 to 50. It is not observed any difference between pregnant and non-pregnant bitches with respect to the maximum progesterone level, for their intervals or for the time of occurrence [10].

At the beginning of diestrus, as a result of corpora lutea secretion [10, 29], plasma progesterone levels reach a maximum of about 20 $ng.mL^{-1}$ and remain high for 25 to 30 days after the LH peak and then decrease until ≤ 2 $ng.mL^{-1}$ after day 50 [4]. LH plasmatic levels in diestrus remain stable at approximately 5 $ng.mL^{-1}$ [29]. The canine luteal progesterone secretion is not autonomous, but is coordinated by the luteotrophic of combined LH and prolactin within 2 weeks of CL formation [35]; the luteotrophic effect of prolactin is more notorious by the middle of diestrus. Small inconsistent peaks of estradiol have been reported to occur during diestrus in dogs [3], but the number of antral follicular structures in the ovaries is limited [4]. The transition from the luteal phase to anestrus is gradual and varies considerably among non-pregnant bitches. The non-pregnant uterus does not determine luteolysis in the bitch, unlike in ruminants or the mare. However, prostaglandin F2α shows a large increase in canine serum in the 24-36 hours preceding parturition, which occurs at day 65 post-LH peak [42-45].

1.6. The endometrial cycle in the dog

The uterus is an important organ in female reproduction. The mammalian endometrium is a highly complex tissue, that must accomplish diverse and often opposing functions during the female lifetime. Its main purpose is to guarantee embryo survival, implantation and the success of pregnancy [46].

After puberty, under the general control of ovarian steroids, the endometrium undergoes cycles of sequential transformations, which include regeneration, remodeling and differentiation of the intercellular matrix. These changes are ultimately controlled by several autocrine and paracrine factors that include cytokines, interleukins and growth factors, among other molecules [47-49]. Moreover, the endometrium is exposed to periodic contact to foreign cells, such allogenic sperm and embryo-associated tissues, which should not be

rejected as any other infectious agent [50, 51], while retaining its ability to react against invading pathogens. In addition the endometrium must also adapt itself to several physiological events such as implantation, pregnancy, parturition and post-partum involution [52, 53]. In mammals, irrespectively of the species, the maintenance of adequate uterine health is a key component for reproductive efficiency, the immunity failure predisposing the uterus to inflammation and thus infertility [54]. Contrasting to other domestic species, available information on the cyclic changes of main regulatory pathways of the endometrial changes in dogs is very limited.

1.6.1. Sex steroid receptors and the endometrial cycle

Most endometrial changes occur in response to systemic concentrations of sex steroids, which is generally believed to be locally coordinated via changes on estrogens and progesterone receptors concentration and its interplay with multiple molecules with paracrine functions. In the uterus, response to blood sex steroid priming involves extensive tissue remodeling, encompassing waves of endometrial cell proliferation, differentiation, recruitment of inflammatory cells, apoptosis and regeneration [55].

Steroids interact with their target cells via specific nuclear receptors, which initiate gene transcription and a cascade of downstream molecular and cellular events when stimulated. Available information suggests that in the mammalian endometrium the expression of estrogen (ER) and progesterone receptors (PR) varies both temporally and spatially across the endometrial cycle, and varies in both distribution and concentration along the estrous cycle [56-59]. Grossly, estrogen stimulates the proliferation of both glandular and stromal cells, whereas progesterone inhibits the growth of glandular cells and stimulates the secretory activity in the endometrial glands. Moreover, it is long recognized that progesterone is critical for endometrial differentiation and implantation.

Several major processes involved in normal endometrial function, such as proliferation and vascularization are regulated by estrogen. In the human endometrium, estrogen up-regulates key genes, including PR or the vascular endothelial growth factor which is a key local mediator of cyclical neovascularization in the functional layer [60].

Recently, it was demonstrated that two forms of estrogen receptor, named as ERα and ERβ, mediate estrogen actions in target tissues. The expression of the former estrogen receptor increases in both glandular and stromal cells in the upper layer of primate endometrium during the proliferative phase and declines in the secretory phase, suppressed

by progesterone; in the basal layer, ERα is expressed in glandular and stromal cells throughout the menstrual cycle in women. The function of ERβ in the uterus remains unclear, though its presence was demonstrated in the endothelium and smooth muscle walls of endometrial vessels [60], suggesting that it may be involved in angiogenesis and the control of endometrial blood flow or vessel permeability.

Though two isoforms have been described for PR, both forms derive from a single gene and function as transcriptional regulators of progestin-responsive genes [60]. In primate endometrium PR decreases in the gland of the upper endometrial layer during the transition from the proliferative to the secretory phase of the cycle; still, these receptors persist in there and are become highly expressed in the stromal cells in close proximity to the uterine vasculature [60]. As for the estrogen receptors, it seems that PR in the basal region are regulated differently, since both the glands and stroma of the deeper layer express PR throughout the cycle. Studies on the pattern of PR localization during the menstrual cycle showed that PR is differentially regulated in stromal and epithelial cells [60]. PR have been associated, via an indirect pathway, to the vasoconstriction of the endometrial vessels that accompanies the progesterone withdrawal.

Most available studies on the expression of estrogen receptors in the canine endometrium were developed prior to the distinction between the two estrogen receptors. Variation in the expression of ER was evidenced across the canine estrous cycle: ER increase during the early proliferative stage (early and mid proestrus), but decrease when estrogenic impregnation is maximal, at the transition to estrus, reaching the lowest levels during the early secretory stage [57, 61, 62]. Increased blood estrogen levels stimulate the ER expression in endometrial luminal epithelium but lead to a decreased ER expression in stroma and glandular epithelium [63]. PR expression is higher in stroma than in endometrial epithelia, supporting the idea that several epithelial functions, in canine or human endometrium, are regulated by stromal cells [64].

It has been shown that estrogens promote the growth, vascularity and edema of the dog endometrium as well as proliferation of the glandular epithelia, while progesterone induces the proliferation of stromal cells and secretory activity of the endometrial glands [27, 59, 64, 65]. The rise in plasma progesterone levels, that accompanies the LH surge and precedes ovulation, in the bitch, is accompanied by decreased expression of ERs and PRs [59, 66]. At the end of the diestrus, when progesterone plasmatic levels withdraw to baseline levels, the overall PR expression increases [61, 64, 67] however, down-regulation of PR receptors is more pronounced in the stroma than in the endometrial epithelia [59].

1.6.2. Proliferative pattern in the canine endometrial cycle

During the estrous cycle, the endometrium undergoes morphological and physiological changes, in response to the physiological ovarian steroid hormones [68]. Simultaneously, it occurs the secretion or synthesis of a variety of several factors such as specific proteins that may induce biochemical, metabolic, and chemical reactions in the epithelial cells, and the expression of growth factors that regulate properly the endometrium [69]. Under the effect of such hormones, there is a proliferative pattern not only on the ovaries, but also in the uterus [27, 59, 65, 70, 71]. The uterine proliferation has been assessed through the proliferative marker, Ki-67, for several species such as the mare, pig and dog [72-74]. As for the canine uterine proliferation, Van Cruchten *et al.* [27] and Srisuwatanasagul *et al.* [72] found a higher proliferation in the surface epithelium during proestrus, and a lower proliferative activity in all uterine cells during diestrus, probably due to downregulation by the increasing progesterone levels observed at this stage. Van Cruchten *et al.* [27] detected two phases of glandular growth, the first one during proestrus in the surface epithelium, the stroma, the blood vessels and the crypts, under estrogenic influence, and the second during estrus in the basal glands, in accordance with canine endometrial proliferation described earlier by Barrau *et al.* [26], and suggested that the proliferation of endometrial basal gland was likely to be regulated by progestin. Progesterone levels may not be the only regulator of endometrial proliferation once, during anestrus, the proliferation was also low although the low levels of plasma progesterone [72]. In their study, Srisuwatanasagul *et al.* [72], found a positive correlation between PR immunopresence and Ki-67 labeling index in the surface epithelium suggesting a parallel regulation of the PR and the proliferation observed in the surface epithelium, as similarly demonstrated in humans by Taylor *et al.* [75] in the glandular epithelium. Since estradiol upregulates the expression of steroid receptors, including PR, and the endometrial cell proliferation [59], it is also likely to influence the proliferation in the surface epithelium during proestrus and estrus and the PR expression around estrus in all endometrial compartments.

Proliferating cell nuclear antigen (PCNA) is often used as a marker for replicating cells undergoing deoxyribonucleic acid (DNA) synthesis. In addition to its role in DNA replication and DNA repair, PCNA was also expressed during tissue regression, such as in the corpus luteum [76, 77]. The role of PCNA in cell proliferation and cell death has also been evaluated in the rat uterus [78]. These authors demonstrated that PCNA is expressed both during uterine growth estrogen-stimulated and in its regression induced by the withdrawal of estrogen.

Apoptosis is a highly distinctive mechanism of programmed cell death in eukaryotic cells [79, 80]. It is critical for normal tissue turnover, tissue modeling during embryogenesis, for the proper development and maintenance of the immune system, and for the elimination of pathogens [79, 81]. Apoptosis is characterized by particular morphological and biochemical events that lead in an efficient elimination of cells from tissue without eliciting an inflammatory reaction, in an extremely well ordered process [80, 82].

Apoptosis, or programmed cell death, is an intricately regulated process [83-86]. Programmed cell death cascade can be divided into at least three phases: signal activation-induction of apoptosis, regulation and execution, and cellular structural alterations [81]. It can be induced by different stimuli such as glucocorticoids, DNA damage, deprivation of growth factors, exposure to ionizing radiation, chemotherapeutic drugs, and/or stress [81].

According to O'Reilly and Strasser [87] there are at least three different models to describe the function of apoptotic molecules. Cell death signals can trigger the activation of both caspases and pre-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family. Protein-protein interactions can cleave and inactivate certain vital cellular proteins leading to apoptosis. The second model presumes the ability of a pre-apoptotic member of the Bcl-2 family to form ion channels in cytoplasmic membranes of mitochondria, nuclear envelope and endoplasmic reticulum [88]. The disruption caused in the mitochondrial membrane results in the release of apoptotic-inducing factors, which activate caspases and subsequently kill cells by apoptosis [89]. This is perhaps the most fundamental biochemical event in apoptosis. This family of cysteine-dependent aspartate-specific proteases known as the caspases cleave a number of cellular proteins, in a process of limited proteolysis with only a small number of cuts (usually only one), in interdomain regions. This cleavage can result either in activation of the protein, or in inactivation, but never in degradation, once their substrate specificity distinguishes caspases as one of the most restricted of endopeptidases [90].

The third model is based on the function of molecules of the tumour necrosis factor receptor family and their corresponding ligands. Some of these receptor's family (e.g. Fas) contain a cytoplasmic region called the `death domain' [91], which, upon activation, undergoes homotypic interaction with a death domain in the adaptor protein FasL, resulting in the initiation of apoptosis [87].

Apoptotic cells are characterized by typical morphological alterations, such as DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage. Cells undergoing apoptosis ultimately disassemble into membrane-enclosed vesicles (apoptotic

bodies) that are engulfed by neighboring cells and phagocytes, thus preventing an inflammatory response [81, 82].

The uterus undergoes extensive remodeling during each reproductive cycle, parturition and uterine involution. The cellular changes in the uterus are regulated by the circulating levels of ovarian sex steroids, progesterone and estrogen [92]. One of the basic events of uterine cellular change is physiological cell turnover, which involves cell death by apoptosis and cell renewal by proliferation [93, 94]. The basic mechanisms leading to degeneration of the luminal epithelium and regression of the glandular epithelium are unknown. It is possible that these mechanisms involve apoptosis, which has been reported to be associated with cell death in the uterus in other species [95].

1.6.3. Angiogenesis pattern during the endometrial cycle

The uterus of the bitch expresses messenger ribonucleic acid for certain growth and angiogenesis factors (platelet activating factor (PAF), epidermal growth factors (EGF), vascular endothelial growth factor (VEGF)) and their receptors.

Two of these growth factors are epidermal growth factor and transforming growth factor (TGF) [96-98]. The EGF family molecules are expressed as well as in many tissues, also in the uterus of women, primates, horses, goats, pigs, mice and rats [99-101]. They are mainly expressed during the pre-implantation and implantation period [102], and influence early embryonic development, cleavage and differentiation of blastocysts [103-106]

TGF and EGF, as well as its receptor, epidermal growth factor receptor (EGFR) systems play an important role in the process of growth regulation, and in the differentiation and regression of endometrial epithelial cells [69, 107].

Vascular endothelial growth factor (VEGF) is an angiogenesis promoter of blood vessels under physiological as well as pathological conditions, and during the sexual cycle, it is involved in the angiogenesis in ovarian and uterine tissue [108, 109].

Platelet-activating factor (PAF) is a potent signal-phospholipid and has an important angiogenic and proliferative effect during the implantation process [110-112] contributing for the successful maternal-fetal interaction [113].

EGF expression is increased during the steroid stimulation phase of the estrous cycle. There is also an increase expression of TGF-alpha and EGFR in luminal and glandular

epithelia at proestrus and estrus. Decreased expression, as well as immunostaining of these proteins, was observed in diestrus and anestrus.[69, 107].

In the diestrus as well as in pregnancy stages, mRNA for EGF, VEGF and PAF or their receptors can be detected in canine uterine tissue. The expression of PAF messenger ribonucleic acid in the uterus is very low [114].

Bukowska *et al.* [115], trough reverse transcription polymerase chain reaction analysis, found a significant increase in the expression of several VEGF transcripts isoforms in the endometrial tissue in pregnant bitches as compared with the non-pregnant ones.

1.6.4. The oxidative stress and the endometrial cycle

1.6.4.1. Reactive oxygen species, cellular antioxidant mechanism and oxidative stress

Energetic metabolism pathways in aerobic cells are oxygen-dependent and physiologically synthetizes a group of pro-oxidant molecules called reactive oxygen species (ROS) [116, 117] that includes high reactive chemical species with unpaired electrons in peripheral orbitals (free radicals) and chemical stable oxygen-derivatives (non-radicals) [118, 119]. Oxygen radicals are short-lived, intermediate, species produced by the reduction of oxygen ultimately forming water [120]. In the intermediate steps of reducing oxygen occurs the formation of superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), corresponding to the stages of reduction by one (e.g.: reaction catalyzed by NADPH oxidase), two (e.g.: reaction catalyzed by glucose oxidase) and three electrons respectively [120, 121]. Oxygen radicals may also occur as alkyl radicals or peroxyl, for example, in lipids [121]. The superoxide radical is formed during the oxidative phosphorylation in the mitochondrial respiratory chain and is normally produced by macrophages (via NADPH oxidase) to destroy bacteria during the process of phagocytosis [116, 122]. Non-radical hydrogen peroxide is less reactive than O_2^-, is more highly diffusible and can cross the plasma membrane [116]. The hydroxyl radical can be produced by homolytic fission from H_2O_2 (Fenton reaction) in the presence of transition elements such as ferrous or cuprous ions. The OH^- radical can also be formed from O_2^- or H_2O_2 and trace elements (Haber-Weiss reaction). The hydroxyl radical is probably the most reactive radical known and reacts so quickly with neighboring molecules, that it is rarely found far from its

place of production [118, 120]. The OH^- radical is a highly reactive oxidant molecule against DNA, lipids and proteins [116].

Cells live in a delicate equilibrium between ROS synthesis [123] and cellular decreasing mechanism including inhibition of its production or enzymatic and non-enzymatic (antioxidants scavenging) catalyzed metabolism [120, 122, 124, 125]. Oxidative stress is a state characterized by the imbalance between the concentrations of ROS (oxidizing agents) and antioxidant defense mechanisms of the organism [117, 126, 127]. Increasing levels of oxidants or/and reducing antioxidants in oxidative stress lead to severe damage at the molecular and cellular level [118, 128, 129]. Direct and indirect ROS effects on cells can be summarized into three distinct categories. RNA and DNA damage, lipid peroxidation, and protein damage [119, 120]. ROS attack the polyunsaturated fatty acids of the fatty acid membrane causing the destruction of membrane lipids by lipid peroxidation. The end-products of this peroxidation are lipid hydroperoxides (LOOH). Many different aldehydes, such as malondialdehyde (MDA), are originated as secondary products [129-131]. Protein oxidation, caused by covalent modifications of a protein, can be induced either directly by ROS or indirectly by reaction with secondary by-products of oxidative stress [132, 133].

Oxidative stress and cellular damage could be avoid by antioxidant enzymatic defenses [117, 126, 127]. Superoxide anion and hydrogen peroxide are degraded by superoxide dismutase (SOD), catalase (CAT) and peroxidases such as glutathione peroxidase (GPx) [120]. The composition of antioxidant defenses differs from tissue to tissue and one type of cell to another within the same tissue [118]. SOD catalyzes the reaction of the superoxide anion to hydrogen peroxide, which plays a key role in antioxidant reactions [116]. In animal cells, most SOD is localized in the cytosol and some is still present in lysosomes, nucleus, mitochondrial intermembrane space, and peroxisomes [118]. Mammals produce three isoenzymes: SOD_1 encodes a Cu, Zn-SOD, containing copper and zinc as metal cofactors and is largely cytosolic; SOD_2 encodes a Mn-SOD, a mitochondrial Mn-containing isoform; SOD_3 encoding the extracellular enzyme (EC-SOD), is structurally similar to Cu, Zn-SOD, and also contains Cu and Zn as cofactors [116, 118, 122]. The SOD-catalyzed dismutation of superoxide anion gives rise to hydrogen peroxide also formed *in vivo* by several other enzymes, particularly oxidases [118]. CAT is the second enzyme which acts on cellular detoxification [116] and is found mostly in the peroxisomes of most cells, particularly in the liver. This metalloenzyme containing iron, catalyzes the conversion of hydrogen peroxide to water and oxygen [118, 120]. Glutathione peroxidase (GPx) plays a key role in the detoxification of peroxides, hydrogen peroxide and lipid peroxides [118, 120] using the reduced glutathione form (GSH) as electron donor. [116, 122]. This enzyme

contains selenocysteine in its active center [120, 122]. GPx also exists in an insoluble form associated to the membrane (phospholipid hydroperoxide glutathione peroxidase) which acts on lipid hydroperoxides [116]. Non-enzymatic antioxidant mechanisms reinforce enzymatic mechanisms action and includes proteins and non-proteins [122, 124, 125]. Antioxidant proteins minimize the availability of pro-oxidants such as iron and copper ions and heme groups (transferrins, haptoglobulins, hemopexin and metallothionein), oxidize ferrous ions (ceruloplasmin) and proteins that protect, by other mechanisms, biomolecules from damage, including oxidative damage (heat shock proteins). Low molecular weight non-protein compounds scavenge ROS (glutathione, alpha-tocopherol, ascorbic acid) [118, 122]. A major source of protection against the deleterious effects of oxygen radicals is provided by antioxidant vitamins, with vitamin E exerting this effect on the membranes and vitamin C in the aqueous compartments [116]. The membranes are the main target of radicals, and the role of vitamin E is crucial in disrupting peroxidation reactions of unsaturated lipid chains [122] Vitamin C recycles oxidized vitamin E to the reduced form and additionally vitamin C radical can be regenerated by transhydrogenases from extracellular sources [120]. Glutathione (GSH) is a tripeptide, γ-L-glutamyl-L-cysteine-L-glycine, representing the most abundant non-protein thiol in the body, found in large amounts in organs exposed to toxins, and in small amounts in body fluids [116]. Glutathione appears either in the reduced form (GSH) or in oxidized form (GSSG) by the formation of a disulfide bond between two molecules and has a pleiotropic capacity, which includes maintaining the cells in a reduced state, forming conjugates with some noxious endogenous compounds and xenobiotics [118, 122]. Moreover reduced glutathione functions as an electron donor for glutathione peroxidase, which reduces peroxides to the corresponding alcohols [122, 134]. The cellular ratio between reduced and oxidized glutathione (GSH/GSSG) is high, so it is necessary a mechanism capable of continuously reducing GSSG to GSH [119]. This function is performed by glutathione reductase (GSR), a membrane-associated flavoprotein. For the GSSG reduction, NADPH is necessary as an electron donor [116, 118, 122]. GSH levels are also maintained by *de novo* synthesis, catalyzed by two enzymes, gamma-glutamylcysteine synthase (GCS-γ) and glutathione synthase (GS) [122]. Glutathione S-transferase (GST) is another enzyme dependent on glutathione metabolism to catalyze its reactions. GST comprises a superfamily of enzymes, mainly cytosolic and widely distributed in all cell types [135, 136], whose main function is to catalyze the conjugation of xenobiotics with GSH [118]. These enzymes catalyze the conjugation of glutathione to electrophilic substrates producing compounds that are more soluble and with less reactive groups, which facilitates their removal and disposal [116]. Some glutathione transferases, given their broad specificity with

respect to the substrate can metabolize cytotoxic aldehydes produced during lipid peroxidation, such as 4-hydroxynonenal [136].

1.6.4.2. Endometrial cycle and oxidative stress

Production of reactive oxygen species (ROS) may change during the endometrial cycle and pregnancy, relying in a fine tune system that when disturbed damages the tissue and predispose to disease [127]. Free radicals have a dual role in the reproductive tract. They are also key signaling molecules, modulating various reproductive functions. Free radicals can influence the oocytes, sperm and embryos in their microenvironments, for example, in follicular, hydrosalpingeal, and peritoneal fluids [117]. Additionally ROS can also be generated by systems such as the cyclooxygenase, which catalyzes the initial oxidation reaction in the conversion of arachidonate to prostanoids and is induced under inflammatory processes, or P450, where ROS generation is important during the metabolic process of steroid hormones synthesis from cholesterol in endocrine organs such as the ovaries and testis [122]. Complexity of the endometrial function correlates with the multitude of small molecules and pathways that cyclically encompasses changes in cell death, proliferation and differentiated product secretion according to the ovarian cycle, aiming the ultimate goal of embryo implantation [137, 138]. These changes affect both the endometrial epithelia and the stroma, pursuing a common goal: the endometrial homeostasis, of supreme importance in mammalian fertility. In the mammalian uterus, cyclic changes in the stromal/epithelial relation and in local molecules acting in a paracrine and autocrine way parallels the changes in sex steroids produced by the ovaries [139-141]. It has also been demonstrated, in humans, that the expression of various antioxidants varies along the endometrial cycle, in response to the alternate secretion of estrogens and progesterone [142]. It has also been demonstrated that the mechanisms of antioxidative protection are disrupted in many uterine diseases [143].

Grossly, a decreased in the oxidative stress has been associated to estrogen dominance while estrogen withdrawn and progesterone dominance have been associated with increased oxidative stress. Sugino *et al.* (1996) reported the existence of a cyclical variation in the expression of superoxide dismutase (SOD) in the human endometrium. In the late secretory stage, preceding the endometrial shedding at menses, SOD activity decreases while lipid peroxidation and ROS levels increase [144]. Increased ROS might originate from the cyclooxygenase activity and the production of prostaglandin F2α. Moreover, SOD is increased in the proliferative stage of the menstrual stages, therefore reducing ROS, in

association to increased levels of estrogen [145]. In the endometrium, controlled levels of ROS have been associated to angiogenesis during endometrial growth and regeneration or in implantation, a role that involve also the local cytokine network. SOD is also increased during decidualization, in humans [146]. The changes of oxidative stress in human endometrium is often indirectly analysed through the study of antioxidants, such as the superoxide dismutase or the glutathione peroxidase, of the lipid and protein peroxidation or by determining the total antioxidant capacity [142, 147]. In the female reproductive system, GSH is called to play a role in reducing oxidative stress, either by direct interaction with ROS, either by transfer of electrons to glutathione peroxidase (GP) [122].

Chapter reference list

[1] Concannon PW. Reproductive cycles of the domestic bitch. Anim Reprod Sci. 2011;124:200-10.

[2] Jochle W, Andersen AC. The estrous cycle in the dog: a review. Theriogenology. 1977;7:113-40.

[3] Concannon PW. Endocrinologic control of normal canine ovarian function. Reprod Domest Anim. 2009;44 Suppl 2:3-15.

[4] Rehm S, Stanislaus DJ, Williams AM. Estrous cycle-dependent histology and review of sex steroid receptor expression in dog reproductive tissues and mammary gland and associated hormone levels. Birth Defects Res B Dev Reprod Toxicol. 2007;80:233-45.

[5] Chandra SA, Adler RR. Frequency of different estrous stages in purpose-bred beagles: a retrospective study. Toxicol Pathol. 2008;36:944-9.

[6] Lord K, Feinstein M, Smith B, Coppinger R. Variation in reproductive traits of members of the genus Canis with special attention to the domestic dog (*Canis familiaris*). Behav Processes. 2013;92:131-42.

[7] Renton JP, Boyd JS, Eckersall PD, Ferguson JM, Harvey MJA, Mullaney J, et al. Ovulation, fertilization and early embryonic development in the bitch (*Canis familiaris*). 1991. p. 221-31.

[8] de Gier J, Kooistra HS, Djajadiningrat-Laanen SC, Dieleman SJ, Okkens AC. Temporal relations between plasma concentrations of luteinizing hormone, follicle-stimulating hormone, estradiol-17beta, progesterone, prolactin, and alpha-melanocyte-stimulating hormone during

the follicular, ovulatory, and early luteal phase in the bitch. Theriogenology. 2006;65:1346-59.

[9] Karre I, Meyer-Lindenberg A, Urhausen C, Beineke A, Meinecke B, Piechotta M, et al. Distribution and viability of spermatozoa in the canine female genital tract during post-ovulatory oocyte maturation. Acta Vet Scand. 2012;54:49.

[10] Concannon PW, Hansel W, Visek WJ. The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. Biol Reprod. 1975;13:112-21.

[11] Root Kustritz MV. Collection of tissue and culture samples from the canine reproductive tract. Theriogenology. 2006;66:567-74.

[12] Concannon PW, Hansel W, Visek WJ. The Ovarian Cycle of the Bitch: Plasma Estrogen, LH and Progesterone. Biol Reprod. 1975;13:112-21.

[13] Gazzano A, Cozzi A, Mariti C, Tidu L, Verri E, Ducci M, et al. Behavioural modifications of bitches during diestrus and anestrus. Vet Res Commun. 2008;32 Suppl 1:S151-3.

[14] Jeffcoate I. Physiology and endocrinology of reproduction in female dogs. Shurdington, Cheltenham, U.K.: BSAVA. 1998.

[15] Wildt DE, Seager SW, Chakraborty PK. Behavioral, ovarian and endocrine relationships in the pubertal bitch. J Anim Sci. 1981;53:182-91.

[16] Jeffcoate IA, Lindsay FE. Ovulation detection and timing of insemination based on hormone concentrations, vaginal cytology and the endoscopic appearance of the vagina in domestic bitches. J Reprod Fertil Suppl. 1989;39:277-87.

[17] Beach FA, LeBoeuf BJ. Coital behaviour in dogs. I. Preferential mating in the bitch. Anim Behav. 1967;15:546-58.

[18] Pal SK, Ghosh B, Roy S. Inter- and intra-sexual behaviour of free-ranging dogs (*Canis familiaris*). Appl Anim Behav Sci. 1999;62:267-78.

[19] Wildt DE, Panko WB, Chakraborty PK, Seager SW. Relationship of serum estrone, estradiol-17beta and progesterone to LH, sexual behavior and time of ovulation in the bitch. Biol Reprod. 1979;20:648-58.

[20] Moxon R, Batty H, Irons G, England GC. Periovulatory changes in the endoscopic appearance of the reproductive tract and teasing behavior in the bitch. Theriogenology. 2012;78:1907-16.

[21] Phemister RD, Holst PA, Spano JS, Hopwood ML. Time of ovulation in the beagle bitch. Biol Reprod. 1973;8:74-82.

[22] Badinand F, Fontbonne A, Maurel MC, Siliart B. Fertilization time in the bitch in relation to plasma concentration of oestradiol, progesterone and luteinizing hormone and vaginal smears. J Reprod Fertil Suppl. 1993;47:63-7.

[23] Post K. Canine vaginal cytology during the estrous cycle. Can Vet J. 1985;26:101-4.

[24] Concannon PW, Temple M, Montanez A, Frank D. Synchronous delayed oestrus in beagle bitches given infusions of gonadotrophin-releasing hormone superagonist following withdrawal of progesterone implants. J Reprod Fertil Suppl. 1993;47:522-3.

[25] Watts JR, Wright PJ, Lee CS. Endometrial cytology of the normal bitch throughout the reproductive cycle. J Small Anim Pract. 1998;39:2-9.

[26] Barrau MD, Abel JH, Jr., Verhage HG, Tietz WJ, Jr. Development of the endometrium during the estrous cycle in the bitch. Am J Anat. 1975;142:47-65.

[27] Van Cruchten S, Van den Broeck W, D'Haeseleer M, Simoens P. Proliferation patterns in the canine endometrium during the estrous cycle. Theriogenology. 2004;62:631-41.

[28] Sunderland SJ, Crowe MA, Boland MP, Roche JF, Ireland JJ. Selection, dominance and atresia of follicles during the oestrous cycle of heifers. J Reprod Fertil. 1994;101:547-55.

[29] Smith MS, McDonald LE. Serum levels of luteinizing hormone and progesterone during the estrous cycle, pseudopregnancy and pregnancy in the dog. Endocrinology. 1974;94:404-12.

[30] Edqvist LE, Johansson ED, Kasstrom H, Olsson SE, Richkind M. Blood plasma levels of progesterone and oestradiol in the dog during the oestrous cycle and pregnancy. Acta Endocrinol. 1975;78:554-64.

[31] Hoffmann B, Riesenbeck A, Klein R. Reproductive endocrinology of bitches. Anim Reprod Sci. 1996;42:275-88.

[32] Chu PY, Lee CS, Wright PJ. Degeneration and apoptosis of endometrial cells in the bitch. Theriogenology. 2006;66:1545-9.

[33] Payan-Carreira R, Pires MA, Strom Holst B, Rodriguez-Martinez H. Tumour necrosis factor in the canine endometrium: an immunohistochemical study. Reprod Domest Anim. 2011;46:410-8.

[34] Payan-Carreira R, Santos C, Miranda S, Pereira RM, Santos D, Pires MA. Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium. Theriogenology. 2014;82:815-26.

[35] Concannon PW. Research challenges in endocrine aspects of canine ovarian cycles. Reprod Domest Anim. 2012;47 Suppl 6:6-12.

[36] Olson PN, Bowen RA, Behrendt MD, Olson JD, Nett TM. Concentrations of reproductive hormones in canine serum throughout late anestrus, proestrus and estrus. Biol Reprod. 1982;27:1196-206.

[37] Hase M, Hori T, Kawakami E, Tsutsui T. Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by ultrasonographic diagnosis system in dogs. J Vet Med Sci. 2000;62:243-8.

[38] Concannon PW, Powers ME, Holder W, Hansel W. Pregnancy and parturition in the bitch. Biol Reprod. 1977;16:517-26.

[39] Nett TM, Akbar AM, Phemister RD, Holst PA, Reichert LE, Jr., Niswender GD. Levels of lutenizing hormone, estradiol and progesterone in serum during the estrous cycle and pregnancy in the beagle bitch . PSEBM (New York, NY). 1975;148:134-9.

[40] Concannon PW, McCann JP, Temple M. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. J Reprod Fertil Suppl. 1989;39:3-25.

[41] Onclin K, Murphy B, Verstegen JP. Comparisons of estradiol, LH and FSH patterns in pregnant and nonpregnant beagle bitches. Theriogenology. 2002;57:1957-72.

[42] Spencer TE, Bazer FW. Conceptus signals for establishment and maintenance of pregnancy. Reprod Biol Endocrinol. 2004;2:49.

[43] Satué K, Gardón JC. A Review of the Estrous Cycle and the Neuroendocrine Mechanisms in the Mare. J Steroids Horm Sci. 2013;04.

[44] Kowalewski MP, Mutembei HM, Hoffmann B. Canine prostaglandin E2 synthase (PGES) and its receptors (EP2 and EP4): expression in the corpus luteum during dioestrus. Anim Reprod Sci. 2008;109:319-29.

[45] Luz M, Bertan C, Binelli M, Lopes M. In vitro PGF2alpha production by endometrium and corpus luteum explants from pregnant and nonpregnant diestrus bitches and placental explants from pregnant bitches. Theriogenology. 2006;66:1442 - 7.

[46] Diedrich K, Fauser BCJM, Devroey P, Griesinger G. The role of the endometrium and embryo in human implantation. Hum Reprod Update. 2007;13:365-77.

[47] Cavagna M, Mantese JC. Biomarkers of endometrial receptivity-a review. Placenta. 2003;24 Suppl B:S39-47.

[48] van Mourik MS, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol. 2009;85:4-19.

[49] Dekel N, Gnainsky Y, Granot I, Mor G. Inflammation and implantation. Am J Reprod Immunol. 2010;63:17-21.

[50] Aflatoonian R, Tuckerman E, Elliott SL, Bruce C, Aflatoonian A, Li TC, et al. Menstrual cycle-dependent changes of Toll-like receptors in endometrium. Hum Reprod (Oxford, England). 2007;22:586-93.

[51] Horne AW, Stock SJ, King AE. Innate immunity and disorders of the female reproductive tract. Reproduction. 2008;135:739-49.

[52] Wira CR, Fahey JV. A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. AIDS (London, England). 2008;22:1909-17.

[53] Kutteh WH MJ, Wira CR. Mucosal immunity in the human female reproductive tract. In: Mestecky J ea, editors, editor. Mucosal Immunology. 3rd ed ed. Amsterdam: Elsevier/Academic Press; 2005. p. 1631–46.

[54] Turner SJ, Rossjohn J. alphabeta T cell receptors come out swinging. Immunity. 2011;35:660-2.

[55] Lam EW, Shah K, Brosens JJ. The diversity of sex steroid action: the role of micro-RNAs and FOXO transcription factors in cycling endometrium and cancer. J Endocrinol. 2012;212:13-25.

[56] Donnay I, Wouters-Ballman P, Devleeschouwer N, Leclercq G, Verstegen J. Changes in oestrogen, progesterone and epidermal growth factor receptor concentrations and affinities during the oestrous cycle in the normal mammary gland and uterus of dogs. Vet Res Commun. 1995;19:101-13.

[57] De Cock H, Ducatelle R, Logghe JP. Immunohistochemical localization of estrogen receptor in the normal canine female genital tract. Domest Anim Endocrinol. 1997;14:133-47.

[58] Dhaliwal GK, England GC, Noakes DE. The influence of exogenous steroid hormones on steroid receptors, uterine histological structure and the bacterial flora of the normal bitch. Anim Reprod Sci. 1999;56:259-77.

[59] Galabova-Kovacs G, Walter I, Aurich C, Aurich JE. Steroid receptors in canine endometrial cells can be regulated by estrogen and progesterone under in vitro conditions. Theriogenology. 2004;61:963-76.

[60] Critchley HO, Kelly RW, Baird DT, Brenner RM. Regulation of human endometrial function: mechanisms relevant to uterine bleeding. Reprod Biol Endocrinol. 2006;4 Suppl 1:S5.

[61] Dhaliwal GK, England GC, Noakes DE. Immunocytochemical localization of oestrogen and progesterone receptors in the uterus of the normal bitch during oestrus and metoestrus. J Reprod Fertil Suppl. 1997;51:167-76.

[62] Vermeirsch H, Simoens P, Lauwers H, Coryn M. Immunohistochemical detection of estrogen receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology. 1999;51:729-43.

[63] Vermeirsch H, Van den Broeck W, Coryn M, Simoens P. Immunohistochemical detection of androgen receptors in the canine uterus throughout the estrus cycle. Theriogenology. 2002;57:2203-16.

[64] Vermeirsch H, Simoens P, Hellemans A, Coryn M, Lauwers H. Immunohistochemical detection of progesterone receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology. 2000;53:773-88.

[65] Galabova G, Egerbacher M, Aurich JE, Leitner M, Walter I. Morphological changes of the endometrial epithelium in the bitch during metoestrus and anoestrus. Reprod Domest Anim. 2003;38:415-20.

[66] Johnston SD, Kiang DT, Seguin BE, Hegstad RL. Cytoplasmic estrogen and progesterone receptors in canine endometrium during the estrous cycle. Am J Vet Res. 1985;46:1653-8.

[67] Fernandes PA, Bowen RA, Sawyer HR, Nett TM, Gorell TA. Concentration of receptors for estradiol and progesterone in canine endometrium during estrus and diestrus. Am J Vet Res. 1989;50:64-7.

[68] Oehler MK, Rees MC, Bicknell R. Steroids and the endometrium. Curr Med Chem. 2000;7:543-60.

[69] Bukowska D, Kempisty B, Jackowska M, Wozna M, Antosik P, Piotrowska H, et al. Differential expression of epidermal growth factor and transforming growth factor beta isoforms in dog endometrium during different periods of the estrus cycle. Pol J Vet Sci. 2011;14:259-64.

[70] Rider V. Progesterone and the control of uterine cell proliferation and differentiation. Front Biosci. 2002;7:d1545-55.

[71] Bigsby RM, Caperell-Grant A, Berry N, Nephew K, Lubahn D. Estrogen induces a systemic growth factor through an estrogen receptor-alpha-dependent mechanism. Biol Reprod. 2004;70:178-83.

[72] Srisuwatanasagul S, Srisuwatanasagul K, Sirividayapong S. Progesterone Receptors and Proliferation in the Bitch Uterus during Different Stages of the Oestrous Cycle. Thai J Vet Med. 2006; 36(3):45-54.

[73] Aupperle H, Özgen S, Schoon HA, Schoon D, Hoppen HO, Sieme H, et al. Cyclical endometrial steroid hormone receptor expression and proliferation intensity in the mare. Equine Vet J. 2000;32:228-32.

[74] Sukjumlong S, Kaeoket K, Dalin AM, Persson E. Immunohistochemical studies on oestrogen receptor alpha (ER alpha) and the proliferative marker Ki-67 in the sow uterus at different stages of the oestrous cycle. Reprod Domest Anim. 2003;38:5-12.

[75] Taylor AH, Guzail M, Wahab M, Thompson JR, Al-Azzawi F. Quantitative histomorphometric analysis of gonadal steroid receptor distribution in the normal human endometrium through the menstrual cycle. Histochem Cell Biol. 2005;123:463-74.

[76] Kiya T, Endo T, Goto T, Yamamoto H, Ito E, Kudo R, et al. Apoptosis and PCNA expression induced by prolactin in structural involution of the rat corpus luteum. J Endocrinol Invest. 1998;21:276-83.

[77] Zheng J, Fricke PM, Reynolds LP, Redmer DA. Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. Biol Reprod. 1994;51:623-32.

[78] Lai MD, Lee LR, Cheng KS, Wing LY. Expression of proliferating cell nuclear antigen in luminal epithelium during the growth and regression of rat uterus. J Endocrinol. 2000;166:87-93.

[79] Ellis RE, Yuan JY, Horvitz HR. Mechanisms and functions of cell death. Annu Rev Cell Biol. 1991;7:663-98.

[80] Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26:239-57.

[81] Harada T, Kaponis A, Iwabe T, Taniguchi F, Makrydimas G, Sofikitis N, et al. Apoptosis in human endometrium and endometriosis. Hum Reprod Update. 2004;10:29-38.

[82] Thornberry N. Caspases: key mediators of apoptosis. Chem Biol. 1998;5:97 - 103.

[83] White E. Death-defying acts: a meeting review on apoptosis. Genes Dev. 1993;7:2277-84.

[84] Green DR, Martin SJ. The killer and the executioner: how apoptosis controls malignancy. Curr Opin Immunol. 1995;7:694-703.

[85] Steller H. Mechanisms and genes of cellular suicide. Science. 1995;267:1445-9.

[86] Nagata S. Apoptosis by death factor. Cell. 1997;88:355-65.

[87] O'Reilly LA, Strasser A. Apoptosis and autoimmune disease. Inflamm Res. 1999;48:5-21.

[88] Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M, Reed JC. Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci USA. 1997;94:5113-8.

[89] Chinnaiyan AM, O'Rourke K, Lane BR, Dixit VM. Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science. 1997;275:1122-6.

[90] Salvesen G, Dixit V. Caspase activation: the induced-proximity model. Proc Natl Acad Sci USA. 1999;96:10964 - 7.

[91] Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell. 1993;74:845-53.

[92] Klinger FG, Battista N, De Felici M, Maccarrone M. Stage-variations of anandamide hydrolase activity in the mouse uterus during the natural oestrus cycle. J Exp Clin Assist Reprod. 2006;3:3.

[93] Spencer SJ, Cataldo NA, Jaffe RB. Apoptosis in the human female reproductive tract. Obstet Gynecol Surv. 1996;51:314-23.

[94] Toki T, Mori A, Shimizu M, Nikaido T, Fujii S. Localization of apoptotic cells within the human endometrium and correlation between apoptosis and p21 expression. Mol Hum Reprod. 1998;4:1157-64.

[95] Chu PY, Wright PJ, Lee CS. Apoptosis of endometrial cells in the bitch. Reprod Fertil Dev. 2002;14:297-305.

[96] Harada T, Fujikawa T, Yoshida S, Onohara Y, Tanikawa M, Terakawa N. Expression of transforming growth factor alpha (TGF-alpha) gene in mouse embryonic development. J Assist Reprod Genet. 1997;14:262-9.

[97] Cai L, Zhang J, Duan E. Dynamic distribution of epidermal growth factor during mouse embryo peri-implantation. Cytokine. 2003;23:170-8.

[98] Beceriklisoy HB, Schafer-Somi S, Kucukaslan I, Agaoglu R, Gultiken N, Ay SS, et al. Cytokines, growth factors and prostaglandin synthesis in the uterus of pregnant and non-pregnant bitches: the features of placental sites. Reprod Domest Anim. 2009;44 Suppl 2:115-9.

