UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

NEW INSIGHTS IN THE IMMUNO-ENDOCRINE REGULATION OF EQUINE REPRODUCTION: *IN VITRO* STUDIES ON LUTEAL AND ENDOMETRIAL FUNCTION

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***Thesis title*:** New insights in the immuno-endocrine regulation of equine reproduction: *in vitro* studies on luteal and endometrial function

Abstract

Coordination of reproductive events in the ovary and uterus demands the action of diverse factors as steroid hormones, eicosanoids, growth factors or cytokines on the regulating of processes such as angiogenesis, cell growth and differentiation, and apoptosis. Thus, the objectives of this study were to evaluate the influence of cytokines tumor necrosis factor α (TNF), interferon gamma (IFNG) and Fas Ligand (FASL) on the regulation of secretory function, angiogenesis, cell viability and apoptosis in the equine: (i) corpus luteum (CL) during luteal establishment and functional and structural regression; and (ii) endometrium during follicular phase (FP) and mid luteal phase (MLP), at cell, gene and molecular levels. All studied cytokine ligands and receptors were expressed in the equine CL, throughout the luteal phase, and in the endometrium, throughout the estrous cycle. During CL growth, TNF was shown to stimulate *in vitro* P_4 and PGE_2, to inhibit $PGF_{2\alpha}$ secretion and to increase VEGF expression and angiogenic factors production. Thus, among all cytokines studied, TNF might give a luteotrophic contribution for CL establishment. Conversely, during CL regression, all cytokines alone reduced P_4 and PGE_2 secretion, while both FASL and TNF stimulated $PGF_{2\alpha}$ secretion. TNF and IFNG reduced angiogenic factors secretion and FASL decreased VEGF expression. Cytokine association (TNF+IFNG+FASL) effectively promoted apoptosis and reduced luteal cell viability. Besides, they stimulated $PGF_{2\alpha}$ and inhibited P_4, PGE_2 secretion and angiogenesis. In conclusion, cytokines interaction appears to coordinate functional and structural luteolysis in the mare. Concerning TNF role on endometrial cells, it can be concluded that during MLP, this cytokine stimulated PGE_2 secretion, promoted angiogenic activity and NO secretion and increased endometrial cells viability. The interaction between TNF, oxytocin and steroid hormones was shown to be determinant for physiologic regulation of equine endometrium.

Key words: TNF, IFNG, FASL, corpus luteum, luteotrophic, luteolysis, endometrium, angiogenesis, mare.

***Título da dissertação*:** Novas perspectivas na regulação imuno-endócrina da reprodução equina: estudos *in vitro* da função lútea e endometrial.

Resumo

A coordenação da função reprodutora no ovário e no útero requer a participação de diversos factores como hormonas esteróides, eicosanóides, factores de crescimento ou citocinas, responsáveis por regular processos biológicos como a angiogénese, o crescimento e diferenciação celular e a apoptose. Assim, o presente estudo teve como objectivo avaliar a influência das citocinas factor de necrose tumoral α (FNT), interferão gama (IFNG) e Fas Ligando (FASL) na regulação da função secretora, angiogénese, viabilidade celular e apoptose: (i) no corpo lúteo (CL) durante o seu estabelecimento e regressão funcional e estrutural; e (ii) no endométrio durante as fases folicular (FF) e lútea média (FLM), a nível celular, molecular e genético na égua. A expressão de todos os ligandos e receptores das citocinas estudadas foi confirmada no CL, ao longo da fase lútea, e no endométrio, ao longo do ciclo éstrico. Durante o crescimento do CL, FNT estimulou a produção *in vitro* de P_4 e PGE_2, inibiu a secreção de $PGF_{2\alpha}$ e aumentou a expressão de VEGF e a produção de factores angiogénicos. Desta forma, de todas as citocinas estudadas, FNT poderá contribuir como factor luteotrófico para o estabelecimento do CL. Contrariamente, aquando da regressão lútea, todas as citocinas individualmente reduziram a secreção de P_4 e PGE_2, enquanto FASL e FNT estimularam a secreção de $PGF_{2\alpha}$. FNT e IFNG inibiram a secreção de factores angiogénicos e FASL diminuiu a expressão de VEGF. A associação de citocinas FNT+IFNG+FASL promoveu de forma efectiva a apoptose e a redução da viabilidade das células lúteas. Além disso, estimulou a secreção de $PGF_{2\alpha}$ e diminuiu as de P_4 e PGE_2, bem como inibiu a angiogénese. Concluindo, a interacção entre as várias citocinas parece coordenar a regressão funcional e estrutural do CL na égua. Considerando o papel do TNF nas células endometriais, conclui-se que, durante a FLM, este factor estimulou a secreção de PGE_2, promoveu a produção de factores angiogénicos e de NO e aumentou a viabilidade das células endometriais. A interacção entre o FNT, a ocitocina e as hormonas esteróides provou ser determinante para a regulação fisiológica do endométrio equino.

***Palavras chave*:** FNT, IFNG, FASL, corpo lúteo, luteotrófico, luteólise, endométrio, angiogénese, égua.

Vivemos tempos sem tempo. O entusiasmo veloz do trabalho, a pressa dos dias no laboratório, define-nos prazos cada vez mais curtos. Tudo se torna para ontem. E aqueles que queremos sempre perto bem presentes, que fazem de nós o que somos, como que os adiamos num para amanhã que acontece menos vezes. Dedico-lhes este trabalho e agradeço-lhes tamanha compreensão, com a certeza que sem eles, simplesmente, este momento não se concretizaria.

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4) Web Publication (GeneBank database), FasL (GQ429291), Galvão A. and G Ferreira-Dias, 2009.

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17β-HSD - 17β-hydroxysteroid dehydrogenase
3βHSD - 3 Beta hydroxysteroid dehydrogenase
A – absorbance
ACH – acetylcholine
ADP- adenosine diphosphate
ANG II - angiotensin II
Ang II - angiotensin II
Ang1 - angiopoietin 1
Ang2 – angiopoietin 2
Apaf 1 - apoptotic peptidase activating factor 1
AU – arbitrary units
B2MG - β2 microglobulin
BAEC - bovine aortic endothelial cells
Bcl2 – B-cell lymphoma 2
Bid - BH3 interacting domain death agonist
bp – base pairs
BSA - bovine serum albumin
cAMP - cyclic adenosine monophosphate
CD36 - thrombospondin 1 receptor
$CD4^+$ - cluster of differentiation 4
$CD8^+$ - cluster of differentiation 8
cDNA - complementary DNA
CHAT - biosynthetic enzyme choline acetyltransferase
CL – corpus luteum
COX-2 - Cyclooxygenase-2
CREB - cAMP response element-binding
CYP17 - cytochrome P450 17
DAB – diaminobenzidine
DAPI - 4',6-diamidino-2-phenylindole
DB – data base
DD - death domain
DED - death effector domain
DISC - death inducing signalling complex
DMEM - dulbecco's modified Eagle's médium
DNA - deoxyribonucleic acid
E2 – estradiol- 17β
early CL – early luteal phase CL
ECM – extracellular matrix
ED50 - half maximal effective dose
EDTA - dthylenediaminetetraacetic acid
EFSA - european food safety authority
EGF - epidermal growth factor
Egr-1 - early growth response protein 1
ELC – endothelial luteal cell
ELEC – epithelial like endometrial cell

eLH – equine luteinizing hormone
eNOS - endothelial NO synthase
ERK - extracellular signal-regulated kinase
ERß - estradiol receptor beta
ERα – estradiol receptor alpha
ET-1 - endothelin-1
ETA – endothelin receptor A
FADD - Fas associated death domain
FAS – Fas Ligand receptor
FASL – Fas Ligand
FBS - fetal bovine serum
FGF - fibroblast growth factor
FP - follicular phase
FPr – PGF2α receptor
GH - growth hormone
HBSS - hank's buffered salt solution
hCG - human chorionic gonadotropin
HDL - high density lipoprotein
HIFA- hypoxia inducible factor alpha
HKG - housekeeping gene
HRP - horseradish peroxidase
IFNG - interferon gamma
IGF-I insulin-like growth factor I
IL-1 - interleukin 1
IRF1 - interferon regulatory factor 1
JaKs - janus Kinase
Kd – kilodalton
late CL – Late luteal phase CL
LDL- low density lipoprotein
LH - luteinizing hormone
LH-R - luteinizing hormone receptor
LLC - large luteal cells
LP – luteal phase
LPA - lypophosphatidic acid
M – molar
M199 – medium 199
MAP - mitogen activated protein
MAPK - mitogen activated protein kinase
MAPKAPK3 - MAPK - activated protein kinase 3
MCP-1 – monocyte chemoattractant protein 1
MgCl2 - magnesium chloride
MHC - major histocompatibility complex
mid CL – mid luteal phase CL
MMP- matrix metalloproteinase
mPR - membrane bound P4 receptor
mRNA – messenger ribonucleic acid
MRP - maternal recognition of pregnancy

NaCl - sodium chloride
$NaNO_2$ - sodium nitrite
NCBI - national center for biotechnology information
NF-κB - nuclear factor-kappa B
NO - nitric oxide
NO_2 – nitrite
NOS - NO synthase
Nur77 - nerve growth factor IB
OXT - oxytocin
OXTR – oxytocin receptor
p38/mapk- p38 mitogen-activated protein kinases
P4 - progesterone
P450scc - cytochrome 450 side-chain cleavage
PARP-1- poly ADP-ribose polymerase-1
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PDGF - platelet derived growth factor
PDGFR ß- platelet derived growth factor receptor beta
PG – prostaglandin
PGE2 – prostaglandin E2
PGES - prostaglandin E2 synthase
PGF2α - prostaglandin F2 alpha
PGFM – 13,14-dihydro-15-keto-PGF2alpha
PGFS - prostaglandin F2 alpha synthase
PKA - protein kinase A
PKC – protein kinase C
PLC -phospholipase C
PR - progesterone receptor
PRA - progesterone receptor A
PRB - progesterone receptor B
PRL - prolactin
R^2 - coefficient of determination
RIA – radioimmunoassay
RIP - receptor interacting protein
RIPA buffer - radio-immunoprecipitation assay buffer
ROS - reactive oxygen species
rRNA – ribosomal ribonucleic acid
RT – room temperature
RT-PCR - real-time-polymerase chain reaction
SDHA - succinate dehydrogenase A
SEM – standard error mean
SLC - small luteal cells
SLEC – stromal like endometrial cell
SMAD - sma and mad related family
SR-BI - scavenger receptor class B, type I
ß actin – beta actin
StAR - steroidogenic acute regulating protein

STAT1 - signal transducer and activator of transcription 1
Tie - tirosine kinase receptor
TNF - tumor necrosis factor alpha
TNFRI - tumor necrosis factor alpha receptor 1
TNFRII - tumor necrosis factor alpha receptor 2
TRADD – TNF receptor-associated death domain
TRAF1 - TNF receptor-associated factor 1
TRAF2 - TNF receptor-associated factor 2
Tris-HCl- tris(hydroxymethyl)aminomethane-hydrochloric acid
TSP1 – thrombospondin 1
V/v - volume fraction
VEGF - vascular endothelial growth factor A
VEGFB - vascular endothelial growth factor B
VEGFR1 - vascular endothelial growth factor receptor 1
VEGFR2 - vascular endothelial growth factor receptor 2
W/v - mass concentration

INTRODUCTION

The ovaries and the endometrium are intimately related during both ovarian cyclicity and early pregnancy. The presence of a viable conceptus determines the disruption of the luteolytic signal, in order to maintain progesterone production by the corpus luteum (CL), needed for an adequate uterine environment, a requisite for a successful pregnancy (Niswender *et al*., 2000). The precise crosstalk between CL and endometrium is crucial during the estrous cycle and at early pregnancy stages in the mare and other animal species. The detailed knowledge of the inter-communication between them is relevant for fertility improvement. In fact, a better understanding of endometrial mechanisms regulating both synthesis and secretion of prostaglandin F2α luteolytic signal and intra-luteal pathways triggered towards structural and functional luteolysis is needed.

Ovarian dysfunctions cause infertility in humans and other species. Despite the seriousness of this problem, the physiologic relevance of most studies on ovarian function in the woman is questionable, since they are based on knowledge generated from abnormal tissue or granulosa cells collected from *in vitro* fertilization women subjected to exogenous supraphysiological doses of gonadotrophins (Matsubara *et al*., 2000). Due to particular similarities between woman and mare (monovulatory species) on many aspects of ovarian function, mare CL is a valuable study model for understanding the regulatory pathways involved on the control of ovarian physiology (Ginther *et al*., 2004). Considering that the CL undergoes extremely rapid cellular and vascular changes only comparable with tumors (Reynolds *et al*., 2002), important factors during angiogenesis, cell migration and invasion, might also play a relevant role on luteal function. Coordination of those biological processes results from a complex cross talk between several factors. Due to their pluripotency, cytokines have recently deserved special attention on the regulating of reproductive function (Jabbour *et al*., 2009).