[99] Twardzik DR. Differential Expression of Transforming Growth Factor-α during Prenatal Development of the Mouse. Cancer Res. 1985;45:5413-6.

[100] Stewart F, Power CA, Lennard SN, Allen WR, Amet L, Edwards RM. Identification of the horse epidermal growth factor (EGF) coding sequence and its use in monitoring EGF gene expression in the endometrium of the pregnant mare. J Mol Endocrinol. 1994;12:341-50.

[101] Flores JM, Sanchez MA, Garcia P, Sanchez B, Nieto A. Immunohistochemical localization of epidermal growth factor, transforming growth factor-alpha and growth factor-beta s in the caprine peri-implantation period. Theriogenology. 1998;50:931-44.

[102] Kennedy TG, Brown KD, Vaughan TJ. Expression of the genes for the epidermal growth factor receptor and its ligands in porcine oviduct and endometrium. Biol Reprod. 1994;50:751-6.

[103] Bonvissuto AC, Lala PK, Kennedy TG, Nygard K, Lee DC, Han VK. Induction of transforming growth factor-alpha gene expression in rat decidua is independent of the conceptus. Biol Reprod. 1992;46:607-16.

[104] Kim JG, Vallet JL, Christenson RK. Characterization of uterine epidermal growth factor during early pregnancy in pigs. Domest Anim Endocrinol. 2001;20:253-65.

[105] Aflalo ED, Sod-Moriah UA, Potashnik G, Har-Vardi I. EGF increases expression and activity of PAs in preimplantation rat embryos and their implantation rate. Reprod Biol Endocrinol. 2007;5:4.

[106] Han VK, Hunter ES, 3rd, Pratt RM, Zendegui JG, Lee DC. Expression of rat transforming growth factor alpha mRNA during development occurs predominantly in the maternal decidua. Mol Cell Biol. 1987;7:2335-43.

[107] Tamada H, Tominaga M, Kida K, Kawate N, Inaba T, Matsuyama S, et al. Detection of transforming growth factor-alpha and epidermal growth factor receptor mRNA and immunohistochemical localization of the corresponding proteins in the canine uterus during the estrous cycle. Histol Histopathol. 2005;20:817-24.

[108] Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, et al. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med. 1998;4:336-40.

[109] Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9:669-76.

[110] Espey LL, Tanaka N, Woodard DS, Harper MJ, Okamura H. Decrease in ovarian platelet-activating factor during ovulation in the gonadotropin-primed immature rat. Biol Reprod. 1989;41:104-10.

[111] Harper MJ. Platelet-activating factor: a paracrine factor in preimplantation stages of reproduction? Biol Reprod. 1989;40:907-13.

[112] Narahara H, Tanaka Y, Kawano Y, Miyakawa I, Johnston J. Platelet-Activating Factor Acetylhydrolase Activity in Human Follicular Fluid. In: Nigam S, Kunkel G, Prescott S, editors. Platelet-Activating Factor and Related Lipid Mediators 2: Springer US; 1996. p. 121-7.

[113] O'Neill C. The role of paf in embryo physiology. Hum Reprod Update. 2005;11:215-28.

[114] Schafer-Somi S, Sabitzer S, Klein D, Reinbacher E, Kanca H, Beceriklisoy HB, et al. Vascular endothelial (VEGF) and epithelial growth factor (EGF) as well as platelet-activating factor (PAF) and receptors are expressed in the early pregnant canine uterus. Reprod Domest Anim. 2013;48:20-6.

[115] Bukowska D, Kempisty B, Jackowska M, Wozna M, Antosik P, Piotrowska H, et al. Analysis of integrins and vascular endothelial growth factor isoforms mRNA expression in the canine uterus during perimplantation period. Pol J Vet Sci. 2011;14:253-8.

[116] Gate L, Paul J, Ba GN, Tew KD, Tapiero H. Oxidative stress induced in pathologies: the role of antioxidants. Biomed Pharmacother. 1999;53:169-80.

[117] Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol. 2006;18:325-32.

[118] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: Oxford University Press; 1999.

[119] Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39:44-84.

[120] Riley JCM, Behrman HR. Oxygen Radicals and Reactive Oxygen Species in Reproduction. Exp Biol Med. 1991;198:781-91.

[121] Sies H. Oxidative stress: oxidants and antioxidants. Experimental physiology. 1997;82:291-5.

[122] Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. Reprod Biol Endocrinol. 2005;3:43.

[123] Rizk B, Badr M, Talerico C. Oxidative Stress and The Endometrium. In: Agarwal A, Aziz N, Rizk B, editors. Studies on Women's Health: Humana Press; 2013. p. 61-74.

[124] Bergendi L, Beneš L, Ďuračková Z, Ferenčik M. Chemistry, physiology and pathology of free radicals. Life Sciences. 1999;65:1865-74.

[125] Rizzo A, Roscino MT, Minoia G, Trisolini C, Spedicato M, Mutinati M, et al. Serum levels of reactive oxygen species (ROS) in the bitch. Immunopharmacol Immunotoxicol. 2009;31:310-3.

[126] Browne RW, Bloom MS, Schisterman EF, Hovey K, Trevisan M, Wu C, et al. Analytical and biological variation of biomarkers of oxidative stress during the menstrual cycle. Biomarkers. 2008;13:160-83.

[127] Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol. 2012;10:49.

[128] Sies H. Role of reactive oxygen species in biological processes. Klinische Wochenschrift. 1991;69:965-8.

[129] Dotan Y, Lichtenberg D, Pinchuk I. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Prog Lipid Res. 2004;43:200-27.

[130] Mylonas C, Kouretas D. Lipid peroxidation and tissue damage. In vivo (Athens, Greece). 1999;13:295-309.

[131] Ayala A, Munoz M, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438.

[132] Shacter E. Quantification and significance of protein oxidation in biological samples. Drug Metab Rev. 2000;32:307-26.

[133] Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J.. 1997;324 (Pt 1):1-18.

[134] Wu G, Fang Y, Yang S, Lupton J, Turner N. Glutathione metabolism and its implications for health. J Nutr. 2004;134:489 - 92.

[135] Ranganathan PN, Whalen R, Boyer TD. Characterization of the molecular forms of glutathione S-transferase P1 in human gastric cancer cells (Kato III) and in normal human erythrocytes. Biochem J. 2005;386:525-33.

[136] Veal EA, Toone WM, Jones N, Morgan BA. Distinct Roles for Glutathione S-Transferases in the Oxidative Stress Response in *Schizosaccharomyces pombe*. 2002. p. 35523-31.

[137] Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. Mol Hum Reprod. 2004;10:879-93.

[138] Sherwin JR, Sharkey AM, Cameo P, Mavrogianis PM, Catalano RD, Edassery S, et al. Identification of novel genes regulated by chorionic gonadotropin in baboon endometrium during the window of implantation. Endocrinology. 2007;148:618-26.

[139] Gomez-Zubeldia MA, Corrales S, Arbues J, Nogales AG, Millan JC. Influence of estradiol and gestagens on oxidative stress in the rat uterus. Gynecol Oncol. 2002;86:250-8.

[140] Hekimoglu A, Bilgin HM, Kurcer Z, Ocak AR. Effects of increasing ratio of progesterone in estrogen/progesterone combination on total oxidant/antioxidant status in rat uterus and plasma. Arch Gynecol Obstet. 2010;281:23-8.

[141] Yuan L, Dietrich AK, Nardulli AM. 17beta-Estradiol alters oxidative stress response protein expression and oxidative damage in the uterus. Mol Cell Endocrinol. 2014;382:218-26.

[142] Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005;3:28.

[143] Dabrowski R, Kostro K, Lisiecka U, Szczubial M, Krakowski L. Usefulness of C-reactive protein, serum amyloid A component, and haptoglobin determinations in bitches with pyometra for monitoring early post-ovariohysterectomy complications. Theriogenology. 2009;72:471-6.

[144] Sugino N. The role of oxygen radical-mediated signaling pathways in endometrial function. Placenta. 2007;28 Suppl A:S133-6.

[145] Sugino N, Shimamura K, Takiguchi S, Tamura H, Ono M, Nakata M, et al. Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. Hum Reprod (Oxford, England). 1996;11:1073-8.

[146] Sugino N, Takiguchi S, Kashida S, Karube A, Nakamura Y, Kato H. Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. Mol Hum Reprod. 2000;6:19-25.

[147] Chiou JF, Hu ML. Elevated lipid peroxidation and disturbed antioxidant enzyme activities in plasma and erythrocytes of patients with uterine cervicitis and myoma. Clinical biochemistry. 1999;32:189-92.

Chapter 2 – Aims

Nowadays, dogs possess great social importance, playing a wide range of roles in human society, used either as working dogs or as pets all over the world. Concurrently a growing interest from breeders and researchers worldwide has prompted the promotion of canine fertility and the advancement of assisted reproductive techniques in this species. Moreover, aging in intact females is frequently associated with pyometra, a life-threatening disease. Although the process of the pyometra development is more or less clear, molecules involved in the early steps of the process remain elusive, which difficult the identification of suitable markers for the precocious diagnosis of the disease. Such inability may derive from the very limited knowledge on the molecular partners involved in the canine endometrial cycle, despite that recently several research team worldwide address this topic.

The main purpose for the research work leading to the present thesis was to highlight some particular aspects of the canine uterine cycle, namely:

- to determine the pattern for neutral endopeptidase/CD10 protein immunoexpression in the normal canine endometrium and to investigate whether this pattern changes during the estrous cycle and in early pregnancy
- to analyse the immunolocalization and temporal changes of the adhesion factors E-cadherin and β-catenin throughout the stages of the canine estrous cycle and at canine embryo apposition and adhesion
- to analyse the immunohistochemical pattern of distribution of antioxidant enzymes, superoxide dismutase 1 (copper-zinc containing SOD) (SOD1) and glutathione peroxidase 1 (GPx1) in the canine endometrium throughout the estrous cycle
- to evaluate oxidative stress through the activity of antioxidant enzymatic system, comprising superoxide dismutase, catalase and glutathione dependent enzymes, and the oxidative damage caused to lipids and proteins, regarding its variation along the canine estrous cycle.

Chapter 3 – General description of material and methods

1. Collection of samples

For the studies developed in this dissertation, frozen and fixated uterine samples would be necessary, as well as to adequately stage the canine estrous cycle and/or early pregnancy. To achieve the necessary number of samples, a protocol was established with private clinics and veterinary hospitals in *Famalicão* and *Mirandela* to collect as many surgical specimens of canine uterus as possible from routine ovariohysterectomy (OVH) or eventual necropsies. These specimens would represent normal canine uteri in different stages of the estrous cycle. Therefore, the female dogs should be submitted to a summary physical exam to exclude systemic disease. Surgery was performed under general anesthesia, and both ovaries, right and left uterine horns and corpus uteri were removed. Excised genital tract was then measured and samples were collected as followed (Figure 1): the apex of the uterine horns and adjacent uterine tubes and ovaries, along with its caudal endings and corpus of the uterus were used for the histological studies; the middle region of the uterine horns was cut in smaller pieces, of *ca.* 1 cm in length, and used for the biochemical studies.

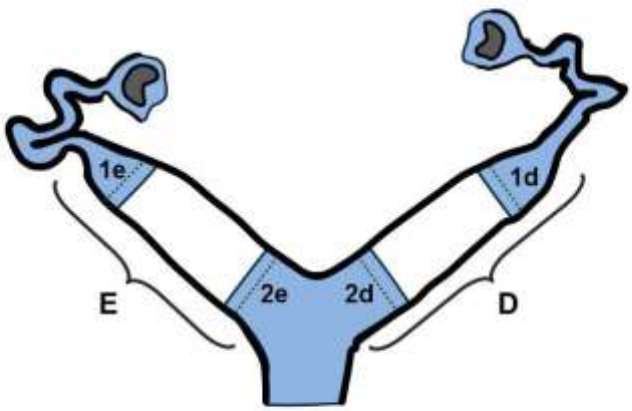


Figure 1 – Drawing of the ovarian-tubal-uterine fragments collected for the study displaying the anatomical points used for measurements (length represented by uppercase letters; diameter corresponding to lowercase letters). Fragments collected for histological techniques are cyan coloured, whereas the fragment representing the middle of the uterine horn (in white) was used for the biochemical studies.

For the histological studies, samples of uterine horns were fixed in 10% buffered formalin immediately after surgery, the left and right sides correctly identify. At the

histopathology laboratory of UTAD (LHAP) these samples were embedded in paraffin wax, sectioned at 3 µm and routinely stained with hematoxylin and eosin for histological evaluation of the endometrium and staging of the estrous cycle. Additional sections with the same thickness were obtained for immunohistochemistry, on silane-coated slides.

For biochemical studies, the samples of uterine horns were cut in fragments with 1 cm in height and snap frozen in liquid nitrogen until reaching the laboratory; thereafter they were stored at -80ºC until analysis.

All biological material was obtained with the owners' informed consent, following the appropriate European Union and National Veterinary Authority guidelines for Animal Care and in accordance to the International Ethical standards. All the surgeries were performed by the owner request, for contraceptive purposes.

As in many cases the previous reproductive history was not known, during collection we also obtained specimens with diverse undiagnosed pathological diseases, pregnancy in distinct moments or with signs of postpartum involution, as well as specimens from prepubertal females. Specimens evidencing macroscopic or histological signs of pregnancy, involution and uterine or ovarian disorders were excluded from the project.

At the end of the collection period we gathered a total of 254 specimens containing the ovarian-uterine segment. From these, only a variable number of selected samples were used in the studies presented herein, depending on the paper or the technique used. All samples do not meeting the inclusion criteria (normal endometrial features, without signs of post-partal involution or unstaged pregnancy, or showing incongurent morphological and clinical data) were removed from the study. The number of samples used, summarized on table 1, will be provided individually in each chapter. The samples used corresponded to mature animals, with ages ranging from 10 months to 8-year old.

Along with the collection of the excised genitalia at OVH or necropsy, for each animal a vaginal cytological specimen and a blood sample were obtained. The vaginal cytology was stained with routine Diff Quick® (Baxter DADE, Switzerland) and used for a preliminary staging of the estrous cycle.

The blood sample was collected by venepuncture from a jugular vein into a controlled vacuum tube (*Serum-gel*, S-Monovette®, Sarstedt, Germany), and promptly centrifuged at 2500 xg for 15 minutes. The serum was stored at -20 ºC until analysis. Serum progesterone levels were determined by chemiluminescent immunoassay system (Immulite®, DPC-Diagnostic Products Corp., Los Angeles, CA, USA). Interassay coefficient of variation was

lower than 6.0%. Peripheral progesterone levels were used to define the luteal stage of the cycle and to delimit the peri-ovulatory events and the day of LH surge in estrus samples.

Table 1– Summary of the distribution of the number of samples used in each chapter according to the cycle stage or status (Cyclic *vs.* Early pregnancy).

Chapter	Stages of the estrous cycle					Early pregnancy
	AN	PE	E	eDI	DI	
4 – Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium	10	9	8	10	10	17
5 – Immunolocalization of E-cadherin and β-catenin in endometrium of female dogs during the estrous cycle	10	8	10	12	10	9
6 – Distribution of superoxide dismutase 1 and glutathione peroxidase 1 in the cyclic canine endometrium	10	10	10	7	9	--
7 – Oxidative stress in canine endometrium across the estrous cycle	5	5	5	5	5	--

2. Staging of the estrous cycle

The staging of estrous cycle was performed on the basis of cumulative information provided by physical examination, vaginal cytology, inspection of the ovaries at OVH, and circulating levels of progesterone (Table 2). This preliminary staging was further confirmed by histological examination of the ovaries and uterus [1].

In brief, anestrus, was defined when vaginal cytology swabs showed more than 90% of the cells being parabasal or intermediate; when the ovarian surface was smooth and without visible structures (or depicting *corpora albicantia* and occasional growing primary and secondary follicles); and when serum progesterone levels remained below 2 $ng.mL^{-1}$. The onset of a clinical follicular stage was suspected when the physical examination revealed the existence of vulvar swelling and the presence of typical vaginal discharge. In proestrus the vaginal cytology presented a variable number of erythrocytes and an increasing percentage of superficial and intermediate cells; large antral follicles with 2-3 mm in diameter were clearly visible in the ovarian cortex, and serum progesterone levels remained below 2 $ng.mL^{-1}$ until the LH surge.

Table 2 – Criteria used to the estrous cycle staging of the canine endometrium. P_4 = progesterone; PF = primary follicle; SF = secondary follicle; TF = tertiary follicle; CH = corpus hemorrhagicum; CL = corpora lutea; LE = endometrial luminal epithelium; GE = endometrial glandular epithelium.

Stage of the cycle	Vaginal cytology	P_4 level	Ovary	Histological parameters	
				Ovary	Endometrium
Anestrus	> 90% of the cells are parabasal or intermediate	Baseline [< 2 $ng.mL^{-1}$]	Smooth ovarian surface, without visible structures (longitudinal cuts may show *corpora albicantia* and occasional growing primary and secondary follicles)	PF SF	Inactive stage [LE and GE cuboidal; straight glands
Proestrus	Presence of erythrocytes and an increasing percentage of superficial and intermediate cells	Below 2 $ng.mL^{-1}$ until the LH surge	Large antral follicles with 2-3 mm in diameter are clearly visible in the ovarian cortex	SF TF	Proliferative stage [Oedema; elongated glands]
Estrus	> 90% of superficial cells, most becoming cornified by ovulation, and only very few erythrocytes	Above 2 $ng.mL^{-1}$ (even reaching 12 to 15 $ng.mL^{-1}$)	Presence of large, luteinized follicles 5-8 mm in diameter with signs of collapse after ovulation	TF CH	Transition from late proliferative stage to an early secretory stage [Maximum oedema and hyperaemia; LE and GE cuboidal-columnar]
Early diestrus	Sharp decrease in superficial cells, while intermediate and parabasal cells, along with neutrophils, are the major cell types visualized	>16 $ng.mL^{-1}$ and rising, originating the initial P4 peak	Dark carmine corpora lutea remained cavitary	Cavitary CL	Great thickness and cellular density. [Coiled basal glands and tall crypt cells]
Diestrus		Progesterone stabilized and remaining high	Corpora lutea were carmine and compact	Mature, compact, and active CL	Regressive uterine changes [vacuolated cytoplasm; flat basal glandular epithelium]

Estrus was staged when the vaginal cytology showed more than 90% of superficial cells, most becoming cornified by ovulation, and with only very few erythrocytes; the gross evaluation of the ovaries would reveal presence of large, luteinized follicles 5-8 mm in diameter, that might be flattened after ovulation; and serum progesterone levels rose above 2 $ng.mL^{-1}$. Diestrus was defined by a vaginal cytology showing a sharp decrease in superficial cells, while intermediate and parabasal cells, along with polymorphonuclear neutrophils would be the major cell types visualised. Circulating progesterone levels would reach an initial peak during the first 30 days post-LH surge (15-90 $ng.mL^{-1}$) and then begin to decrease. For the study purpose, we further divided this stage into two phases: early diestrus, corresponding to the first 20 days of diestrus, during which dark carmine corpora lutea remained cavitary and that would represent the pre-implantation period in the dog [2]; and full diestrus, where corpora lutea were carmine and compact, and the production of progesterone has stabilized and remain high [3]. Samples in diestrus matching with peripheral progesterone levels below 5 $ng.mL^{-1}$ were not used in the present project.

Samples from females with unwanted pregnancy, submitted to OVH up to 3 weeks post-coitus, were used for neutral endopeptidase/CD10 and E-Cadherin and β-Catenin immunoexpression studies. For staging pregnancy samples, the animal history of known unwanted breeding and the progesterone levels were used as a first approach for the possibility of a pregnancy. After OVH, the macroscopic appearance of the uterus and the histoarchitecture of the uterus allowed to establish and to date the existence of pregnancy. When pregnancy has shorted than 2.5 to 3 weeks, when small-sized (<3 cm) uterine swellings may be seen, flushing of the uterine tubes and uterine horns were used to recover the embryos [4]; the faillure to collect embryos by oviductal or uterine flushing was regarded as corresponding to unexisting pregnancies and the sample was discarted from the study.

When necessary for the study purposes, pregnancies were divided into two groups. Group 1 corresponded to pregnancies days 11 to 13 and included the samples with free-floating morulae or young blastocysts, recovered from the oviducts or the uterus. Group 2, representing pregnancy days 15 to 23, included the embryos adherent or invading the endometrium. In this period, the endometrium shows some important morphological changes in the endometrium and some of the stuctures that will form the canine placenta may be identify. For the histological staging of the early pregnancy events, Barrau *et al.* [5] descriptions were used. More detailed information on the landmarks used for the pregnancy staging will be provided in the corresponding chapters.

3. Immunohistochemistry

For detection of the targeted molecules in the canine endometrium, it was used an indirect immunohistochemistry method based on the streptavidin-biotin-peroxidase technique (UltraVision, Lab Vision, Fremont, CA, USA). The adaptation of the method to each particular molecule in analysis is given individually in each chapter. Below it is presented a brief description of the technique.

The general procedure included routine deparaffinization of tissue sections in xylene and its rehydration in graded alcohol. Antigen retrieval was performed after hydration; the thermal treatment adopted varied with the antibody in use, but it included either the microwave irradiation at 750W or the steamer, with slides immersed in the appropriated solution (a 0.05% Extran solution for E-cadherin and β-catenin; citrate buffer for the other molecules). The exact combination of the thermal method, time and solution used for antigen retrieval is provided within the correspondent chapter, and is summarized on table 3.

After cooling, the sections were immersed in 3% hydrogen peroxide for 30 min to block the endogenous peroxidases; prior to incubation with the correspondent primary antibody, to preventing non-specific binding the slides were incubated with a blocking serum (Ultra V Block®, LabVision Corporation) for 5 min.

A list of the primary antibodies used in the present project is given below (table 3), along with their reference, dilution and incubation data. Thereafter, tissue sections were incubated with Biotinylated Goat Polyvalent Plus® antibody (LabVision Corporation), followed by incubation with Streptavidin-peroxidase Plus® (LabVision Corporation). Colour was developed with 3.3´diaminobenzidine and sections were counterstained with Gill's or Mayer´s hematoxylin, dehydrated and mounted for light microscopy evaluation.

Negative controls were obtained by omitting primary antibodies in anestrus samples, which were replaced with PBS and by the appropriated mouse or rabbit control immunoglobulins (normal mouse and rabbit immunoglobulin G (IgG); Santa Cruz Biotechnology, Heidelberg, Germany). No positive structures or cells were found in such sections.

As positive controls samples appropriated canine tissues were used; these varied with the target molecule and are detailed in individual chapters.

Table 3 – Primary antibodies used for immunohistochemistry

Antibody	Clone (ref.)	Source	Dilution	Thermal treatment	Incubation
anti-SOD1	Rabbit polyclonal (ab13498)	Abcam®	1:300	Microwave irradiation 750W; 3x5 min Citrate buffer pH 6.0	Overnight at 4ºC
anti-GPx1	Rabbit polyclonal (ab59546)	Abcam®	1:200		
anti-CD10	Mouse Monoclonal (56C6)	Novocastra™	1:50	Steamer (ca. 94ºC) Citrate buffer pH 6.0	
anti-E-cadherin	Mouse Monoclonal (4A2C7)	Invitrogen™,	1:100	Microwave irradiation 750W; 3x5 min 0.05% Extran solution	
anti-β-catenin	Mouse Monoclonal (pY86)	Invitrogen™	1:100		
IgG	Rabbit (sc-2027)	Santa Cruz	Used at an assay dependent concentration	Same method as for the corresponding target primary antibodies	
	Mouse (sc-2025)				

3.1. Immunohistochemical scoring

A blind assessment of the degree of staining was performed with a NIKON photomicroscope. Positivity was indicated by the presence of a distinct brown staining. Two independent observers performed a blind semi-quantitative assessment of the intensity of staining, in a representative area of the slide, usually covering the following structures:

1) the epithelial elements – individually assessing the immunoreaction in the surface epithelium (SE) and the glandular epithelium (GE), which might further be differentiated in superficial and deep glandular epithelium (SGE and DGE), whenever they behave differently;

2) the stroma (Str), which could be assessed globally or considering the following layers, according to the study: deep basal layer (DbS) (equivalent to the *stratum basalis* of the human endometrium), the intermediate and superficial layers (UpS)

(equivalent to the *stratum functionalis* of the human endometrium) and a sub-surface, adluminal layer (SSL), located just beneath the endometrial surface epithelium.

A positive immunoreaction was analysed considering the intensity of immunoreaction; whenever considered necessary further parameters were also evaluated, such as the subcellular dislocation of the immunolabeling or the percentage of labelled cells. Particularities for the scoring system used are provided in individual chapters.

4. Endometrial extracts – preparation of tissue homogenates

The assessment of the antioxidant enzymes in the canine endometrium (by biochemical methods) and the determination of the suitability of CD10 antibody (using western blots) demanded the use of tissue extracts, which were prepared from frozen samples.

Unthawed sampled tissues were first dissected to separate the endometrium from the surrounding myometrium and connective tissue. The suitability of the physical separation was confirmed in 3 samples that were subsequently preserved in formalin and processed for routine histological examination to confirm the inexistence of tissues other than the endometrium. After establishing the procedure, all the samples in analysis were prepared for the biochemical assessment of antioxidant enzymes activity.

After dissection, the endometrium was manually sliced into small pieces using a surgical blade and homogenized separately in 500 µL of saline phosphate buffer (PBS) (in mM: 1.76 KH_2PO_4, 10 NaH_2PO_4, 2.7 KCl, 137 NaCl; pH 7.0).

For western blotting analysis, PBS was supplemented with a cocktail of protease and phosphatase inhibitors (1 µl.mL^{-1} Sigma P4830 protease inhibitor cocktail). For the biochemistry methods, namely for the oxidative stress studies, the homogenates were further submitted to sonication (4 cycles of 5'' with 15'' interval at 80% amplitude; under refrigeration) for cell disruption. Thereafter, resulting extracts were centrifuged (Sigma 3K30, Rotor 12153) at 5000 xg for 10 min at 4°C. Protein was quantified by the method of Bradford [6] using bovine serum albumin as standard. To assess oxidative status, superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to the method of Paya and Halliwell [7], catalase (CAT, EC 1.11.1.6) activity was determined with a Clark type oxygen electrode, according to Del Rio *et al.* [8], glutathione reductase (GSR, EC 1.6.4.2) activity was assayed according to the method of Carlberg and Mannervik [9], glutathione peroxidase

(GPx, EC 1.11.1.9) activity was determined by the modified method of Paglia and Valentine [10], and glutathione S-transferase (GST, EC 2.5.1.18) activity was measured as described by Habig *et al.* [11]; to assess the lipid peroxidation index and the protein oxidation index we used the quantification of the as well as the formation of thiobarbituric acid-reactive species (TBARS) as described by Buege and Aust [12], and the total thiol content following the Ellman [13] as modified by Sedlak and Lindsay [14] and Suzuki *et al.* [15].

Further details on the methods used for western blot analysis are given on chapter 4, and those concerning the measurements of antioxidant enzymes and of lipid and protein oxidation are given on chapter 7.

5. Chapter reference list

[1] Rehm S, Stanislaus DJ, Williams AM. Estrous cycle-dependent histology and review of sex steroid receptor expression in dog reproductive tissues and mammary gland and associated hormone levels. Birth Defects Res B Dev Reprod Toxicol. 2007;80:233-45.

[2] Holst PA, Phemister RD. The prenatal development of the dog: preimplantation events. Biol Reprod. 1971;5:194-206.

[3] Payan-Carreira R, Pires MA, Strom Holst B, Rodriguez-Martinez H. Tumour necrosis factor in the canine endometrium: an immunohistochemical study. Reprod Domest Anim. 2011;46:410-8.

[4] Tsutsui T, Hori T, Okazaki H, Tanaka A, Shiono M, Yokosuka M, et al. Transfer of canine embryos at various developmental stages recovered by hysterectomy or surgical uterine flushing. J Vet Med Sci. 2001;63:401-5.

[5] Barrau MD, Abel JH, Jr., Verhage HG, Tietz WJ, Jr. Development of the endometrium during the estrous cycle in the bitch. Am J Anat. 1975;142:47-65.

[6] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.

[7] Paya M, Halliwell B, Hoult JR. Interactions of a series of coumarins with reactive oxygen species. Scavenging of superoxide, hypochlorous acid and hydroxyl radicals. Biochem Pharmacol. 1992;44:205-14.

[8] Del Rio LA, Ortega MG, Lopez AL, Gorge JL. A more sensitive modification of the catalase assay with the Clark oxygen electrode. Application to the kinetic study of the pea leaf enzyme. Anal Biochem. 1977;80:409-15.

[9] Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol. 1985;113:484-90.

[10] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med. 1967;70:158-69.

[11] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem. 1974;249:7130-9.

[12] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol. 1978;52:302-10.

[13] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82:70-7.

[14] Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 1968;25:192-205.

[15] Suzuki Y, Lyall V, Biber TU, Ford GD. A modified technique for the measurement of sulfhydryl groups oxidized by reactive oxygen intermediates. Free Radic Biol Med. 1990;9:479-84.

Chapter 4 – Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium

THE CONTENT OF THIS CHAPTER WAS PUBLISHED IN:

Payan-Carreira R, Santos C, Miranda S, Pereira RM, Santos D, Pires MA. Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium. Theriogenology. 2014;82:815-26.

TEMPORAL CHANGES IN NEUTRAL ENDOPEPTIDASE/CD10 IMMUNOEXPRESSION IN THE CYCLIC AND EARLY PREGNANT CANINE ENDOMETRIUM

R. Payan-Carreira[a]*, C.Santos[a], S. Miranda[a,b], R.M.L.N. Pereira[b,c], D. Santos[d], M. A. Pires[a]

Running Head: Neutral endopeptidase in dog endometrium

[a] CECAV, Universidade de Trás-os-Montes e Alto Douro, UTAD, 5000-801 Vila Real, Portugal

[b] Dept. Veterinary Medicine, University School Vasco da Gama, Coimbra, Portugal

[c] Strategic Unit for Biotechnology and Genetic Resources, National Institute of Agrarian and Veterinary Research (INIAV) – Pole at Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal

[d] CITAB, Universidade de Trás-os-Montes e Alto Douro, UTAD, 5000-801 Vila Real, Portugal

* Corresponding author: Rita Payan-Carreira, CECAV, Zootecnia Department, University of Trás-os-Montes and Alto Douro; Vila Real, Portugal; Fax: +351.259350482.

E-mail addresses: rtpayan@gmail.com; ritapay@utad.pt

Abstract

CD10 is a multifunctional transmembrane neutral endopeptidase (NEP), considered to be a reliable marker of ectopic human endometrial stroma. Available information in NEP/CD10 protein expression in animal endometria is scarce. This study focused on the immunolocalization of NEP/CD10 in the canine uterus and on its temporal changes during the estrous cycle and early pregnancy (days 11 to 23 post-LH surge) in healthy females. NEP/CD10 expression was found in the canine endometrial stroma in all stages of the estrous cycle, showing cyclic differences both in intensity and in distribution pattern. A small population of negative stromal cells in subsurface position was also observed. This

population shared some morphological characteristics with the human predecidual cells, which became positive in progesterone-associated stages of the cycle. In addition, positive immunolabelling was also observed in canine myometrial stroma. In early pregnancy, the basal glandular epithelia and the syncytium cords remained negative to this marker, contrasting with the trophoblast and the lacunar epithelium. A weak to moderate intensity of immunolabelling was observed in the decidual cells, while stromal immunolabelling was more intense at the delimitation of the syncytium cords. In conclusion, CD10 is consistently expressed in the canine endometrial stroma and myometrium, but not in endometrial epithelia. The characteristic pattern seen in early pregnancy also suggests a role for this molecule in the process of embryo invasion at implantation.

Keywords: Neutral endopeptidase; CD10; endometrial stroma; estrous cycle; early pregnancy; immunohistochemistry; female dog.

1. Introduction

The mammalian endometrium is a highly complex tissue that undergoes accurately defined, cyclic morphological changes in response to sex steroids stimulation. The ultimate goal is to guarantee embryo survival, implantation and the success of pregnancy [1, 2].

Although under the control of sex steroids, endometrial cyclic changes are ultimately controlled by several autocrine and paracrine factors that include a multitude of local molecules that determine proliferation of the epithelial endometrial elements, epithelial-stromal cells interaction and invasiveness, angiogenesis, apoptosis, differentiation, as well as immune cells infiltration, among others [3-5]. A correct equilibrium of these molecules, both in sequential changes and in quantity is essential for fertility [6].

Endometrial stroma cell functions are not limited to maintaining the endometrial structure; the stroma is involved in epithelial development and proliferation [7], cell adhesion, tissue remodelling and organ immune competence [8]. These are notorious during the cyclic changes and at implantation, particularly in species with deciduae placenta.

CD10 protein is a membrane-associated neutral peptidase, also known as neprylisin, enkephalinase, common acute lymphoblastic leukemia antigen or neutral endopeptidase (NEP) [9, 10]. CD10 is a 90- to 110-kDa cell-surface zinc-dependent metalloprotease, shown to be expressed by a widely variety of cell types and tissues, including the uterus [11]. CD10 functions as a cell surface enzyme, acting to reduce the cell response to some peptide factors, including oxytocin, endothelins and interleukin 1 [12]; through cleavage and inactivation of those peptides, NEP/CD10 reduces its local concentrations and decreases their effects [13-15].

NEP/CD10 has been implicated in the regulation of growth and differentiation in many cellular systems, in which it plays an important role in the maintenance of homeostasis [16-18], as well as in carcinogenesis and tumour progression [19-23], possibly mediated through its role on angiogenesis [24], in cell cycle activity [25] and apoptosis [26].

In human, NEP/CD10 is frequently used as a reliable immunohistochemical marker of normal endometrial stroma [27, 28], and is used for diagnosis of several neoplasic [28-30] and non-neoplasic [31, 32] gynaecological conditions. Yet, NEP/CD10 functions in the endometrium remain poorly understood.

Although clinical conditions such as endometriosis are not proven to exist in dogs, additional knowledge of the location and cyclic variation of this endopeptidase in the canine endometrium may be valuable, especially in pathological alterations such as cystic endometrial hyperplasia or when fertility may be compromised. In domestic animals, although previous work by Riley *et al.* [33] reported the presence of this enzyme in the sheep uterus, limited information is available on uterine pattern of the CD10/NEP protein expression.

The purpose for this study was to determine the pattern for NEP/CD10 protein expression in the normal canine endometrium by using an immunohistochemical technique and to investigate whether this pattern changes during the estrous cycle and in early pregnancy (days 11 to 23 post-LH surge). By establishing the normal pattern of CD10 expression in canine endometrium, this study will further allow provide reference data that might be essential in the study of endometrial diseases, especially in angiogenesis and stromal-epithelial cross-talk.

2. Material and methods

Tissue collection and preparation

Forty-seven post-pubertal, healthy non-pregnant bitches, and 17 pregnant females of different breeds and ages ranging from 10 months to 6-year old, were used in this study. Endometrial tissue, collected at ovariohysterectomy (OVH), was used with the owners' informed consent, in accordance to the International Ethical standards.

For immunohistochemistry, samples from the uterus were fixed in 10% formalin immediately after the surgery. Transversal fragments were collected from each uterine horn, embedded in paraffin wax, sectioned at 3 µm and stained with haematoxylin and eosin for histological staging of the estrous cycle and for excluding uterine disease. Samples showing histological signs of delayed uterine involution (glandular dysplasia and increased number of macrophages in the presence of large vessels within the *stratum vasculare*) or endometrial disease (such as cystic endometrial hyperplasia or pyometra) were excluded from the study. For the pregnant group, transversal samples were collected from zonary invasion areas and interplacental areas (or paraplacenta). When those were not distinguishable, longitudinal sections were obtained. For western blotting, adjacent 1 cm thick uterine sections were collected from non-pregnant samples and immediately snap frozen in liquid nitrogen before being stored at -70ºC, until analysis.

Before surgery, a vaginal cytological specimen was obtained and a blood sample was collected from the jugular vein into a controlled vacuum tube (*Serum-gel*, S-Monovette®, Sarstedt, Nümbrecht, Germany), centrifuged and stored at -20ºC until analysis. Serum progesterone levels were determined by chemiluminescence immunoassay system (Immulite®; DPC-Diagnostic Products Corp., Los Angeles, CA, USA).

Estrous cycle and pregnancy staging

Non-pregnant animals were initially selected on the basis of the vaginal cytology. At OVH, the stage of the estrous cycle for each bitch was determined by ovaries inspection and later confirmed upon the histological examination of the ovaries and by progesterone levels, which were used in the fine tuning the histological staging [8]. Uterine samples for the proestrus (n=9), estrus (n=8), diestrus (n=20) and anestrus (n=10) were used in this study.

Considering that, in carnivores, implantation is not an early event [34], with dog embryos interacting with the endometrium around post-ovulatory day 16 [35], the diestrus was further divided in two stages: an early diestrus period (n=10), with rising progesterone levels and with young, cavitary corpora lutea in the ovaries; and a full diestrus period (n=10), with high progesterone levels and mature, compact, active corpora lutea in the ovaries.

Pregnancy samples were obtained from females with unwanted pregnancies up to 3 weeks post-coitus, submitted to OVH. Pregnancy samples were then staged on the basis of cumulative information gathered from diestrus-compatible cytology, known unwanted breeding plus high progesterone levels and the co-existence of small-sized (<3 cm) uterine swellings. When uterine swellings were not noticed, but knowledge of coitus existed, pregnancy estimated of less than 17 days was determined upon embryo collection by flushing each uterine tube and uterine horns separately, as described by Tsutsui *et al.* [35]. Pregnancies were further divided in two groups: group 1 (PGr1; n=5) corresponded to the period previous to embryo attachment to the maternal endometrium and mainly corresponded to pregnancies days 11 to 13, were morulae or young blastocysts were found in the oviducts or in the uterus; group 2 (PGr2; n=12) corresponded to pregnancy days 15 to 23. Between pregnancy days 16 to 21, morphological changes in the endometrium are visible although embryos are still non-adherent. After day 22 embryos attach and invasion begins. The chronology of the pregnancy was confirmed according to the histological descriptions of canine early pregnancy events [8] and aligned to the day from pre-ovulatory surge [37]. Briefly, by day 13 to 15 some changes in the superficial endometrium are observed, including increased interstitial edema and depth of the endometrial crypts. Around day 17 the embryo is apposed to the endometrium; the trophoblast grows down and wedges the maternal surface epithelium (SE). Only small lacunae are visible. By day 20, the trophoblast continues to spread down, and the syncytial cells penetrate deeper in the endometrium appearing as strong, linear cords, frequently presenting mitotic figures. After day 22, the crypts at implantation sites elongate, and became tortuous and closely packed, with enlarged lacunae below. The deep endometrial glands start to grow [34, 36].

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 3 µm thick tissue sections on silane-coated slides were used for the immunohistochemistry study of CD10 expression in the bitch endometrium, using a streptavidin-biotin-peroxidase technique (UltraVision Detection System, Fremont, CA, USA) with a monoclonal antibody to CD10 (clone 56C6, reference NCL-CD10-270; Novocastra®, New Castle Upon Tyne, UK). Sections were routinely deparaffinized in xylene and hydrated through graded alcohol and water. Antigen retrieval was performed in a steamer, with slides immersed in boiling citrate buffer (pH 6.0; *ca*. 94ºC) for 2 minutes. After blocking the endogenous peroxidases in 3% hydrogen peroxide/PBS for 30 minutes and non-specific binding by incubation with Ultra V-Block® for 5 min, the slides were incubated overnight with the primary antibody at a 1:50 dilution in PBS at 4ºC, in a humid chamber. Thereafter, samples were incubated with a biotin conjugated secondary antibody and then incubated using streptavidin-biotin system, for 10 minutes each, at room temperature. Reactions were visualized using DAB (3.3'-diaminobenzidine) as chromogen. Sections were then counterstained with Gill's haematoxylin, dehydrated and mounted.

Sections from canine ovaries were included as negative controls, since ovarian stroma is negative for CD10/NEP. Additional negative controls were used, whereby endometrial specimens were submitted to the same procedure, with the exception that the primary antibody was omitted and replaced by PBS or by a normal mouse IgG (sc-2025; Santa Cruz Biotechnology Inc., Europe, Heidelberg, Germany). In neither negative control was CD10-immunoreactivity observed.

Immunohistochemical scoring

Microscopic examination at low magnification (40x) was performed to examine the overall pattern of immunoreaction against NEP/CD10 in the canine uterus. Thereafter, staining results were evaluated at a higher magnification (200x and 400x) to define the intensity and the pattern of the immunoreaction. Positivity was indicated by the presence of a distinct golden-brown cytoplasmic labelling.

Two independent observers performed a blind semi-quantitative assessment of the intensity of staining, using a three-point score classification (weak, moderate and strong), operator-wise. The repeatability of the results from both observers, in all the 3-point scales

used for scoring, was assessed in selected samples from anestrus, estrus and diestrus; only cases providing repeatable scores in immunostaining were used.

In cyclic endometrial samples, positive reaction was scored independently for each endometrial component (Stroma – S, Surface Epithelium – SE and Glandular Epithelia – GE). According to the pattern perceived under small magnification, the endometrial stroma in cyclic samples was further evaluated individually for the deep basal layer (DbS; equivalent to the *stratum basalis* of the human endometrium), the intermediate and superficial layers (equivalent to the *stratum functionalis* of the human endometrium and thereafter named as upper stromal layer - UpS) and a sub-surface, adluminal layer (SSL), located just beneath the endometrial surface epithelium, which could correspond to the endometrial *stratum compactum*.

The same stromal regions were used to analyse NEP/CD10 immunoreaction in samples from pre-attachment pregnancy (PGr1). In the samples from the attachment period (PGr2), individual scoring was performed for the trophoblast and the epithelium of the syncytium cords (or *lamellae*), the *lacunae* and the deep glands, as well as for the adluminal decidualized stroma, the syncytial and the intermediate and deep endometrial stromal layers.

Western Blot analysis

Western blotting analysis was used to test the specificity of the human CD10 antibody labelling in canine endometrium. For western blotting analysis, fragments of five frozen unthawed tissues corresponding to different stages of the canine estrous cycle (anestrus, early diestrus and diestrus) were homogenized separately in ice-cold phosphate buffer saline (PBS in mM: 1.76 KH_2PO_4, 10 NaH_2PO_4, 2.7 KCl, 137 NaCl; pH 7.0) supplemented with a cocktail of protease and phosphatase inhibitors (1 $\mu l.mL^{-1}$ Sigma P4830 protease inhibitor cocktail). Protein concentration was determined by the Bradford method.

After denaturation at 95 ºC for 5 min in a Laemmli buffer (in mM: 25 Tris, 182 glycine, 0.1% SDS; pH 8.3), equal protein (25 µg) of each sample were loaded in duplicate and electrophoresed on SDS–polyacrylamide gel for 90 min at a constant 100 V and transferred onto polyvinylidene difluoride membrane at 100 V for 90 min at 4ºC. Membranes were blocked for 60 min at room temperature, in Tris buffered saline (in mM: 20 Tris–HCl; 137 NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% skimmed milk. Blots were then incubated for 1 h with the primary antibody (NCL-CD10-270; Novocastra®, New Castle Upon Tyne, UK) diluted at 1:1000, with gentle agitation. The solution of primary antibody was

prepared in 1% fat free dry milk in TBS-T. After extensive washing with 0.5% fat free dry milk in TBS-T solution, immunodetection was performed with WesternDot 625 goat anti-mouse western blot kit. Membranes were imaged using a Versa Doc instrument (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

The IBM SPSS Statistics Base 19.0 statistical software for Windows® was used to perform statistical comparisons. Statistical analysis of the differences in the intensity of immunoexpression for CD10/NEP between the stages of the estrous 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. The Z-test was performed for group comparisons (stage of the estrous cycle; early diestrus *vs.* pregnancy; distribution for the diverse stromal layers).

3. Results

Specificity of the CD10/NEP antibody, developed in mouse for the human molecule, was assessed by Western blot analysis in canine endometrial cell lysates. On those blots, the antibody used recognised a molecular band of approximately 100 kDa, consistent with the reported molecular weight of CD10 protein (Figure 1).

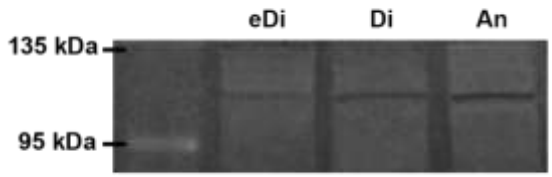


Figure 1 – Western blot for anti-CD10 showing bands corresponding in size to the 100 kDa protein in extracts of canine endometrium.

CD10 immunoexpression in the canine uteri was present in all samples analysed. Data gathered for this study on the canine endometrial expression of CD10 molecule are summarized in Tables 1 and 2.

Table 1 – NEP/CD10 immunoreactivity scores in the endometrial samples of female dogs throughout the stages of the estrous cycle and in pre-attachment PGr1.

	Scores	Anestrus (n=10)	Proestrus (n=9)	Estrus (n=8)	Early Diestrus (n=10)	Diestrus (n=10)	PGr1 (n=5)
SSL	neg	10	9	6	0	0	0
	1	0	0	2	10	8	2
	2	0	0	0	0	2	3
	3	0	0	0	0	0	0
UpS	neg	0	0	0	0	0	0
	1	0	1	4	9	1	2
	2	2	7	4	1	8	3
	3	8	1	0	0	1	0
DbS	neg	0	0	0	0	0	0
	1	2	1	3	3	3	0
	2	1	2	4	5	3	5
	3	7	6	1	2	4	0
SE	neg	10	9	8	10	10	5
	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
GE	neg	10	9	8	10	10	5
	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0

(SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DbS – deep basal layer; SE - Surface Epithelium; GE - Glandular Epithelia).

Table 2 – NEP/CD10 immunoreactivity scores in the implantation area in samples from canine pregnancy days 16 to 23 (attachment period, PGr2).

Scores	**Trophoblast**	**Decidual cells**	**Lamellar stroma**	**Lamellar epithelium**	**Intermediate stroma**	**Lacunar epithelium**	**DGE**	**Deep stroma**
neg	0	0	2	12	0	0	12	0
1	5	4	8	0	1	12	0	9
2	7	8	2	0	2	0	0	3
3	0	0	0	0	9	0	0	0

Assessment of endometrial samples at low magnification showed that during all stages of the estrous cycle positive immunoreaction existed both at the endometrial stroma

and the myometrium, but not in the epithelial elements of the endometrium (Figure 2; Table 1; Graph 1).

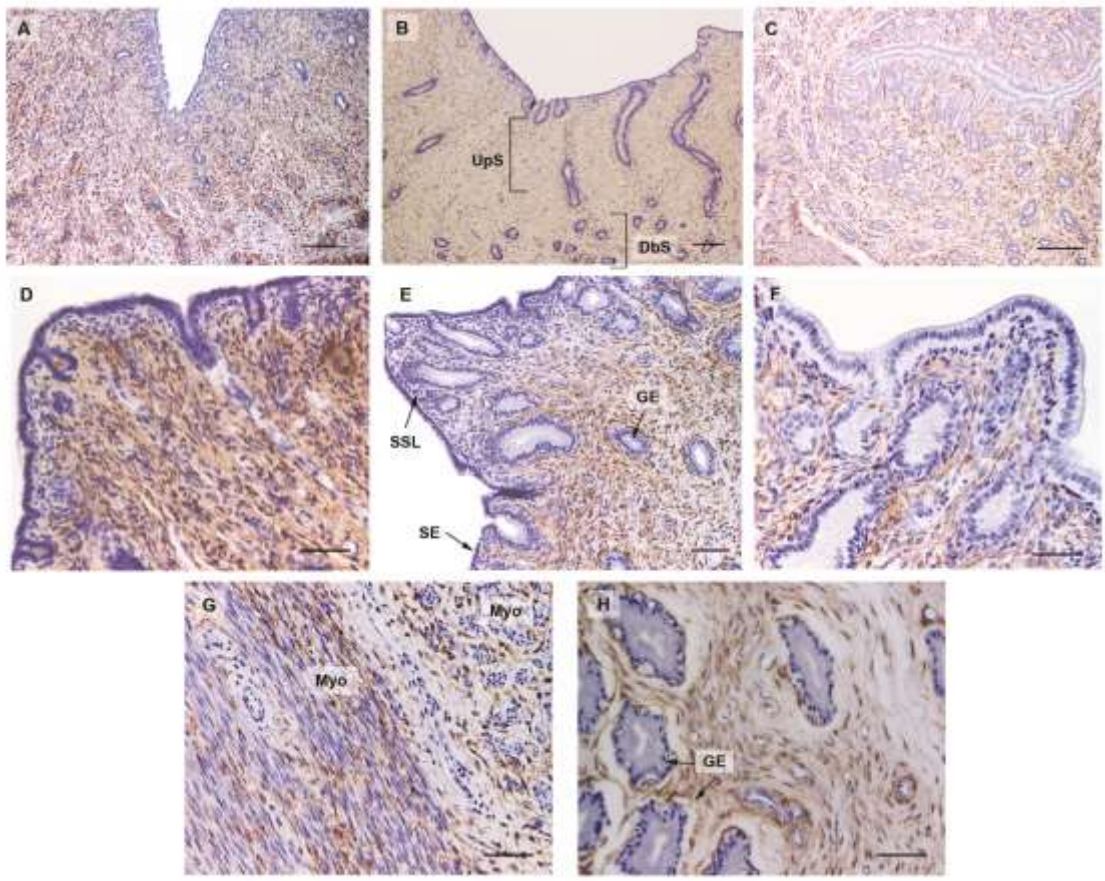
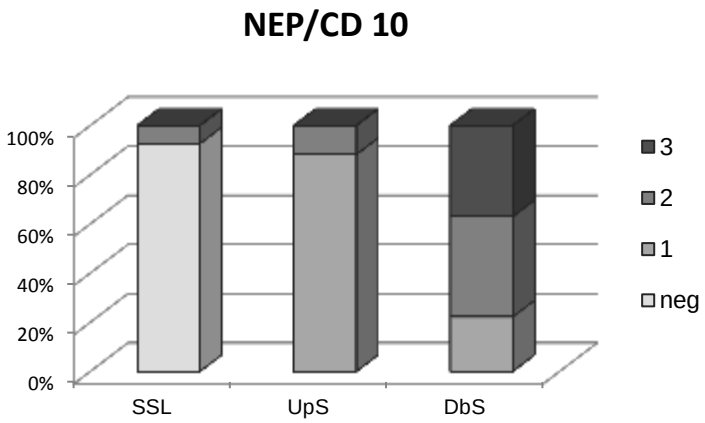


Figure 2 – Immunohistochemical expression of NEP/CD10 in normal canine endometrium (counterstained with Gill´s Haematoxylin; bar: 100 µm). Overall, epithelial endometrial cells are negative for this protein. (**A**) In anestrus, an uniform strong immunoreaction against NEP/CD10 was observed in the endometrium. (**B**) In proestrus, though a homogeneously strong to moderate intensity of immunostaining was observed in the endometrium, the intense oedema difficult the visualization of the immunostaining. On the image are depicted the upper stromal (UpS) and the deep basal (DbS) stromal areas. (**C**) In early diestrus and diestrus, NEP/CD10 expression was decreased compared to non-progesterone stages of the cycle. (**D**) In higher magnifications, in anestrus as in proestrus it was clearly identified a subsurface layer negative for NEP/CD10. (**E**) The subsurface stromal layer (SSL) remains negative for most of the estrus length; the surface epithelium (SE) and of the glandular epithelium (GE) are negative for NEP/CD10. (**F**) In early diestrus and diestrus all the stromal cells in the subsurface layer are positive to NEP/CD10. (**G**) Strong to moderate intensity of immunolabelling was found in myometrium (Myo), were positivity was identified in cells perimysium, while the myocytes remained negative. (**H**) Detail of the deep endometrial layer showing a moderate intensity of immunolabelling; it was also often found that an increased density of stromal cells around the deep glandular elements (GE) may confer to this particular area the appearance of increased intensity.

In the myometrium, smooth muscle cells were negative for NEP/CD10, the immunoreaction being restricted to the cells perimysium (Figure 2G). In general, the intensity of myometrial immuno labelling tended to match that of deep basal stroma, and did not vary significantly during the estrous cycle (P = 0.054; Fisher = 14.353).

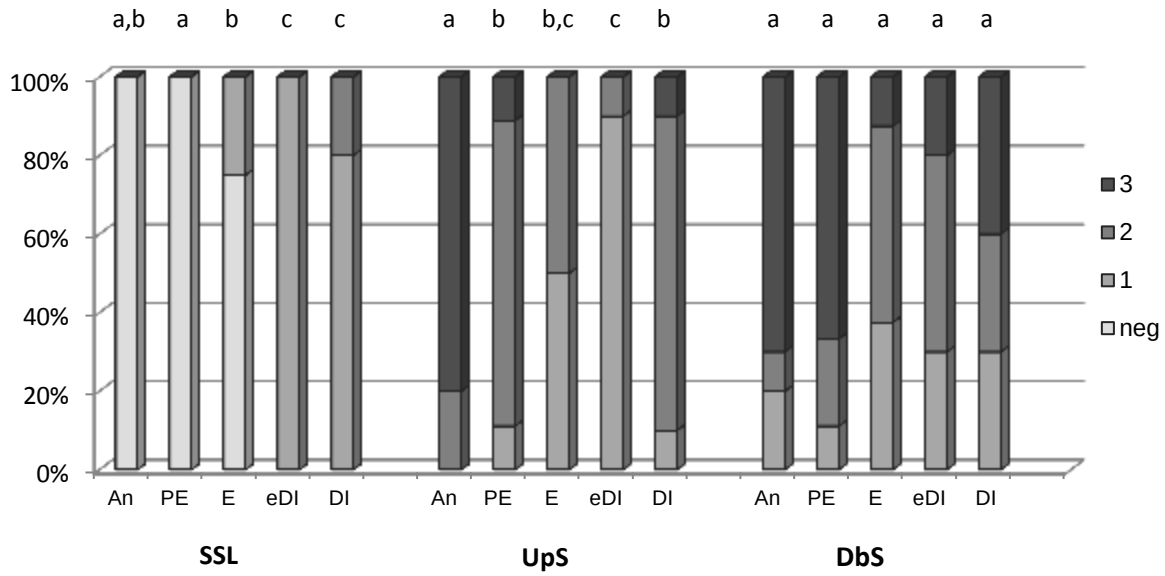
Within the endometrium, cytoplasmic NEP/CD10 expression was consistently found in the endometrial stroma throughout all stages of the estrous cycle of the bitch (Table 1). However, immunolabelling differences in intensity and distribution pattern were seen in different stages of the estrous cycle (Graph 2; Figure 2). When comparing the relative overall intensity of immunostaining in the canine endometrial stroma (Table 1; Graph 1), the differences among the cycle stages were greater in the subsurface and the upper stromal layers (P < 0,001; Fisher = 40.499 and 33.427, respectively), which also presented lower overall intensity scores than the deep basal layer (P = 0,050; Fisher = 13.357). Furthermore, overall staining scores were higher in anestrus and proestrus, than in other stages, when suprabasal levels of progesterone exist (P < 0.001; Fisher = 98.417).



Graph 1 – Graphic representation of the overall intensity scores for NEP/CD10 in canine endometrium (SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DbS – deep basal layer).

In anestrus, strong immunoreaction against NEP/CD10 was observed (Graph 2) in a rather uniform distribution pattern throughout the endometrial stroma (Figure 2.A), with the exception of a negative stromal population located beneath the surface epithelium. NEP/CD10 negative population was composed mainly of fusiform cells, while scant in

cytoplasm, with poorly defined cell borders and ovoid or elongated, dense nuclei without visible nucleoli (Figure 2.D).



Graph 2 – Graphic representation of the temporal variations in the relative intensity scores for NEP/CD10 in the different endometrial layers (SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DBS – deep basal layer) during the canine estrous cycle.

During proestrus, a diffuse stromal immunoexpression was observed in lower magnifications, partly due to endometrial oedema and red blood cells infiltration. At higher magnifications, upper stromal cells showed a decrease in the intensity of immunolabelling in comparison to anestrus (P = 0.020; Fisher = 6.798), with prevalence of a moderate intensity of immunostaining and a homogeneous pattern observed in the entire endometrium. In this stage, the subsurface stromal area beneath the surface epithelium remained negative for NEP/DC10 (Figure 2.D). In general, overall immunoreaction against this neutral endopeptidase in proestrus was not different from that found in anestrus (P = 0.115; Fisher = 5.912).

In the estrus stage, a relative, non-significant decrease on NEP/CD10 expression was observed in the upper and deep basal endometrial stroma, when compared to those of proestrus (P > 0.104; Fisher = 3.310 and 4.930, respectively). Furthermore, an apparent increase in the endometrial stroma intensity of staining was perceived at lower

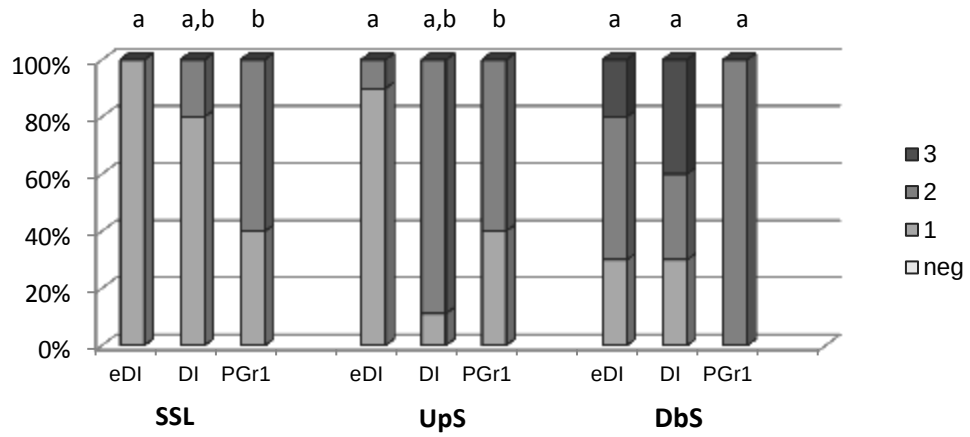
magnifications (Table 1). The deep basal stromal layer showed a relatively stronger cytoplasmic expression than the upper layer, particularly around deep basal glands (Figure 2.B). A predominance of fusiform NEP/CD10 negative stromal cells was observed underneath the surface epithelium (Figure 2.E), although in some of the samples (n=2; 25%) a faint NEP/CD10 positivity was observed. Overall immunoreaction against this molecule in estrus differed from that found in proestrus (P = 0.005; Fisher = 12.465).

In early diestrus, a decrease in NEP/CD10 expression was detected in the upper and deep basal endometrial stroma (Table 1; Figure 2.C). The upper stromal layer cells tend to show lower NEP/CD10 expression than the deep basal stromal layer (P = 0.05; Fisher = 17.084). All stromal cells in the subsurface layer are now positive to this protein, contrasting to the observed in previously described stages (P = 0.005; Fisher = 7.976) (Figure 2.F); the majority of these cells, with scanty cytoplasm, now present round to ovoid nuclei and rough chromatin, with the nucleoli seldom visible. The overall intensity of immunoreaction against NEP/CD10 was significantly different from the one evidenced in estrus (P = 0.038; Fisher = 7.434).

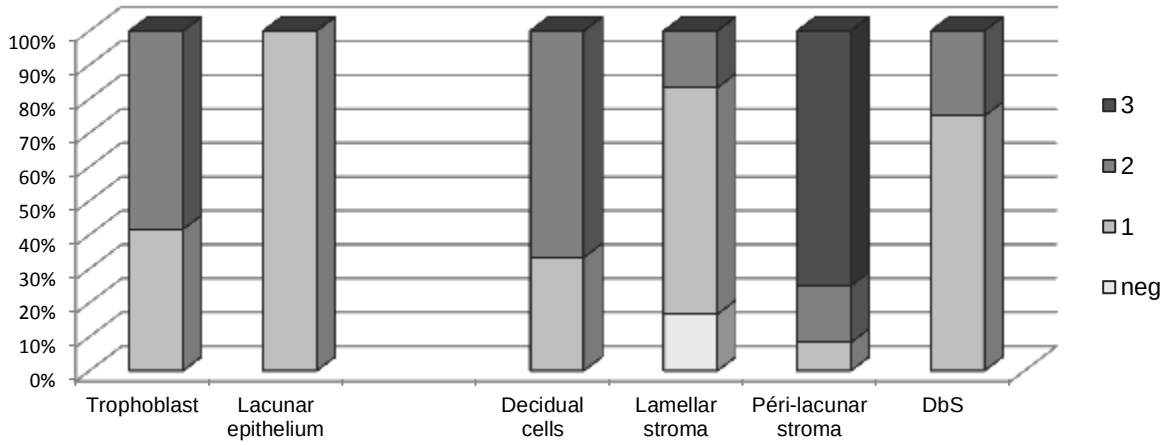
In full diestrus, apart from the subsurface stromal layer, NEP/CD10 immunoexpression observed in the endometrial stroma resembled that of the proestrus and estrus stages. Stromal cells adjacent to the surface epithelium showed morphological features similar to those described for early diestrus, maintaining identical positive immunoreaction for NEP/CD10. No statistical differences were found in the intensity of immunolabelling between early diestrus and full diestrus for the subsurface and deep stromal layers (Figure 2.H), despite the slight increase in the overall staining for these layers; the upper layer stroma showed a marked increase in the intensity of the immunoreaction when compared to the one observed in early diestrus (P = 0.001; Fisher = 12.847). Nonetheless, the overall intensity of immunostaining was considered different between these two stages (P = 0.032; Fisher = 6.647).

NEP/CD10 immunoreaction was also found in all the samples from canine pregnancy days 11 to 13 (PGr1; Table 1 and Graph 3) and 16 to 23 (PGr2; Table 2 and Graph 4). In the pre-attachment period (PGr1), no morphological changes in the endometrium developed in comparison to early diestrus, despite the differences found in the overall NEP/CD10 immunolabelling (P = 0.002; Fisher = 11.415). There was a relative increase in the intensity of immunoreaction against NEP/CD10 in the subsurface and upper stromal layers compared to that of early diestrus (respectively P = 0.004, Fisher = 8.282 and P = 0.031; Fisher = 7.166; Graph 3), but not to that of full diestrus. In contrast, non-significant differences in immunostaining were observed in the deep basal stroma between groups. In contrast to the

observed throughout the canine estrous cycle, a moderate to strong immunoreaction against NEP/CD10 was observed in apical position in the epithelial cells of more than 75% of the endometrial glands, although no cytoplasmic immunostaining was visible.



Graph 3 – Graphic representation of the relative intensity scores for NEP/CD10 in the pre-attachment period of canine pregnancy (PGr1) and the non-pregnant endometrium in early and full diestrus (SSL – sub-surface, adluminal layer of endometrium; UpS – Upper stromal layer; DBS – deep basal layer).



Graph 4 – Graphic representation of the relative intensity scores for NEP/CD10 in the attachment period of canine pregnancy (PGr2).

In samples from the attachment period (PGr2), in the implantation area, the intermediate peri-lacunar stroma was found to be more compact than the basal stroma (Graph 4), and displayed a strong intensity of immunostaining when compared to equivalent endometrial layer in PGr1 (*P* = 0.09; Fisher = 8.055), thus giving to the overall pattern of the organ the appearance of a ring or a barrier (Figure 3.A to 3.D).

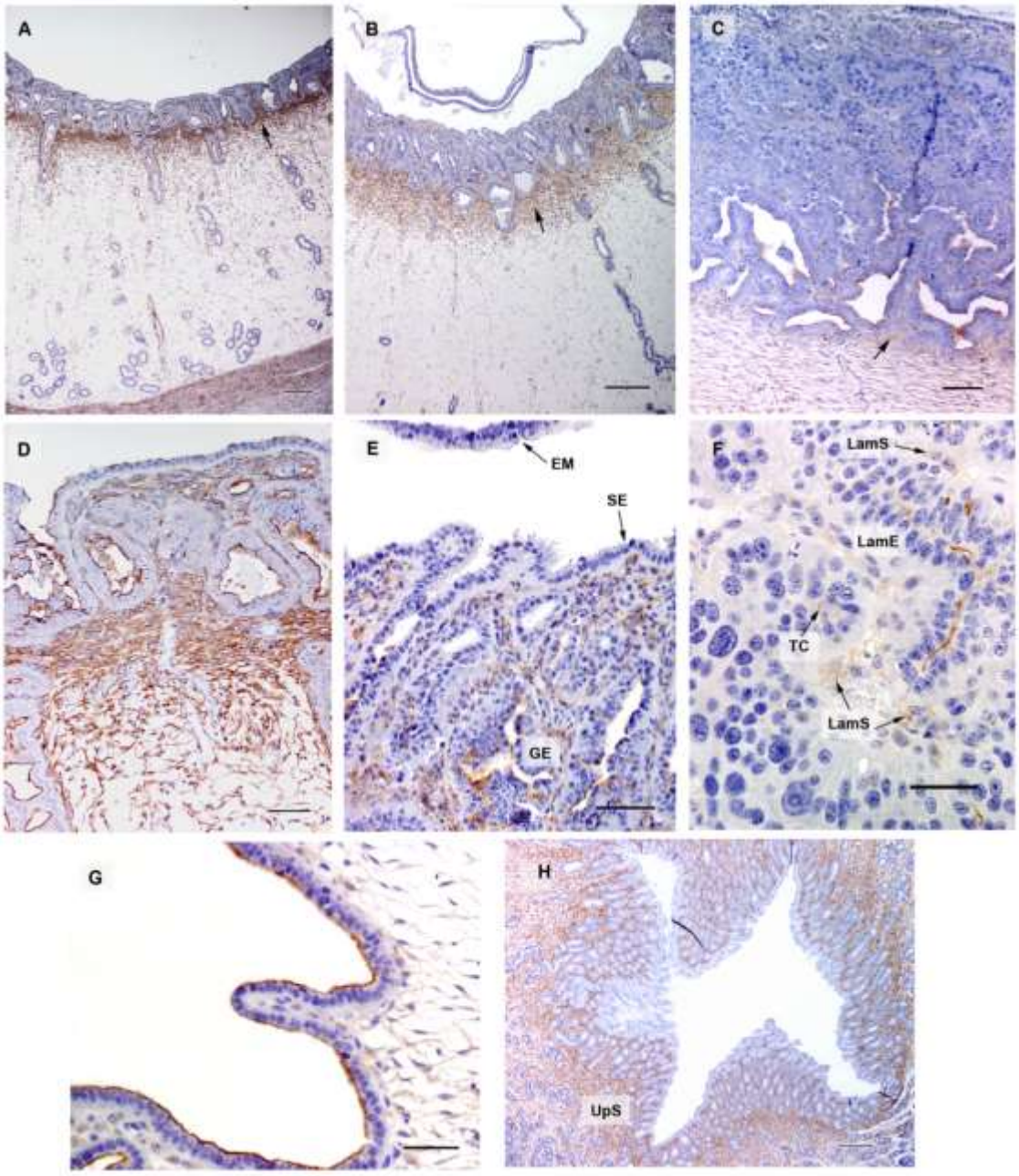


Figure 3 – Immunohistochemical expression for NEP/CD10 in the early stages of canine pregnancy (PGr2) (counterstained with Gill´s Haematoxylin; bar: 100 µm). In low magnifications (images A to C), the immunoreaction against NEP/CD10 showed a ring-like area of increased intensity of immunostaining (arrow) surrounding the invading trophoblast, compared to the faint intensity of labeling in the remainder of the endometrial stroma. (**A**) On pregnancy day 15. (**B**)

Around pregnancy day 17. (**C**) After pregnancy day 22, this ring-like area was reduced. (**D**) A detail of the superficial area in the implantation area on day 15, focusing the ring-like area, displaying increased intensity of immunolabelling along with an increased density of stromal cells. (**E**) Detail of the upper endometrial area on day 17 with the decidual cells showing strong intensity of immunolabelling, while the endometrial epithelia (SE – surface epithelium; GE – Glandular epithelium) remain negative for NEP/CD10; embryo membranes (EM), still unattached, show faint intensity of immunolabelling. (**F**) After day 22, cells from the cords or lamellae (LamE) are negative to NEP/CD10. A faint positive reaction was recorded in the invading trophoblast (TC), while the lamellar stroma (LamS) showed faint to moderate immunoreaction. (**G**) By day 22, the epithelial cells of lacunae displaying an apical reinforcement of immunostaining. (**H**) In the interplacental areas the upper endometrial stroma (UpS) showed a strong immunostaining similar to the observed surrounding the invading trophoblast in matched samples.

In comparison to subsurface stromal layer pattern in PGr1, no differences were found between pre-attachment and attachment periods, although the decidual cells located closer to the foetal-maternal interface showed an increased intensity of immunolabelling. Furthermore, the scores for decidual cells were significantly higher than the recorded for stroma at the syncytium cords (P = 0.038; Fisher = 6.553) (Figure 3.D to 3.F). The embryo trophoblast showed a weak to moderate intensity of immunolabelling (Graph 4). However, the cells from the cords or lamellae are negative to NEP/CD10 (Figure 3.F). A faint cytoplasmic immunoreaction for this molecule was observed in lacunar epithelial cells, which displayed an apical reinforcement (Figure 3.G), although the deep glandular epithelium remained negative for this molecule. This same fact was observed during the canine estrous cycle. Sporadically, apical moderate to strong positivity was found in DGE cells.

In the interplacental areas, an increased intensity of immunostaining was observed in the upper stroma (Figure 3.H) similar in intensity to the one registered for the area adjacent to the syncytium cords in matched samples. Both surface and glandular epithelia were negative to the NEP/CD10.

3. Discussion

NEP, a neutral metalloendopeptidase, also named as CD 10 antigen, has been proposed as a marker for normal endometrial stroma in women [27, 28] and is considered a valuable marker for ectopic endometrial stroma identification [28, 31]. In this study, we localized for the first time NEP/CD10 expression in the normal bitch endometrial stroma

where it showed a diffuse cytoplasmic staining, similar to that described in human normal endometrium [13, 37]. It was also possible to demonstrate the existence of a temporal variation in the expression pattern during the canine estrous cycle. In opposition to the described in women, we found NEP/CD10 expression in the canine myometrium. Myometrial positivity for this molecule has also been reported in sheep [33] and in the uterus of pregnant mice [38]. In contrast to that described in sheep [33], a positive reaction for NEP/CD10 in the canine myometrium was found only in the perimysium of the smooth muscle cells, whatever of the myometrial layer considered. A possible explanation is that the differences in the immunostaining intensity observed in small magnifications are due to differences in the arrangement of bundles of smooth muscle fibres in myometrium layers. It has been proposed that NEP/CD10 functions in smooth muscle layers might include oxytocin cleavage and the regulation of uterine contractibility [33, 38]. However, in the present study no significant changes were found between the stages, thus impairing any inference.

The present work also shows that NEP/CD10 expression is observed in cyclic variations according to the phase of the canine estrous cycle. In women, diffuse NEP/CD10 expression is observed in the functional and basal layer of the endometria, although more intense staining was detected in the deeper portion of the functional layer and in basal layer [13, 29, 37]. In the canine endometrium, the lowest variations in the intensity scores were found for the deep basal stromal layer, which is in accordance with that reported in women, where the basal endometrial stroma has been described as relatively independent of sex steroids influences [37]. In dogs, estrogen associated stages and anestrus were the stages with higher NEP/CD10 expression.

The current study also allowed the identification of a small population of NEP/CD10 negative stromal cells located beneath the surface epithelium. As far as we know, there are no previous reports on the existence of a consistently NEP/CD10 negative population of stromal cells in non-luteal stages, despite the report by Riley *et al.* [33] on the presence of a stromal layer of decreased intensity in sheep endometrium, in both the caruncular and intercaruncular areas of cyclic endometrium. However, the authors did not comment on putative cyclic changes in this pattern of immunostaining. Such a layer would correspond to the stromal compartment described by Johnson *et al.* [39], proving morphological characteristics of decidualization during early pregnancy, which was also osteopontin (OTP) negative.

In the bitch, NEP/CD10 negative cells, located underneath the surface epithelium, are fusiform cells showing an ovoid nucleus of dense chromatin that tend to become round and rough during the progestagenic stages of the estrous cycle. These NEP/CD10 negative cells

in the endometrial stroma of the bitch then share some morphological resemblances with predecidual cells, which are detected in the women endometrium in middle to late secretory phase [40, 41]. The canine NEP/CD10 negative stromal cells express vimentin during the entire estrous cycle and also desmin during the estrus and diestrus (Payan-Carreira, data not shown); furthermore, they are negative for OTP, except during the early diestrus stage. Vimentin and desmin expression was also found in predecidual and decidual stromal cells of women and mouse [42-44]. Considering that, in dogs, experimental deciduoma formation is possible in the progesterone-primed uterus [46,47], inducing placenta-like lesions, we consider the possibility of a spontaneous pre-decidualization differentiation of this particular population of stromal cells occurring in the canine endometrium during progesterone-associated stages, favouring decidualization during canine embryo implantation as a part of the endometrial receptivity mechanism in dogs. This pre-differentiation would further progress into decidualization in a process possibly mediated by the embryo.

In the endometrium of the bitch, major temporal differences in NEP/CD 10 expression included: 1) an increased intensity of immunostaining against NEP/CD10 in canine anestrus, when basal estrogen and progesterone levels exists in the canine estrous cycle [45]. This result is similar to the reported by Riley *et al.* [33] for ovariectomized non-supplemented sheep; 2) a decrease in the overall expression for this molecule during early diestrus, the phase corresponding to the implantation period for the species. Iwase *et al.* [46] reported a similar decrease in NEP/CD10 expression in the human endometrium using a semi-quantitative western blot technique. A decrease in NEP/CD10 expression has also been reported in sheep [33], in early diestrus and again around luteolysis.

Riley *et al.* [33] report a different pattern for NEP/CD10 expression in the caruncular and intercaruncular areas of the sheep endometrium. In contrast, in canine pregnant endometrium no differences were found from scorings in matching longitudinal cuts, which correlates with the uniform distribution of the glandular elements across the canine endometrium and suggests the inexistence of pre-determined implantations sites in this species. Evidences obtained with different markers, such as integrin αvβ3 [47], tumour necrosis factor [8] and interleukin 18 [48] further support this feature.

In early pregnant endometrium in human and sheep, a decrease in the NEP/CD10 overall expression has been reported [33]. However, available data is still insufficient to determine the regional pattern of immunoexpression of NEP/CD10 in peri-implantation endometria. In our study, a relative decrease in the intensity of immunolabelling was found in the endometrial stroma at the syncytial cords areas, adjacent to the faint to moderate positivity of trophoblastic cells, similar to human early pregnancies [42]. However, a new fact

is the observation of a ring-like stronger positivity for NEP/CD10 at the intermediate peri-lacunar area, suggesting a possible barrier to the progress of embryo invasiveness.

Although useful as a marker of endometrial stroma in human endometriosis, the role of NEP/CD10 in endometrial function remains unclear and needs to be further studied. Initially, the role proposed for this molecule in the endometrial stroma was limited to its catalytic activity over small bioactive peptides, thus decreasing its concentration and inhibiting its local function. A broad range of peptides have been proposed to serve as substrates to NEP/CD 10, such as bradykinin, substance P, atrial natriuretic peptide, interleukin 1β, oxytocin and endothelin [13-15]. Endothelin, interleukin 1β and oxytocin are known to be present in the endometrium of different species [46]. However, additional roles have been advanced in past years for NEP/CD10 besides the regulation of peptide bioactivity, including its interplay with different signalling mechanisms related to cell growth and proliferation, apoptosis and angiogenesis [24, 49, 50]. Studies developed on distinct malignancies and on several immuno-mediated pathologies revealed that NEP/CD10 is involved in the regulation of inflammation [16, 18, 51], in angiogenesis [24], and also in the regulation of cell proliferation [25], migration [15, 19] and apoptosis [20-22].

The endometrial cycle, in women as in other mammal females, integrates a highly precise and orchestrate events that, drove by cyclic sex hormone changes, also include mechanisms of regeneration, cell growth, proliferation and migration, modulation of angiogenesis and neovascularization, as well as the control of invasiveness during embryo implantation. Furthermore, epithelial and stromal interactions in the uterus are major determinants in the success of the endometrial cycle [52]. Moreover, several molecules known to interact with NEP/CD10, such as endothelins, integrins, interleukin 1β and PTEN, have been located in the endometrium of different species [4, 53].

The transforming growth factor beta1 (TGF-β1) is an important stimulator of NEP/CD10 activity [54]. In dog endometrial stroma, TGF-β1 immunoexpression presents the temporal changes described herein for NEP/CD10 [55]: its immunoexpression was higher in anestrus and proestrus and the lowest in early diestrus, while estrus and diestrus presented intermediate scores. Considering the close association between the temporal pattern between these two molecules, and the important role of TGF β family in endometrial remodelling in human endometrium [56], the possibility that NEP/CD10 may also play an important role in cyclic endometrial regeneration, cell proliferation and differentiation phenomena during the endometrial cycle can not be excluded, thus exceeding the functions of a simple stromal marker usually assigned to this molecule.

Many NEP/CD10 actions involve the interplay with signal transduction via AkT (also called as protein kinase B) pathways [49], through catalytic-dependent and independent mechanisms. AkT are important pathways for numerous cellular functions in the uterus, such as proliferation, adhesion, migration, angiogenesis and apoptosis [57]. It is important to acknowledge that modulation of AkT pathways in the stromal cell decidualization has been previously demonstrated [58, 59]. Moreover, AkT has been located in human and mice endometrium, in both stroma and epithelial cells, and the phosphorylation of this protein presents temporal variations in the endometrium [60, 61]. Additionally, NEP/CD10 negatively regulates angiogenesis via different signalling mechanisms [24, 49] and small increased vascularization in the superficial stromal layer is observed in the canine endometrium in the initial diestrus, which becomes more evident when embryos are present [62]. Although not assessed in the present study, it would be reasonable to conjecture that these two molecules might present complementary functions in canine endometrium. Thus, when NEP/CD10 expression in endometrial stroma is strong, as we found in canine endometrium during anestrus, an important stage for endometrial remodelling, impairment of AkT pathways would restrict cell proliferation; reduce cell protection against apoptosis and limited vascularization in the endometrial stroma. Future studies will be endorsed to explore the involvement of AkT pathways in the mechanisms of the canine endometrial cycle.

An interesting feature observed in samples from the attachment period concerns the existence of a stronger immunoreaction against NEP/CD10 in the stromal area neighbouring the *lacunae*, while in the syncytium crypts stromal cells are negative. The former feature assumes the appearance of a barrier to embryo invasiveness and may be part of a strategy to limit the superficial endometrial changes during the formation of placenta. The later coexists in an area of intense morphological changes and trophoblast invasion. NEP/CD10 possesses inhibitory effects on cell migration that have been described in different organs and neoplasia, where a decrease in NEP/CD10 is envisaged as a prognosis for malignancy and invasiveness [24, 49]. Surprisingly, in samples from days 22 and 23 of pregnancy, positive apical staining was noticed in epithelial cells of lacunae. NEP/CD10 is expressed as a typical brush-border enzyme in enterocytes and renal tubules and glomeruli [10]; it is also associated with human trophoblast or chorion tumours [63]. Moreover, some soluble variants of NEP/CD10 have been detected in human urine [64], although it remains unclear if they are produced in a soluble form or are shed from a membrane-bound form. In contrast to the negative expression of NEP/CD10 protein in normal endometrial epithelial cells during the canine cycle, in an early report on endometrial adenocarcinoma, sporadic expression of this protein was found in epithelial cells of the tumour [65]. The exact significance for this finding in canine placental lacunae remains unclear and needs additional studies for clarification.

In this study, we characterized the distribution and the immunostaining pattern of NEP/CD10 expressing cells during the estrous cycle of the bitch and described changes during early pregnancy. Diffuse NEP/CD10 immunoexpression is consistently observed in the endometrial stroma throughout the estrous cycle, showing particular changes at different stages of the cycle. In addition, the existence of a sub-surface layer of NEP/CD10 negative stromal cells sharing some morphological characteristics with human predecidual cells was detected. The results from this study raise interesting questions on the participation of NEP/CD10 in events characterizing the canine endometrial cycle and implantation, although additional studies are needed to highlight the possible down-stream mechanisms and pathways involved in NEP/CD10 function in the canine uterus.

5. Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Acknowledgements

This work was sponsored by the Portuguese Science and Technology Foundation (FCT) under the Project PEst-OE/AGR/UI0772/2014 and the project PTDC/CVT/66587/2006.

The authors acknowledge Ann Barreiro for her kind assistance in revising this manuscript.

7. Chapter reference list

[1] Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. Mol Hum Reprod. 2004;10:879-93.

[2] Sherwin R, Catalano R, Sharkey A. Large-scale gene expression studies of the endometrium: what have we learnt? Reproduction. 2006;132:1-10.

[3] Cavagna M, Mantese JC. Biomarkers of endometrial receptivity - A review. Placenta. 2003;24.

[4] van Mourik MS, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol. 2009;85:4-19.

[5] Dekel N, Gnainsky Y, Granot I, Mor G. Inflammation and implantation. Am J Reprod Immunol. 2010;63:17-21.

[6] Aplin JD. Adhesion molecules in implantation. Rev Reprod. 1997;2:84-93.

[7] Arnold JT, Kaufman DG, Seppälä M, Lessey BA. Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. Hum Reprod. 2001;16:836-45.

[8] Payan-Carreira R, Pires MA, Ström Holst B, Rodriguez-Martinez H. Tumour necrosis factor in the canine endometrium: an immunohistochemical study. Reprod Domest Anim. 2011;46:410-8.

[9] Shipp MA, Vijayaraghavan J, Schmidt EV, Masteller EL, D'Adamio L, Hersh LB, *et al.* Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 ("enkephalinase"): direct evidence by cDNA transfection analysis. Proc Natl Acad Sci U S A. 1989;86:297-301.

[10] McIntosh GG, Lodge AJ, Watson P, Hall AG, Wood K, Anderson JJ, *et al.* NCL-CD10-270: a new monoclonal antibody recognizing CD10 in paraffin-embedded tissue. Am J Pathol. 1999;154:77-82.

[11] Chu P, Arber DA. Paraffin-section detection of CD10 in 505 nonhematopoietic neoplasms. Frequent expression in renal cell carcinoma and endometrial stromal sarcoma. Am J Clin Path. 2000;113:374-82.

[12] Albrecht M, Mittler A, Wilhelm B, Lundwall A, Lilja H, Aumuller G, *et al.* Expression and immunolocalisation of neutral endopeptidase in prostate cancer. Eur Urol. 2003;44:415-22.

[13] Imai K, Kanzaki H, Mori T. Cell surface peptidases in human endometrium. Mol Hum Reprod. 1996;2:425-31.