Horse industry in Europe has an estimated total economic effect of 100 billion Euros on GDP and creates 400.000 full time equivalent jobs mainly on rural areas. Considering the impact of horse production, a greater understanding of the mechanisms controlling ovarian and endometrial function will improve reproductive efficiency in horses, increasing economical sustainability, in addition to advancing scientific knowledge.

Therefore, the present research work aimed at the study of some of the mechanisms involved on the control of luteal vascular and non vascular development, as well as on the dichotomy between luteal structural (apoptosis) and functional regression (hormonal impairment) at

gene, molecular and cellular levels. The interactions between the immune system (mainly mediated by cytokines), angiogenesis, and secretory luteal function were addressed. Furthermore, some pathways involved on the control of endometrial prostaglandin secretion regulation by cytokines, steroid hormones and oxytocin were also studied.

CHAPTER I. LITERATURE REVIEW

1. THE OVULATION PROCESS

Before ovulation, estradiol is the primary steroid secreted by the ovary (Niswender *et al*., 2000). In ruminants its production is coordinated between follicular granulosa and thecal cells (Bao & Garverick, 1998; Fortune & Quirk, 1988), the so called "two cells theory" (Bao & Garverick, 1998). Thecal cells express the enzymes needed to convert cholesterol in androgens but lack the enzymes necessary for the convertion of androgens to estradiol. Granulosa cells possess the aromatase needed for estradiol synthesis (Bao & Garverick, 1998). Conversely, in the mare, and other species (pigs) (Conley *et al*., 1997), granulosa cells from estrous follicles have the active aromatase system (Jarrel, 1982), showing that estrogens can be produced by isolated granulosa cells. Nevertheless, equine combined theca and granulosa cells were more effective on *in vitro* estradiol-17β production than isolated granulosa cells (Mahajan & Samuels, 1974).

Ovulation process in the mare is not very deeply investigated, concerning biochemical and biophysical aspects. Ovulation is likely to occur due to controled focal weakening of the follicular wall caused by apoptosis rather than an increase in intrafollicular pressure (Murdoch, 1995). As reported by Carnevale *et al*. (1988), a tear can be visualized by ultrasonography in the follicle wall before ovulation, which is thought to be due to a breakdown of ovarian stroma. More recently, Riley *et al*. (2001) have shown the presence of proteases responsible for matrix reorganization, members of the matrix metalloprotease (MMP) family, the MMP-2 and MMP-9 in the follicular fluid, indicating that they are likely to be required for follicular growth and development. The increase in the concentration of MMP-9 in the larger follicles suggests that MMP-9 is involved in this ovulatory process. Collagenolytic enzymes are thought to be crutial for ovulation in other species like rat and macaque (Espey, 1994). In this process, prostaglandins should be mentioned as important mediators of the process (Reich *et al*., 1991). Interestingly, reports on mice showed that P_4 receptor regulates the expression of proteolytic enzymes determinant for follicule rupture (Robker *et al*., 2000).

Comparing with other species, in the mare, a distinctive feature of ovulatory process, in the mare, is the occurrence of ovulation in a specialized area, the ovulation fossa, where the restricted location of germinal epithelium and infundibulum can be found (Ginther, 1992).

Although several works reported that the preoovulatory follicle may reach the fossa by special mechanisms, there are no objective evidences that the preovulatory follicle reaches ovulation fossa besides the increasing in its size (Ginther, 1992). Localization and secretion of MMPs (MMP-2 and MMP-9) in cells of the ovarian stroma indiates a possible role in the migration of the follicle to the ovulation fossa (Riley *et al*., 2001).

After ovulation, the pyriform follicular cavity becomes luteinized. The process starts from the ovulation fossa and spreads to the remainder of the gland, resulting in a mushroom-shaped CL. All the described events around ovulatory process (ovulation time determined as the day 0) are intrinsically dependent on interrelationship between estradiol, progesterone (P_4), LH and FSH. As reported by Ginther *et al*. (2008a), estradiol from the dominant follicle increases in plasma that reaches a peak on day 2 before ovulation and then recedes. Its highest level is coincident with the lowest FSH level. Besides, it is by that time the preovulatory follicle reaches its biggest size and stops growing. The LH concentration in the ovulatory surge increases slowly and then more rapidly, with the transition between a slow and a rapid increase occurring at the peak of the estradiol surge (2 days before ovulation) (Ginther *et al*., 2006). The enhanced output of LH reaches its maximum on day 1. The prolonged ascending arm of the LH surge (10 days) with a peak occurring after ovulation contrasts with the short ascending arm (about 4 hours) with the peak before ovulation in cattle (Haughian *et al*., 2004).

The increase in LH, 2 days before the ovulation, is determinant for estradiol reduction and cessation of follicular growth (Ginther *et al*., 2008a). Another consequence of LH surge is luteinization of the follicular cells and alteration of the steroidogenic pathway towards P_4 secretion (Niswender *et al*., 2000). A slight but significant increase in circulating P_4 occurs with consistency among mares on the day of detection of ovulation or a collapsed follicle.

2 .THE CORPUS LUTEUM

It was near the end of 19th century that corpus luteum (CL) was initialy associated with pregnancy. Later on, it was made clear that the luteal product necessary for pregnancy success was a progestin (Allen & Wintersteiner, 1934).

The main targets of progesterone (P_4) are the reproductive tract, mammary gland and the hypothalamo-pituitary axis (Niswender *et al*., 2000). Generaly, on the reproductive tract, P_4 prepares the uterus for initiation and maintenance of pregnancy (further discussed on detail). In every ovarian cycle, soon after ovulation, a complex process of luteinization leads to CL formation. Thus the CL, simply represents a transient endocrine organ that mainly secretes P_4, a hormone required for for implantation (Allen & Wintersteiner, 1934).