[14] Erdös EG, Skidgel RA. Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. FASEB J. 1989;3:145-51.

[15] Sumitomo M, Shen R, Walburg M, Dai J, Geng Y, Navarro D, *et al.* Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling. J Clin Invest. 2000;106:1399-407.

[16] Di Maria GU, Bellofiore S, Geppetti P. Regulation of airway neurogenic inflammation by neutral endopeptidase. Eur Respir J. 1998;12:1454-62.

[17] van der Velden VH, Naber BA, van der Spoel P, Hoogsteden HC, Versnel MA. Cytokines and glucocorticoids modulate human bronchial epithelial cell peptidases. Cytokine. 1998;10:55-65.

[18] Scholzen TE, Steinhoff M, Bonaccorsi P, Klein R, Amadesi S, Geppetti P, *et al.* Neutral Endopeptidase Terminates Substance P-Induced Inflammation in Allergic Contact Dermatitis. J Immunol. 2001;166:1285-91.

[19] Iwaya K, Ogawa H, Izumi M, Kuroda M, Mukai K. Stromal expression of CD10 in invasive breast carcinoma: a new predictor of clinical outcome. Virchows Arch. 2002;440:589-93.

[20] Freedland SJ, Seligson DB, Liu AY, Pantuck AJ, Paik SH, Horvath S, *et al.* Loss of CD10 (neutral endopeptidase) is a frequent and early event in human prostate cancer. Prostate. 2003;55:71-80.

[21] Bilalovic N, Sandstad B, Golouh R, Nesland JM, Selak I, Torlakovic EE. CD10 protein expression in tumor and stromal cells of malignant melanoma is associated with tumor progression. Mod Pathol. 2004;17:1251-8.

[22] Braham H, Trimeche M, Ziadi S, Mestiri S, Mokni M, Amara K, *et al.* CD10 expression by fusiform stromal cells in nasopharyngeal carcinoma correlates with tumor progression. Virchows Arch. 2006;449:220-4.

[23] Dall'Era MA, Oudes A, Martin DB, Liu AY. HSP27 and HSP70 interact with CD10 in C4-2 prostate cancer cells. Prostate. 2007;67:714-21.

[24] Goodman OB, Febbraio M, Simantov R, Zheng R, Shen R, Silverstein RL, *et al.* Neprilysin inhibits angiogenesis via proteolysis of fibroblast growth factor-2. J Biol Chem. 2006;281:33597-605.

[25] Cutrona G, Tasso P, Dono M, Roncella S, Ulivi M, Carpaneto EM, *et al.* CD10 is a marker for cycling cells with propensity to apoptosis in childhood ALL. Br J Cancer. 2002;86:1776-85.

[26] Bai M, Agnantis NJ, Skyrlas A, Tsanou E, Kamina S, Galani V, *et al.* Increased expression of the bcl6 and CD10 proteins is associated with increased apoptosis and proliferation in diffuse large B-cell lymphomas. Mod Pathol. 2003;16:471-80.

[27] Imai K, Kanzaki H, Fujiwara H, Kariya M, Okamoto N, Takakura K, *et al.* Expression of aminopeptidase N and neutral endopeptidase on the endometrial stromal cells in endometriosis and adenomyosis. Hum Reprod. 1992;7:1326-8.

[28] McCluggage WG, Sumathi VP, Maxwell P. CD10 is a sensitive and diagnostically useful immunohistochemical marker of normal endometrial stroma and of endometrial stromal neoplasms. Histopathology. 2001;39:273-8.

[29] Toki T, Shimizu M, Takagi Y, Ashida T, Konishi I. CD10 is a marker for normal and neoplastic endometrial stromal cells. Int J Gynecol Pathol. 2002;21:41-7.

[30] Nascimento AF, Hirsch MS, Cviko A, Quade BJ, Nucci MR. The role of CD10 staining in distinguishing invasive endometrial adenocarcinoma from adenocarcinoma involving adenomyosis. Mod Pathol. 2003;16:22-7.

[31] Groisman GM, Meir A. CD10 is helpful in detecting occult or inconspicuous endometrial stromal cells in cases of presumptive endometriosis. Arch Pathol Lab Med. 2003;127:1003-6.

[32] Potlog-Nahari C, Feldman AL, Stratton P, Koziol DE, Segars J, Merino MJ, *et al.* CD10 immunohistochemical staining enhances the histological detection of endometriosis. Fertil Steril. 2004;82:86-92.

[33] Riley SC, Wong E, Findlay JK, Salamonsen LA. Localization of neutral endopeptidase in the ovine uterus and conceptus during the oestrous cycle and early pregnancy. Reprod Fertil Dev. 1995;7:27-33.

[34] Barrau MD, Abel JH, Torbit CA, Tietz WJ. Development of the implantation chamber in the pregnant bitch. Am J Anat. 1975;143:115-30.

[35] Concannon P, Tsutsui T, Shille V. Embryo development, hormonal requirements and maternal responses during canine pregnancy. J Reprod Fertil Suppl. 2001;57:169-79.

[36] Amoroso EC. Placentation. In: Parkes AS, editor. Marshall's Physiology of Reproduction. London: Longmans Green and Co.; 1952. p. 127-311.

[37] Head JR, MacDonald PC, Casey ML. Cellular localization of membrane metalloendopeptidase (enkephalinase) in human endometrium during the ovarian cycle. J Clin Endocrinol Metab. 1993;76:769-76.

[38] Ottlecz A, Walker S, Conrad M, Starcher B. Neutral metalloendopeptidase associated with the smooth muscle cells of pregnant rat uterus. J Cell Biochem. 1991;45:401-11.

[39] Johnson GA, Burghardt RC, Bazer FW, Spencer TE. Osteopontin: roles in implantation and placentation. Biol Reprod. 2003;69:1458-71.

[40] Ferenczy A. Anatomy and histology of the uterine corpus. In: Kurman R, editor. Blanstein's Pathology of the Female Genital Tract. 4th Edition ed. New York: Springer-Verlag; 1994. p. 327-66.

[41] Benirsch K, Kaufmann P. Decidua. In: Benirsch K, Kaufmann P, editors. Pathology of the Human Placenta. 4th Edition ed. New York: Springer-Verlag; 2000. p. 188-96.

[42] Oliver C, Montes MJ, Galindo JA, Ruiz C, Olivares EG. Human decidual stromal cells express alpha-smooth muscle actin and show ultrastructural similarities with myofibroblasts. Hum Reprod. 1999;14:1599-605.

[43] Oliveira SF, Greca CP, Abrahamsohn PA, Reis MG, Zorn TM. Organization of desmin-containing intermediate filaments during differentiation of mouse decidual cells. Histochem Cell Biol. 2000;113:319-27.

[44] Korgun ET, Cayli S, Asar M, Demir R. Distribution of laminin, vimentin and desmin in the rat uterus during initial stages of implantation. J Mol Histol. 2007;38:253-60.

[45] Concannon PW. Reproductive cycles of the domestic bitch. Anim Reprod Sci. 2011;124:200-10.

[46] Iwase A, Ando H, Nagasaka T, Goto M, Harata T, Kikkawa F. Distribution of endothelin-converting enzyme-1 and neutral endopeptidase in human endometrium. J Histochem Cytochem. 2007;55:1229-35.

[47] Payan-Carreira R. Endometrial Expression of Integrin alpha V beta 3 in the Bitch. Reprod Domest Anim. 2009;44:76-.

[48] Miranda S, Pereira M, Pires MA, Neto RL, Payan-Carreira R. IL-18 localization in canine endometrium. Reprod Biol. 2013;13, Supplement 2:32-3.

[49] Sumitomo M, Shen R, Nanus DM. Involvement of neutral endopeptidase in neoplastic progression. Biochim Biophys Acta. 2005;1751:52-9.

[50] Maguer-Satta V, Besançon R, Bachelard-Cascales E. Concise review: neutral endopeptidase (CD10): a multifaceted environment actor in stem cells, physiological mechanisms, and cancer. Stem Cells. 2011;29:389-96.

[51] Defea K, Schmidlin F, Déry O, Grady EF, Bunnett NW. Mechanisms of initiation and termination of signalling by neuropeptide receptors: a comparison with the proteinase-activated receptors. Biochem Soc Trans. 2000;28:419-26.

[52] Pierro E, Minici F, Alesiani O, Miceli F, Proto C, Screpanti I, *et al.* Stromal-epithelial interactions modulate estrogen responsiveness in normal human endometrium. Biol Reprod. 2001;64:831-8.

[53] Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev. 2007;19:65-78.

[54] Casey ML, Smith JW, Nagai K, MacDonald PC. Transforming growth factor-beta 1 inhibits enkephalinase (EC 3.4.24.11) gene expression in human endometrial stromal cells and sex skin fibroblasts in culture. J Clin Endocrinol Metab. 1993;77:144-50.

[55] Payan-Carreira R, Ström-Holst B. TGF-beta isoforms in the canine endometrium. Am J Reprod Immunol. 2011;66:111 [Abstr.].

[56] Jones RL, Stoikos C, Findlay JK, Salamonsen LA. TGF-beta superfamily expression and actions in the endometrium and placenta. Reproduction. 2006;132:217-32.

[57] Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci. 2011;4:51.

[58] Yoshino O, Osuga Y, Hirota Y, Koga K, Yano T, Tsutsumi O, *et al.* Akt as a possible intracellular mediator for decidualization in human endometrial stromal cells. Mol Hum Reprod. 2003;9:265-9.

[59] Iwase A, Ando H, Nagasaka T, Shibata D, Harata T, Shimomura Y, *et al.* Neutral endopeptidase expressed by decidualized stromal cells suppresses akt phosphorylation and deoxyribonucleic acid synthesis induced by endothelin-1 in human endometrium. Endocrinology. 2006;147:5153-9.

[60] Dery MC, Leblanc V, Shooner C, Asselin E. Regulation of Akt expression and phosphorylation by 17beta-estradiol in the rat uterus during estrous cycle. Reprod Biol Endocrinol. 2003;1:47.

[61] Guzeloglu Kayisli O, Kayisli UA, Luleci G, Arici A. In vivo and in vitro regulation of Akt activation in human endometrial cells is estrogen dependent. Biol Reprod. 2004;71:714-21.

[62] Schäfer-Somi S, Sabitzer S, Klein D, Reinbacher E, Kanca H, Beceriklisoy HB, *et al.* Vascular endothelial (VEGF) and epithelial growth factor (EGF) as well as platelet-activating factor (PAF) and receptors are expressed in the early pregnant canine uterus. Reprod Domest Anim. 2013;48:20-6.

[63] Ordi J, Romagosa C, Tavassoli FA, Nogales F, Palacin A, Condom E, *et al.* CD10 expression in epithelial tissues and tumors of the gynecologic tract: a useful marker in the diagnosis of mesonephric, trophoblastic, and clear cell tumors. Am J Clin Path. 2003;27:178-86.

[64] Aviv R, Gurbanov K, Hoffman A, Blumberg S, Winaver J. Urinary neutral endopeptidase 24.11 activity: modulation by chronic salt loading. Kidney Int. 1995;47:855-60.

[65] Pires MA, Seixas F, Palmeira C, Payan-Carreira R. Histopathologic and immunohistochemical exam in one case of canine endometrial adenocarcinoma. Reprod Domest Anim. 2010;45:545-9.

Chapter 5 – Immunolocalization of E-cadherin and β-catenin in the cyclic and early pregnant canine endometrium

THE CONTENT OF THIS CHAPTER WAS PREPARED FOR SUBMISSION TO:

Theriogenology

IMMUNOLOCALIZATION OF E-CADHERIN AND β-CATENIN IN THE CYCLIC AND EARLY PREGNANT CANINE ENDOMETRIUM

R. Payan-Carreira[a]*, M. A. Pires[a], C. Santos[b], B. Ström Holst[c], J. Colaço[a]; H. Rodriguez-Martinez[d]

[a] CECAV, University of Trás-os-Montes and Alto Douro, P.O. Box 1013, 5001-801 Vila Real, Portugal.

[b] DEBA, University of Trás-os-Montes and Alto Douro, P.O. Box 1013, 5001-801 Vila Real, Portugal.

[c] SLU, Dept. of Clinical Sciences, Box 7054, 750 07 Uppsala, Sweden

[d] Linköping University, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Sweden

* Corresponding author: Rita Payan-Carreira, CECAV, Department of Zootecnics, Universidade de Trás-os-Montes e Alto Douro; Vila Real, Portugal; Fax: +351.259350480.

E-mail address: rtpayan@gmail.com

Abstract

E-cadherin and β-catenin were immunolocalised in the canine cyclic and early pregnant endometrium, using monoclonal antibodies. Relevant for the integrity of the epithelia, it is of interest to study the putative changes during implantation in dogs. Both molecules were detected in all types of endometrial epithelia (surface, superficial glandular and deep glandular epithelia) at all stages of the estrous cycle and in early placental structures. E-cadherin depicted a gradient of immunoreaction, being lowest in the surface epithelium and progressively increasing towards the deepness of endometrial glands, regardless of the stage of estrous cycle. The overall immunostaining was, however, weaker at diestrus. In pregnant samples, the trophoblast immunolabelling was higher than in the

observed in endometrial surface epithelium; in the later, the cytoplasmic pattern predominated over the membranar, as it also happened in the giant decidual cells in the labyrinth. At the early placenta, only trophoblast cells and lacunae retained a membrane signal. β-catenin membrane labelling was relatively constant through the cycle, although a tendency towards a decrease in intensity was detected at the secretory stages of the cycle. In addition, a dislocation of the immunoreaction to the cytoplasm was observed in both the surface and the glandular epithelia in particular stages of the cycle. In early pregnancy, a loss of the membranar pattern was observed in the surface epithelium and labyrinth, but not on trophoblast cells nor in lacunae. Results suggest that a softening of adherens junctional complex exists in progestagen stages favouring embryo-maternal interactions and endometrial invasion during implantation.

Keywords: E-cadherin; β-catenin; canine endometrium; estrous cycle; immunohistochemistry; dog.

1. Introduction

In the bitch, like in other mammals, the endometrium consists of a mesenchymal stroma, composed of individual fusiform cells surrounded by extracellular matrix, and two epithelial cell populations: the glandular epithelium and the surface or luminal epithelium (SE). The glandular epithelium is usually further classified into superficial glandular epithelium (SGE), located at the crypt zone, and deep glandular epithelium (DGE), located at the basal zone of the endometrium [1]. Both epithelial types are secretory and of utmost relevance for the integrity of the organ, the interactions with the spermatozoa and with the embryo, and for the establishment and maintenance of pregnancy. A complex sequence of signalling events, encompassed by the crosstalk between endometrial epithelium and stroma, is crucial to the establishment of pregnancy [2, 3].

In response to cyclic variations in sex hormones, the mammalian endometrium evidences recurrent structural and morphological modifications in what is frequently named the endometrial cycle [4]. Several molecules have been shown to display cyclic patterns of expression, responding differently to the dominance by estrogen or progesterone influence

on target tissues, contributing to normal functioning of the endometrium and the endometrial receptivity to the embryo [4-7]. The endometrial remodelling processes, controlled by sex steroids, entail complex changes in epithelial cell-cell interactions, which are required to maintain the continuity and integrity of the endometrium [8].

In the endometrium, the epithelial lining creates a protective barrier against bacterial or viral pathogens as well as against different antigens while in particular periods, such as at the window of implantation, it display a permissive status that allow embryo-maternal interactions culminating with the establishment of placenta. Adhesive interactions between neighbouring epithelial cells are also crucial for tissue morphogenesis and renewal [9]. Endometrial modifications in the expression of different adhesion molecules during the cycle or early pregnancy have been reported in humans [8, 10-12], sheep [13] and pigs [12, 14]. Adherens junctions play important functions in the maintenance of intercellular connections, which defines the characteristic epithelial architecture. Through cadherin–mediated epithelial cell–cell connexions, the integrity and strength of the epithelial layer [15, 16], as well as the polarity of the epithelia [17] are maintained. Thus it can be presumed that the plasticity of cell-to-cell adhesion of endometrial epithelial cells is crucial to female fertility.

E-cadherin, member of the cadherin family of calcium-dependent adhesion molecules, is a cell surface glycoprotein and is an important determinant of tissue processes related to selective cell adhesion or detachment [15, 16], which in the uterus involves changes designed to support the implantation and growth of an embryo [8, 18, 19].

The cyclic tissue remodelling occurring in the endometrium during the cycle requires complex changes in cell-cell and cell-matrix interactions. Spatiotemporal changes in E-cadherin adhesion system may be associated with the morphologic changes observed in the mammals' endometrial cycle, a dynamic process that comprises epithelial cell rearrangements, proliferation, renewal and movement that involves the permanent breaking and reforming of cell–cell adhesion [10, 20]. Furthermore, down-regulation of E-cadherin has been associated to invasive processes, which in the uterus includes the disruption of the epithelial barrier and the progression through a permissive extracellular mesenchymal matrix, as occurs at implantation in species with decidual placenta [21]. Supporting this premise, Dawood *et al.* [22] showed that E-cadherin and its gene transcripts were expressed in peri-implantation phase endometrium in women and Liu *et al.* [23] found a relationship between E-cadherin and metalloproteinase-2 and -9 during mice embryo implantation process. As endometrial invasion is a tightly controlled process, E-cadherin expression at the window of implantation could be associated to the regulation of the migrating and invasive potential of the trophoblast. It was demonstrated that E-cadherin expression is significantly reduced

close to the maternal recognition period in sheep [13] and pigs [14], species where it may also play a favorable role in embryo elongation. In dogs, Guo and collaborators [24], using *in situ* hybridization, describe a reduction in E-cadherin mRNA expression until pregnancy day 20 compared with the expression levels recorded in estrus, thereafter showing a strong signal in the glandular epithelium. Based on these findings the authors suggest a role for this molecule in the canine implantation process.

The E-cadherin junctional complex also includes several other molecules, such as β-catenin, by which the cadherin molecule is anchored to the cytoskeleton. In the cytoplasm, β-catenin pairs bind strongly to the E-cadherin domain, binding the complex to the actin skeleton through α-actin molecule [25-27]. In addition to its role in cell-to-cell adhesion, β-catenin also plays an important role in the Wnt/Wg growth factor signalling pathway [28, 29], which has been implicated in the endometrial cycle regulation [30] and implantation [31]. β-catenin participation in either cell adhesion or the Wnt pathway depends on the existence of a competitive binding of this molecule to E-cadherin and Wnt-signalling molecules, which is determined by the activity of different kinases [28, 29, 31].

Epithelial functional or physical integrity is a major issue regarding embryo invasion in early pregnancy. In dogs, embryo implantation process involves embryo adhesion to the maternal epithelium, quickly followed by the invasion and transformation of the upper half of the endometrium to establish an endotheliochorial placenta [2, 32]. Thereby, changes in E-cadherin/β-catenin adhesive complex are expected to occur at the implantation, in dogs, as it is currently accepted that changes in the components of this complex will result in lateral cell-cell dissociation [2, 33], supporting embryo invasion or tumour progression. On other hand, a reduction in E-cadherin expression or the compromised polarity of epithelia during the luteal or secretory stage might also favour the development of pyometra during progesterone dominance, facilitating the colonization of the endometrium by pathogen bacteria ascending from the vagina. These could contribute to a transitory fragility in the endometrial epithelial barrier mainly in species with a physiologically long diestrus, as it happens in dogs.

Despite extensive research reported on cadherin and β-catenin activity in the uterus of different species [8, 13, 18, 34], information about the immunolocalization of E-cadherin and β-catenin in the canine endometrium is sparse. Dogs reproductive physiology comprises several characteristics that distinguish the species from that of other domestic animals, including: a relatively long estrogenic stage, the preovulatory increase in progesterone, a rather long luteal stage, similar in pregnant and non-pregnant cycles [35] and a endotheliochorial placenta, that is accompanied by a certain degree of invasion by the trophoblast and of the destruction of the luminal epithelium in the process [36, 37].

Consequently, the spatiotemporal pattern in E-cadherin and β-catenin molecules in the canine endometrium may differ from the established in other species. The aim of this work was: 1/ to analyse E-cadherin and β-catenin protein immunolocalization throughout the stages of the canine estrous cycle and to determine whether temporal changes exits during the uterine cycle; 2/ and to ascertain possible modifications of cadherin/β-catenin adhesion pathways in the canine embryo apposition and adhesion.

2. Material and methods

Animals

A total of 50 mature, healthy bitches, submitted to elective ovariohysterectomy were used. Additionally nine pregnancy samples were obtained from females with unwanted 3 weeks pregnancies, submitted to elective OVH. 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), and promptly centrifuged at 2500g for 15 minutes. The serum was stored at -20 ºC 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 the expressed consent of the animals' owners, and in respect to the International Ethical standards. Excised genital tracts were fixed in 10% buffered formalin immediately after surgery, for no more than 3 days. A section from each uterine horn was collected at its caudal ending, at approximately 1 cm above the uterine body, and thereafter embedded in paraffin wax, sectioned at 3 µm for routine staining with haematoxylin and eosin for histological staging of the estrous cycle and for excluding uterine disease. In this study, only one of the uterine samples was used.

Staging of the estrous cycle and early pregnancy

The staging of the estrous cycle was performed after the genital tract collection, at the laboratory, based on the cumulative information provided by vaginal cytology, inspection of the ovaries at OVH, and circulating levels of progesterone. Vaginal cytology allowed a

preliminary staging of the cycle and was particularly useful to distinguish proestrus and estrus. The ovary and uterine morphology and the serum progesterone were used for further staging, as previously described [38, 39]. Uterine samples for the proestrus (n=8), estrus (n=10), early diestrus (n=12), full diestrus (n=10) and anestrus (n=10) were used in this study.

Nine samples representing pregnancy days 17 to 20 were selected. The pregnancy samples were staged on the basis of cumulative information gathered from a diestrus-compatible cytology, the date of acknowledged coitus plus the histological changes observed in the endometrium, according to the histological descriptions of canine early pregnancy events [36, 40], and aligned to the day from pre-ovulatory LH surge. Up to day 17 the embryo is not yet adhered to the endometrium, although some changes are found in the superficial layers of the endometrium, including increased interstitial edema and deepen of the endometrial crypts. On day 17, the trophoblast grows down and wedges the maternal surface epithelium (SE). Only small lacunae are visible. Between days 18 and 20, the embryo adherence prompts the endometrial invasion; the trophoblast continues to spread down, and the syncytial cells penetrate deeper in the endometrium forming the labyrinth that appear as strong, linear, closely packed cords, often presenting mitotic figures. Until this moment, limited development of the basal glands is observed.

Immunohistochemistry

Immunohistochemistry analysis was performed on tissue sections 3 µm thick on silane-coated slides and a streptavidin-biotin-peroxidase technique (UltraVision, Lab Vision, Fremont, CA, USA).

The primary antibodies used were mouse monoclonal antibodies raised against E-cadherin (Clone 4A2C7, Invitrogen) and β-catenin (Clone CAT-5H10, Invitrogen), at 1:100 dilution in PBS, as previously established for canine tissues [41].

The slides were submitted to routine deparaffinization and rehydration in graded alcohol, and pre-treated to enhance antigen retrieval (microwave irradiation; 750W, three cycles of 5 min, with slides immersed in a 0.05% Extran solution); thereafter, the endogenous peroxidases blocked by 30 minutes immersion in 3% hydrogen peroxide/PBS, followed by inhibition of non-specific binding by incubation with Large Volume Ultra V-Block (UltraVision, Lab Vision, Fremont, CA, USA) for 5 min. Incubation with primary antibodies was performed in a humid chamber, overnight at 4ºC. Afterwards, the labelled slides were

incubated with a biotin conjugated secondary antibody, followed by incubation with enzyme-labelled streptavidin, for 10 minutes each (Biotinylated Goat Polyvalent Plus® antibody and Streptavidin-peroxidase Plus® - UltraVision, Lab Vision, Fremont, CA, USA - respectively), at room temperature. The reactions were visualized using DAB (3,3'-diaminobenzidine) as a chromogen, and the slides were counterstained with haematoxylin, dehydrated and mounted for light microscopy evaluation.

For negative controls, primary antibodies were replaced by either the normal mouse IgG (sc-2025; Santa Cruz Biotechnology, Inc., Europe, Heidelberg, Germany) or PBS. In neither negative control immunoreaction against the two molecules was detected. Samples from a canine mammary carcinoma were used as positive controls.

Immunohistochemical scoring

Two independent observers blindly assessed the immunoreaction for E-cadherin and β-catenin, in a NIKON Eclipse 80i (Nikon Instruments Europe, BV) photomicroscope. The distribution of E-cadherin and β-catenin immunoreaction was studied for each one of the endometrial epithelial types: the surface epithelium (SE), the superficial glandular epithelium (SGE) and the deep glandular epithelium (DGE). Positivity was indicated by the presence of a distinct brown membrane labelling. For both these molecules, the evidence of a membrane staining was semiquantitatively scored using a 4-point scale: weak (1), moderate (2), strong (3) or very strong (4).

Cytoplasmic staining was frequently seen in addition to membranous staining for both molecules. For E-cadherin, membrane labelling extending to the cytoplasm was frequently found; it has been considered to correspond to its cytoplasmic domain and it was not scored independently whenever it escorted membrane labelling. When cells failed to evidence membrane immunoreaction, which was located exclusively in the cytoplasm, the pattern was scored as cytoplasmic.

For β-catenin the membrane dislocation of the immunoreaction was assessed due to its role at the cell-to-cell junctions and at the Wnt/Wg signalling pathways. The subcellular pattern and the differences in the membrane and cytoplasmic staining were scored independently for intensity and the pattern, the later being further scored as diffuse, scattered (or dot-like), apical or supranuclear.

The immunoreaction in pregnancy samples was graded using the same semiquantitative scoring system, which was applied individually for the trophoblast, the surface epithelium and the epithelium of the labyrinth (or syncytium cords) and the *lacunae*, as well as for the deep endometrial glands.

Statistical analysis

Statistical comparisons were performed by using the statistical software IBM SPSS Statistics version 22.0 for Mac OS X 10.8. Associations between the intensity and patterns of immunoreaction for E-cadherin and β-catenin and the categorical variables (stage of the estrous cycle and epithelial type) were performed using the chi-square and Fisher exact tests. The Z-test was performed for group comparisons, the P-values adjusted by Bonferroni method. Comparisons between early diestrus and pregnancy samples were only established for the surface and the deep glandular epithelia. A P value ≤ 0.05 was regarded as statistically significant.

3. Results

The non-pregnant canine endometrium

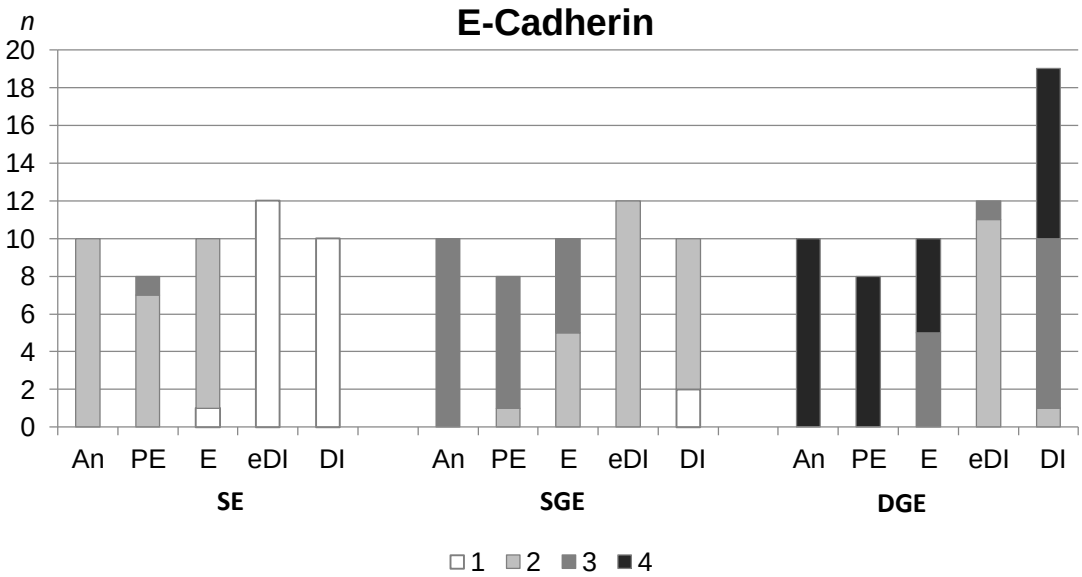
The scoring results for the immunohistochemistry distribution of E-cadherin and β-catenin in the non-pregnant endometrium are shown in graphs 1 and 2 as well as in tables 1 and 2. For both molecules, a typical membranous labelling was evident in all samples and in all the epithelial types. Moreover, changes of the intensity of the immunolabelling were detected in different stages of the estrous cycle. In addition to the membrane immunolabelling, different cytoplasmic patterns of immunolabelling were observed for β-catenin, and scored accordingly (Table 2).

In general, the E-cadherin immunolabelling in the canine endometrium showed a gradient of intensity between the different epithelial types ($P < 0.001$; Fisher = 115.376); it was lower in the superficial epithelium and progressively increasing towards the deep endometrial glands (Graph 1).

Table 1 – E-cadherin and β-catenin membrane immunoreactivity scores in the canine endometrium throughout the stages of the estrous cycle

		SE				SGE				DGE			
	Stages	1	2	3	4	1	2	3	4	1	2	3	4
E-Cadherin	Anestrus	0	10	0	0	0	0	10	0	0	0	0	10
	Proestrus	0	8	0	0	0	1	7	0	0	0	0	8
	Estrus	1	9	0	0	0	5	5	0	0	0	5	5
	Early diestrus	12	0	0	0	0	12	0	0	0	11	1	0
	Diestrus	10	0	0	0	2	8	0	0	0	1	9	0
β-catenin	Anestrus	8	2	0	0	3	7	0	0	0	10	0	0
	Proestrus	7	1	0	0	3	5	0	0	1	7	0	0
	Estrus	10	0	0	0	6	4	0	0	3	7	0	0
	Early diestrus	11	1	0	0	8	4	0	0	3	9	0	0
	Diestrus	9	1	0	0	4	6	0	0	0	10	0	0

SE – surface endometrium; SGE – superficial glandular endometrium; DGE – deep glandular endometrium

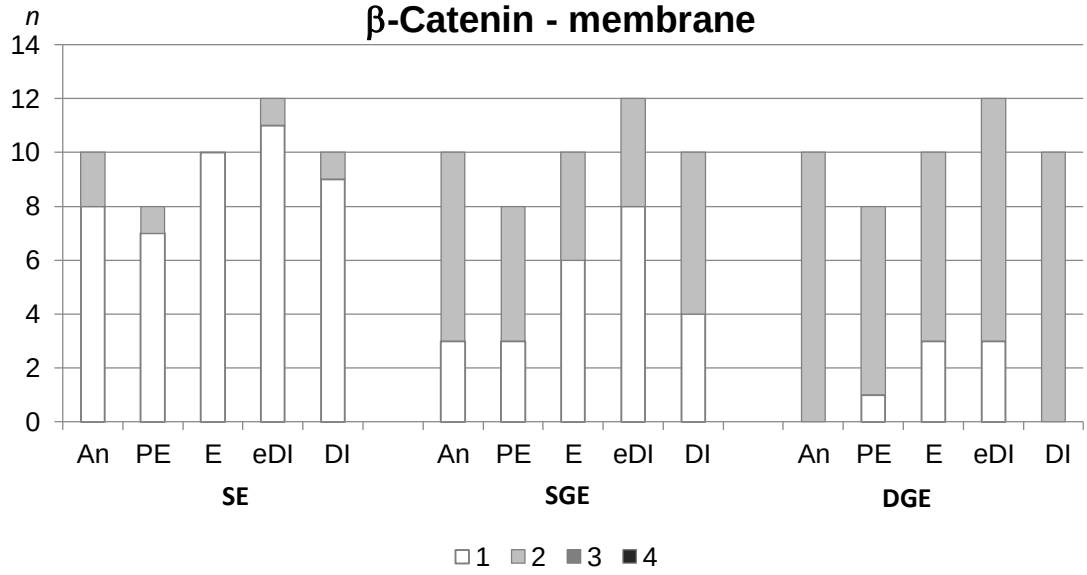


Graph 1 – Graphic representation of the endometrial membrane intensity scoring for E-cadherin during the canine estrous cycle.

Table 2 – Cytoplasmic intensity and pattern of immunoreaction against β-catenin in the cyclic canine endometrium.

		Score	Anestrus	Proestrus	Estrus	Early Diestrus	Diestrus
Intensity score	SE	1	2	1	2	11	10
		2	8	7	8	1	0
		3	0	0	0	0	0
		4	0	0	0	0	0
	SGE	1	1	2	1	5	8
		2	9	6	9	7	2
		3	0	0	0	0	0
		4	0	0	0	0	0
	DGE	1	0	0	0	0	1
		2	8	8	2	9	9
		3	2	0	8	3	0
		4	0	0	0	0	0
Pattern	SE	diffuse	9	4	2	8	6
		apical	1	4	2	3	4
		scattered	0	0	0	0	0
		supranuclear	0	0	6	1	0
	SGE	diffuse	10	5	0	1	4
		apical	0	3	0	3	4
		scattered	0	0	2	6	1
		supranuclear	0	0	8	2	1
	DGE	diffuse	10	8	2	8	6
		apical	0	0	0	1	4
		scattered	0	0	0	1	0
		supranuclear	0	0	8	2	0

SE – surface endometrium; SGE – superficial glandular endometrium; DGE – deep glandular endometrium



Graph 2 – Graphic representation of the endometrial membrane intensity scoring for β-catenin throughout the canine estrous cycle.

When studied individually, the different endometrial epithelial types tend to show differences in E-cadherin membrane intensity of immunolabeling during the different stages of the estrous cycle. The SE showed similar immunostaining scores for anestrus, proestrus (Figure 1A) and estrus (Figure 1C), evidencing a moderate immunopositivity (Graph 1; Table 1). In contrast, during early diestrus and diestrus (Graph 1; Figure 1E), a clear reduction of the intensity of immunolabelling was noticed ($P < 0.001$; Fisher = 50.681). For the SGE, a stronger immunoreaction was detected in anestrus and proestrus (Graph 1; Figure 1A), which tended to decrease in estrus (Figure 1C). A reduction of the intensity of the immunolabelling ($P < 0.001$; Fisher = 42.224) was recorded in early diestrus and diestrus (Graph 1; Figure 1E). The intensity of the DGE immunostaining was found to be similar between anestrus and proestrus (Graph 1), with epithelial cells evidencing a very strong labelling for E-cadherin. In estrus, a tendency for a decrease in the intensity of labelling was found (Graph 1), with a clear decrease in early diestrus and diestrus (Graph 1; Figure 1G) ($P < 0.001$; Fisher = 61.143), most obvious in early diestrus. Overall, stronger immunolabelling for E-cadherin was observed during anestrus, proestrus and estrus compared with early diestrus and diestrus ($P < 0.001$; Fisher = 154.265) (Table 1; Graph 1). When the variations in the intensity score for all the three epithelial types were evaluated together, a significant difference was found between the different stages of the canine estrous cycle ($P < 0.001$;

Fisher = 154.265) with a decrease of the E-cadherin immunoreaction in the progesterone-mediated stages (early diestrus and diestrus).

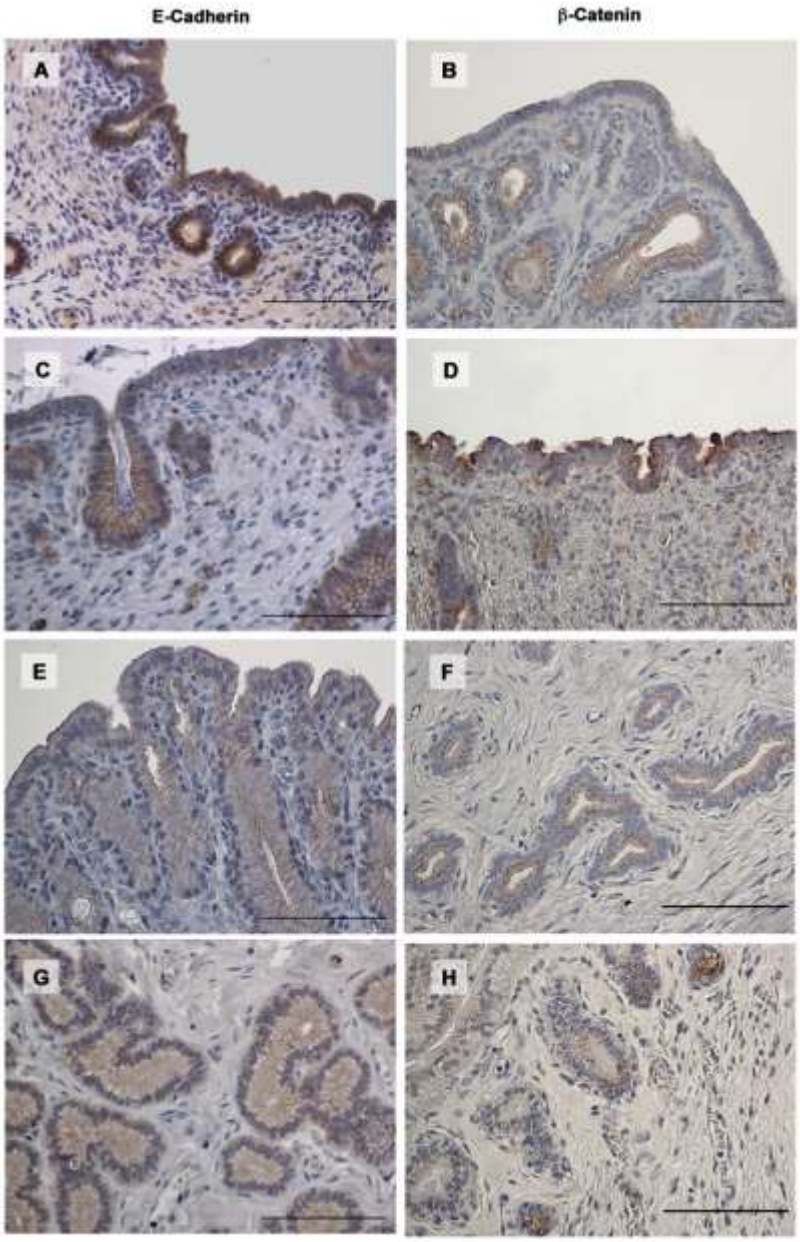
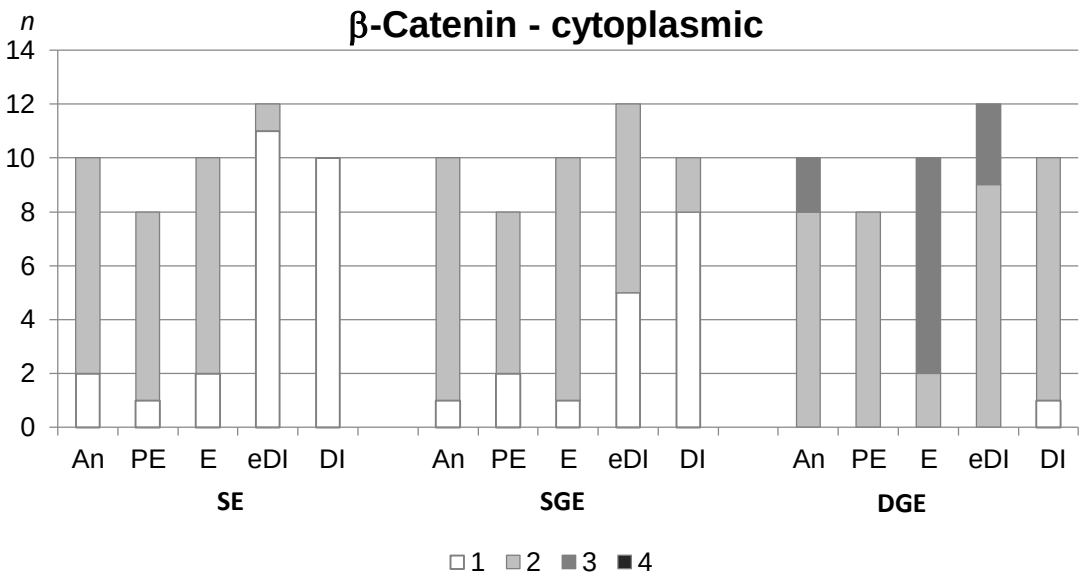


Figure 1 – Immunohistochemical expression of E-cadherin and β-catenin in the normal canine endometrium throughout the estrous cycle (bar: 100μm). In the plansch, the left column of images corresponds to the membranar immunoreaction against E-cadherin in the superficial endometrial layers in proestrus (**A**), estrus (**C**) and early diestrus (**E**) or to DGE immunolabelling in full diestrus (**G**), while the column in the right depicts the diverse patterns associated with β-catenin immunolabeling: the membranar pattern (**B**), here observed in the, SE and SGE in full diestrus samples; the apical cytoplasmic pattern in the SE of a proestrus sample (**D**); the supranuclear cytoplasmic pattern prevailing in estrus samples (**F**); and the scattered cytoplasmic pattern evidenced in some early diestrus samples (**H**).

The membrane intensity scores for β-catenin varied between 1 and 2 for all the endometrial epithelia (Graph 2; Figure 1B; Table 1). In general, the epithelium type influenced the intensity of β-catenin immunolabelling ($P < 0.001$; Fisher = 63.989): it was observed a tendency for lower intensity scores in SE and SGE, like it happened for E-cadherin, whilst the DGE tended to present stronger intensity scores. However, no significant changes in the membrane staining intensity along the cycle stages were found within epithelial types ($P = 0.901$; Fisher = 12.676).