Giving a brief picture of the whole process, luteal tissue formation is characterized by differentiation and proliferation of cells derived from the postovulatory follicle (Murphy, 2000), and changes in microvascularization (Ferreira-Dias *et al*., 2006a). Angiogenesis seems to be controlled by stimulating and inhibiting factors (Redmer *et al*., 1996) that might regulate luteal vascularization and function (Redmer *et al*., 1996; Ferreira-Dias *et al*., 2006a). Vascular endothelial growth factor (VEGF) is the main angiogenic factor on CL angiogenesis (Redmer *et al*., 1996). Progesterone itself and a number of growth/angiogenic factors and prostaglandins might also be involved in luteal survival (Fig. 1). Since CL growth and its endocrine function are closely dependent on the formation of new capillaries, a deficient production of P_4 due to luteal insufficiency might be related to a deficient vascularization, and results in early embryonic death. In the absence of pregnancy, luteolysis allows for the resumption of a new ovarian cycle, and a decrease in P_4 production is followed by structural regression of the CL. A large number of factors has been pointed out as responsible for luteolysis in several species, such as prostaglandin $F_{2\alpha}$, tumor necrosis factor α (TNF) (Friedman *et al*., 2000) and other cytokines like Fas Ligand, interferon gamma (IFNG) (Davis & Rueda, 2002), nitric oxide (NO) (Vega *et al*., 2000), and leukotrienes (Skarzynski *et al*., 2005; Fig. 2). In the mare, luteal angiogenesis and vascular regression might be regulated by many different factors and are coordinated with the development of non-vascular tissue (Ferreira-Dias *et al*., 2006a).

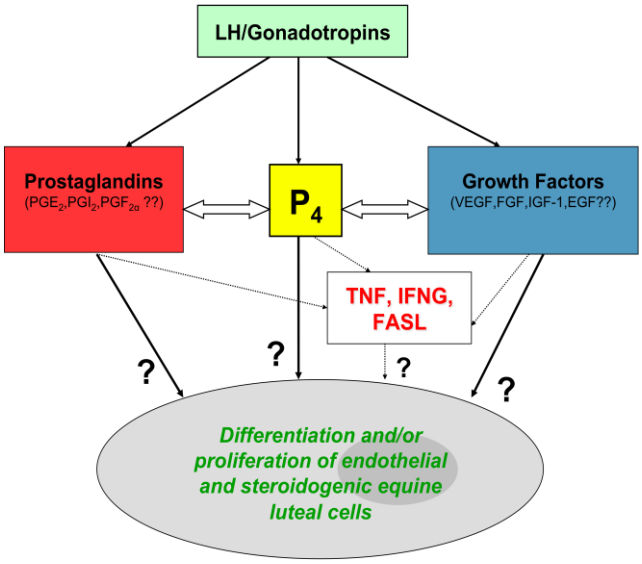


Figure 1: Schematic representation of the interactions in the CL during luteal establishment.

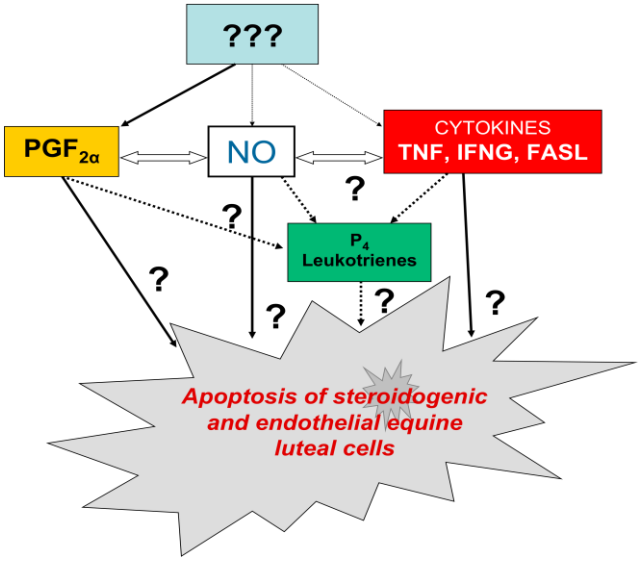


Figure 2: Schematic representation of the interactions in the CL during luteal regression.

2.1 Microscopic characteristics

Descriptive studies on microscopic characteristics of equine corpus luteum are scarce. Further investigations are needed, mainly concerning luteinization of theca versus granulosa cells. According to van Niekerk *et al.* (1975) the theca interna from the follicle does not contribute to the luteal tissue, as in other species. The theca cells are supposed to degenerate and are replaced by hypertrophied fibroblasts. More recently these findings were confirmed

(Watson & Sertich, 1990). As described by Ginther (1992), the chronologic events leading to CL formation are as follows: (i) few days before ovulation, fibroblastic cells of the theca interna proliferate and enlarge into round cells and the granulosa cells stop dividing (also motivated by estradiol drop); and (2) just before ovulation, theca cells are in different stages of degeneration (showing cytoplasm condensation, eosinophilia, pycnosis, fragmentation and phagocytosis) and granulosa cells change from a compact mass to a spindle-shaped cells. After ovulation, granulosa cells are about 10 μm diameter. The collapsed inner wall of the follicle consists of stroma cells and distended blood vessels, while 24 h after the ovulation, granulosa cells are 15 μm diameter and possess vesiculated nuclei and cytoplasm with vacuoles, indicating luteinization and secretory activity. Also, by this time, P_4 level in peripheric circulation is increasing. Folds of stromal tissue start to grow into the granulosa of luteinizing tissue followed by proliferating capillaries, which are surrounded by the hypertrophied fibroblasts that apparently replace the degenerating theca cells. These fibroblasts differ from large luetal cells since they are more vesicular, have no prominent nucleoli and have small spindle shaped cytoplasm. Conversely, large luteal cells are polyhedral, with vacuolated cytoplasm and single eccentric nucleus, containing one or more nucleoli. On day 3 after ovulation, luteinization of granulosa cells is complete. Around day 5-6, 46% of luteal cells (small and large) are large luteal cells (Watson & Sertich, 1990); and on day 9 maximum hypertrophy of granulosa cells (mean diameter around 40 μm) was observed. At this time, small luteal cells account for about 15% of the total cells (Watson & Sertich, 1990). These small cells (diameter 11μm) have homogeneous eosinophilia and dense, dark staining, often elongated nucleus. It has been stated that these cells represent a predecessor stage of large luteal cells (Niekerk *et al*., 1975). By this stage the number of hypertrophied fibroblasts is reduced, suspecting that they probably contribute for the stroma of the CL. On day 12, large luteal cells begin to decrease in diameter. The proportion of small luteal cells increase to 25%, being no longer converted in large luteal cells, what indicates that the percentage of these cells increase in aged corpora lutea (Watson & Sertich, 1990). This feature contrasts with other species, where the proportion of small to large luteal cells decreases as the cycle advances (Fitz *et al*., 1981; Niswender *et al*., 1985). Also different fate is seen for theca and granulosa follicular cells. Specifically, theca and granulosa-derived luteal cells originate two distinct luteal cells that differ morphologically and physiologically. Granulosa follicular cells originate the large luteal cells and theca cells the small luteal cells (Niswender & Nett, 1994). As established for other species, in addition to steroidogenic cells, CL contains endothelial cells, fibroblasts, pericytes and cells originating from the bloodstream

(Channing, 1966). Among the few studies performed on equine CL, different cell types besides the steroidogenic small and large luteal cells, like fibroblastic, immune system or endothelial cells have been reported (Al-zi'abi *et al*., 2002; Al-zi'abi *et al*., 2003; Kelly *et al*., 1988; Roberto da Costa *et al*., 2005).