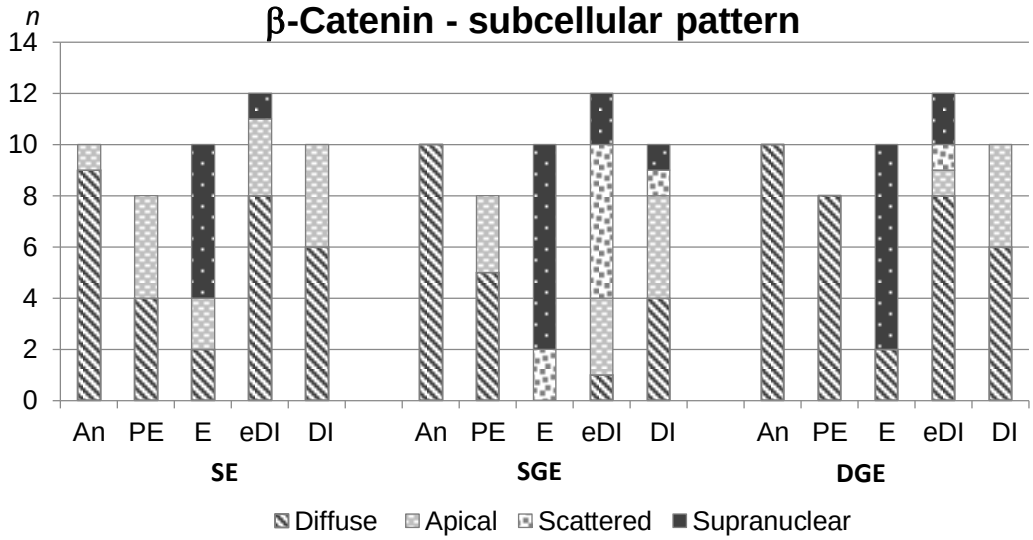
Diverse cytoplasmic immunoreactivity was observed for β-catenin (Graph 3; Table 2). Overall, lower intensity scores were found for the cytoplasmic immunoreaction in early diestrus and in diestrus, compared to anestrus, proestrus or estrus ($P < 0.001$; Fisher = 44.311). In addition, the cytoplasmic pattern was diffuse in all cycle stages with exception for the estrus, when the supranuclear pattern predominated in all the three epithelial types ($P < 0.001$; Fisher = 108.770). The supranuclear and apical patterns (Figures 1D and 1F) usually co-existed with higher cytoplasmic intensity scores ($P = 0.006$; Fisher = 19.309).



Graph 3 – Graphic representation of the endometrial cytoplasmic intensity scoring for β-catenin during the canine estrous cycle.

β-catenin cytoplasmic immunolabelling along the canine estrous cycle presented a significant association between immunostaining and epithelial type ($P < 0.001$; Fisher = 67.749) as well as between the cytoplasmic intensity and pattern ($P < 0.001$; Fisher =

108.770). The SE had a low intensity cytoplasmic immunostaining in early and mid diestrus, and a moderate intensity in all other stages (Graph 3). In both diestrus stages the diffuse pattern dominated over the apical or the supranuclear areas (Graph 4).



Graph 4 – Graphic representation of the cytoplasmic pattern of immunoreaction for β-catenin throughout the canine estrous cycle.

The SGE showed high membranar and cytoplasmic intensity scores in all cycle stages except during diestrus. A diffuse cytoplasmic pattern was found in anestrus and proestrus, while in diestrus the diffuse and apical pattern were equally common; a scattered cytoplasmic pattern (Figure 1H) was the most prevalent pattern in early diestrus samples, while in estrus the supranuclear staining (Graph 4) was the most frequently observed. In DGE, the intensity of the cytoplasmic immunostaining did not varied between cycle stages with exception of estrus, when higher intensity scores were observed. A diffuse cytoplasmic pattern of labelling was found predominantly during anestrus and proestrus (Graph 4); the supranuclear pattern predominating during estrus while in early diestrus (Figure 1H) the scattered cytoplasmic pattern prevailed over the apical location pattern (50% *Vs.* 25% of the samples). In diestrus (Figure 1D) the apical and diffuse patterns prevailed.

Intensity of β-catenin membrane and cytoplasmic staining was significantly lower in progesterone associated-stages of the estrous cycle ($P < 0.001$, Fisher = 16.112 and $P < 0.001$, Fisher = 60.245, respectively). Furthermore, the supranuclear pattern of strong intensity was consistently found in the cycle stage where estrogens peak ($P = 0.003$; Fisher

= 25.661). Furthermore, a significant association between the intensity of the membrane immunolabelling for E-cadherin and β-catenin was found ($P < 0.001$; Fisher = 274.471).

The early pregnant canine endometrium

Table 3 summarizes the information concerning the E-cadherin and β-catenin immunolabelling in the canine pregnant endometrium (days 17 to 20).

Table 3 – E-Cadherin and β-Catenin membrane immunoreactivity scores in early pregnant canine endometrium (pregnancy days 17 to 20) (n=9).

		Labyrinth epithelium					
Marker	**Scores**	**Trophoblast**	**SE**	**Trophoblast**	**Decidual cells**	**Lac E**	**DGE**
E-Cadherin	1	0	5	0	9	0	2
	2	0	1	3	0	0	6
	3	5	0	6	0	4	1
	4	4	0	0	0	0	0
β-Catenin	1	6	1	6	0	1	3
	2	3	5	3	0	3	5
	3	0	0	0	0	0	1
	4	0	0	0	0	0	0

SE – surface endometrium; DGE – deep glandular endometrium; Lac E – Lacunar epithelium.

The trophoblast evidenced intensity score of 3 and 4 against E-cadherin (Figure 2A; Table 3), in a notorious contrast with the maternal SE that predominantly showed a weak immunostaining and often failed to evidence a membrane pattern for this molecule (Figure 2A; Table 3). No differences were observed in the immunoreaction for E-cadherin in the SE in early pregnant and early diestrus stage.

The E-cadherin membranar pattern was also heterogeneously presented in the labyrinth: the trophoblast population displayed a moderate to strong membrane pattern (Figure 2A), whereas in the giant decidual cells the membrane pattern was absent and a

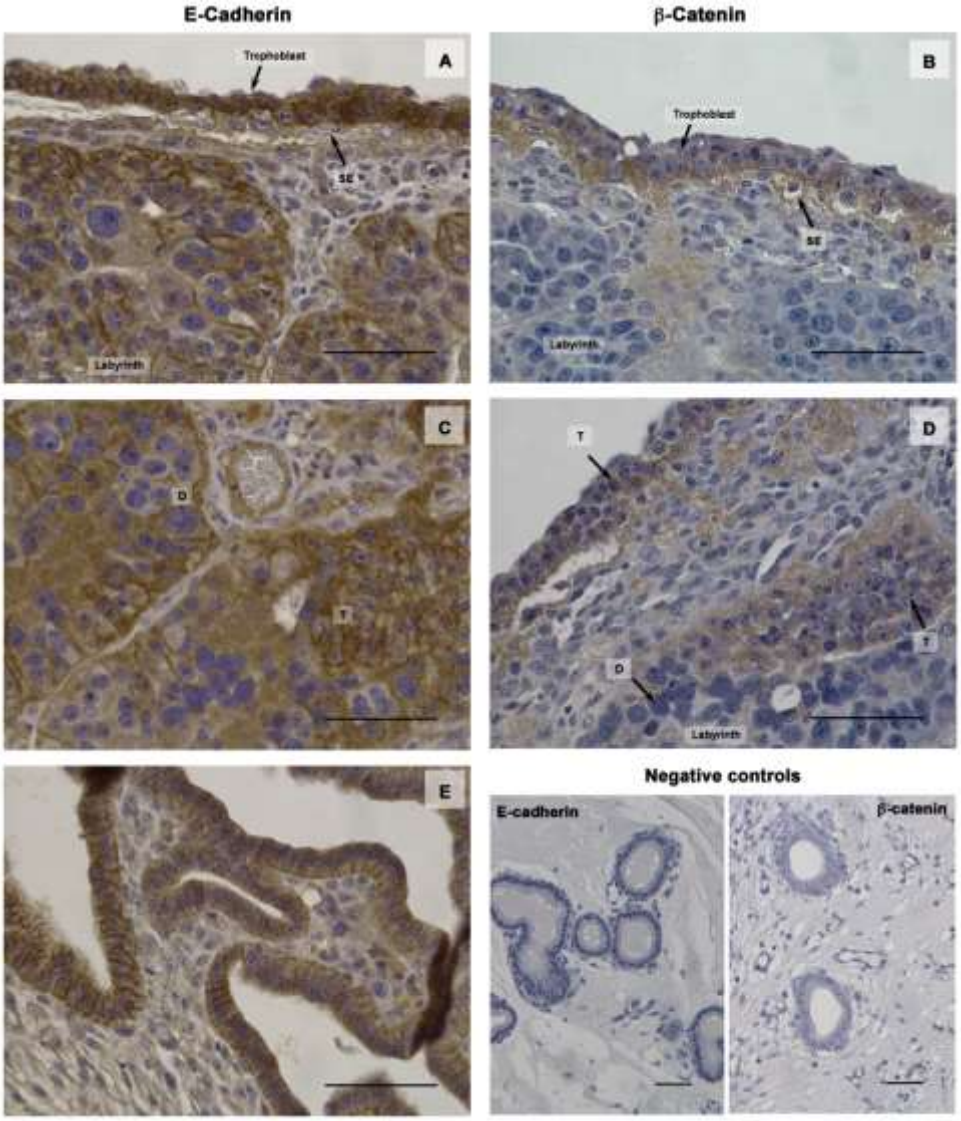


Figure 2 – Immunohistochemical expression of E-cadherin and β-catenin in early pregnant (days 17 to 20) endometrium (bar: 50μm). In the plansch, the left column of images corresponds to the immunoreaction against E-cadherin during the early steps of endometrial invasion: At the endometrial surface (**A**), the trophoblast, showing a strong intensity score, lined the SE that fail to evidence a membrane reaction for E-Cadherin. In the labyrinth (**C**), trophoblast cells showed a strong membrane pattern contrasting to the absence E-cadherin membrane immunolabeling shown by decidual cells. In lacunae (**E**), the epithelial cells evidenced strong membrane immunoscoring. The right column represents the β-catenin immunolabeling. (**B**) Weak to moderate membranar intensity score was evidenced in the trophoblast, while the SE displayed a moderate cytoplasmic pattern, no membrane pattern being discernible. In the labyrinth (**B** and **D**) a moderate intensity of immunostaining, either membrane and cytoplasmic, was observed in the trophoblast contrasting to the negative decidual cells. The images on the left bottom represent the negative controls for E-cadherin and β-catenin, respectively. T – Trophoblast; D – Decidual cells.

weak cytoplasmic pattern was noticed (Figure 2A and 2C). When lacunae were formed, closer to pregnancy day 20, the membrane immunolabeling for E-cadherin was strong (Figure 2E). No differences were found for DGE intensity scores between early pregnant and early diestrus stage (Tables 1 and 3); in both the stages a moderate intensity of immunolabelling was observed.

Considering the immunostaining for β-catenin, in the epithelial types represented in the early pregnant canine endometrium the moderate intensity score prevailed (Table 3). The trophoblast showed a moderate membrane intensity (Figure 2B), which is also represented in the trophoblast populations located in the labyrinth cords. Contrasting, the giant decidual cells were negative for β-catenin or displayed a weak, cytoplasmic labelling (Figure 2D). The intensity of the immunolabeling for this molecule in DGE was not different from the described for the early diestrus stage (Tables 1 and 3); the most represented intensity score was the moderate.

4. Discussion

Previous work in human and rodents showed that adhesion signalling molecules change during the endometrium cycle in response to the sex steroid dynamics [10, 19, 34]. Moreover, it has been proposed that E-cadherin/catenin complexes may play a role in initial attachment of the embryo during implantation [42], besides the maintenance of the endometrial architectures [34].

Results from the present study clearly show the existence of differences of the intensity or pattern for E-cadherin and β-catenin immunolabelling according to sex steroid milieu. The immunostaining was evidenced for both molecules in all canine endometrial samples and on all the epithelial elements (SE, SGE and DGE). E-cadherin was abundantly expressed in anestrus and in the proliferative stages of the bitch estrous cycle (proestrus and estrus), but its immunolabelling decreased during progesterone dominance, in particular in full diestrus, when progesterone levels are high. A similar down-regulation of E-cadherin expression during the progesterone phase of the cycle was reported in ewes [13] and in humans [34, 42]. However, a small increase in E-cadherin mRNA was observed following exogenous progesterone administration to ovariectomized bitches [24]. This apparent slight up-regulation of E-cadherin mRNA, which seems to contradict the immunohistochemical results presented in the present study, might be associated to the acute progesterone administration upon a non sex steroid-primed endometrium, while in the present report

uterine samples from normally cycling bitches were used. The acute progesterone administration could induce a down-regulation of the endometrial progesterone receptors, mimicking a late diestrus phase. On the other hand, that study focused on the activation of the E-cadherin synthesis pathway, whereas the protein was evidenced in the experiment presented herein. Differences could also be explained by a transcription regulatory mechanism.

The present study revealed that β-catenin membrane immunolabelling changes during the canine estrous cycle, with a tendency to decreased for a lower expression during the secretory stages of the cycle (diestrus stages). Such changes significantly correlated with decreased immunohistochemical expression of E-cadherin in the membrane. This may suggest a reduction of the E-cadherin/ β-catenin complexes, which could be associated with a modification of the adherens junction properties, in order to facilitate embryo interaction with the endometrium. An apparent decrease in the intercellular junctional strength, that was more pronounced in the canine SE and the SGE, would allow trophoblast cells to migrate between the epithelial cells during implantation. Evidences collected from early pregnancy samples representing the adhesion and initial invasion steps of the implantation process support that hypothesis. These showed a loss of the membrane pattern and a rather weak intensity of E-cadherin immunoreaction in the SE during trophoblast invasion, which is escorted by an exclusive diffuse cytoplasmic pattern for β-catenin immunolabeling. Adhesive intercellular interactions are crucial for endometrial epithelial differentiation [43]. Taken together these findings support the loss of lateral cell-to-cell adherence, preceding the apposition and endometrial invasion of the canine embryo. The loss of E-cadherin/β-catenin adherence at early pregnancy, as observed herein in the SE and in the giant decidual cells in the labyrinth, is suggestive of the existence of a transitional process in these cells, occurring during invasion and establishment of placenta in dogs, alike the proposed by Bartley and colleagues [44] to occur in human endometriosis, which would allow endometrial cells to detach from their primary site and adhesion and invade the implantation sites to form endometriotic lesions.

For most mammalian species with invading trophoblast, embryo strategies towards implantation mimic those of tumour tissues [45, 46]. Involvement of E-cadherin/β-catenin complexes in invasion suppression has been widely proven, in particular concerning tumour progression and invasiveness. It has been demonstrated that a decrease in E-cadherin membrane expression and of E-cadherin/βcatenin complexes along with the existence of aberrant β-catenin expression result in loss of the epithelial phenotype accompanying increased invasiveness and hence a higher metastatic ability [34, 45-47]. A decrease in E-

cadherin membrane expression usually accompanies a weakened attachment to the cellular skeleton and destabilization of the cell-to-cell adhesion to each other [25, 27, 28]. In parallel with a decrease in its expression, an increase in the E-cadherin instability and degradation in association with a decrease in the active cadherin complex has been described [26, 28, 48]. Based on the findings reported in the current study, a comparable situation could be hypothesized to occur in the endometrial epithelia at embryo implantation, as in the present study a decrease in E-cadherin junctional complexes during progesterone-associated stages and at early pregnancy was shown.

As seen in the present study, a reduction in cadherin/catenin membrane expression during early and full diestrus, by interfering with the strength of the epithelial endometrial barrier may also modulate the local susceptibility to invading pathogens and inflammation. Associated to a decrease in MUC1 expression occurring during canine diestrus [49], the reduction in the epithelial E-cadherin active complex might contribute to the onset of canine pyometra in diestrus.

Concerning the cytoplasmic immunostaining for β-catenin in the present study, a nuclear immunostaining for this molecule was not found, contradicting sporadic nuclear immunolabelling reported in humans [34, 50]. In the uterus, cytoplasmic β-catenin expression should not be considered abnormal, even when nuclear expression is detected, as this molecule is involved in cellular pathways other than the E-cadherin junctional complexes [8]. The Wnt/Wg signaling pathway has been demonstrated to be present in the endometrium [30, 50], and β-catenin is an active partner in such mechanism [29].

In the present study, cytoplasmic β-catenin was found to vary in intensity and pattern. These temporal variations seemed associated to sex steroid peripheral levels. Decreased cytoplasmic intensity was detected in early diestrus and in diestrus, when compared to the other cycle stages. The most frequently cytoplasmic pattern found was the diffuse type that has been observed in all stages with exception to estrus, where a supranuclear immunolabelling pattern predominated. This pattern could correspond to the cytosolic pool of inactive β-catenin that does not interact to either cadherin or the Wnt pathway [29]. Among all other stages, estrus displayed the strongest immunolabelling, associated with supranuclear location. Such a strong supranuclear immunolabelling might be associated with a transitory stimulation of the Wnt signaling pathway, in non-pregnant endometrium, as it clearly contrasts to the usual β-catenin cytoplasmic immunostaining. In consequence, the Wnt-associated pathways, demonstrated in humans to be dependent on high levels of estrogens and with cell proliferation [30], might also be activated in the canine endometrium during estrus.

In conclusion, this study showed a gradient of the E-cadherin immunolabelling in the canine endometrium, with a weaker immunolabelling observed in the surface epithelium against a stronger labeling in the deep glandular epithelium. Furthermore, a marked decrease in the immunolabeling was observed in secretory stages, associated with high progesterone levels. A decrease in the membrane and cytoplasmic intensity of immunolabelling for β-catenin during the secretory stages, accompanying the reduction in E-cadherin immunostaining, was also found. We speculate that a softening of the lateral intercellular connections during diestrus would favour embryo implantation. The cyclic changes in the cytoplasmic immunopattern for β-catenin might also be suggestive of a possible activation of the Wnt signalling pathway during the proliferative stages of the canine estrous cycle.

5. Acknowledgements

The authors thank Mrs. Ligia Lourenço (UTAD) and Mrs. Annika Rickberg for their expert technical assistance.

The Portuguese Science and Technology Foundation (FCT) sponsored this work under the Project PEst-OE/AGR/UI0772/2011, and granted Rita Payan Carreira with a sabbatical fellowship (SFRH/BSAB/938/2009).

6. Chapter reference list

[1] Barrau MD, Abel JH, Jr., Verhage HG, Tietz WJ, Jr. Development of the endometrium during the estrous cycle in the bitch. Am J Anat. 1975;142:47-65.

[2] Rahnama F, Thompson B, Steiner M, Shafiei F, Lobie PE, Mitchell MD. Epigenetic regulation of E-cadherin controls endometrial receptivity. Endocrinology. 2009;150:1466-72.

[3] Aplin JD. Adhesion molecules in implantation. Rev Reprod. 1997;2:84-93.

[4] Salamonsen LA, Nie G, Hannan NJ, Dimitriadis E. Society for Reproductive Biology Founders' Lecture 2009. Preparing fertile soil: the importance of endometrial receptivity. Reprod Fertil Dev. 2009;21:923-34.

[5] Aplin JD. Embryo implantation: the molecular mechanism remains elusive. Reprod Biomed Online. 2006;13:833-9.

[6] Cavagna M, Mantese JC. Biomarkers of endometrial receptivity - A review. Placenta. 2003;24.

[7] Bauersachs S, Mitko K, Ulbrich SE, Blum H, Wolf E. Transcriptome studies of bovine endometrium reveal molecular profiles characteristic for specific stages of estrous cycle and early pregnancy. Exp Clin Endocrinol Diabetes. 2008;116:371-84.

[8] Singh H, Aplin JD. Adhesion molecules in endometrial epithelium: tissue integrity and embryo implantation. J Anat. 2009;215:3-13.

[9] Halbleib JM, Nelson WJ. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. Genes Dev. 2006;20:3199-214.

[10] van der Linden PJ, de Goeij AF, Dunselman GA, Erkens HW, Evers JL. Expression of cadherins and integrins in human endometrium throughout the menstrual cycle. Fertil Steril. 1995;63:1210-6.

[11] Fujimoto J, Ichigo S, Hori M, Tamaya T. Alteration of E-cadherin, alpha- and beta-catenin mRNA expression in human uterine endometrium during the menstrual cycle. Gynecol Endocrinol. 1996;10:187-91.

[12] Ryan PL, Baum DL, Lenhart JA, Ohleth KM, Bagnell CA. Expression of uterine and cervical epithelial cadherin during relaxin-induced growth in pigs. Reproduction. 2001;122:929-37.

[13] Satterfield MC, Dunlap KA, Hayashi K, Burghardt RC, Spencer TE, Bazer FW. Tight and adherens junctions in the ovine uterus: differential regulation by pregnancy and progesterone. Endocrinology. 2007;148:3922-31.

[14] Kiewisz J, Kaczmarek MM, Andronowska A, Blitek A, Ziecik AJ. Gene expression of WNTs, β-catenin and E-cadherin during the periimplantation period of pregnancy in pigs-involvement of steroid hormones. Theriogenology. 2011;76:687-99.

[15] Leckband D. Beyond structure: mechanism and dynamics of intercellular adhesion. Biochem Soc Trans. 2008;36:213-20.

[16] Wirtz-Peitz F, Zallen JA. Junctional trafficking and epithelial morphogenesis. Curr Opin Genetics Dev. 2009;19:350-6.

[17] Nejsum LN, Nelson WJ. A molecular mechanism directly linking E-cadherin adhesion to initiation of epithelial cell surface polarity. J Cell Biol. 2007;178:323-35.

[18] Li Q, Wang J, Armant DR, Bagchi MK, Bagchi IC. Calcitonin down-regulates E-cadherin expression in rodent uterine epithelium during implantation. J Biol Chem. 2002;277:46447-55.

[19] Jha RK, Titus S, Saxena D, Kumar PG, Laloraya M. Profiling of E-cadherin, beta-catenin and Ca^{2+} in embryo-uterine interactions at implantation. FEBS Lett. 2006;580:5653-60.

[20] Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, *et al.* ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. Proc Natl Acad Sci U S A. 2005;102:9182-7.

[21] Perry JK, Lins RJ, Lobie PE, Mitchell MD. Regulation of invasive growth: similar epigenetic mechanisms underpin tumour progression and implantation in human pregnancy. Clin Sci. 2010;118:451-7.

[22] Dawood MY, Lau M, Khan-Dawood FS. E-cadherin and its messenger ribonucleic acid in periimplantation phase human endometrium in normal and clomiphene-treated cycles. Am J Obstet Gynecol. 1998;178:996-1001.

[23] Liu G, Zhang X, Lin H, Wang H, Li Q, Ni J, *et al.* Effects of E-cadherin on mouse embryo implantation and expression of matrix metalloproteinase-2 and -9. Biochem Biophys Res Commun. 2006;343:832-8.

[24] Guo B, Han BC, Tian Z, Zhang XM, Jiang LX, Liu JX, *et al.* Expression and Hormonal Regulation of E-Cadherin in Canine Uterus During Early Pregnancy. Reprod Domest Anim. 2009.

[25] Lilien J, Balsamo J, Arregui C, Xu G. Turn-off, drop-out: functional state switching of cadherins. Dev Dyn. 2002;224:18-29.

[26] Nelson WJ. Regulation of cell-cell adhesion by the cadherin-catenin complex. Biochem Soc Trans. 2008;36:149-55.

[27] Yang S, Guo X, Debnath G, Mohandas N, An X. Protein 4.1R links E-cadherin/beta-catenin complex to the cytoskeleton through its direct interaction with beta-catenin and modulates adherens junction integrity. BBA Biomembranes. 2009;1788:1458-65.

[28] Huber AH, Weis WI. The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. Cell. 2001;105:391-402.

[29] Gottardi CJ, Gumbiner BM. Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. J Cell Biol. 2004;167:339-49.

[30] Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, Veldscholte J, *et al.* Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. Clin Cancer Res. 2009;15:5784-93.

[31] Chen Q, Zhang Y, Lu J, Wang Q, Wang S, Cao Y, *et al.* Embryo-uterine cross-talk during implantation: the role of Wnt signaling. Mol Hum Reprod. 2009;15:215-21.

[32] Wooding P, Burton G. Endotheliochorial Placentation: Cat, Dog, Bat. Comparative placentation: structures, functions and evolution. Heidelberg, Germany: Springer-Verlag Berlin Heidelberg; 2008. p. 169-83.

[33] Matsuzaki S, Darcha C, Maleysson E, Canis M, Mage G. Impaired down-regulation of E-cadherin and beta-catenin protein expression in endometrial epithelial cells in the mid-secretory endometrium of infertile patients with endometriosis. J Clin Endocrinol Metab. 2010;95:3437-45.

[34] Shih HC, Shiozawa T, Miyamoto T, Kashima H, Feng YZ, Kurai M, *et al.* Immunohistochemical expression of E-cadherin and beta-catenin in the normal and malignant human endometrium: an inverse correlation between E-cadherin and nuclear beta-catenin expression. Anticancer Res. 2004;24:3843-50.

[35] Concannon PW. Reproductive cycles of the domestic bitch. Anim Reprod Sci. 2011;124:200-10.

[36] Barrau MD, Abel JH, Torbit CA, Tietz WJ. Development of the implantation chamber in the pregnant bitch. Am J Anat. 1975;143:115-30.

[37] Holst PA, Phemister RD. The prenatal development of the dog: preimplantation events. Biol Reprod. 1971;5:194-206.

[38] Payan-Carreira R, Pires MA, Holst BS, Rodriguez-Martinez H. Tumour Necrosis Factor in the Canine Endometrium: An Immunohistochemical Study. Reprod Domest Anim. 2011;46:410-8.

[39] Payan-Carreira R, Santos C, Miranda S, Pereira RMLN, Santos D, Pires MA. Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium. Theriogenology. 2014;82:815-26.

[40] Amoroso EC. Placentation. In: Parkes AS, editor. Marshall's Physiology of Reproduction. London: Longmans Green and Co.; 1952. p. 127-311.

[41] Gama A, Paredes J, Gartner F, Alves A, Schmitt F. Expression of E-cadherin, P-cadherin and beta-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival. Vet J. 2008;177:45-53.

[42] McEwan M, Lins RJ, Munro SK, Vincent ZL, Ponnampalam AP, Mitchell MD. Cytokine regulation during the formation of the fetal-maternal interface: focus on cell-cell adhesion and remodelling of the extra-cellular matrix. Cytokine Growth Factor Rev. 2009;20:241-9.

[43] Jeong JW, Lee HS, Franco HL, Broaddus RR, Taketo MM, Tsai SY, *et al.* beta-catenin mediates glandular formation and dysregulation of beta-catenin induces hyperplasia formation in the murine uterus. Oncogene. 2009;28:31-40.

[44] Bartley J, Jülicher A, Hotz B, Mechsner S, Hotz H. Epithelial to mesenchymal transition (EMT) seems to be regulated differently in endometriosis and the endometrium. Arch Gynecol Obstet. 2014;289:871-81.

[45] Birchmeier W, Behrens J. Cadherin Expression in Carcinomas - Role in the Formation of Cell-Junctions and the Prevention of Invasiveness. BBA Rev Cancer. 1994;1198:11-26.

[46] Bremnes RM, Veve R, Hirsch FR, Franklin WA. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis, and prognosis. Lung Cancer. 2002;36:115-24.

[47] Yoshida R, Kimura N, Harada Y, Ohuchi N. The loss of E-cadherin, alpha- and beta-catenin expression is associated with metastasis and poor prognosis in invasive breast cancer. Int J Oncol. 2001;18:513-20.

[48] Delva E, Kowalczyk AP. Regulation of cadherin trafficking. Traffic. 2009;10:259-67.

[49] Ishiguro K, Baba E, Torii R, Tamada H, Kawate N, Hatoya S, *et al.* Reduction of mucin-1 gene expression associated with increased *Escherichia coli* adherence in the canine uterus in the early stage of dioestrus. Vet J. 2007;173:325-32.

[50] Nei H, Saito T, Yamasaki H, Mizumoto H, Ito E, Kudo R. Nuclear localization of beta-catenin in normal and carcinogenic endometrium. Mol Carcinog. 1999;25:207-18.

Chapter 6 – Distribution of superoxide dismutase 1 and glutathione peroxidase 1 in the cyclic canine endometrium

THE CONTENT OF THIS CHAPTER WAS SUBMITTED TO PUBLICATION AT:

Theriogenology (submission ref.: THERIO-D-15-00716)

DISTRIBUTION OF SUPEROXIDE DISMUTASE 1 AND GLUTATHIONE PEROXIDASE 1 IN THE CYCLIC CANINE ENDOMETRIUM

Celso Santos[1*], Maria dos Anjos Pires[1*]; Dario Santos[2]; Rita Payan-Carreira[1#]

[1] CECAV, Centro de Ciência Animal e Veterinária, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal;

[2] CITAB, Centro de Investigação e Tecnologias Agroambientais e Biológicas, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal;

*These authors contributed equally to this work

[#] Corresponding author: Rita Payan-Carreira, CECAV, Zootecnia Department, University of Trás-os-Montes and Alto Douro; Vila Real, Portugal; Fax: +351.259350480.

E-mail address: rtpayan@gmail.com

Abstract

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are two important antioxidant enzymes integrating the scavenging system for reactive oxygen species (ROS) in cells or tissues. These enzymes, either directly or by controlling the local amount ROS, are involved in tissue homeostasis, including in the endometrium. Information concerning antioxidant enzymes in dog uterus is almost inexistent. Therefore, this work intends to establish the pattern of distribution of SOD1 and GPx1 immunoreaction in canine endometrium. Using 46 samples of canine healthy endometrium representing different cycle stages (anestrus-10; proestrus-10; estrus-10; early diestrus-7 and diestrus-9), cyclic changes in tissue distribution of positive cells and subcellular dislocation of immunostaining were analysed. SOD1 distribution in canine endometrium showed cyclic variations ($p \leq 0.001$), the progesterone-associated stages presenting the higher immuno-scores. Changes in immunoreaction also interested the different epithelial structures considered (surface,

superficial glandular and deep glandular epithelia, respectively SE, SGE and DGE) ($p \leq 0.001$), but it was always higher than in the stroma ($p \leq 0.001$). Moreover, DGE cells usually showed higher scores of immunoreaction compared to the other epithelial cells. Interestingly, in epithelial cells, distinct subcellular patterns for SOD1 immunostaining: the nuclear labelling was observed in estrus and early diestrus ($p \leq 0.001$), whereas an apical reinforcement was observed in estrus ($p=0.011$) in the glandular epithelia but not in the SE. In general, GPx1 distribution in canine endometrium remained relatively unchanged throughout the estrous cycle ($p=0.169$), despite the slight decrease observed from proestrus to early diestrus. The highest scores were found in anestrus and diestrus ($p<0.05$), varying with of the structure considered. An apical reinforcement pattern was also found for this molecule, which peaked in proestrus and estrus ($p<0.005$). Collectively, the present study showed that SOD1 and GPx1 are consistently distributed in the canine endometrium. The cyclic changes registered for both molecules suggest they may play important roles in endometrial physiology.

Keywords: reactive oxygen species; oxidative stress; antioxidant enzymes; immunohistochemistry; uterus; dogs

1. Introduction

The mammalian endometrium is a complex and highly dynamic tissue, with the ultimate purpose to guarantee embryo survival, implantation and a successful pregnancy. In response to the alternancy in sex steroids, the endometrium undergoes cyclic remodelling, integrating morphological and functional changes. This process, named as the *endometrial cycle* in humans, is ultimately controlled by diverse cytokines, interleukins and growth factors, among other molecules [1-3].

As in other aerobic systems, endometrial cells continuously generate reactive oxygen species (ROS), as a consequence of their normal metabolism. The term reactive oxygen species refers to radical and non-radical oxygen species formed by the partial reduction of oxygen, such as the superoxide anion ($O_2^{\bullet -}$), the hydrogen peroxide (H_2O_2), or the hydroxyl radical ($OH^{\bullet}$) [4]. Physiological amounts of ROS are necessary for normal tissue functioning,

as they are involved in the regulation of different cellular signalling pathways [4, 5], that affect different cellular processes such as proliferation, differentiation, and programed cell death (apoptosis).

ROS control is an important process sought to maintain tissue homeostasis [6]. The amount of ROS produced in tissues is maintained within physiological balanced levels by a network of highly complex and integrated defence mechanisms, through specific scavenger reactions and detoxification pathways, that include endogenous enzymatic and non-enzymatic antioxidant systems [7]. The key endogenous enzymes directly involved in the control of ROS production include the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPxs), glutathione reductase (GSR) and glutathione-S-transferase (GST), whereas the glutathione, the nicotinamide adenine dinucleotide phosphate ($NADP^+$) and the reduced form of $NADP^+$ (NADPH) integrate the non-enzymatic endogenous antioxidant systems [7, 8]. A variety of other dietary antioxidant substances also exist, such as vitamin C, vitamin E, carotenoids or the natural flavonoids [7].

In a first step of the scavenger defense mechanism that maintain the redox homeostasis occurs the dismutation of the superoxide anion ($O_2^{\bullet -}$) into H_2O_2 and O_2, by the superoxide dismutase (SOD). In mammals, SOD possess three different isoforms: a copper-zinc containing SOD (Cu,Zn-SOD or SOD1), a dimeric protein found in the cell cytoplasm; a manganese containing SOD (Mn-SOD or SOD2), located in the mitochondrial matrix; and a Cu- and Zn containing SOD (ecSOD or SOD3), a tetrameric glycoprotein that constitutes the major SOD isoform in extracellular fluids [9]. In a second step to avoid ROS propagation, the produced H_2O_2 is quickly converted into H_2O and O_2 by GPxs, typically through the oxidation of glutathione. There are several known GPxs isoforms (GPx1 to 8), some of them identified in specific tissues such as the gastro-intestinal or the male reproductive tract, or the lungs [10, 11]. GPx1, located in the cell cytosol and mitochondria, is ubiquitously expressed in tissues and represents the major antioxidant enzyme of GPx family [12].

A delicate and complex balance between ROS and the protective antioxidant mechanisms exists in cells and tissues. ROS are not only toxic for cells but rather exert crucial roles in tissue homeostasis. When ROS accumulates, overwhelming a physiological threshold, oxidative stress occurs. Oxidative stress may damage cell structures, nucleic acids, lipids and proteins. It has been implicated in diverse degenerative diseases, aging, infertility and carcinogenesis [4].

In the reproductive system, alike diverse other body systems, ROS are maintained under a delicate balance by the antioxidant mechanisms. It is now commonly accepted that ROS have a crucial role within the female reproductive system, actively contributing to fertility

[13, 14] and early pregnancy events [15, 16]. In humans, under physiological levels, ROS mediates a number of significant roles in the endometrium, including the hormone signalling, angiogenesis, apoptosis, cell proliferation and prostaglandin secretion [17, 18]. It has been demonstrated ROS involvement with progesterone-mediated physiological events, such as decidualization or menstruation [17, 19].

Changes in the expression of ROS and antioxidants enzymes have been evidenced during the endometrial cycle in women [14, 15, 20]. References on ROS and antioxidants enzymes expression in the endometrium of domestic animals are however scarce. Nevertheless, the work of Al-Gubory *et al.* [16] in sheep and that of Ramos *et al.* [21] in cows are suggestive of the existence of changes in antioxidant enzymes throughout the estrous cycle.

The interest in studying the role of oxidative stress in female reproduction is increasing, particularly regarding its possible association with infertility and the development of uterine diseases. Dogs present a typical hormonal profiling during their estrous cycle, that is considerably different from those of other domestic species. Dogs are monoestric species, meaning that a stage of anestrus separates two consecutive reproductive cycles [22], which is fundamental for the maintenance of female dog fertility [23]. Further unique features of the canine reproductive biology include a relatively prolonged follicular stage, the pre-ovulatory luteinisation of the dominant ovarian follicles, the ovulation of an immature oocyte that takes close to 3 days to became fertilizable, and a relatively long diestrus, similarly lengthened in cyclic or pregnant diestrus [22]. The characteristic extended luteal stage favours the incidence of progesterone-associated diseases, such as deciduoma formation [24] or the cystic endometrial hyperplasia and pyometra complex [25].

The balance of oxidative stress within the canine endometrium is poorly known, despite that recently Kobayashi and colleagues [26] described an increase in SOD activity in the uterine fluid of bitches in diestrus compared to anestrus or estrus. Therefore, this work intends to analyse the immunohistochemical pattern of distribution of two antioxidant enzymes, the copper-zinc containing SOD (SOD1) and the glutathione peroxidase 1 (GPx1) in the canine endometrium throughout the estrous cycle.

2. Material and methods

Tissue collection and preparation

This study included forty-six post-pubertal, healthy non-pregnant bitches. Most animals were mongrels (n=33) or crossbreds [n= 7; distributed as follows: Poodle (3), Portuguese Podenco (2), Pincher (1), Pequinois (1) crosses]; purebreds were represented by Siberian husky (n=3), Cocker Spaniel (n=2) and Labrador retriever (n=1). The ages ranged from 10 months to 8 years old, with an average of 2.5 years, distributed in the following age groups: 34 females from 10 months to <3 years old, nine females with ages between 3 and <6 years old, and three females being 6 to 8 years old.

Samples of canine endometrial tissue were collected during elective ovariohysterectomy (OVH), requested for contraception purposes, and used with the owners' informed consent, in accordance to the International Ethical standards. Transversal fragments from each uterine horn, along with a transversal hemi-section from each ovary, were collected immediately after the surgery. After fixed in 10% formalin, embedded in paraffin wax, sectioned at 3 μm and stained with haematoxylin and eosin, the uterine and ovarian sections were used for histological staging of the estrous cycle and for excluding uterine disease.

Before surgery a blood sample was collected from the jugular vein into a controlled vacuum tube (Serum-gel, S-Monovette®, Sarstedt, Nümbrecht, Germany), centrifuged and stored at -20ºC until analysis. Serum progesterone levels were determined by chemiluminescence immunoassay system (Immulite®; DPC-Diagnostic Products Corp., Los Angeles, CA, USA).

Staging of estrous cycle

Non-pregnant animals were initially selected on the basis of the vaginal cytology, collected prior to surgery. At OVH, the stage of the estrous cycle for each bitch was determined both by inspection of the ovaries and later confirmed upon the histological examination; progesterone levels were used to confirm the histological staging [27].

Uterine samples used in this study represented anestrus (n=10), proestrus (n=10), estrus (n=10), while the diestrus was further divided in early diestrus (n=7) and diestrus (n = 9), according to Payan-Carreira and colleagues [28].

Immunohistochemistry

To analyse the SOD1 and GPx1 distribution pattern in the canine endometrium, we used tissue sections 3 µm thick on silane-coated slides and a streptavidin-biotin-peroxidase technique (UltraVision, Lab Vision, Fremont, CA, USA). The primary antibodies used were rabbit polyclonal antibodies raised against superoxide dismutase 1 (SOD1, ab13498; Abcam®, Cambridge, UK) and glutathione peroxidase 1 (GPx1, ab59546, Abcam® Cambridge, UK), respectively at the dilutions of 1:300 and 1:200.

The slides were routinely deparaffinised and hydrated, pre-treated to enhance antigen retrieval (microwave irradiation; 750W for 3x5 minutes, the slides immersed in citrate buffer pH 6.0) and the endogenous peroxidases blocked by 30 minutes immersion in 3% hydrogen peroxide/PBS, followed by inhibition of non-specific binding by incubation with Large Volume Ultra V-Block (UltraVision, Lab Vision, Fremont, CA, USA) for 5 min. Incubation with the primary antibodies was performed in a humid chamber, overnight at 4ºC. Thereafter, the labelled slides were incubated with a biotin conjugated secondary antibody, followed by incubation with enzyme-labelled streptavidin, for 10 minutes each, at room temperature. The reactions were visualized using DAB (3,3'-diaminobenzidine) as a chromogen, and then counterstained with Gill's haematoxylin, dehydrated and mounted.

For negative controls, anestrus samples were incubated with rabbit control immunoglobulin (rabbit IgG; Santa Cruz Biotechnology) or PBS in substitution to SOD or GPx primary antibodies. In neither negative control positive immunoreaction was observed.

Immunohistochemical scoring

Two independent observers blindly assessed the distribution pattern and sub-cellular dislocation of the immunoreaction against SOD1 and GPx1. A low magnification (objective of x4) was firstly used to examine the overall pattern of the tissue section, whereas a higher magnification (objective of x40) was used to determine the proportion of positive cells, to define the intensity of immunolabelling and to detail the sub-cellular distribution of the

immunoreaction. Positivity was indicated by the presence of distinct brownish staining. For scoring, ten non-overlapping fields of view were examined at 400x magnification, in a systematic random sampling pattern.

Immunoreactivity to the target molecules was assessed independently for each endometrial component (Stroma – S, Luminal Epithelium – LE, Superficial Glandular Epithelia – SGE and Deep Glandular Epithelia – DGE). Stroma labelling was represented, particularly for GPx1, by both the stromal matrix and cell staining; also SOD1 immunoreaction was observed in some resident immune cells. However, only the cell labelling in stromal fibroblast was accounted for this study.

The percentage of positive epithelial cells was assessed semiquantitatively, according to the marks: 0 (negative, <10%), 1 (10-25% positive cells), 2 (26-50% positive cells), 3 (51-90% positive cells) and 4 (>90% positive cells). When immunoreaction existed, either in the epithelia or the stroma, its intensity was graded as weak (1+), moderate (2+) or strong (3+). These two parameters (intensity and percentage of labelled cells) were used to compute a total labelling graded as negative (N) weak (W), moderate (M), or strong (S) positive (Table 1). Figure 1 shows examples of the total scoring grades for both the SOD1 and GPx1 immunoreactions.

Table 1 - Criteria for grading SOD1 and GPx1 Immunoreactivity

Scores	Intensity		
Percentage of labelled cells	1+	2+	3+
Negative (<10%)		Negative	
1 (10-25% positive cells)	Weak	Weak	Weak
2 (26-50% positive cells)	Weak	Moderate	Moderate
3 (51-90% positive cells)	Weak	Moderate	Strong
4 (>90% positive cells)	Moderate	Strong	Strong

A diffuse cytoplasmic labelling with evidences of membrane staining was considered the normal pattern for both SOD1 and GPx1. Dislocation of the normal pattern of the immunoreaction to different sub-cellular areas were also annotated, namely the existence of apical reinforcement (Figure 1.A and 1.D for SOD1 and GPx1 labelling, respectively) or nuclear labelling (Figure 1.C).

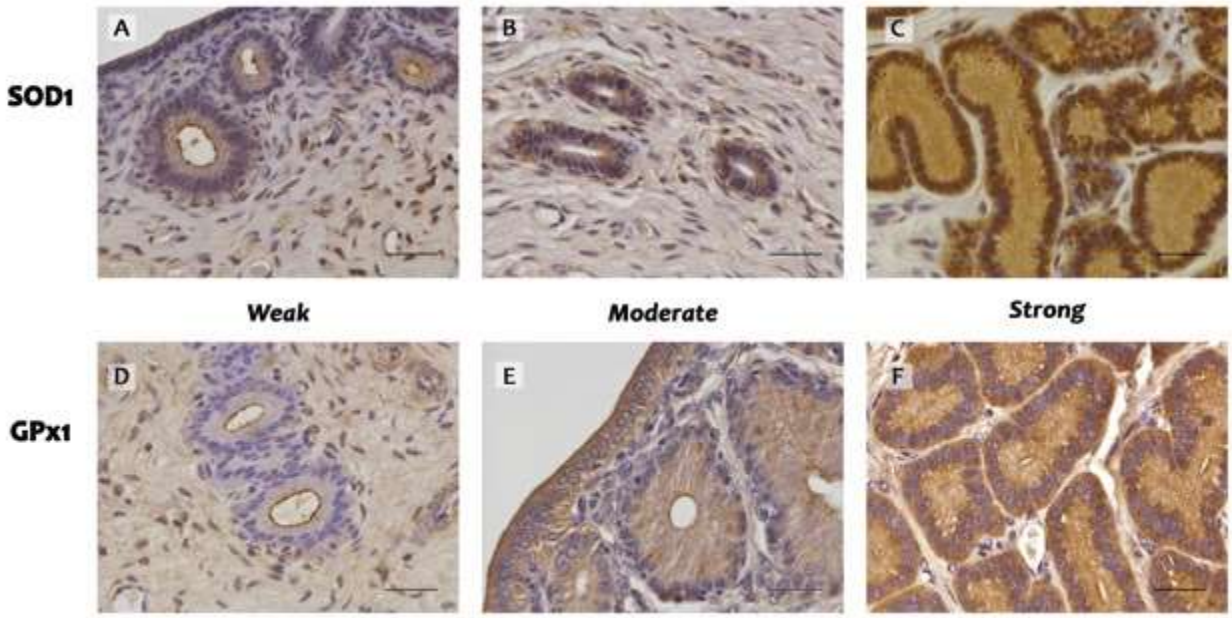


Figure 1 – Total scoring grades for SOD1 (images A to C) and GPx1 (images D to F) molecular expression and examples of the subcellular dislocation of the immunoreaction in the canine endometrium. The immunoreaction was counterstained with Gill´s Haematoxylin. Scale bar = 300 µm. **A** – A weak immunoreaction against SOD1 showed in the epithelial cells of the surface and the superficial glandular epithelia; the later also presented apical reinforcement. It is also noticeable the reduced expression of SOD1 in the subluminal area of the endometrial stroma (endometrial sample in estrus). **B** – This image shows the moderate grade of SOD1 immunolabeling in the superficial glandular epithelium (endometrial sample in proestrus). **C** – An example of a strong total score found in deep glandular epithelial cells, which also showed nuclear labelling for SOD1 (endometrial sample in diestrus). **D** – This image illustrate a weak total score for GPx1 evidenced in the superficial epithelial cells as well as in the stroma; the epithelial cells also showed apical dislocation of the labelling (endometrial sample in estrus). **E** – The moderate grade of GPx1 labelling is illustrated in this image, either in the surface epithelium and the superficial glandular epithelium (endometrial sample in diestrus). **F** – This image illustrates the strong total score for GPx1 immunoreaction evidenced in the deep glandular endometrium (endometrial sample in diestrus).

Statistical analysis

The statistical software IBM SPSS Statistics version 22.0 for Mac OS X 10.8 was used to perform statistical comparisons. All statistical analyses were performed using the chi-square and Fisher's exact tests. The Z-test was performed for group comparisons, the *P* -

values adjusted by Bonferroni method. A P value ≤ 0.05 was regarded as statistically significant.

3. Results

SOD and GPx immunoexpression were present in all samples of canine endometrium analyzed. Data registered for this study on canine endometrial expression of SOD and GPx are summarized in Tables 2 (SOD1) and 3 (PGx1).

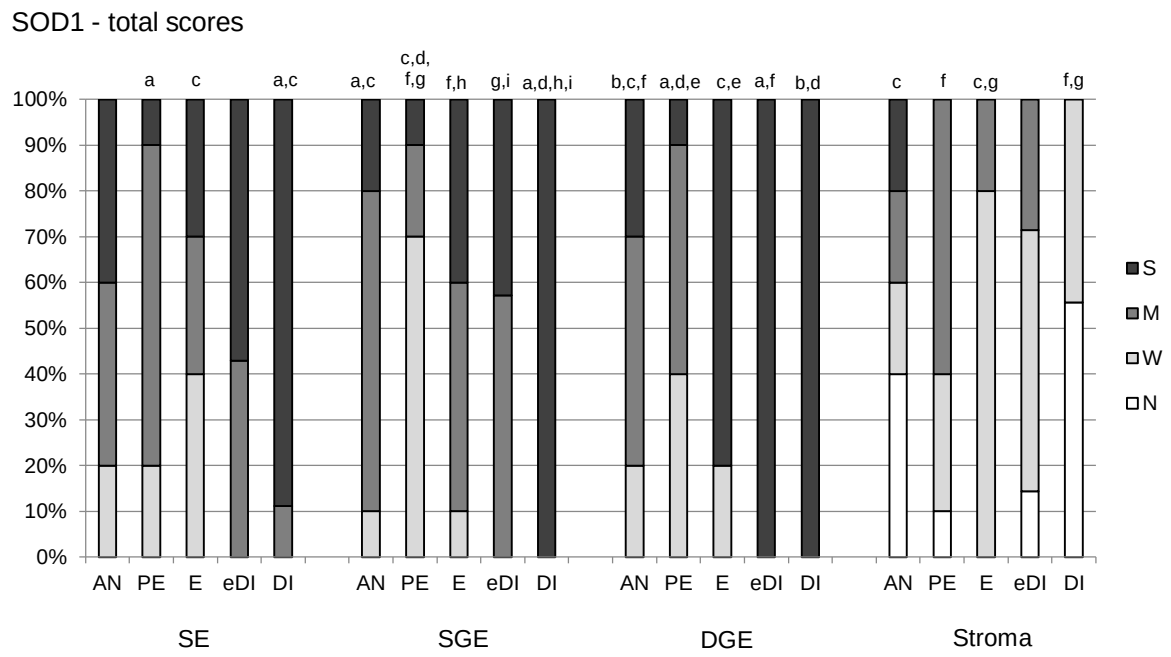
SOD1

In general, SOD1 immunoreaction in the canine endometrium changed during the estrous cycle (P ≤ 0.001). The immunohistochemistry scores decreased from anestrus to proestrus (P = 0.015) and thereafter it increased from estrus to diestrus (P < 0.01; Table 2). Early diestrus presented an intensity of immunolabeling similar to anestrus and estrus (P = 0.306 and P = 0.121, respectively); diestrus was the stage presenting the highest scores (P < 0.005; Table 2).

The stage of the cycle affected the immunoexpression of SOD1 molecule among the represented types of endometrial epithelia (P ≤ 0.001). The total SE scores varied during the canine estrous cycle (P = 0.020; Table 2; Graph 1). Albeit the decrease observed in estrogen-associated stages (proestrus and estrus) or the increase in scores recorded in progesterone-associated stages (early diestrus and diestrus), the differences were devoid of significant except between diestrus and proestrus or estrus (P < 0.05). Diestrus was the stage showing the highest immune scores (Table 2; Graph 1).

Table 2 - Immunoreactivity scores for SOD1 – the intensity of immunolabeling and the percentage of labeled cells – in canine endometrial samples throughout the stages of the estrous cycle.

	Scores	Stage					*P* -Value
		AN (n = 10)	PE (n = 10)	E (n = 10)	EDi (n = 7)	Di (n = 9)	
	Intensity of immunoreaction						
SE	Weak	2	2	4	0	0	0.020
	Moderate	4	7	3	3	1	
	Strong	4	1	3	4	8	
SGE	Weak	1	7	1	0	0	≤0.001
	Moderate	7	2	5	4	0	
	Strong	2	1	4	3	9	
DGE	Weak	2	4	2	0	0	≤0.001
	Moderate	5	5	0	0	0	
	Strong	3	1	8	7	9	
Str	Negative	4	1	0	1	5	0.006
	Weak	2	3	8	4	4	
	Moderate	2	6	2	2	0	
	Strong	2	0	0	0	0	
	Apical reinforcement pattern						
SE	No	10	10	10	7	9	1
	Yes	0	0	0	0	0	
SGE	No	10	10	4	5	9	≤0.001
	Yes	0	0	6	2	0	
DGE	No	10	10	4	6	9	≤0.001
	Yes	0	0	6	1	0	
	Nuclear localization						
SE	No	8	4	6	2	7	0.138
	Yes	2	6	4	5	2	
SGE	No	9	9	2	3	8	≤0.001
	Yes	1	1	8	4	1	
DGE	No	9	10	0	3	8	≤0.001
	Yes	1	0	10	4	1	



Graph 1 - Graphic representation of the intensity scores for SOD1 in the different endometrial structures during the canine estrous cycle. Within each endometrial structure, different column superscripts indicate statistical significance at $P < 0.05$: a,d → $P \leq 0.001$; b,e → $P \leq 0.01$; c,f,g,h,i → $P \leq 0.05$. [N – Negative; W – Weak; M – Moderate; S – Strong].

The scores for SGE showed important changes throughout the estrous cycle ($P \leq 0.001$; Table 2; Graph 1). A marked decrease in the SGE total scores was noticed from anestrus to proestrus ($P = 0.022$), proestrus being the stage with the lowest scores ($P < 0.03$). From estrus towards diestrus, the SOD1 total scores (Graph 1) increased, once again the diestrus being the stage presenting the strongest labelling scores ($P < 0.02$). Also the DGE presented cyclic changes during the canine estrous cycle ($P \leq 0.001$; Table 2; Graph 1). In general, the DGE scores were higher compared with those of the SGE ($P \leq 0.001$). A non-significant decrease in the total immunochemistry scores was found from anestrus to proestrus; thereafter, the scores significantly increased towards diestrus ($P < 0.003$; Table 2; Graph 1), albeit no differences existed for scores in estrus, early diestrus and diestrus.

The immunoreaction against SOD1 in endometrial stroma (Table 2; Graph 1) presented generally lower scores than those evidenced by epithelial cells ($P \leq 0.001$). Moreover, the immunoreaction in stroma cells significantly varied during the canine estrous cycle ($P = 0.006$). Anestrus displayed higher cell labelling scores, although differences were established only between anestrus and estrus ($P = 0.012$). The stromal cells in diestrus samples showed lower immunoreaction scores (Table 2; Graph 1) that differed only from

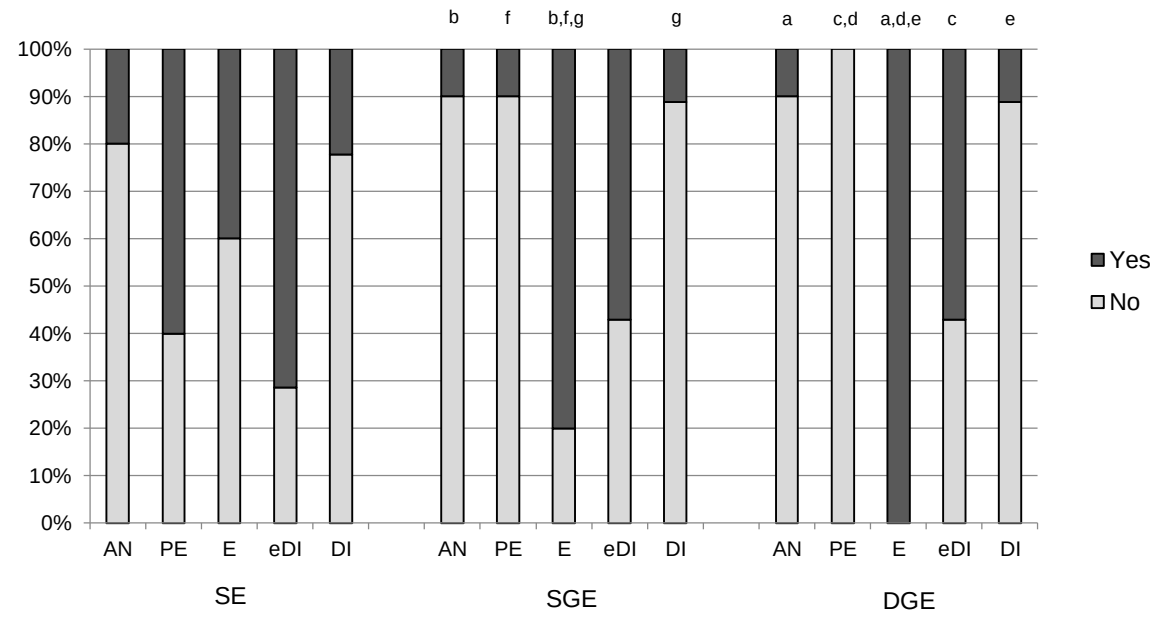
those of proestrus and estrus samples (P = 0.017 and P = 0.013, respectively). A tendency for lower scores was perceived between proestrus and estrus (P = 0.071) and between early diestrus and diestrus (P = 0.069). Interestingly, in estrus and early diestrus the sub-epithelial stroma cells showed weaker immunoreaction than the remainder of the endometrial stroma, while in the other stages of the cycle (Figure 1.A), the stromal cell labelling was rather uniform.

Interestingly, the subcellular pattern of SOD1 in the epithelial cells changed in estrus and early diestrus compared to all other stages of the cycle ($P \leq 0.001$). The nuclear localization of SOD1 immunostaining prevailed in estrus and early diestrus samples (Graph 2), but was lower in all other stages. These changes interested particularly the glandular epithelium, and appeared in estrus samples (P = 0.001 and $P < 0.01$, respectively for DGE and SGE) and in a lesser extent the early diestrus (P = 0.05) compared with the nuclear labelling evidenced in anestrus and proestrus samples (Graph 2). In addition, a pattern of cytoplasmic immunolabeling with apical reinforcement was observed almost exclusively in estrus (P = 0.011), in both the SGE and DGE, but was practically absent from the SE (Graph 2). Moreover, the apical reinforcement pattern was absent from the glandular epithelium in anestrus, proestrus and diestrus.

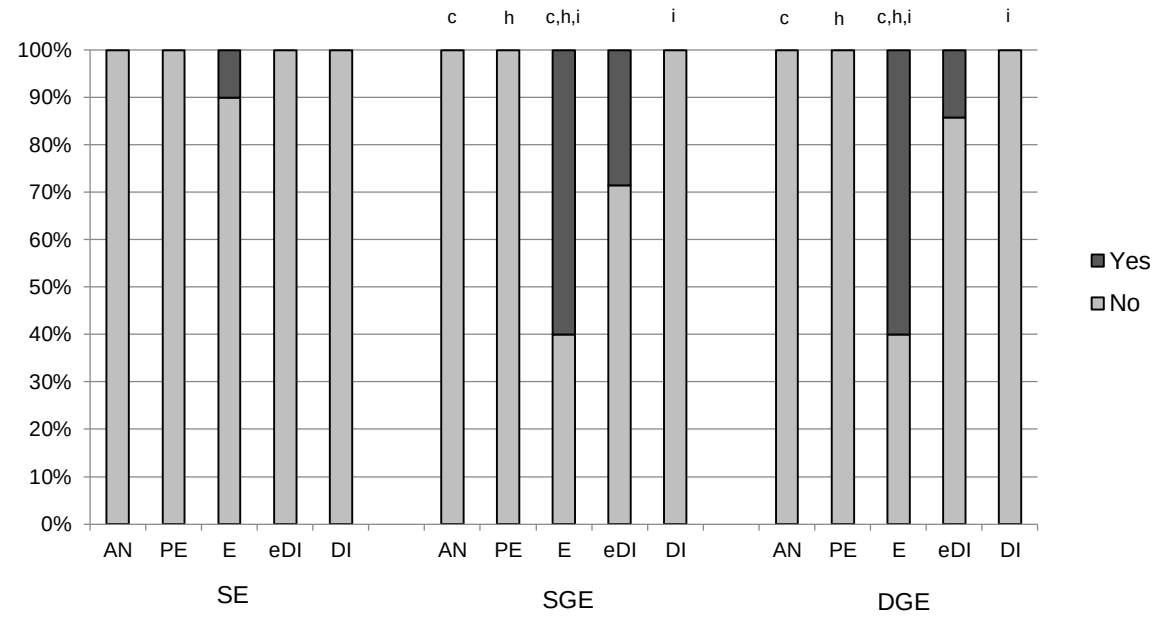
GPx1

In general, the immunostaining for GPx1 in canine endometrium remains relatively unchanged throughout the estrous cycle (P = 0.169). Nevertheless, occasional tendencies were found respecting a decrease in the total scores in estrus compared to anestrus (P = 0.069) or diestrus (P = 0.072) (Table 3).

SOD1 - Nuclear staining



SOD1 - Apical reinforcement pattern



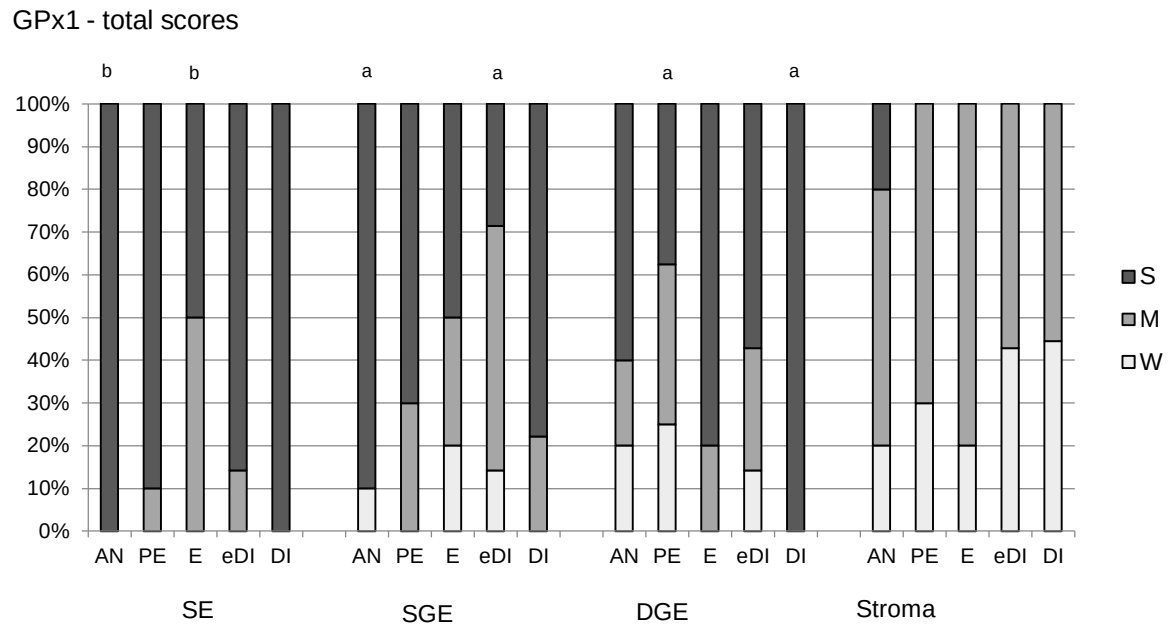
Graph 2 - Prevalence of nuclear staining (upper) and the apical reinforcement (bottom) patterns in SOD1 in the different endometrial epithelia throughout the canine estrous cycle. Within each endometrial structure,, different column superscripts indicate statistical significance at $P < 0.05$: a,d,e → $P \leq 0.001$; b,f,g → $P \leq 0.01$; c,h,i → $P \leq 0.05$.

Table 3 - Immunoreactivity scores for GPx1 – the intensity of immunolabeling, the percentage of labeled cells and the number of cases displaying apical reinforcement – in canine endometrial samples throughout the stages of the estrous cycle.

Scores		Stage					*P* -Value
		AN (n = 10)	PE (n = 10)	E (n = 10)	EDi (n = 7)	Di (n = 9)	
Intensity of immunoreaction							
SE	Weak	0	0	0	0	0	0.029
	Moderate	0	1	5	1	0	
	Strong	10	9	5	6	9	
SGE	Weak	1	0	2	1	0	0.050
	Moderate	0	3	3	4	2	
	Strong	9	7	5	2	7	
DGE	Weak	2	2	0	1	0	0.115
	Moderate	2	3	2	2	0	
	Strong	6	3	8	4	9	
Str	Negative	2	3	2	3	4	0.594
	Weak	6	7	8	4	5	
	Moderate	2	0	0	0	0	
	Strong	0	0	0	0	0	
Apical reinforcement pattern							
SE	No	3	1	0	3	8	0.001
	Yes	7	9	10	4	1	
SGE	No	3	0	0	3	6	0.004
	Yes	7	10	10	4	3	
DGE	No	8	3	0	4	9	≤0.001
	Yes	2	7	10	3	0	

Moreover, the total immunolabeling scores varied during the cycle in the SE (*P* = 0.029) and SGE (*P* = 0.050), but not in DGE (*P* = 0.115; Graph 3). Higher immunoreaction scores were generally obtained in SE compared with the glandular epithelium (*P* = 0.034). SE total scores decreased in estrus compared to anestrus (*P* = 0.033); a tendency for a reduction in scores (Table 3; Graph 3) was also observed between estrus and diestrus (*P* = 0.082), but differences were not found in all other stages of the cycle. The scores for SGE showed a decrease from proestrus towards early diestrus (Graph 3). However, these changes only showed statistical significance between early diestrus and anestrus (*P* = 0.007), while a tendency for a difference in scores was noticed between anestrus and estrus (*P* = 0.069). The total scores registered for DGE endured during the estrous cycle; DGE

scores differed only between proestrus and diestrus (P = 0.010), in spite of the tendency for a difference between early diestrus and diestrus (P = 0.077; Table 3; Graph 3).

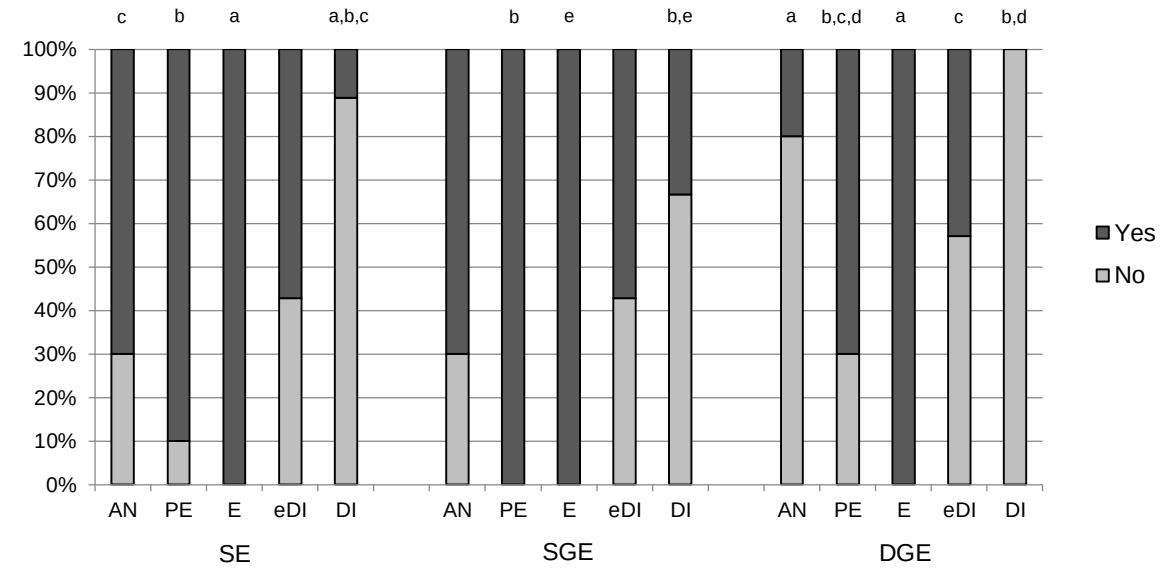


Graph 3 - Graphic representation of the intensity scores for GPx1 in the different endometrial structures during the canine estrous cycle. Within each endometrial structure, different column superscripts indicate statistical significance at $P < 0.05$: a → $P \leq 0.01$; b → $P \leq 0.05$. [W – Weak; M – Moderate; S – Strong].

The immunoreaction against GPx1 was always stronger in the endometrial epithelia than in stroma ($P \leq 0.001$). The stromal GPx1 scores remained unchanged throughout the canine estrous cycle (Table 3; Graph 3). Both the stromal matrix and cells labelled for GPx1. Though the stromal matrix showed a rather constant immunolabelling, a small non-significant decrease was observed in the stromal cells in progesterone-associated stages contrasting to the observed in the epithelial elements of the canine endometrium.

On what respect the subcellular pattern, never the dislocation of the immunolabelling into the cell nucleus was found in GPx1 immunostaining. Contrasting, the apical reinforcement pattern was present in canine endometrial epithelium and changed throughout the estrous cycle (P = 0.001; Table 3; Graph 4).

GPx1 - Apical reinforcement pattern



Graph 4 - Graphic representation of the expression of apical reinforcement pattern in GPx1 immunolabeling in the different endometrial epithelia during the canine estrous cycle. Within each endometrial structure, different column superscripts indicate statistical significance at $P < 0.05$: a,d → $P \leq 0.001$; b,e → $P \leq 0.01$; c → $P \leq 0.05$.

In the SE, the apical reinforcement pattern increased from anestrus to estrus, to decrease in early diestrus and bottomed in diestrus (Graph 4; Table 3). Statistical differences for the apical pattern expression at SE were only observed between diestrus and the other stages of the canine cycle ($P < 0.05$) with exception to early diestrus. In SGE, the apical reinforcement pattern was less frequently observed in diestrus than in proestrus ($P = 0.007$) or estrus ($P = 0.009$). Despite the tendency for a decreased observed in early diestrus compared to proestrus or estrus ($P = 0.051$ and $P = 0.063$), the apical reinforcement pattern did not differed between all other cycle stages (Table 3; Graph 4). The DGE apical reinforcement pattern was absent from the diestrus samples; in general this pattern was increased in estrus (Graph 4; Table 3), compared with anestrus ($P = 0.001$), early diestrus ($P = 0.019$) and diestrus ($P = 0.007$), or between proestrus and diestrus samples ($P = 0.004$). A tendency for differences in the apical pattern prevalence was found between early diestrus and diestrus ($P = 0.077$) and between anestrus and proestrus ($P = 0.077$).

4. Discussion

This work presents the endometrial distribution of SOD1 and GPx1 positive cells and reports both spatial and temporal changes during the canine estrous cycle. Cu,Zn-superoxide dismutase (SOD1) is a major cytoplasmic antioxidant enzyme that metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide; the latter will be then reduced into water and oxygen by the glutathione peroxidase (GPx), glutathione-dependent enzyme ubiquitously expressed in tissues [10]. Both SOD1 [29, 30] and GPx1 [31] molecules have been localised in human endometrium. These studies showed that the pattern of localization and the protein content varies during the menstrual cycle. Other studies also refer to cyclic differences in the activity of SOD or GPx in women endometrium [20, 29]. Similarly, changes in the content of different antioxidant enzymes were reported in sheep endometrium [16]. Recently, it was also referred a variation in SOD activity in the peri-ovulatory period in canine uterine fluid [26], but not in the cow uterine fluid or the endometrium [21] suggesting that differences among species may rather reflect particularities of the species reproductive physiology.

The uterus, alike other organs, presents defence mechanisms controlling the reactive oxygen species [19]. So it would be expectable that changes in the distribution pattern of antioxidant enzymes would reflect the changes associated to morphological events and metabolic activities within the tissue.

In this work, stromal SOD 1 or GPx1 immunoreaction displayed lower scores compared to the endometrial epithelia, as it was also reported in the women endometrium [29, 31, 32]. However, existing reports are somehow conflicting on respect to changes in the SOD immunoreaction in human endometrium, particularly on respect to its content in epithelial cells. Sugino *et al.* [32] described the immunolabelling as having a constant moderate intensity, while Narimoto and colleagues [29] reported an increase in the intensity of immunostaining in the secretory phase, compared to the proliferative. Data gathered in the current study are in accordance with the described by Narimoto and colaborators [29]. In canine endometrium, the immunoreaction against SOD was lower in proestrus, accompanying the typical increase in estrogen peripheral levels. Thereafter, it increases towards diestrus, the stage with highest scores. Sugino and colleagues [32], in women, refered to an increase of SOD1 intensity in mid- to late- secretory phases, resembling the findings described herein.

Contrasting to the cyclic changes reported by Ohwada *et al.* [30] and Serviddio *et al.* [20] on GPx localization and activity in human endometrium, in dogs the endometrial scores

for GPx1 remained relatively constant throughout the estrous cycle. While in women a decrease in the intensity of immunolabeling occurred in the glandular epithelium in the early proliferative phase, followed by a peak of intensity in the early secretory phase that bottomed thereafter [30], in dogs, as shown in here, only a slight decrease in the immuno-scores was recorded in endometrial epithelial cells in estrus. This is a stage where it is observed the transition from an estrogen-driven environment to one with increasing levels of progesterone, due to the preovulatory follicular luteinisation in the ovaries [22]. In addition, in the canine endometrium, the scores peaked in diestrus, corresponding to the mid-to-late secretory stage in humans.

There have been previous indications for a relation between SOD1 and GPx1 and the local cytokines network, such as the tumour necrosis factor-alpha (TNF) or particular transforming growth factor (TGF) beta isoforms, which are also expressed in the endometrium. Through these pathways, SOD1 and GPx1 may play important roles in the organ immune response [33], in apoptosis or in proliferation [9, 11, 17, 34, 35]. Afonso *et al.* [36] showed that TNF depresses SOD1 in monocytes, reducing the resistance of these cells to the toxic effects of TNF. It was also found that a decrease in GPx1 accompanies a TNF reduction [35]. In canine endometrium, TNF presented higher immunoreaction scores in anestrus and proestrus [27], the stages that in the current study also showed decreased scores for SOD1. Both SOD and GPx, by controlling ROS concentrations, can affect the dynamics of metaloproteinases (MMP) 2 or 9, whereby they modulate also cell proliferation and migration events or extracellular matrix remodelling [9, 35]. Whether or not this effect is exerted through TNF is yet to be established.

It was also shown that in some cellular linages, accumulation of ROS are associated with TGF-β driven differentiation and proliferation [37]. In particular, it was mentioned that TGF-beta 1 inhibits SOD1 expression in rat hepatocytes [8] and in human lung fibroblasts [34]. In dogs, the immunoreaction for TGFβ1 decrease in intensity at early diestrus, specially in the epithelium [38], coinciding with the stage were the scores for SOD1 start to increase in the present work.

SOD and GPx activity have been associated to apoptosis, migration and angiogenesis (SOD) or differentiation (GPx1) in several tissues [9, 34, 35, 39], generally due to variation in local concentrations of superoxide, mainly the H_2O_2. Apoptosis may be triggered by SOD1 down-regulation and consequently to local accumulation of ROS [9]. Recently, it was demonstrated that H_2O_2 accumulation would dislocate SOD1 to the cell nucleus [40], where the molecule regulates the expression involved in oxidative resistance and repair genes, promoting genomic stability and thereby preventing apoptosis. Although

SOD1 is mainly cytosolic enzyme, it may also be found in the nucleus of different cell types [41], including the endometrial epithelium [29]. The present work also showed SOD1 nuclear labelling in epithelial cells. This was more evident in estrus and early diestrus. The pattern of apoptosis in the canine endometrium was described by Van Cruchten and colleagues [42], who found it to be increased in the glandular epithelia in diestrus and anestrus, while referring a very low apoptotic index in the surface epithelium and in the stroma. This cyclic pattern is rather reflected by the SOD1 nuclear immunolabeling described herein, which was expressed more frequently during estrus and early diestrus, and presented a decreased prevalence in diestrus and anestrus, particularly in SGE and DGE. Comparing the pattern of nuclear SOD1 immuno-expression in estrus and early diestrus, it was also found in the present study a small decrease in the GPx1 immunolabelling scores, particularly in the glandular epithelia, which was reverted in diestrus, reinforcing the hypothesis of an existing accumulation of H_2O_2 during these stages of the cycle. Taken together, the SOD1 nuclear labelling and the decreased GPx1 scores are suggestive of an increased production of H_2O_2 and also of a phase-related protective effect against apoptosis. The reduction of SOD1 and GPx activity seems to play a crucial role in early pregnancy event, including the trophoblast differentiation, the decidualization of stromal cells, the regulation of vascular permeability and trophoblast migration within the maternal endometrium [12, 15, 43]. This might possibly explain the subtle changes in SOD1 immunoreaction in the sub-surface area of the endometrial stroma registered in early diestrus, when implantation and decidualization occurs in dogs. This area also shows differentiated pattern of labelling with other markers that have been associated to a pre-decidualization of the endometrial stroma [28].

Proliferation increases the cellular metabolism, and thereby challenges the antioxidant enzyme and ROS interplay. GPx1 has been associated with growth factor-mediated proliferation, the estrogens being proposed to act as a posttranscriptional mediator of GPX1 [39]. Al-Gubory and co-workers [16] reported a SOD1 and GPx activities in estrogen treated spayed ewes, whereas they were unaffected by progesterone treatment. Similarly, in the present study it was found a decrease in the scores for this molecule during the canine follicular phase (comprising the proestrus and estrus), accompanying the increase of estrogens in the peripheral blood [22], particularly in SE and SGE. Proestrus and estrus are also the stages of the estrous cycle presenting the peak of proliferation, as described by Van Cruchen and colleagues [44], for the surface and the cryptal epithelia, whereas proliferation in DGE increased in estrus and early diestrus. A clear connection cannot be established between the reported proliferation pattern in canine endometrium and the changes in the scores for GPx1 reported in here. It is possible that the molecular content in the GPx1 in the endometrium estimated from the immunohistochemical molecular localization does not

corresponds similar changes in the enzymes activities, as it was previously patent in the study by Ohwada et al [30] in human endometrium.

In the uterus, a decrease in SOD1 activity was also connected to prostaglandin F2-alpha synthesis and the endometrial shedding at progesterone withdrawal and luteolysis [14, 15]. Also, GPx1 is known to inhibit COX activity and modulate prostaglandin E2 activity, which is a major molecule in regeneration and survival of epithelial cells [35]. The phenomena of luteolysis remains poorly characterized in dogs; nevertheless, Silva *et al.* [45] describe an increased production of prostaglandins in the canine endometrium by the end of diestrus, compared with all other cycle stages. This seems to contrast with data reported herein. One possible explanation is that diestrus samples used in the present study represented endometrial samples at mid-to-late progestagenic stage, not the final diestrus phase of the canine cycle. Sugino [15] described an increase in SOD1 immunostaining in early and mid proliferative phases of the menstrual cycle of women. Another explanation could be related with the nature of the study itself: the present study refers to the molecule distribution on the canine endometrium and describes variations in its relative concentration during the cycle, but does not allow estimating the enzyme activity; moreover, antioxidant enzymes have the ability to compensate each other, strengthening the scavenger ability of the protective system against the noxious effects of ROS. Thus future work sought to examine the enzymatic activity of SOD in the endometrium throughout the canine estrous cycle to give a more complete picture of the antioxidant enzymes interplay during the endometrial cycle in the canine endometrium.

The apical reinforcement pattern was observed for both SOD1 and GPx1 in women endometrium [29, 30], alike the described in the present work for the canine endometrium. It has also been referred that SOD and GPx may be secreted into the extracellular space and can be found lining epithelial cells and in diverse body fluids. It was detected in the uterine fluid of the bitch [26] and cow [21]. It is plausible that the antioxidant enzymes found in the uterine fluid originated from the epithelial cells that release these molecules into the uterine lumen. The apical reinforcement observed in particular stages in the canine endometrium complies with this hypothesis. This hypothesis is supported when the results presented by Kobayashi et al [26], who found increased SOD activity in estrus, are compared to data shown herein, regarding the increased prevalence of apical reinforcement in epithelial cells in the dog estrogen-associated stages (proestrus and estrus) for both antioxidant molecules.

5. Conclusions

To our knowledge, this study provides the first description of the immunohistochemical distribution of SOD1 and GPx1 in the canine endometrium throughout the estrous cycle. The fluctuations in the expression of these molecules concentrations varies in response to sex steroids, suggesting that both SOD and GPx may participate in different pathways regulating the endometrial cycle in dogs, by acting either directly or through regulated local ROS accumulation. This work starts unveiling some aspects of SOD1 and GPx1 molecules in canine endometrium, but does not exhaust the subject as antioxidant enzymes present many compensatory mechanisms that may influence their enzymatic activity. Also it would be of interest for the practitioner to address the age-related changes and it relation with endometrial disease in future studies and the way it may relate to disease predisposition, for which a larger cohort of samples will be need.

6. Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could raise a potential conflict of interest.

7. Acknowledgements

The authors thank Inês Santana for her help during immunohistochemistry. This work was sponsored by the Portuguese Science and Technology Foundation (FCT) under the Projects PEst-OE/AGR/UI0772/2011 and PEst-OE/AGR/UI0772/2014.

8. Chapter reference list

[1] Cavagna M, Mantese JC. Biomarkers of endometrial receptivity - A review. Placenta. 2003;24.

[2] Dekel N, Gnainsky Y, Granot I, Mor G. Inflammation and implantation. Am J Reprod Immunol. 2010;63:17-21.

[3] van Mourik MSM, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol. 2009;85:4-19.

[4] Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012;24:981-90.

[5] Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. Oxidative stress and cell signalling. Curr Med Chem. 2004;11:1163-82.

[6] Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82:47-95.

[7] Al-Gubory K. Maternal Nutrition, Oxidative Stress and Prenatal Devlopmental Outcomes. In: Agarwal A, Aziz N, Rizk B, editors. Studies on Women's Health: Humana Press; 2013. p. 1-31.

[8] Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39:44-84.

[9] Fukai T, Ushio-Fukai M. Superoxide Dismutases: Role in Redox Signaling, Vascular Function, and Diseases. Antioxid Redox Signal. 2011;15:1583-606.

[10] Brigelius-Flohe R, Maiorino M. Glutathione peroxidases. Biochim Biophys Acta. 2013;1830:3289-303.

[11] Khan AA, Rahmani AH, Aldebasi YH, Aly SM. Biochemical and pathological studies on peroxidases -an updated review. Glob J Health Sci. 2014;6:87-98.

[12] Ufer C, Wang CC. The roles of glutathione peroxidases during embryo development. Front Mol Neurosci. 2011;4.

[13] Agarwal A, Gupta S. Role of reactive oxygen species in female reproduction. Part I. Oxidative stress: a general overview. Agro Food Ind Hi Tech. 2005;16:21-5.

[14] Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: From molecular mechanisms to health implications. Antioxid Redox Signal. 2008;10:1375-403.

[15] Sugino N. The role of oxygen radical-mediated signaling pathways in endometrial function. Placenta. 2007;28 Suppl A:S133-6.

[16] Al-Gubory KH, Bolifraud P, Garrel C. Regulation of key antioxidant enzymatic systems in the sheep endometrium by ovarian steroids. Endocrinology. 2008;149:4428-34.

[17] Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H. Reactive oxygen species stimulate prostaglandin F2 alpha production in human endometrial stromal cells in vitro. Hum Reprod. 2001;16:1797-801.

[18] Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005;3:28.

[19] Rizk B, Badr M, Talerico C. Oxidative Stress and The Endometrium. In: Agarwal A, Aziz N, Rizk B, editors. Studies on Women's Health: Humana Press; 2013. p. 61-74.

[20] Serviddio G, Loverro G, Vicino M, Prigigallo F, Grattagliano I, Altomare E, *et al.* Modulation of endometrial redox balance during the menstrual cycle: relation with sex hormones. J Clin Endocrinol Metab. 2002;87:2843-8.

[21] Ramos RS, Oliveira ML, Izaguirry AP, Vargas LM, Soares MB, Mesquita FS, *et al.* The periovulatory endocrine milieu affects the uterine redox environment in beef cows. Reprod Biol Endocrinol. 2015;13:39.

[22] Concannon PW. Reproductive cycles of the domestic bitch. Anim Reprod Sci. 2011;124:200-10.