2.2 Steroidogenesis in luteal cells

In the ovary, a dramatic change in steroidogenic role is seen during different stages of the estrous cycle. It is generally accepted that follicles are associated with estrogen production and CL with progestins. During the estrous cycle, estradiol-17β (E_2) is the major ovarian estrogen in the mare, and P_4 is the main progestin (Ginther, 1992). Steroid hormones differ in the nature of attached groups and in the location of double bounds. The critical substrate for steroidogenesis is cholesterol, whose main source is the liver (Krisans, 1996), being transported in the plasma in the form of lipoproteins (low density lipoprotein – LDL and high density lipoprotein - HDL) to steroidogenic organs, like the CL (besides follicle, testis and adrenal cortex), (Ohashi *et al*., 1982). Under conditions of lipid deprivation (like in almost all *in vitro* conditions) luteal cells are able to synthesize cholesterol from acetate (Cook *et al*., 1967). The uptake of LDL by the cell is done by receptor mediated endocitosis (Brown & Goldstein, 1986). Once internalized, endosomes are combined with lysosomes where cholesterol is released, being available to the cell (Grummer & Carroll, 1988). The HDL is classically uptaken by membrane-bound HDL binding protein (Lestavel & Fruchart, 1994). More recently, the scavenger receptor class B, type I (SR-BI) has been described as another receptor responsible to mediate the process (Connelly & Williams, 2003). Once free in the cell, cholesterol can be used for steroidogenesis, for cell membranes formation or can be esterified with fatty acids, to form cholesterol esters (Johnson *et al*., 1997).

For steroidogenesis to be accomplished, cholesterol should be transported to the mitochondria and then from the outer to the inner mitochondrial membrane, where cholesterol side-chain cleavage enzyme complex transforms cholesterol in pregnenolone (Stone & Hechter, 1954). This process requires initially intact cytoskeleton (Crivello & Jefcoate, 1978). Moreover, sterol binding proteins also appear to be crucial for the transport to the mitochondria (Scallen *et al*., 1985). But the rate-limiting step in steroidogenic process seems to be the transport from outer to inner mitochondrial membrane (Stevens & Lambent, 1993), performed by the steroidogenic acute regulating protein (StAR) (Clark *et al*., 1994). This step appears to be the primary negative/positive site of acute regulation of steroidogenesis by second messenger

systems (Simpson & Waterman, 1983). However, it has been subsequently reported that peripheral-type benzodiazepine receptor, which is present in the mitochondrial membrane of steroidogenic cells, is able to regulate the transport of cholesterol and influence steroidogenesis (Papadopoulos & Brown, 1995). Thus StAR, benzodiazepine receptors and the endogenous ligand of this receptor, all appear to be determinant for the transport of cholesterol from the outer to the inner mitochondrial membrane, the site of cholesterol side-chain cleavage.

Once in mitochondrial matrix, cytochrome P-450 complex ($P\text{-}450_{scc}$), adrenodoxin and adrenodoxin reductase cleave the side chain of cholesterol to form pregnenolone (Stone & Hechter, 1954). Pregnenolone is then transported to smooth endoplasmatic rethiculum, where 3□-hydroxysteroid dehydrogenase/ 5,□4 isomerase (3β-HSD) converts pregnenolone to progesterone (reviewed in Hanukoglu, 1992). Progesterone is then thought to diffuse from the cell.

The preovulatory LH surge results in luteinization of granulosa follicular cells (Ginther *et al*., 2008a), altering the steroidogenic pathway so that P_4 becomes the primary steroid hormone produced. After ovulation, the granulosa cell is fully luteinized and the conversion of P_4 to 17α-hydroxyprogesterone is retarded and P_4 is accumulated with some conversion to 20α-hydroxyprogesterone (Ginther, 1992) (Fig. 3). Nevertheless, the CL of several species, including humans, pigs, and rats, retains the ability to produce some estradiol (Richards & Hiden, 1988). The same ability was demonstrated for equine CL (al-Timimi *et al*., 1989). Further studies evidenced that the equine CL was capable of aromatization (Amri *et al*., 1993), through another enzyme involved in estradiol biosynthetic pathway, the cytochrome P450 17 (CYP17), which was considered not being expressed in the equine CL of nonpregnant mare by other authors (Rodger *et al*., 1998). In another work conducted by Albrecht and Daels (1997), the presence of CYP17 in small luteal cells was clearly shown, providing direct evidences of the contribution of these cells on steroidogenesis. Another group of the same workers showed also that both small and large luteal cells stained identically for 3β-HSD (Albrecht & Daels, 1997). Thus, existent evidences demonstrate that both small and large equine luteal cells are potentially capable to carry out steroidogenesis. Finally, steroid producing tissues possess the intermediates for synthesis of all steroid molecules depending on the availability of certain enzymes (Ghinter, 1992).

A simplified version of the main routes of biosynthesis of steroid hormones is depicted (Fig. 3) for a better understanding of the consider pathways (based on the review - Niswender, 1974).

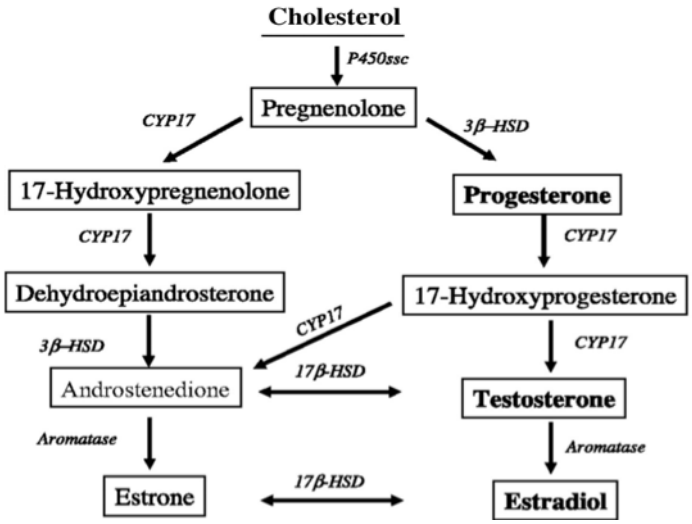


Figure 3: Overview of the sex steroid biosynthetic pathway: P450ssc, cytochrome P450 complex or the side-chain cleaving enzyme; CYP17, cytochrome P450 17; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase (adapted from Niswender *et al.*, 1974).

2.3 Luteal establishment

Complexity of CL establishment as an endocrine organ can be seen as a bidimentional paradigm. On one hand, a profound tissular reorganization and growth dinamized by cellular proliferation should be accomplished in few days (Jablonka-Shariff *el al.*, 1993). On the other hand, P_4 secretion and its circulating levels should ensure implantation for pregnancy progression (Graham & Clarke, 1997). The differentiation and proliferation of cells derived from the postovulatory follicle (Murphy, 2000) is followed by changes in microvascularization (Ferreira-Dias *et al.*, 2006a). Naturally, it would be impossible to mention here all the factors until the moment described as participant in luteal establishment. Nevertheless, a wide picture of the process is presented here.

First and foremost, molecular regulation of CL formation is strongly dependent on pituitary gland (Hutchison & Zeleznik, 1984). Studies in ewes, where pituitary was removed, evidenced a drop in steroidogenic enzymes StAR, 3β-HSD and P450ssc (Juengel *et al.*,

1995a). Both LH and GH were shown to be determinant for CL establishment and maintenance not only in ruminants (Niswender & Nett, 1994), but also in the mare (Ginther, 1992; Watson *et al*., 2000a). Neutralization of LH with antibodies attested the dependency of equine CL on pituitary hormones (Pineda *et al*., 1972). The luteotrophic role of LH was also evident when administration of GnRH to diestrus mares caused an increase in P_4 production (Johnson *et al.*, 1988). Besides, *in vivo* and *in vitro* studies confirmed that luteal cells increase the secretion of P_4 when exposed to LH or hCG (Kelly *et al*., 1988). Indeed, as reported by Ghinter (1992), in mid diestrus LH concentration is low, but the occurrence of ocasional LH pulses should keep LH in levels required for CL maintenance in the mare. Nevertheless, controversial results were found in other studies with tissue culture systems, where LH did not stimulate P_4 secretion (Watson & Sertich, 1990). Several works in other species reported that expression of key factors is transiently induced after LH action over the CL. Examples are genes of P_4 receptor (Natraj & Richards, 1993), cyclooxygenase-2 (COX-2) (Lim *et al*., 1999), early growth response protein-1 (Egr-1) (Espey *et al*., 2000) and nerve growth factor IB (Nur77) (Park *et al*., 2001). Both Egr-1 and Nur77 are transcription factors responsible for activation of signaling cellular pathways. The Egr-1 activates protein kinase A (PKA) and MAPK pathway and Egr-1 knockout mice lack ovulation and CL formation (Russell *et al*., 2003). Concerning Nurr77, it was shown to regulate transcriptional activity of steroidogenic cells (Chang & Chung, 1995) and it has been associated with apoptosis in nonovarian cells (Liu *et al*., 1994). But, studies in Nurr77 knockout mice did not show aberrant reproductive phenotype, suggesting alternative pathways may compensate the absence of Nur77 (Crawford, 1995). Besides transient pathways activated by LH, permanent activated pathways were clearly reviewed by Stocco and colleagues (2007). Figure 4 summarizes the LH mediated signaling mechanisms occurring in granulosa cells during luteinization. Activation of LH-R leads to an increase in the intracellular messenger for signaling transduction cAMP and subsequent PKA activation, whose catalytic subunit phosphorylates the transcription of several nuclear factors (reviewed in Stocco *et al*., 2007). The cAMP response element-binding (CREB), known as a transcription factor, is one of the phosphorylated proteins involved in the activation of gonadotrophin regulated ovarian genes (Mukherjee *et al*., 1996). The mitogen-activated protein kinase (MAPK) has been also associated to activation of CREB (Hazzalin & Mahadevan, 2002). MAPKs comprise a superfamily of kinases described in CL of species like cow, rat or human (Chen *et al*., 2001; Maizels *et al*., 2001). As shown by Hunzicker-Dunn and colleagues (Maizels *et al*., 2001), LH-R phosphorylates another member of this family, the p38/MAPK and its downstream protein activated is MAPK-activated

protein kinase 3 (MAPKAPK3). Finally, MAPKAPK3 seems to be determinant for CREB phosphorylation during luteinization (Maizels *et al*., 2001). It is speculated also that growth factors like VEGF and IGF-I as well as LH may participate on MAPK activation, but this needs further investigation (Stocco *et al*., 2007).

LH surge not only increases cAMP, but also activates enzymes involved in steroidogenesis, like phospholipase C (PLC) (Gudermann *et al*., 1992; Lee *et al*., 2002) or protein kinase C (PKC) (Morris & Richards, 1995).

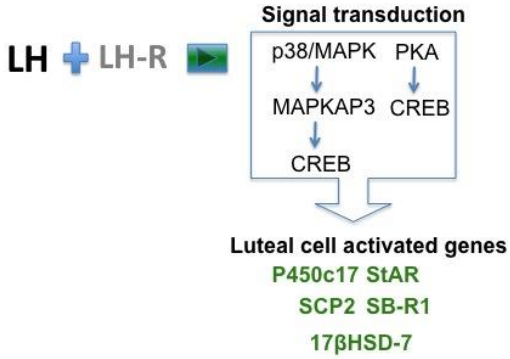


Figure 4: LH signaling mechanisms and target genes activated in luteal function.