[23] Okkens AC, Kooistra HS. Anoestrus in the dog: A fascinating story. Reprod Domest Anim. 2006;41:291-6.

[24] Nomura K. Induction of canine Deciduoma in some reproductive stages with the different conditions of Corpora lutea. J Vet Med Sci. 1997;59:185-90.

[25] Noakes DE, Dhaliwal GK, England GC. Cystic endometrial hyperplasia/pyometra in dogs: a review of the causes and pathogenesis. J Reprod Fertil Suppl. 2001;57:395-406.

[26] Kobayashi M, Wada M, Hori T, Kawakami E. Superoxide dismutase activity in the oviductal and uterine fluid of the bitch and the effects of the enzyme on viability, motility and hyperactivation of canine sperm in vitro. J Vet Med Sci. 2014;76:741-3.

[27] Payan-Carreira R, Pires MA, Holst BS, Rodriguez-Martinez H. Tumour Necrosis Factor in the Canine Endometrium: An Immunohistochemical Study. Reprod Domest Anim. 2011;46:410-8.

[28] Payan-Carreira R, Santos C, Miranda S, Pereira RMLN, Santos D, Pires MA. Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium. Theriogenology. 2014;82:815-26.

[29] Narimoto K, Noda Y, Shiotani M, Tokura T, Goto Y, Takakura K, *et al.* Immunohistochemical Assessment of Superoxide-Dismutase Expression in the Human Endometrium Throughout the Menstrual-Cycle. Acta Histochem Cytochem. 1990;23:487-98.

[30] Ohwada M, Suzuki M, Sato I, Tsukamoto H, Watanabe K. Glutathione peroxidase activity in endometrium: Effects of sex hormones and cancer. Gynecol Oncol. 1996;60:277-82.

[31] Ota H, Igarashi S, Kato N, Tanaka T. Aberrant expression of glutathione peroxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Fertil Steril. 2000;74:313-8.

[32] Sugino N, Shimamura K, Takiguchi S, Tamura H, Ono M, Nakata M, *et al.* Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. Hum Reprod. 1996;11:1073-8.

[33] Marikovsky M, Ziv V, Nevo N, Harris-Cerruti C, Mahler O. Cu/Zn superoxide dismutase plays important role in immune response. J Immunol. 2003;170:2993-3001.

[34] Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 2000;279:L1005-28.

[35] Brigelius-Flohe R, Kipp A. Glutathione peroxidases in different stages of carcinogenesis. Biochim Biophys Acta. 2009;1790:1555-68.

[36] Afonso V, Santos G, Collin P, Khatib AM, Mitrovic DR, Lomri N, *et al.* Tumor necrosis factor-alpha down-regulates human Cu/Zn superoxide dismutase 1 promoter via JNK/AP-1 signaling pathway. Free Radic Biol Med. 2006;41:709-21.

[37] Proell V, Carmona-Cuenca I, Murillo MM, Huber H, Fabregat I, Mikulits W. TGF-beta dependent regulation of oxygen radicals during transdifferentiation of activated hepatic stellate cells to myofibroblastoid cells. Comp Hepatol. 2007;6:1.

[38] Payan-Carreira R, Ström-Holst B. TGF-beta isoforms in the canine endometrium. Am J Reprod Immunol. 2011;66:111 [abstr.].

[39] Lubos E, Loscalzo J, Handy DE. Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. Antioxid Redox Signal. 2011;15:1957-97.

[40] Tsang CK, Liu Y, Thomas J, Zhang YJ, Zheng XFS. Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. Nature Communications. 2014;5.

[41] Crapo JD, Oury T, Rabouille C, Slot JW, Chang LY. Copper, Zinc Superoxide-Dismutase Is Primarily a Cytosolic Protein in Human-Cells. Proc Natl Acad Sci USA. 1992;89:10405-9.

[42] Van Cruchten S, Van den Broeck W, Duchateau L, Simoens P. Apoptosis in the canine endometrium during the estrous cycle. Theriogenology. 2003;60:1595-608.

[43] Al-Gubory KH, Fowler PA, Garrel C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol. 2010;42:1634-50.

[44] Van Cruchten S, Van den Broeck W, D'Haeseleer M, Simoens P. Proliferation patterns in the canine endometrium during the estrous cycle. Theriogenology. 2004;62:631-41.

[45] Silva E, Henriques S, Brito S, Ferreira-Dias G, Lopes-da-Costa L, Mateus L. Oestrous cycle-related changes in production of Toll-like receptors and prostaglandins in the canine endometrium. J Reprod Immunol. 2012;96:45-57.

Chapter 7 – Oxidative stress in the canine endometrium during the estrous cycle

THE CONTENT OF THIS CHAPTER HAS BEEN INTENT FOR SUBMISSION TO:

Molecular Reproduction and Development

OXIDATIVE STRESS IN THE CANINE ENDOMETRIUM DURING THE ESTROUS CYCLE

R Payan-Carreira[a], C Santos[b], D. Santos[c]*

Running head: Oxidative stress in cyclic canine endometrium

[a] CECAV, University of Trás-os-Montes and Alto Douro, P.O. Box 1013, 5001-801 Vila Real, Portugal.

[b] DEBA, University of Trás-os-Montes and Alto Douro, P.O. Box 1013, 5001-801 Vila Real, Portugal.

[c] CITAB, University of Trás-os-Montes and Alto Douro, P.O. Box 1013, 5001-801 Vila Real, Portugal.

* Corresponding author: Dario Loureiro dos Santos, CITAB, Department of Biology and Environment, University of Trás-os-Montes and Alto Douro; Vila Real, Portugal.

E-mail address: darios@utad.pt

Abstract

Reactive oxygen species (ROS) are physiologically synthetized in endometrium and normal redox state is controlled by the antioxidant enzyme system consisting in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR) and glutathione-S-transferase (GST). Enzyme activities were measured in five estrous cycle phases: anestrus, proestrus, estrus, early diestrus and diestrus. SOD activity decrease from anestrus to diestrus in opposition to CAT activities that increase along the cycle. In contrast, no variations in the enzyme activity were detected for the glutathione-dependent enzymes GPx, GSR and GST. Analysis of thiobarbituric reactive species (TBARS) indicated lipid peroxidation associated to oxidative stress, which in canine endometrium were only

significantly increased in proestrus. In the other hand, thiol cell content remained unchanged throughout the stages of the cycle. Results indicated an efficient oxidative stress control without lipid or proteins attack as results of excessive reactive oxygen species on endometrium. SOD and catalase fluctuations during the estrous cycle should be associated with endometrium regulatory mechanisms and estrogen-progesterone levels during the estrous cycle stages that may reflect the species particular physiology.

Keywords: Oxidative stress; antioxidant enzymes; TBARS; thiols; estrous cycle; dog

1. Introduction

In normal physiological conditions, the metabolism of oxygen in aerobic organisms leads to the generation of dangerous and very reactive compounds known as reactive oxygen species (ROS) [1, 2]. Reactive oxygen species is a collective term used to include not only the oxygen radicals' superoxide (O_2•−) and hydroxyl (OH•) but also some non-radical derivatives of oxygen hydrogen peroxide (H_2O_2). The former molecules contain one or more unpaired electrons in atomic or molecular orbitals giving a considerable degree of reactivity to the free radical oxidizing almost all classes of biologically important macromolecules, including proteins, lipids and nucleic acids [3].

ROS are products of a normal cellular function involved in signal transduction to sustain life [4-6]. The physiological level of oxidants is usually regulated by antioxidant defense mechanisms, which control the flux of ROS through specific scavenger reactions and detoxification pathways, and has the function of inhibiting and/or reducing the damage caused by the deleterious reactive oxygen species [7, 8]. This antioxidant system is divided into enzymatic and non-enzymatic [7]. Active oxygen scavenging enzyme systems includes superoxide dismutase (SOD), catalase, and glutathione-related enzymes glutathione peroxidase (GPx), glutathione reductase (GSR) and glutathione-S-transferase (GST) [9]. The non-enzymatic system consists of a variety of antioxidant substances, which may be endogenous or dietary: glutathione (GSH), vitamin C, vitamin E, carotenoids, natural flavonoids, melatonin, and other compounds [3]. Anti-oxidizing defense systems are compensation mechanisms that antagonize ROS-induced cellular damage. However, the

damage will take place if the production of deleterious oxygen species far exceeds the capacity of these mechanisms. Oxidative stress is often defined as the imbalance between oxidants (which are formed as a normal product of aerobic metabolism) and antioxidants, either by overproduction of reactive oxygen species, or by dysfunction of the antioxidant systems [1, 3]. Oxidative stress has been implicated in several diseases, such as tumor development, viral expression, neurological diseases, diabetes mellitus, atherosclerosis, hypertension, and chronic kidney disease [3, 10].

In the female reproductive tract, cyclic changes in the stromal/epithelial interplay orchestrated by sex steroid hormones are crucial to endometrial function in the uterus [11, 12]. It has been demonstrated that the expression of various antioxidants varies along the endometrial cycle [13, 14]. Reactive oxygen species should play a number of significant diverse roles in female reproductive biology including modulation of the uterine environment and embryo-maternal interaction at implantation [15-17].

Reactive oxygen species may modulate the growth of endometrial stroma. Studies in humans showed that under pathologic conditions such as endometriosis, increased oxidative stress and depletion of antioxidants might contribute to the excessive growth of endometrial stromal cells. Oxidative stress was also associated with non-physiological modification of endometrium [18, 19] and antioxidant mechanisms are disrupted in many uterine diseases [20]. However, they may also be associated with physiologic events, such as menstruation or decidual implantation [21].

Dogs have a rather different estrous cycle compared to other domestic animals. Dogs are monoestric species [22] meaning that a stage of anestrus, with basal levels of estrogens or progesterone, separates two consecutive reproductive cycles. The transition to a new follicular phase starts in the last third of anestrus, when follicular recruitment takes place. Anestrus is also fundamental for the maintenance of female dog fertility [23], representing the stage of endometrial regeneration. Other typical features of the canine estrous cycle are the relatively prolonged follicular stage, culminating with the pre-ovulatory luteinisation of the dominant ovarian follicles, and the rather long diestrus, similarly extended in cyclic or pregnant diestrus [22]. The characteristics of the canine estrous cycle, in particular those associated to the endometrial stimulation by sex steroids, favour the incidence of progesterone-associated diseases, such as deciduoma formation [24] or the cystic endometrial hyperplasia and pyometra complex [25].

There is an increased interest in studying the role of oxidative stress in female reproduction, and in particular its association with infertility and the development of uterine diseases. Nevertheless, limited information exists regarding the endometrial oxidative stress

in domestic species, both in normal and in pathological conditions. Nonetheless, limited information exists on oxidative stress in the uterus of domestic species [26, 27] in health or disease.

The purpose of this study was to evaluate oxidative stress in the normal canine endometrium analysing levels of protein and lipid oxidation and antioxidant enzyme activities throughout the canine estrous cycle. This study will also provide reference data for potential use in the diagnosis of endometrial pathology.

2. Material and methods

Tissue collection and preparation

Twenty-five healthy post-pubertal, non-pregnant bitches with ages ranging from 10 months to 6-year old, submitted to elective ovariohysterectomy (OVH) were used in this study. Surgical specimens were used with the owners' informed consent, in accordance with the International Ethical Standards. The stage of the estrous cycle was determined for each bitch by the clinical history, physical examination, vaginal cytology and macroscopic observation of the ovaries, and was later confirmed upon the histological examination of the uterus and progesterone levels, as described by [28]. From each uterine horn, at its middle portion, one fragment with 1.5 cm in length was collected immediately after the surgery and snap frozen in liquid nitrogen until reaching the laboratory; thereafter it was maintained frozen at -80 ºC until analysis. Also an adjacent uterine segment was collected for histological analysis, fixed at 10 % buffered formalin, embedded in paraffin wax, sectioned at 2 µm and stained with haematoxylin and eosin, for the histological staging of the estrous cycle and for excluding uterine disease.

Uterine samples for the proestrus (n=5), estrus (n=5), diestrus (n=10) and anestrus (n=5) were used in this study. Since implantation occurs in dogs at post-ovulatory day 16 [29, 30] the diestrus was further divided in two stages as previously described [31]: an early diestrus period (n=5), corresponding to the phase of embryo implantation in a pregnant cycle, and a full diestrus period (n=5), equivalent to the mid-secretory stage.

Preparation of dog endometrial extracts

Frozen unthawed sampled tissues were dissected to separate the endometrium from the surrounding myometrium and connective tissue. Dissected endometrium was finely chopped using a surgical blade and then homogenized in refrigerated phosphate buffer saline (PBS – 1.76 mM KH_2PO_4, 10 mM NaH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.0). Homogenates were further submitted to sonication (4 cycles of 5'' with 15'' interval at 80% amplitude; under refrigeration) for cell disruption. Thereafter, they were centrifuged at 1.500 *xg* for 15 min at 4 °C. The resulting supernatant was used for determination of protein concentration and for the measurement of enzymatic activities and assessment of oxidative damages.

Protein determination

Protein was quantified by the method of Bradford [32] using bovine serum albumin (BSA) as standard.

Antioxidant enzyme activity assays

All the enzyme activities were performed in two replicates, and the result of each sample represented the mean value of replicates.

SOD activity - SOD (SOD, EC 1.15.1.1) activity was determined as described previously [33]. Briefly, the method is based on the inhibition of nitroblue tetrazolium (NBT) reduced by the xanthine/xanthine oxidase system as a superoxide generator. Assays were conducted in the presence of 100 nM potassium phosphate buffer (pH 7.8), EDTA 10 mM, NBT 10 mM, hypoxanthine 10 mM, and xanthine oxidase 0,023 $U.mol^{-1}$. The reduction of NBT was measured at 560 nm and constant temperature (25 °C). One unit of SOD was defined as the enzyme amount causing 50 % inhibition in the NBT reduction rate. The rate of NBT reduction in the absence of tissue was used as reference rate and activity was expressed in terms of %.

CAT activity - CAT (CAT, EC 1.11.1.6) activity was determined with a Clark type oxygen electrode at 25 ºC [34]. Catalase activity was expressed in term of units of catalase activity:

one unit of CAT activity being the amount of enzyme that catalysis the decomposition of 1 μmol H_2O_2 per minute.

GST activity - GST (GST, EC 2.5.1.18) activity was measured as previously described [35] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 1 mM CDNB and 2 mM GSH. The incubation time was 2 min and enzyme activity was assayed during 2 min at 25 °C. The assay kinetics was calculated by using a molar absorptivity for CDNB of 9.6 x 10^{-3} $M^{-1}.cm^{-1}$ at 340 nm. GST activity was expressed in terms of nM CDNB conjugated glutathione.$min^{-1}.mg^{-1}$ of protein.

GSR activity - GSR (GSR, EC 1.6.4.2) activity was assayed according to the method Smith *et al.* [36]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.5), 2 mM EDTA, 0.1 mM NADPH and 1 mM GSSG. The incubation time was 2 min and the rate of NADPH oxidation was monitored during 2 min at 25 °C. The assay kinetics was calculated by using a molar absorptivity for NADPH of 6.22 x 10^3 $M^{-1}.cm^{-1}$ at 340 nm. Enzyme activity was expressed in terms of nmol NADPH oxidized.$min^{-1}.mg^{-1}$ of protein.

GPX activity - GPX (GP, EC 1.11.1.9) activity was determined by the modified method of Paglia and Valentine [37] using *tert*-butyl hydroperoxide as substrate. Enzyme activity was measured by a coupled assay system in which oxidation of GSH was coupled to NADPH oxidation catalysed by GSR. The rate of decrease in NADPH concentration was recorded at 340 nm. The reaction mixture consisted of 1 mM of *tert*-butyl hydroperoxide, 0.24 units of yeast GSR, 0.5 mM of NADPH, 2 mM EDTA, 0.1 mM in 100 mM potassium phosphate buffer (pH 7.5). The incubation time was 2 min and the rate of NADPH oxidation was monitored during 2 min at 30 °C. The assay kinetics was calculated by using a molar absorptivity for NADPH of 6.22 x 10^3 $M^{-1}.cm^{-1}$ at 340 nm. GPx activity was expressed in terms of nmol NADPH oxidized.$min^{-1}mg^{-1}$ of protein.

Lipid and thiol group oxidation

For assessment of the lipid peroxidation index we use the formation of thiobarbituric acid-reactive species (TBARS) during an acid-heating reaction, as described by Buege and Aust [38] and modified by Doktorovova *et al.* [39]. Briefly, sample aliquots (0.5 mL) were mixed with 2.5 mL of thiobarbituric acid (TBA) reagent containing 0.375 % (w/v) TBA, 0.25 M HCl, 15 % (w/v) trichloroacetic acid (TCA) and 6.8 mM butylated hydroxytoluene (BHT). The mixture was then heated in a water bath set at 95 ºC for 15 min. After cooling, flocculent precipitate was removed by centrifugation at 10.000 *xg* for 2 min and the supernatant was

collected. TBARS were determined in a spectrophotometer at 530 nm by the absorbance of the MDA-TBA complex (absorbance co-efficient = 1.56 x 10^5 $M^{-1}.cm^{-1}$). Results were expressed as nmol of TBARS.mg^{-1} protein.

The total thiol group (-SH) oxidation was assayed by assessing of the total thiol group content on dog endometrial extracts using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), following the Ellman´s method [40] as modified by Sedlak & Lindsay [41] and Susuki *et al.* [42]. In brief, the previously prepared endometrial suspension (0.5 mL) was mixed with 1 mL of 4 % (w/v) sulfosalicylic acid and centrifuged at 24.000 *xg* for 15 min. The pellet was resuspended and homogenated in 100 mM phosphate buffer (pH 8); the obtained protein suspension (0.5 mL) was incubated with agitation during 15 min with 4.5 mL of phosphate buffer and 70 μL of 10 mM DTNB, freshly prepared in ethanol absolute. Tubes were vortexed and incubated 15 min at room temperature. Absorbance was determined at 412 nm and thiol concentration was calculated using an extinction coefficient of 1.36 x 10^5 $M^{-1}cm^{-1}$, and expressed in terms of nmol thiol.mg^{-1} protein.

Statistical analysis

The results from biochemical analysis are expressed as mean ± standard error of the mean. For each sample, run in replicate for each assay, the mean value for an assay was used for pooling the data. Basic descriptive statistics was used to characterize the samples and the estrous stages used in this study (mean, standard error and standard error of the mean). The significance of differences between groups was determined using the software IBM SPSS Statistics version 22.0 for Mac OS X 10.8. Data was analysed by one-way ANOVA followed by the nonparametric Tukey's multiple comparison test and differences between groups were considered to be statistically significant when $P<0.05$.

3. Results

The activities of antioxidant enzymes in bitch endometrium homogenate were performed by spectrophotometic analysis of substrates or derived products (SOD, GPX, GSR, GST) or polarographic analysis of reaction products (CAT). Table 1 summarizes the results for the activity of each antioxidant enzyme assayed as well as for the protein and lipid oxidation.

Table 1. Basic statistics descriptive for endometrial oxidative stress enzymes and thiols and lipid oxidation (TBARS) in female dogs throughout the estrous cycle. Number of animals per group, n = 5.

	Stage	Mean	SEM	SD	Minimum	Maximum	*P* value
SOD	AN	92,12	5,12	11,44	78,90	106,22	0.002
	PE	87,50	1,58	3,53	83,57	92,00	
	E	64,75	2,54	5,67	57,57	72,96	
	eDI	69,44	7,18	14,36	56,22	89,59	
	DI	69,79	6,38	14,26	51,96	87,91	
CAT	AN	3,37	0,60	1,33	1,94	4,93	≤0.001
	PE	3,29	0,47	1,05	2,32	4,97	
	E	9,77	3,31	7,40	4,94	22,64	
	eDI	16,71	4,51	9,03	4,32	24,54	
	DI	25,24	3,85	8,61	16,32	35,26	
GPx	AN	169,30	12,22	27,33	137,77	208,39	0.409
	PE	182,56	24,33	54,40	109,04	249,02	
	E	150,09	22,94	51,29	111,04	229,05	
	eDI	126,81	19,22	38,45	96,97	181,26	
	DI	150,27	18,83	42,11	116,00	215,20	
GSR	AN	10,00	3,73	8,34	3,19	23,60	0.110
	PE	6,60	1,29	2,89	4,47	11,58	
	E	11,35	1,25	2,80	8,16	15,18	
	eDI	17,87	4,59	9,17	12,63	31,57	
	DI	14,67	2,69	6,02	9,39	21,66	
GST	AN	17,76	5,41	12,10	6,52	37,29	0.010
	PE	18,99	3,94	8,82	9,20	32,48	
	E	60,89	13,08	29,24	21,77	91,74	
	eDI	59,73	12,82	25,63	43,98	98,00	
	DI	43,50	11,36	25,41	21,07	84,68	
TBARS	AN	4,24	0,14	0,31	3,84	4,62	0.298
	PE	9,69	2,16	4,82	4,65	15,79	
	E	6,00	1,48	3,31	2,47	10,72	
	eDI	6,46	1,02	2,04	4,49	8,78	
	DI	5,71	2,64	5,91	0,84	15,79	
Thiols	AN	445,30	102,34	228,84	142,66	745,56	0.491
	PE	585,59	53,44	119,49	466,07	720,33	
	E	473,66	43,97	98,32	374,10	614,93	
	eDI	458,17	83,75	167,49	307,83	645,56	
	DI	429,75	32,06	71,69	365,32	535,33	

Stages of the cycle: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). SOD – Superoxide dismutase; CAT – Catalase; GPx – Glutathione Peroxidase; GSR – Glutathione reductase; GST – Glutathione-S-transferase; Thiols – Total sulfhydryls (-SH).

The stage of the cycle significantly affected the activity of total superoxide radical −scavenging antioxidant enzyme (*P* = 0.002; Table 1). SOD activity was increased in anestrous and proestrus samples (Figure 1) compared to the others stages (estrus, early diestrus and diestrus). SOD activity was similar between anestrus and proestrus (*P* = 0.956), but was higher than in estrus (*P* = 0.005) and early diestrus and diestrus (*P* < 0.01). In contrast, SOD activity was different only proestrus and estrus (*P* = 0.022). No differences were observed among stages where the progesterone levels raised above basal levels (estrus, early diestrus and diestrus), reflecting a general decrease in activity in those stages (Figure 1).

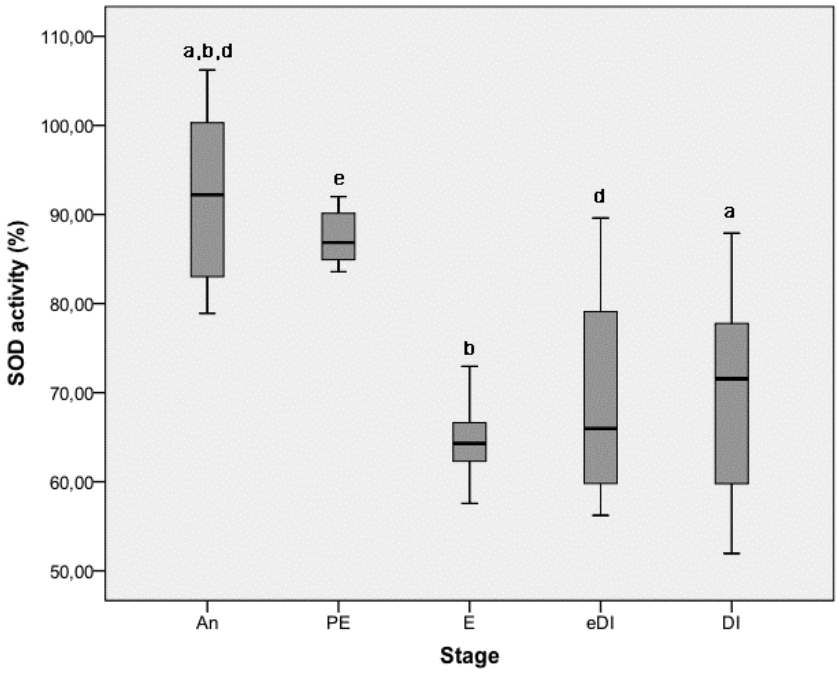


Figure 1. Box-plot graph for Superoxide dismutase (SOD) activity in the different stages of estrous cycle of bitch: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). Number of animals used per group, n = 5. Values sharing the same letter are significantly different: *P*<0.05 (a/d/e); *P* <0.01 (b).

H_2O_2 scavenging antioxidant enzymes, CAT and GPX, activities are presented in figures 2 and 3. Catalase activities varied throughout the cycle in the canine endometrium (*P* ≤ 0.001; Table 1). CAT activity was very low at stages of the cycle when progesterone is at its basal levels (anestrus and proestrus), no differences found between them (Figure 2). In estrus, a slight increase in CAT activity was observed, but no differences were found towards anestrus, proestrus or even to early diestrus (Figure 2). However, CAT enzyme activity drastically increased when cycle enters in early diestrus (*P* < 0.05 for early diestrus *vs*.

anestrus or proestrus) and diestrus ($P < 0.001$ for diestrus *vs.* anestrous/proestrous and $P < 0.01$ for diestrus *vs.* estrus).

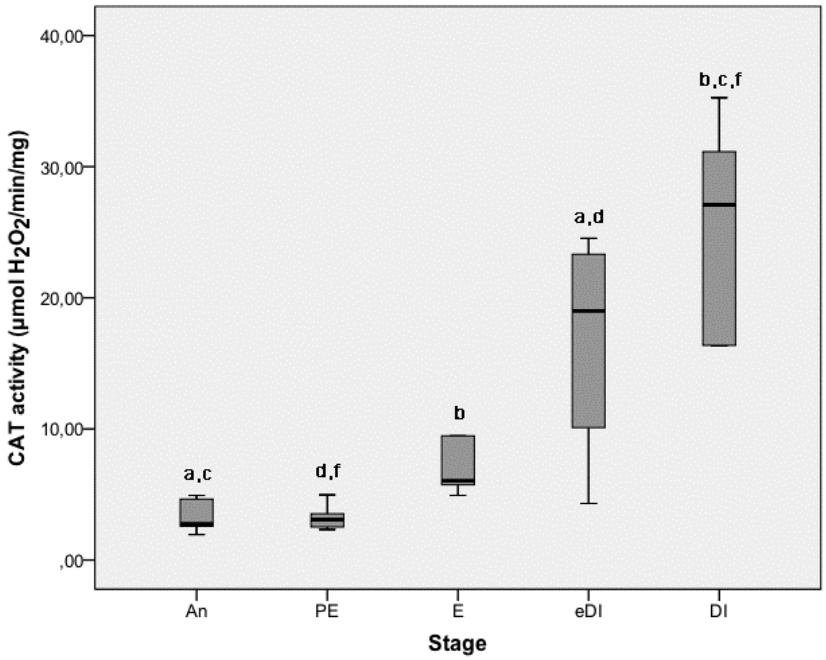


Figure 2. Box-plot graph for Catalase (CAT) activity in different stages of estrous cycle of bitch: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). Number of animals used per group, n = 5. Values sharing the same letter are significantly different: $P<0.05$ (a/d); $P<0.01$ (b); $P<0.001$ (c/f).

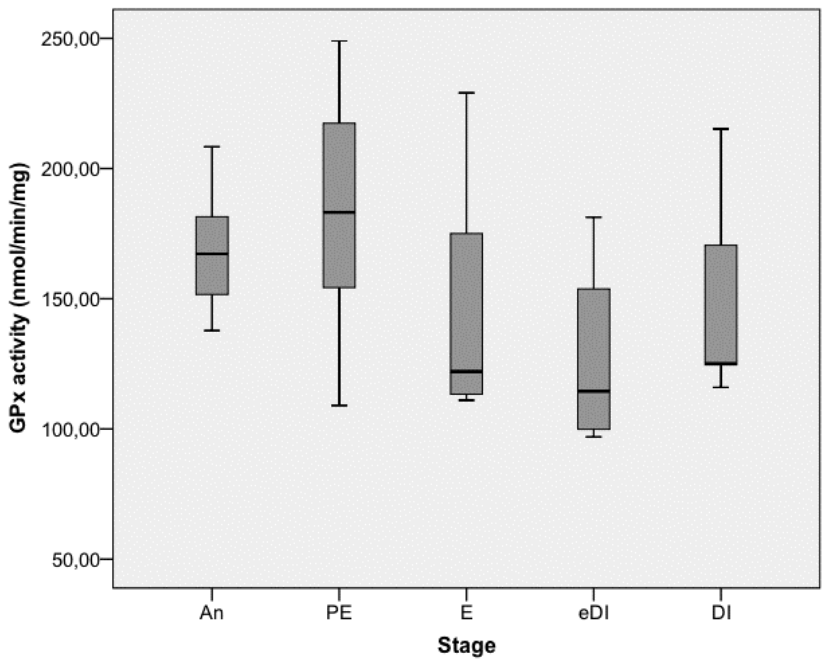


Figure 3. Box-plot graph for Glutathione Peroxidase (GPx) activity in different stages of estrous cycle of bitch: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). Number of animals used per group, n = 5.

The endometrial activity of GPx did not varied with the stage of the estrous cycle (*P* = 0.409; Table 1) in the population studied, despite the numeric variations observed in each stage measurements (Figure 3).

Conversely, the enzymatic activity of GSR was not influenced by the stage of estrous cycle (*P* = 0.110; Table 1; Figure 4). Still, its activity is increased in stages presenting suprabasal levels of progesterone (*P* = 0.038), particularly when early diestrus and diestrus were compared with anestrus and proestrus (*P* = 0.030).

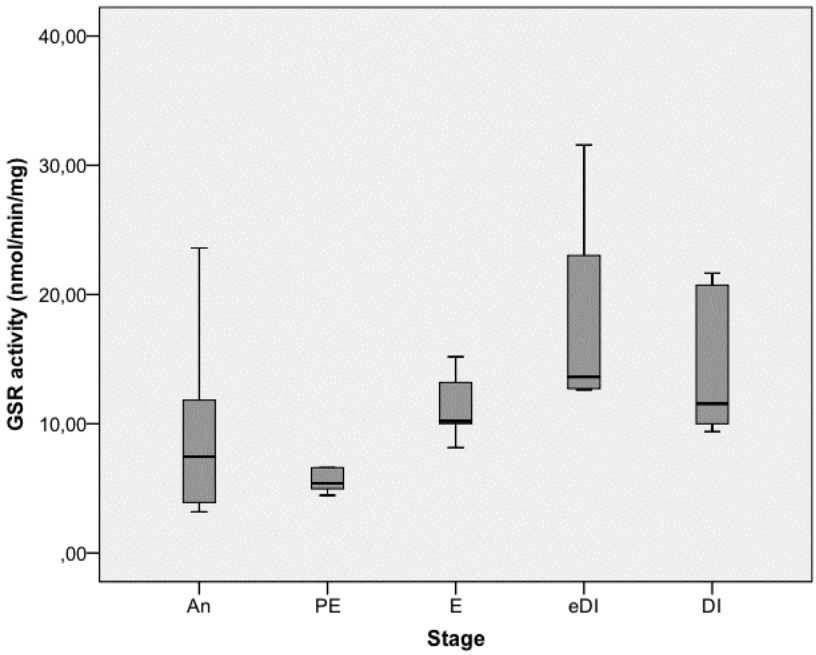


Figure 4. Box-plot graph for Glutathione reductase (GSR) activity in different stages of estrus cycle of bitch: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). Number of animals used per group, n = 5.

Glutathione-S-transferase microsome-linked activity was affected by the stage of the canine estrous cycle (*P* = 0.010). It was reduced in anestrus and proestrus compared to the three other stages of estrous cycle (estrus, early diestrus and diestrus) (Figure 5). The increase in GST activity was more notorious in estrus and early diestrus that corresponds to the stages of the cycle with rising progesterone peripheral values, as shown in figure 5. Statistical significances were only observed between anestrus or proestrus and estrus (*P* = 0.037 and *P* = 0.044, respectively for anestrus and proestrus), whilst the GST activity in early diestrus only showed a tendency to differ towards the evidenced in anestrus and proestrus (*P* = 0.062 and *P* = 0.074, respectively for anestrus and proestrus).

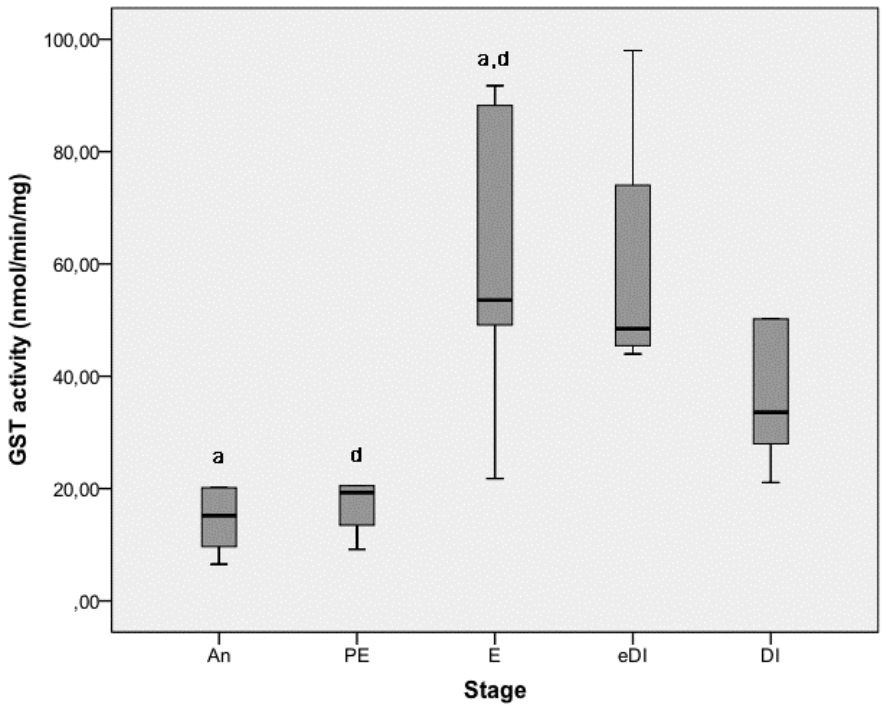


Figure 5. Box-plot graph for Glutathione-S-transferase (GST) activity in different stages of estrous cycle of bitch: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). Number of animals used per group, n = 5. Values sharing the same letter are significantly different: *P*<0.05 (a/d).

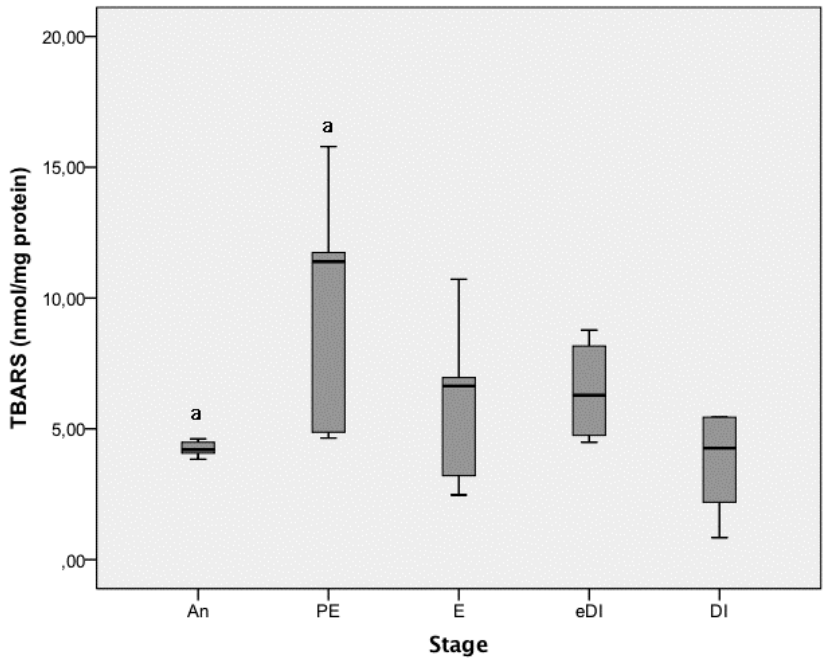


Figure 6. Box-plot graph for TBARS levels in different stages of estrous cycle of bitch: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). Number of animals used per group, n = 5. Values sharing the same letter are significantly different: *P*<0.05 (a).

TBARS content in endometrial homogenates reflecting ROS-induced lipid damage at different phases of the estrous cycle of bitch are independent of the stage of the canine cycle (*P* = 0.298; Table 1), as shown in figure 6. Nevertheless, TBARS content was significantly higher in proestrus compared with anestrus (*P* = 0.040); however, no differences were obtained among the other stages of the cycle analyzed.

Similarly, the stage of the cycle did not affect the total sulfhydryls (-SH) levels in the canine endometrium (*P* = 0.491; Table 1), as shown in Figure 7, despite the slight higher the values obtained in proestrus compared to the other stages of the cycle.

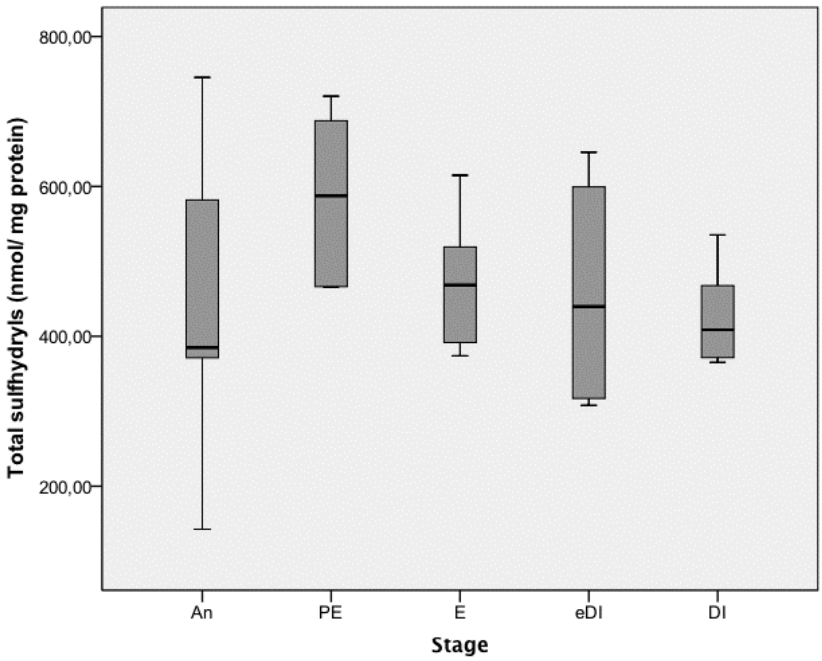


Figure 7. Box-plot graph for total sulfhydryls (-SH) levels in different stages of estrous cycle of bitch: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). Number of animals used per group, n = 5.

4. Discussion

A low concentration of ROS in endometrial cells has an essential role in its physiology and metabolic regulation [14, 43]. These levels are maintained by cellular systems that promote the monitoring of levels of ROS within well-defined limits. These antioxidant defense systems encompass compounds with intrinsic antioxidant capacity and a set of enzymes whose main function is to eliminate surplus quantities of these reactive species. The enzymes of the cell system known as enzymatic antioxidant catalyze reactions that whole

convert ROS (as superoxide radical and hydrogen peroxide) in water, avoiding the unwanted formation of hydroxyl radical which presents itself with high oxidizing capacity and detrimental of chemical and/or cellular structures.

Our results showed there the total activity of glutathione-dependent enzymes in endometrium remained relatively unchanged throughout the estrous cycle. However, anestrus and proestrus stages presented increased lipid peroxidation and SOD total activity while exhibiting reduced CAT enzyme activity. Superoxide radical degradation is accomplished to a superoxide dismutase activity enzyme that occurs in cells in two forms, a cytoplasmatic (SOD 1) and a mitochondrial (SOD 2) form. The activity of this enzyme appears often associated with the reproduction in mammals [44]. Anestrus was the stage presenting the highest values for SOD activity and the lowest for CAT activity. Anestrus in dogs is a stage of intense remodeling in the canine endometrium. Apoptosis is increased in dog endometrium in late diestrus and anestrus [45]. Together, CAT lower activity and SOD increased activity in anestrus and proestrus it is likely to provided a local environment with increased oxidative potential triggering a compensatory mechanism that promote cell survival [46]. Low level of lipid peroxidation in cells mounts an adaptive stress response to increasing antioxidant enzymatic systems [47]. An alternative explanation for our results is that high SOD activity in the presence of reduced CAT activity would increase the levels of free hydroxyl radicals, which are highly reactive and could increase lipid peroxidation [46]. Contrasting to the described in humans, where increasing total SOD activity existed from the proliferative until mid-secretory stages [48], this study showed that in dogs SOD activity is inhibited by increasing levels of progesterone (progesterone levels start increasing in dogs in estrus and remain above basal levels through the early and full diestrus [49]). In humans, estrogen and progesterone withdrawal in endometrial cells cultured *in vitro* leads to a decrease in SOD activity, thereby increasing ROS concentrations [50], which would trigger prostaglandin F2α, luteolysis and the endometrial shedding at menstruation. In dogs, the phenomena of luteolysis remains poorly characterized; nevertheless, Luz *et al.* [51] showed that PGF2α in late diestrus in dogs remained in low values, even when progesterone peripheral levels are below 1-2 ng/mL, which is considered rather low compared to the other species where the prostaglandins play a role in the pathways. Therefore, it is possible that differences between human and dogs in respect to SOD activity profiles during the reproductive cycle will be due to differences in the species physiology.