Some studies have started to focus on the importance of paracrine factors for luteal establishment (Fig.1). Supportive role of catecholamines (epinephrine and norepinephrine) on luteal establishment is well documented in the cow (Skarzynski & Okuda, 2000). Nevertheless, the same conclusions were not obvious for the mare (Condon *et al*., 1979). Cytokines and growth factors are present as well in the CL during its development. Specifically, TNF and TNFR are present in early stages of CL from pig (Wuttke *et al*., 1997), cow (Sakumoto *et al*., 2000) and human (Vaskivuo *et al*., 2002). In the cow, TNF is a potent stimulator of prostaglandins (PG)s (PGE_2 and PGI_2), known to stimulate P_4 secretion (Benyo & Pate, 1992; Sakumoto *et al*., 2000), evidencing a luteotrophic role. Also in the mare was reported an increased secretion of PGE_2 from early CL by Watson and Sertich (1990), but a luteotrophic role for PGE_2 in equine CL was not described until the moment. Likewise, PGE_2 secretion was increased in mare early CL by NO and TNF (Ferreira-Dias *et al*., 2011), being suggested that TNF mediates the interaction between NO and PGE_2 in its hypothetical luteoprotective role during CL establishment in the mare. But NO role in the CL is unclear. Several reports have demonstrated that NO expression increases during luteolysis in humans (Friden *et al*., 2000), rats (Motta *et al*., 1999), cows (Jaroszewski & Hansel, 2000; Skarzynski

et al., 2003b) and mare (Ferreira-Dias *et al*., 2011). In spite of that, it was described that NO stimulates P_4 secretion by rat ovarian cells (Dong *et al*., 2000) and by early CL equine luteal tissue (Ferreira-Dias *et al*., 2011). Data concerning NO action in the CL suggest that it may act in a dualistic manner, depending on the dose and phase of the cycle (Keator *et al*., 2008).

Not only PGE_2, but also P_4 was increased by NO and TNF in early equine CL (Ferreira-Dias *et al*., 2011). The same group of authors have shown, in a previous work, the increase in P_4 receptor (PR) staining intensity from early to mid CL in the mare (Roberto da Costa *et al*., 2005), indicating an increase in PR expression and emphasizing P_4 action on luteal establishment. In bovine CL auto-, paracrine luteotrophic role of P_4 was objectively shown when treatment of luteal cells with P_4 increased mRNA and protein expression for StAR, cytochrome P450scc and 3β-HSD (Rekawiecki *et al*., Kotwica, 2005). Moreover, P_4 appears to be a required auto-, paracrine factor for maintenance of full endocrine function, including P_4 secretion and prevention of apoptosis (Skarzynsky & Okuda, 1999).

The intra-ovarian interaction of factors like insulin growth factor, leukotrienes or neurotransmitters also deserves special attention. The insulin growth factor (IGF) I and II are polypeptide growth factors involved in cellular growth and development (Armstrong & Webb, 1997). Both IGF-I and IGF-II were shown to regulate ovarian function in ewe (Webb *et al*., 2002) and IGF-I increased P_4 secretion in bovine CL (McArdle & Holtorf, 1989). Hence, regulation of luteal function by IGF proteins may not be restricted to secretory function, but may include control of vascular growth and function and regulation of vascular and steroidogenic cell interaction (Webb *et al*., 2002). Considering IGF-I regulation of luteal steroidogenesis, a speculative mediatory role has been ascertained to growth hormone (GH), being proposed that GH may influence luteal function directly or indirectly via IGF-I in humans (Taketani *et al*., 2008). Preliminary studies in the mare demonstrated the stimulatory role of IGF-I and GH on angiogenic factors production and NO secretion (Tramontano *et al*., 2010), showing that these factors (IGF-I and GH) might regulate luteal vascular activity in equine CL. Another group of factors shown to be involved on luteal growth are the pro-inflammatory lipid mediators leukotrienes. Studies in bovine ovary described the local production and regulation of PGs and P_4 secretion by CL *in vitro* (Korzekwa *et al*., 2010a) and *in vivo* (Korzekwa *et al*., 2010b). Besides leukotrienes, acetylcholine (ACH) and its biosynthetic enzyme choline acetyltransferase (CHAT) were shown to be expressed in bovine CL (Al-Zi'abi *et al.*, 2009). Moreover, ACH stimulated P_4 secretion by bovine luteal cells and

prevented apoptosis of these cells, being postulated that ACH acts as a signal molecule, controlling basic cellular processes, such as cell differentiation (Al-Zi'abi *et al*., 2009).

Finally, the importance of the ovarian steroids estrogens on luteal establishment should be mentioned. In several species, such as rats, pigs or rabbits, estrogens are luteotrophic (Niswender *et al*., 2000). As an example, in rabbit E_2 plays a key role on luteal growth and LH mainly stimulates E_2 synthesis in follicles (reviewed in Holt, 1989). Similarly, in rat E_2 is primarily involved in P_4 secretion, and LH and prolactin (PRL) play secondary roles, where PRL maintains E_2 and LH-R while LH stimulate E_2 secretion from the CL (reviewed in Gibori *et al*., 1988).

As stated before, luteal growth and establishment is followed by the development of blood vessels, entirely necessary for normal transport of nutrients, oxygen, hormones and other factors. Due to the dimension of the angiogenic process in the CL, a chapter will be further dedicated to elucidate the regulation of this process during luteal establishment and regression.

2.4 Luteal regression

The importance of the uterus for luteolysis was initially established by Loeb (1923), after studies in guinea pig where histerectomised animals stopped having ovarian cyclicity and started showing abnormal luteal persistence. Subsequently, the same was observed for cyclic sheep, cow, pig, mare, rabbit and rat (Anderson *et al*., 1969). In the mare, it became clear that cyclic luteolysis was triggered by endometrial pulses of $PGF_{2\alpha}$ during approximately days 14-16 after ovulation, originating a drop in P_4 in three or four hours (Douglas & Ginther, 1976; Kindahl *et al*., 1982).