In bitches little is known about redox status and oxidative stress in the endometrium or uterine environment and their modulation by sex steroids hormones. In sheep activity of antioxidants enzymes, such as SOD and GPx, are up-regulated during the window of

implantation [14]; such increase might be a survival response during the transition from the implantation period to the post-implantation period [14] that is important to prevent a possible oxidative insult in early pregnancy [52]. In pigs it has been demonstrated that estrogen exerts antioxidant activity by inhibiting the H_2O_2 synthesis in luteal and follicular cells [53]. In mice, estrogen reduced the total SOD activity in the uterus [54]. Estrogen-mediated reduction in SOD activity appeared to be associated with an increase in the membrane fluidity of endometrial cells [54]. During the process of embryo implantation a slight increase in lipid peroxidation increases fluidity of the membranes of endometrial cells helping the fusion of the trophectoderm with the endometrial cells [55]. Physiological levels of estrogen and progesterone in sheep reduced the activity of SOD1 in the endometrium [27]. Data gathered in the present study also showed a decrease in SOD activity from proestrus to estrus; in dogs, rising levels of estrogens occurs during proestrus to peak in the beginning of estrus when the levels of progesterone start rising escorting the pre-ovulatory LH surge [22, 49].

It has been suggested that oxidative stress plays a role in the induction of apoptosis through mitochondrial dysfunction. ROS-mediated mitochondrial membrane damage and cytochrome c release would be followed by apoptotic cell death [56]. Our results suggest that an increased SOD activity plus decreases CAT activity at anestrus phase are responsible for increase H_2O_2 and should be related with higher levels of endometrial cells regeneration [56].

In the endometrium, CAT activity may interfere in the proliferation and apoptosis pathways. In other tissues, it has been demonstrated that an increase in CAT expression aborts the activation of cell proliferation [57]. The increase in CAT activity observed in the current study may contribute to a reduction in the proliferate activity of the canine endometrium. In the canine endometrium, proliferation peaks in proestrus and early estrus, particularly in the superficial and intermediate layers of the canine endometrium [58]. Moreover, CAT activity seems to protect tumour cells from apoptosis, in a mechanism mediated by the hydrogen peroxide [59]**.** A similar role has been attributed to GST [60]. The increased activity found in stages when the progesterone dominates, in particular in estrus post-LH surge, as it happened in the current study, may reflect an involvement in the cell proliferation and apoptosis phenomena in canine endometrium.

The role of individual antioxidant enzymes may have different outcomes in tissue homeostasis, and each enzyme may play a distinct role in different cell populations, as in the case of the endometrium, where stroma and epithelial populations modulate each other functions. Further studies are foreseen to detail the individual participation of antioxidant enzymes and ROS in the endometrial function in dogs.

5. Conclusions

This study provided the first data on antioxidant status and lipid and thiol group compounds oxidation in endometrial tissues of dogs. Herein we showed that in the cyclic canine endometrium exists an efficient control of oxidative stress without lipid or proteins attack in results of excessive reactive oxygen species. Moreover, cyclic fluctuations in antioxidant enzymes, in particular SOD and catalase, should be associated with endometrium regulatory mechanisms and the sex steroid cyclic variations thus reflecting the species particular physiology.

6. Acknowledgements

This work was sponsored by the Portuguese Science and Technology Foundation (FCT) under the Project UID/AGR/04033/2013

7. Chapter reference list

[1] Sies H. Oxidative Stress - from Basic Research to Clinical-Application. Am J Med. 1991;91:S31-S8.

[2] Stanczyk M, Gromadzinska J, Wasowicz W. Roles of reactive oxygen species and selected antioxidants in regulation of cellular metabolism. Int J Occup Med Environ Health. 2005;18:15-26.

[3] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford-New York: Oxford University Press; 1999.

[4] Finkel T. Oxygen radicals and signaling. Curr Opin Cell Biol. 1998;10:248-53.

[5] Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82:47-95.

[6] Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. Oxidative stress and cell signalling. Curr Med Chem. 2004;11:1163-82.

[7] Yu BP. Cellular Defenses against Damage from Reactive Oxygen Species. Physiol Rev. 1994;74:139-62.

[8] Pamplona R, Costantini D. Molecular and structural antioxidant defenses against oxidative stress in animals. Am J Physiol-Reg I. 2011;301:R843-R63.

[9] Zhu RZ, Wang YJ, Zhang LQ, Guo QL. Oxidative stress and liver disease. Hepatol Res. 2012;42:741-9.

[10] Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell B. 2007;39:44-84.

[11] Agarwal A, Gupta S. Role of reactive oxygen species in female reproduction. Part I. Oxidative stress: a general overview. Agro Food Ind Hi Tech. 2005;16:21-5.

[12] Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: From molecular mechanisms to health implications. Antioxid Redox Sign. 2008;10:1375-403.

[13] Cornelli U, Belcaro G, Cesarone MR, Finco A. Analysis of oxidative stress during the menstrual cycle. Reprod Biol Endocrinol : RB&E. 2013;11:74.

[14] Al-Gubory KH, Fowler PA, Garrel C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell B. 2010;42:1634-50.

[15] Riley JCM, Behrman HR. Oxygen Radicals and Reactive Oxygen Species in Reproduction. P Soc Exp Biol Med. 1991;198:781-91.

[16] Rizzo A, Roscino MT, Binetti F, Sciorsci RL. Roles of Reactive Oxygen Species in Female Reproduction. Reprod Domest Anim. 2012;47:344-52.

[17] Ufer C, Wang CC. The Roles of Glutathione Peroxidases during Embryo Development. Front Mol Neurosci. 2011;4:12.

[18] Van Langendonckt A, Casanas-Roux F, Donnez J. Oxidative stress and peritoneal endometriosis. Fertil Steril. 2002;77:861-70.

[19] May KE, Villar J, Kirtley S, Kennedy SH, Becker CM. Endometrial alterations in endometriosis: a systematic review of putative biomarkers. Hum Reprod Update. 2011;17:637-53.

[20] Szczubial M, Dabrowski R. Activity of Antioxidant Enzymes in Uterine Tissues of Bitches with Pyometra. B Vet I Pulawy. 2009;53:673-6.

[21] Rizk B, Badr M, Talerico C. Oxidative Stress and The Endometrium. Studies on Women's Health: Humana Press; 2013. p. 61-74.

[22] Concannon PW. Reproductive cycles of the domestic bitch. Anim Reprod Sci. 2011;124:200-10.

[23] Okkens AC, Kooistra HS. Anoestrus in the dog: a fascinating story. Reprod Domest Anim. 2006;41:291-6.

[24] Nomura K. Induction of canine deciduoma in some reproductive stages with the different condition of corpora lutea. J Vet Med Sci. 1997;59:185-90.

[25] Noakes DE, Dhaliwal GK, England GC. Cystic endometrial hyperplasia/pyometra in dogs: a review of the causes and pathogenesis. J Reprod Fertil Suppl.2001;57:395-406.

[26] Ramos RS, Oliveira ML, Izaguirry AP, Vargas LM, Soares MB, Mesquita FS, *et al.* The periovulatory endocrine milieu affects the uterine redox environment in beef cows. Reprod Biol Endocrinol. 2015;13:39.

[27] Al-Gubory KH, Bolifraud P, Garrel C. Regulation of key antioxidant enzymatic systems in the sheep endometrium by ovarian steroids. Endocrinology. 2008;149:4428-34.

[28] Payan-Carreira R, Pires MA, Holst BS, Rodriguez-Martinez H. Tumour Necrosis Factor in the Canine Endometrium: An Immunohistochemical Study. Reprod Domest Anim. 2011;46:410-8.

[29] Barrau MD, Abel JH, Torbit CA, Tietz WJ. Development of Implantation Chamber in Pregnant Bitch. Am J Anat. 1975;143:115-29.

[30] Concannon P, Tsutsui T, Shille V. Embryo development, hormonal requirements and maternal responses during canine pregnancy. J Rep Fer S. 2001:169-79.

[31] Payan-Carreira R, Pires MA, Holst BS, Rodriguez-Martinez H. Tumour Necrosis Factor in the Canine Endometrium: An Immunohistochemical Study. Reprod Domest Anim. 2011;46:410-8.

[32] Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. Anal Biochem. 1976;72:248-54.

[33] Paya M, Halliwell B, Hoult JRS. Interactions of a Series of Coumarins with Reactive Oxygen Species - Scavenging of Superoxide, Hypochlorous Acid and Hydroxyl Radicals. Biochem Pharmacol. 1992;44:205-14.

[34] Delrio LA, Gomezortega M, Leallopez A, Lopezgorge J. More Sensitive Modification of Catalase Assay with Clark Oxygen-Electrode - Application to Kinetic Study of Pea Leaf Enzyme. Anal Biochem. 1977;80:409-15.

[35] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-Transferases - First Enzymatic Step in Mercapturic Acid Formation. J Biol Chem. 1974;249:7130-9.

[36] Smith IK, Vierheller TL, Thorne CA. Assay of Glutathione-Reductase in Crude Tissue-Homogenates Using 5,5'-Dithiobis(2-Nitrobenzoic Acid). Anal Biochem. 1988;175:408-13.

[37] Paglia DE, Valentin WN. Studies on Quantitative and Qualitative Characterization of Erythrocyte Glutathione Peroxidase. J Lab Clin Med. 1967;70:158-&.

[38] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol. 1978;52:302-10.

[39] Doktorovova S, Santos DL, Costa I, Andreani T, Souto EB, Silva AM. Cationic solid lipid nanoparticles interfere with the activity of antioxidant enzymes in hepatocellular carcinoma cells. Int J Pharm. 2014;471:18-27.

[40] Ellman GL. Tissue Sulfhydryl Groups. Arch Biochem Biophys. 1959;82:70-7.

[41] Sedlak J, Lindsay RH. Estimation of Total Protein-Bound and Nonprotein Sulfhydryl Groups in Tissue with Ellmans Reagent. Anal Biochem. 1968;25:192-&.

[42] Suzuki Y, Lyall V, Biber TUL, Ford GD. A Modified Technique for the Measurement of Sulfhydryl-Groups Oxidized by Reactive Oxygen Intermediates. Free Radical Bio Med. 1990;9:479-84.

[43] Sugino N. The role of oxygen radical-mediated signaling pathways in endometrial function. Placenta. 2007;28:S133-S6.

[44] Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. Reprod Biol Endocrinol. 2005;3:43.

[45] Van Cruchten S, Van den Broeck W, Duchateau L, Simoens P. Apoptosis in the canine endometrium during the estrous cycle. Theriogenology. 2003;60:1595-608.

[46] Ramos RS, Oliveira ML, Izaguirry AP, Vargas LM, Soares MB, Mesquita FS, *et al.* The periovulatory endocrine milieu affects the uterine redox environment in beef cows. Reprod Biol Endocrin. 2015;13.

[47] Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438.

[48] Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H. Differential regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase by progesterone withdrawal in human endometrial stromal cells. Mol Hum Reprod. 2002;8:68-74.

[49] Concannon PW. Endocrinologic control of normal canine ovarian function. Reprod Domest Anim. 2009;44 Suppl 2:3-15.

[50] Sugino N, Karube-Harada A, Taketani T, Sakata A, Nakamura Y. Withdrawal of ovarian steroids stimulates prostaglandin F2alpha production through nuclear factor-kappaB activation via oxygen radicals in human endometrial stromal cells: potential relevance to menstruation. J Reprod Develop. 2004;50:215-25.

[51] Luz MR, Bertan CM, Binelli M, Lopes MD. In vitro PGF2alpha production by endometrium and corpus luteum explants from pregnant and nonpregnant diestrus bitches and placental explants from pregnant bitches. Theriogenology. 2006;66:1442-7.

[52] Al-Gubory KH, Arianmanesh M, Garrel C, Bhattacharya S, Cash P, Fowler PA. Proteomic analysis of the sheep caruncular and intercaruncular endometrium reveals changes in functional proteins crucial for the establishment of pregnancy. Reproduction. 2014;147:599-614.

[53] Murdoch WJ. Inhibition by oestradiol of oxidative stress-induced apoptosis in pig ovarian tissues. J Reprod Fertil. 1998;114:127-30.

[54] Jain S, Saxena D, Kumar PG, Koide SS, Laloraya M. Effect of estradiol and selected antiestrogens on pro- and antioxidant pathways in mammalian uterus. Contraception. 1999;60:111-8.

[55] Laloraya M. Fluidity of the phospholipid bilayer of the endometrium at the time of implantation of the blastocyst--a spin label study. Biochem Biophys Res Commun. 1990;167:561-7.

[56] Kruman I, Bruce-Keller AJ, Bredesen D, Waeg G, Mattson MP. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. J Neurosci. 1997;17:5089-100.

[57] Yano S, Yano N. Regulation of catalase enzyme activity by cell signaling molecules. Mol Cell Biochem. 2002;240:119-30.

[58] Van Cruchten S, Van den Broeck W, D'Haeseleer M, Simoens P. Proliferation patterns in the canine endometrium during the estrous cycle. Theriogenology. 2004;62:631-41.

[59] Bechtel W, Bauer G. Catalase protects tumor cells from apoptosis induction by intercellular ROS signaling. Anticancer Res. 2009;29:4541-57.

[60] Singh S. Cytoprotective and regulatory functions of glutathione S-transferases in cancer cell proliferation and cell death. Cancer Chemother Pharmacol. 2015;75:1-15.

Chapter 8 – General discussion

Gradually, information on the molecular interplay in the canine endometrium becomes nowadays available [1-9]. Still, information covering the changes in molecular markers throughout the canine endometrial cycle remains rather limited [1-3] Most authors investigating molecular markers in the canine uterus focus their interests on the differences between diestrus and early pregnancy [4] or pyometra [5], because either phenomena develops during diestrus.

However the study of a given stage of the cycle or a particular phase of a disease may be quite limitative, once most endometrial changes at a molecular level derive from the alternancy of estrogen and progesterone influences over time and in intact animals, sex steroids fluctuations may interfere with the course of disease. Moreover, it may also be of interest to compare the morphological changes during the endometrial cycle to changes in the molecular interplayers in the canine endometrium, expecting to contribute to the identification of a clinical marker for the early canine pregnancy or the early phases of the cystic endometrial hyperplasia/pyometra, while highlighting the pathogenesis of the process.

The first work presented herein, on neutral endopeptidase (NEP/CD10) expression in the canine endometrium, showed that the canine endometrium presents a difuse stromal cytoplasmatic staining, resembling that described in women [10, 11], which varied along the estrous cycle. Estrogen associated stages and anestrus were the stages with higher NEP/CD10 expression. In the endometrium, the lowest labelling variations were found for the deep basal stromal layer, which had been also refered in women, where this layer is mentioned as relatively independent of sex steroids influence [10, 12]. This study also identified for the first time, to our best knowledge, a population of NEP/CD10 negative cells in the stroma, just beneath the surface epithelium, in non-luteal stages of the estrous cycle. These are fusiform cells with an ovoid nucleus of dense chromatin that tend to become round and rough when under progesterone influence, that resemble in their morphology predecidual cells detected in women endometrium in middle to late secretory phase [13, 14]. Expression of NEP/CD10 occurred also in the perimysium of the smooth muscle cells constituting the myometrium, similarly to the reported for pregnant mice [15] and for sheep [16], but in contrast to women. The differences found between these cases, migth be related to differences in the arrangement of bundles of smooth muscle fibres in myometrium layers.

In early pregnancy, at the implantation sites, a reinforcement of NEP/CD10 immunoreaction display a ring-like peri-lacunar disposition limiting the embryo invasiveness;

the NEP/CD10 intensity of immunoreaction was higher in the subluminal stroma, in decidual cells, than in the the basal layer of the endometrial stroma, whereas the syncytium cords in the labirynth were negative for this marker. Before implantation, no differences were found in the longitudinal cuts of the canine endometrium suggesting the inexistence of pre-determined implantation sites, as it was already evidenced with other markers, such as integrin αvβ3 [17], tumour necrosis factor [18] and interleukin 18 [19].

Data gathered in the NEP/CD10 study also indicates that this molecule might have other useful functions that go far beyond those currently referred in human medicine, *i.e.* as a reliable marker for the identification of *ex-situ* endometrial stroma. The molecule has been associated to the regulation of several bioactive peptides and the interplaying with different signalling mechanisms related to cell growth and proliferation, angiogenesis [11, 20-22], regulation of inflammation [23-25], and apoptosis [26-28]. Therefore, we consider that the role for NEP/CD10 in endometrial function remains unclear and needs to be further studied.

E-cadherin and β- catenin integrates the adherens junctional complex, thereby contributing to the integrity and polarity of epithelia in tissues. Grossly, in the uterus as in other organs, the epithelium constitutes a barrier at the surface of the organ. Previous work in human and rodents showed variations of these molecules during the endometrium cycle in response to the sex steroid dynamics [29-31]. On the other hand, it has been suggested that E-cadherin/β-catenin complexes may play a role in initial attachment of the embryo during implantation [32], besides the maintenance of the endometrial architectures [31].

The studies in E-cadherin and β-catenin, herein presented in chapter 4, showed that the strength of E-cadherin/β-catenin complexes change across the canine cycle according to sex steroid influence. The immunoexpression of E-cadherin was higher from anestrus to estrus but decreased particularly in full diestrus, when under progesterone influence, just like reported in ewes [33] and in humans [31, 32]. β-catenin membrane immunolabelling showed a tendency to decreased for a lower expression during the diestrus stages. These changes significantly correlate with the decreased immunohistochemical expression of E-cadherin in the membrane, suggesting a reduction of the E-cadherin/ β-catenin complexes that could lead to a modification of the adherens junction properties, facilitating the embryo-maternal interaction. This slight softening of the intercellular junctional complexes, which in our study was more evident in the surface epithelium and in the superficial glandular epithelia, should facilitate the trophoblast migration through the epithelial cells. This hypothesis is supported from evidences collected from early pregnancy samples where adhesion and initial invasion are taking place. Those facts support the occurrence of a transition process during

trophoblast invasion, leading to the establishment of the placenta, alike it was proposed for human endometriosis [34], and mimetizing those of tumour invasion in tissues [35, 36]

Moreover, the decrease in E-cadherin/β-catenin membrane expression during early and full diestrus, as seen in the current study, would also interfere with the strength of the epithelial endometrial barrier, which would increase the local susceptibility to invasion of pathogens and the development of inflammation, as well as some particular degeneartive changes, such as cystic endometrial hyperplasia. Once there is also a decrease in MUC-1 expression during canine diestrus [37], this might contribute also to facilitate the onset of canine pyometra in diestrus.

The endometrial distribution of SOD1 and GPx1 positive cells and the spatial and temporal changes in the relative amount of these molecules during the canine estrous cycle was addressed in chapter 6. Both SOD1 [38, 39] and PGx1 [40] molecules have been reported in human endometrium, with variations in their pattern of localization and protein content during the menstrual cycle [38, 41]. Changes in the content of different antioxidant enzymes have also been reported in sheep endometrium [42]. Once the uterus, as other organs, presents defence mechanisms against reactive oxygen species [43], changes in morphological and metabolic events are expected to reflect variations in the distribution pattern of antioxidant enzymes. Lower stromal SOD 1 or GPx1 immunoreaction scores were found in comparison with endometrial epithelia. Similar findings have been described in the women endometrium [38, 40, 44], although those reports are not coincident regarding the epithelial cells. Sugino *et al.* [44] describe a constant moderate intensity, while Narimoto and colleagues [38] refer an increase in the intensity in the secretory phase. Our data is in accordance with the described by Narimoto *et al.* [38]

In canine endometrium, SOD immunoreaction was lower in proestrus, increasing towards diestrus, when it showed the highest scores, accompanying the typical increase in progesterone peripheral levels. These results resemble the ones described by Sugino and colleagues [44], who refer to an increase of SOD1 intensity in mid- to late- secretory phases. The endometrial scores for GPx1 remained relatively constant throughout the estrous cycle, contrasting with previous reports in human endometrium [39, 41]. Variations in women are much more noticeable [39] than in dogs, where it was observed only a slight decrease in the immuno-scores in endometrial epithelial cells in estrus, coincident with the transition from an estrogen-driven environment to one with increasing levels of progesterone [45]. Furthemore, corresponding to the mid-to-late secretory stage described in humans, the labelling scores peaked in diestrus in the dog endometrium.

SOD1 and GPx1 may be another player in the local cytokines network, such as the tumour necrosis factor-alpha (TNF-α) or particular transforming growth factor (TGF)-beta isoforms, which presence has also been demonstrated in the canine endometrium. In this way, SOD1 and GPx1 may have important roles in the organ immune response [46], in apoptosis or in proliferation [47-51]. SOD1 showed decreased scores in anestrus and proestrus, the same stages where TNF presented higher immunoreaction scores [3]. The interaction of several different mechanisms can affect the dynamics of other markers and pathways, but these effects need further studies in order to be established.

Interestingly, SOD1 nuclear immunolabelling in epithelial cells was noticed in estrus and early diestrus, although this enzyme is mainly cytosolic. This could be explained by H_2O_2 accumulation driven a dislocation of SOD1 to the cell nucleus [52] in order to prevent apoptosis. This is furthermore supported by the pattern of apoptosis in canine endometrium described by Van Cruchten *et al.* [53]. The present study also found a relation of SOD1 and GPx immunoexpression, which demonstrated a small decrease, particularly in the glandular epithelia, that was reverted in diestrus. These findings reinforce the hypothesis of an existing accumulation of H_2O_2 during these stages of the cycle, and of a phase-related protective effect against apoptosis.

The roles of SOD1 and GPx1 in the canine endometrium could not be drawn from the study developed herein. However, as deffended for other species, SOD1 and GPx activity may foster the trophoblast differentiation, decidualization of stromal cells, regulation of vascular permeability and trophoblast migration within the maternal endometrium [54-56]; these may be reflected in the subtle changes in SOD1 labelling in the sub-surface area of the endometrial stroma registered in early diestrus, when implantation and decidualization take place. Also GPx1 scores were decreased during the proestrus and estrus (follicular phase), while the peripheral levels of estrogens increase in dogs [45], which correspond to the stages of the estrous cycle presenting a peak of proliferation, as described by Van Cruchen *et al.* [1]. Another putative role for SOD1 and GPx1 could relate with the prostaglandin F2-alpha synthesis and the endometrial remodelling at progesterone withdrawal and luteolysis, that in the uetrus have been associated to a decrease in SOD1 activity [51, 55, 57]. Data reported here need to be completed to establish such association, either because diestrus samples used in this study represented endometrial samples at mid-to-late progestagenic stage or because the present study refers to the molecule distribution on the canine endometrium and describes variations in its relative concentration during the cycle, but does not allow estimating the enzyme activity. Further studies will be necessary to enlight the antioxidant enzymes interplay during the endometrial cycle in the canine endometrium.

In the present study, an increased apical reinforcement pattern in epithelial cells was evidenced for both SOD1 and GPx1, alike the described for in women endometrium [38, 39], in proestrus and estrus (estrogen-associated stages). This may be related to the secretion of the enzymes from the epithelial cells into the extracellular space or the lumen of the uterus. These enzymes have already being identify in the uterine fluid in the bitch [58] and cow [59]. This hypothesis is reinforced by Kobayashi *et al.* [58], who also reported an increase in SOD activity in estrus in dogs.

As already seen in the previous study, there seems to be an adequate balance between oxidant species and antioxidant defences, preventing oxidative stress to occur. In here it was shown that the activity of glutathione-dependent enzymes remains relatively unchanged throughout the estrous cycle, although there was a slight increase in lipid peroxidation in anestrus and proestrus, as well as in SOD activity, contrasting with a reduced CAT activity in theses stages. Canine endometrium presents a deep remodeling at anestrus, and increased apoptosis in late diestrus and anestrus [60]. In regards to these fenomena, these enzymes might act as compensatory mechanism to cellular survival in face to increased oxidative stress [61], and avoiding lipid peroxidation [62]. Although in humans total SOD activity increased from the proliferative until mid-secretory stages [63], and Sugino *et al.* [64] , using *in vitro* cultured cells, showed that estrogen and progesterone withdrawal induces lower SOD activity and consequently higher ROS levels, triggering, for example, prostaglandin F2α and luteolysis. However, as shown herein, data gathered in here showed that, in dogs, SOD activity is depressed by increasing levels of progesterone. Moreover, in dogs, PGF2α levels in late diestrus remain low compared to other species that depend on prostaglandins for luteolysis. Hence, this sort of differences between dogs and species with corpus luteum of short duration might probably reflect differences and particularities in dog physiology.

Although some knowledge exists for sheep, pig, mice, little is know about oxidative status in bitches, and its relation with sex steroids. In those species, SOD and GPx increase may favour the implantation process and successful pregnancy [56, 65] or inhibits H_2O_2 synthesis in luteal and follicular cells [66]. In mice, as in dogs, these enzymes are regulated by sex steroids. In mice, decreased SOD activity and increased lipid peroxidation estrogen mediated, facilitates membrane fluidity of endometrial cells and thus implantation [67, 68]. The present work showed a decrease in SOD activity from proestrus to estrus, when estrogen levels are rising from proestrus to estrus and progesterone levels raise after the pre-ovulatory LH surge [45, 69]. The increased SOD activity together with decreased CAT activity verified at anestrus, may lead to increased H_2O_2 levels and higher rates of

endometrial regeneration [70]. The increased CAT activity registered may be involved to a decreased proliferation in the canine endometrium, as CAT activity may abort the activation of cell proliferation, by interfering with proliferation and apoptosis pathways [71]. Bechtel and collegues [72] proposed that CAT could protect tumour cells from apoptosis via a mechanism mediated by H_2O_2**.** In dogs the increased activity measured under high progesterone levels may due to interference in cell proliferation and apoptosis mechanisms.

Chapter reference list

[1] Van Cruchten S, Van den Broeck W, D'Haeseleer M, Simoens P. Proliferation patterns in the canine endometrium during the estrous cycle. Theriogenology. 2004;62:631-41.

[2] Vermeirsch H, Simoens P, Hellemans A, Coryn M, Lauwers H. Immunohistochemical detection of progesterone receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology. 2000;53:773-88.

[3] Payan-Carreira R, Pires MA, Holst BS, Rodriguez-Martinez H. Tumour Necrosis Factor in the Canine Endometrium: An Immunohistochemical Study. Reprod Domest Anim. 2011;46:410-8.

[4] Schafer-Somi S. Early canine pregnancy--a battle for successful growth and angiogenesis. Reprod Domest Anim. 2012;47 Suppl 6:165-8.

[5] Silva E, Leitao S, Ferreira-Dias G, Lopes da Costa L, Mateus L. Prostaglandin synthesis genes are differentially transcripted in normal and pyometra endometria of bitches. Reprod Domest Anim. 2009;44 Suppl 2:200-3.

[6] Kowalewski MP, Mutembei HM, Hoffmann B. Canine prostaglandin E2 synthase (PGES) and its receptors (EP2 and EP4): expression in the corpus luteum during dioestrus. Anim Reprod Sci. 2008;109:319-29.

[7] Kowalewski M, Beceriklisoy H, Aslan S, Agaoglu A, Hoffmann B. Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch. Anim Reprod Sci. 2009;116:129 - 38.

[8] Kowalewski M, Fox B, Gram A, Boos A, Reichler I. Prostaglandin E2 functions as a luteotrophic factor in the dog. Reproduction. 2013;145:213 - 26.

[9] Kowalewski M, Kautz E, Hogger E, Hoffmann B, Boos A. Interplacental uterine expression of genes involved in prostaglandin synthesis during canine pregnancy and at induced prepartum luteolysis/abortion. Reprod Biol Endocrinol. 2014;12:46.

[10] Shipp MA, Vijayaraghavan J, Schmidt EV, Masteller EL, D'Adamio L, Hersh LB, et al. Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 ("enkephalinase"): direct evidence by cDNA transfection analysis. Proc Natl Acad Sci USA. 1989;86:297-301.

[11] McIntosh GG, Lodge AJ, Watson P, Hall AG, Wood K, Anderson JJ, et al. NCL-CD10-270: a new monoclonal antibody recognizing CD10 in paraffin-embedded tissue. Am J Pathol. 1999;154:77-82.

[12] Imai K, Kanzaki H, Mori T. Cell surface peptidases in human endometrium. Mol Hum Reprod. 1996;2:425-31.

[13] Sumitomo M, Shen R, Walburg M, Dai J, Geng Y, Navarro D, et al. Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling. J Clin Invest. 2000;106:1399-407.

[14] Di Maria GU, Bellofiore S, Geppetti P. Regulation of airway neurogenic inflammation by neutral endopeptidase. Eur Respir J. 1998;12:1454-62.

[15] Ottlecz A, Walker S, Conrad M, Starcher B. Neutral metalloendopeptidase associated with the smooth muscle cells of pregnant rat uterus. J Cell Biochem. 1991;45:401-11.

[16] Riley SC, Wong E, Findlay JK, Salamonsen LA. Localization of neutral endopeptidase in the ovine uterus and conceptus during the oestrous cycle and early pregnancy. Reprod Fertil Dev. 1995;7:27-33.

[17] Payan-Carreira R. Endometrial Expression of Integrin alpha V beta 3 in the Bitch. Reprod Domest Anim. 2009;44:76-.

[18] Payan-Carreira R, Pires MA, Ström Holst B, Rodriguez-Martinez H. Tumour necrosis factor in the canine endometrium: an immunohistochemical study. Reprod Domest Anim. 2011;46:410-8.

[19] Miranda S, Pereira M, Pires MA, Neto RL, Payan-Carreira R. IL-18 localization in canine endometrium. Reprod Biol. 2013;13, Supplement 2:32-3.

[20] Bilalovic N, Sandstad B, Golouh R, Nesland JM, Selak I, Torlakovic EE. CD10 protein expression in tumor and stromal cells of malignant melanoma is associated with tumor progression. Mod Pathol. 2004;17:1251-8.

[21] Bai M, Agnantis NJ, Skyrlas A, Tsanou E, Kamina S, Galani V, et al. Increased expression of the bcl6 and CD10 proteins is associated with increased apoptosis and proliferation in diffuse large B-cell lymphomas. Mod Pathol. 2003;16:471-80.

[22] Toki T, Shimizu M, Takagi Y, Ashida T, Konishi I. CD10 is a marker for normal and neoplastic endometrial stromal cells. Int J Gynecol Pathol. 2002;21:41-7.

[23] Nascimento AF, Hirsch MS, Cviko A, Quade BJ, Nucci MR. The role of CD10 staining in distinguishing invasive endometrial adenocarcinoma from adenocarcinoma involving adenomyosis. Mod Pathol. 2003;16:22-7.

[24] Groisman GM, Meir A. CD10 is helpful in detecting occult or inconspicuous endometrial stromal cells in cases of presumptive endometriosis. Arch Pathol Lab Med. 2003;127:1003-6.

[25] Potlog-Nahari C, Feldman AL, Stratton P, Koziol DE, Segars J, Merino MJ, et al. CD10 immunohistochemical staining enhances the histological detection of endometriosis. Fertil Steril. 2004;82:86-92.

[26] Concannon P, Tsutsui T, Shille V. Embryo development, hormonal requirements and maternal responses during canine pregnancy. J Reprod Fertil Suppl. 2001;57:169-79.

[27] Amoroso E. Placentation. Marshall's physiology of reproduction. 1952:127 - 316.

[28] Head JR, MacDonald PC, Casey ML. Cellular localization of membrane metalloendopeptidase (enkephalinase) in human endometrium during the ovarian cycle. The J Clin Endocrinol Metab. 1993;76:769-76.

[29] van der Linden PJ, de Goeij AF, Dunselman GA, Erkens HW, Evers JL. Expression of cadherins and integrins in human endometrium throughout the menstrual cycle. Fertil Steril. 1995;63:1210-6.

[30] Jha RK, Titus S, Saxena D, Kumar PG, Laloraya M. Profiling of E-cadherin, beta-catenin and Ca2+ in embryo-uterine interactions at implantation. FEBS Lett. 2006;580:5653-60.

[31] Shih HC, Shiozawa T, Miyamoto T, Kashima H, Feng YZ, Kurai M, et al. Immunohistochemical expression of E-cadherin and beta-catenin in the normal and malignant human endometrium: an inverse correlation between E-cadherin and nuclear beta-catenin expression. Anticancer Res. 2004;24:3843-50.

[32] McEwan M, Lins RJ, Munro SK, Vincent ZL, Ponnampalam AP, Mitchell MD. Cytokine regulation during the formation of the fetal-maternal interface: focus on cell-cell adhesion and remodelling of the extra-cellular matrix. Cytokine Growth Factor Rev. 2009;20:241-9.

[33] Satterfield MC, Dunlap KA, Hayashi K, Burghardt RC, Spencer TE, Bazer FW. Tight and adherens junctions in the ovine uterus: differential regulation by pregnancy and progesterone. Endocrinology. 2007;148:3922-31.

[34] Bartley J, Julicher A, Hotz B, Mechsner S, Hotz H. Epithelial to mesenchymal transition (EMT) seems to be regulated differently in endometriosis and the endometrium. Arch Gynecol Obstet. 2014;289:871-81.

[35] Birchmeier W, Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta. 1994;1198:11-26.

[36] Bremnes RM, Veve R, Hirsch FR, Franklin WA. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis, and prognosis. Lung cancer (Amsterdam, Netherlands). 2002;36:115-24.

[37] Ishiguro K, Baba E, Torii R, Tamada H, Kawate N, Hatoya S, et al. Reduction of mucin-1 gene expression associated with increased *Escherichia coli* adherence in the canine uterus in the early stage of dioestrus. Vet J. 2007;173:325-32.

[38] Narimoto K, Noda Y, Shiotani M, Tokura T, Goto Y, Takakura K, et al. Immunohistochemical Assessment of Superoxide-Dismutase Expression in the Human Endometrium Throughout the Menstrual-Cycle. Acta Histochem Cytochem. 1990;23:487-98.

[39] Ohwada M, Suzuki M, Sato I, Tsukamoto H, Watanabe K. Glutathione peroxidase activity in endometrium: Effects of sex hormones and cancer. Gynecol Oncol. 1996;60:277-82.

[40] Ota H, Igarashi S, Kato N, Tanaka T. Aberrant expression of glutathione peroxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Fertil Steril. 2000;74:313-8.

[41] Serviddio G, Loverro G, Vicino M, Prigigallo F, Grattagliano I, Altomare E, et al. Modulation of endometrial redox balance during the menstrual cycle: relation with sex hormones. J Clin Endocrinol Metab. 2002;87:2843-8.

[42] Al-Gubory KH, Bolifraud P, Garrel C. Regulation of key antioxidant enzymatic systems in the sheep endometrium by ovarian steroids. Endocrinology. 2008;149:4428-34.

[43] Rizk B, Badr M, Talerico C. Oxidative Stress and The Endometrium. In: Agarwal A, Aziz N, Rizk B, editors. Studies on Women's Health: Humana Press; 2013. p. 61-74.

[44] Sugino N, Shimamura K, Takiguchi S, Tamura H, Ono M, Nakata M, et al. Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. Hum Reprod. 1996;11:1073-8.

[45] Concannon PW. Reproductive cycles of the domestic bitch. Anim Reprod Sci. 2011;124:200-10.

[46] Marikovsky M, Ziv V, Nevo N, Harris-Cerruti C, Mahler O. Cu/Zn superoxide dismutase plays important role in immune response. J Immunol. 2003;170:2993-3001.

[47] Fukai T, Ushio-Fukai M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid Redox Signal. 2011;15:1583-606.

[48] Khan AA, Rahmani AH, Aldebasi YH, Aly SM. Biochemical and pathological studies on peroxidases -an updated review. Glob J Health Sci. 2014;6:87-98.

[49] Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H. Reactive oxygen species stimulate prostaglandin F2 alpha production in human endometrial stromal cells in vitro. Human reproduction (Oxford, England). 2001;16:1797-801.

[50] Thannickal V, Fanburg B. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 2000;279:L1005 - 28.

[51] Brigelius-Flohe R, Kipp A. Glutathione peroxidases in different stages of carcinogenesis. Biochim Biophys Acta. 2009;1790:1555-68.

[52] Tsang CK, Liu Y, Thomas J, Zhang YJ, Zheng XFS. Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. Nature Communications. 2014;5.

[53] Van Cruchten S, Van den Broeck W, Duchateau L, Simoens P. Apoptosis in the canine endometrium during the estrous cycle. Theriogenology. 2003;60:1595-608.

[54] Ufer C, Wang CC. The roles of glutathione peroxidases during embryo development. Front Mol Neurosci. 2011;4.

[55] Sugino N. The role of oxygen radical-mediated signaling pathways in endometrial function. Placenta. 2007;28 Suppl A:S133-6.

[56] Al-Gubory K, Fowler P, Garrel C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol. 2010;42:1634 - 50.

[57] Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. Antioxid Redox Signal. 2008;10:1375 - 403.

[58] Kobayashi M, Wada M, Hori T, Kawakami E. Superoxide dismutase activity in the oviductal and uterine fluid of the bitch and the effects of the enzyme on viability, motility and hyperactivation of canine sperm in vitro. J Vet Med Sci. 2014;76:741-3.

[59] Ramos RS, Oliveira ML, Izaguirry AP, Vargas LM, Soares MB, Mesquita FS, et al. The periovulatory endocrine milieu affects the uterine redox environment in beef cows. Reprod Biol Endocrinol. 2015;13:39.

[60] Van Cruchten S, Van den Broeck W, Duchateau L, Simoens P. Apoptosis in the canine endometrium during the estrous cycle. Theriogenology. 2003;60:1595-608.

[61] Ramos R, Oliveira M, Izaguirry A, Vargas L, Soares M, Mesquita F, et al. The periovulatory endocrine milieu affects the uterine redox environment in beef cows. Reprod Biol Endocrinol. 2015;13:39.

[62] Ayala A, Munoz M, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438.

[63] Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H. Differential regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase by progesterone withdrawal in human endometrial stromal cells. Mol Hum Reprod. 2002;8:68 - 74.

[64] Sugino N, Karube-Harada A, Taketani T, Sakata A, Nakamura Y. Withdrawal of ovarian steroids stimulates prostaglandin F2alpha production through nuclear factor-kappaB activation via oxygen radicals in human endometrial stromal cells: potential relevance to menstruation. J Reprod Develop. 2004;50:215-25.

[65] Al-Gubory K, Arianmanesh M, Garrel C, Bhattacharya S, Cash P, Fowler P. Proteomic analysis of the sheep caruncular and intercaruncular endometrium reveals changes in functional proteins crucial for the establishment of pregnancy. Reproduction. 2014;147:599 - 614.

[66] Murdoch W. Inhibition by oestradiol of oxidative stress-induced apoptosis in pig ovarian tissues. J Reprod Fertil. 1998;114:127 - 30.

[67] Jain S, Saxena D, Kumar GP, Laloraya M. NADPH dependent superoxide generation in the ovary and uterus of mice during estrous cycle and early pregnancy. Life Sci. 2000;66:1139-46.

[68] Laloraya M, Jain S, Thomas M, Kopergaonkar S, Pradeep Kumar G. Estrogen surge: a regulatory switch for superoxide radical generation at implantation. Biochem Mol Biol Int. 1996;39:933-40.

[69] Concannon PW. Endocrinologic control of normal canine ovarian function. Reprod Domest Anim. 2009;44 Suppl 2:3-15.

[70] Kruman I, Bruce-Keller AJ, Bredesen D, Waeg G, Mattson MP. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. J Neurosci. 1997;17:5089-100.

[71] Yano S, Yano N. Regulation of catalase enzyme activity by cell signaling molecules. Mol Cell Biochem. 2002;240:119-30.

[72] Bechtel W, Bauer G. Catalase protects tumor cells from apoptosis induction by intercellular ROS signaling. Anticancer Res. 2009;29:4541-57.

Chapter 9 – General conclusions

Today, most research on molecular markers in the canine endometrium focus the identification of molecules involved in early embryo-endometrial cross-talk and on the pathogenesis of uterine diseases. However, the target is long to be achieved, and the veil over the molecular mechanism controlling the endometrial cycle and their changes in early pregnancy or in the early steps of uterine disease still exposed little of the reality.

The studies reunited under this thesis intent to generate essential information on molecular interplayers on the healthy canine endometrium. Several markers, either for stroma and epithelium, were used for the study of the cyclic changes that may be associated to endometrial proliferation, epithelial integrity and oxidative stress.

Our work demonstrated that NEP/CD10 might be an important regulator of embryo invasion, possibly by interaction with MMP/TIMP, while also allowing the identification of a subsurface stromal layer that was up to now unknowned to exist in canine endometrium.

The studies in the E-cadherin/β-catenin adherence complexes showed that in early diestrus, the strenght of the epithelial barrier is reduced in progesterone associated stages, which lead us to hypothesize that it is intend to favour embryo implantation and may strongly contribute to a higher susceptibility to inflammation evidenced by dogs in diestrus.

The immunohistochemical distribution of SOD1 and GPx1 in the canine endometrium throughout the estrous cycle showed a variation in the expression of these enzymes of the enzymatic defense mechanism against oxidative stress, which may be correlated with the participation of both SOD and GPx in different pathways that regulate the endometrial changes observed at different stages of the cycle. The information was completed by the analysis of the activity of antioxidant enzymes and the levels of lipid and protein oxidation along the estrous cycle allowed to estlabish the antioxidant status of the canine endometrium as well as the oxidation of lipids and proteins. This study evidence the existence of an efficient oxidant/antioxidant balance exists once there was no lipid or proteins attack by reactive oxygen species.

Most cyclic variations recorded for the markers used in this thesis were related to sex steroids dynamics reflecting the species particular physiology, integrating each molecule in different regulatory pathways addressing the endometrial homeostasy. Further studies with a large cohort of samples, particularly inciding on the effect of age and parity, are still foresee to detail possible partners involved in infertility, in age-related changes and with endometrial inflammation.