The sheep has long been used as a model to study luteolysis. Several studies on transplantation of uterus and ovary carried out in the sheep allowed for the conclusion that both organs had to be linked for the occurrence of cyclic regression (Anderson *et al*., 1969). By the same time, Pharris and Wyndgarden (1969) discovered that $PGF_{2\alpha}$ shortened pseudopregnancy in rats and diminished luteal content in P_4. Later, the contra current mechanism to transfer $PGF_{2\alpha}$ from the uterine vein to the ovarian artery was demonstrated in the sheep (McCracken *et al*., 1972; McCracken *et al*., 1971). Studies in the mare confirmed that, at the end of the luteal phase, $PGF_{2\alpha}$ determined a decrease in plasma P_4, defined as functional luteolysis (Douglas & Ginther, 1972; Henderson & McNatty, 1975). But in

contrast with ruminants, no difference between intrauterine and intramuscular routes was found in the mare (Douglas & Ginther, 1975). Anatomical variations between mare and cow or sheep account for the absence of an uteroovarian pathway, since the ovarian artery does not have a major contact with the uteroovarian vein (Ginther, 1976). Naturally, sensitivity of the CL to luteolytic $PGF_{2\alpha}$ should be also very different, since the systemic route implies that 90% of PGs are metabolized in lungs (Ferreira & Vane, 1967). As reported by Ginther (1992), sensitivity of equine CL is 18 times higher than sheep and affinity of equine CL for $PGF_{2\alpha}$ is 10 times greater than cow corpora lutea (Kimball & Wyngarden, 1977). Another characteristic of the mare is the responsiveness of the CL to the luteolytic eicosanoid, since a rapid drop in plasma P_4 is seen after a single $PGF_{2\alpha}$ administration in mid luteal phase (Johnson *et al*., 1988; Noden *et al*., 1978).

The cellular mechanisms of luteolysis diverge among species. The way $PGF_{2\alpha}$ exerts luteolysis in the mare is not obvious, but it is assumed that the main steps include a reduced intracellular (luteal cell) cholesterol transport (functional luteolysis), vasoconstriction and stimulation of an influx of leukocytes responsible for cytokines secretion, capable of triggering luteal cell apoptosis and luteal matrix remodeling (structural luteolysis) (Stout, 2011). Action of PGF2α is transduced via the phospholipase C (PLC) and protein kinase C (PKC) pathway (Pate & Keyes, 2001).

2.4.1 Triggering luteolysis

In ruminants, one of the first responses of the CL to uterine $PGF_{2\alpha}$ is the secretion of luteal oxytocin (OXT) (Jones & Flint, 1989; Theodosis *et al*., 1986), an episode not seen in the mare (Stevenson *et al*., 1991). Another consequence, is the increased secretion of intra luteal $PGF_{2\alpha}$ seen *in vitro* for the cow as loop to amplify the luteolytic signal (Tsai & Wiltbank, 1997). In this regard, production of $PGF_{2\alpha}$ by luteal tissue has been reported also in sheep (Rexroad & Guthrie, 1979), pigs (Guthrie *et al*., 1978) and horses (Watson & Sertich, 1990). Even though the $PGF_{2\alpha}$ output by the equine CL was not quantified, a three fold increase in the mRNA expression of the rate limiting enzyme involved in PGs synthetic pathway, the cyclooxygenase (COX) 2, after exogenous treatment with $PGF_{2\alpha}$ was reported (Beg *et al*., 2005). The present findings support for the mare the hypothesis of an intraluteal autocrine loop to augment the luteolytic signal (Tsai & Wiltbank, 1997), as well. Moreover, the same author reported as additional consequences of $PGF_{2\alpha}$ treatment the reduction in mRNA

expression of both StAR and LH receptors (LH-R) (Beg *et al*., 2005). In the mare, the equivalent decrease in LH-R together with luteal and plasmatic P_4 was reported in natural (Roser & Evans, 1983) and $PGF_{2\alpha}$ induced (Roser *et al*., 1982) luteolysis.

It was evidenced that the luteolytic action of $PGF_{2\alpha}$ is mediated by a specific plasma membrane receptor (Powell *et al*., 1975), $PGF_{2\alpha}$ receptor (FPr). As a consequence of uterine $PGF_{2\alpha}$ action on the CL, FPr expression can be up regulated, as shown in rat (Olofsson *et al*., 1996), cow (Rao *et al*., 1979) or pig (Gadsby *et al*., 1990) during late luteal phase. In horses, besides being reported in follicular cells before and after ovulation (Sayasith *et al*., 2006), its mRNA transcription has been shown in the CL (Beg *et al*., 2005). However, in equine CL, $PGF_{2\alpha}$ treatment did not increase mRNA transcription of its receptor as shown in other species. But, luteolysis onset appears not to be exclusively dependent on the presence of FPr, since pigs CL was shown to be resistant to a single dose of $PGF_{2\alpha}$ until day 13 after ovulation (Guthrie & Polge, 1976), in spite of expressing FPrs since day 4 (Gadsby *et al*., 1990). Also in cows, $PGF_{2\alpha}$ was not effective before day 5 of luteal phase (Henricks *et al*., 1974). However, in this species $PGF_{2\alpha}$ receptors are already expressed on day 2 (Wiltbank *et al*., 1995). An additional mechanism must be needed. Moreover, differential affinity of receptors to $PGF_{2\alpha}$ throughout the estrous cycle may contribute for the refractoriness seen in the early stages of the CL. As demonstrated for ruminants, discordant sensitivity of the CL to $PGF_{2\alpha}$ could be due to the existence of high- and low-affinity states of the FPr (McCracken *et al*., 1999). Thus, refractoriness to $PGF_{2\alpha}$ evidenced by different species in the early stage of the luteal phase is another intriguing issue under the scope of luteolytic molecular pathways. Specifically in the mare, studies in the last decades tend to contradict previous reports where the absence of luteolytic effect of $PGF_{2\alpha}$ was seen in early luteal phase, until day 5 after ovulation (Douglas & Ginther, 1972; Paccamonti *et al*., 1991). More recently, early administration of $PGF_{2\alpha}$ on day 2 after ovulation did impair luteal function and shorten interovulatory interval (Nie *et al*., 2003; Troedsson *et al*., 2001). Nevertheless, because of the diversity of luteolytic agents and doses used, relationship between dosage and efficacy of luteolytic treatments among different reports is difficult to compare studies, since determination of dosage per kg of body mass is impossible because body weights were not reported in all the studies (Handler *et al*., 2004). Finally, the luteoprotective role of P_4 can be also a reason for the absence of luteolytic capacity of $PGF_{2\alpha}$. The luteoprotective role of P_4 was evidenced and is well established, preventing structural luteolysis and protecting against apoptosis (Ferreira-Dias *et al*., 2007; Liszewska *et al*., 2005).

Despite of diversity on molecular pathways involving the action of the luteolytic agent, among species, the most recent findings in bovine CL surprisingly demonstrate that $PGF_{2\alpha}$ effect is dependent on the stage of luteal phase (Shirasuna *et al*., 2010). It starts being evident that $PGF_{2\alpha}$ may have a dual function, acting as antiluteolytic, since it was able to stimulate angiogenic factors in early CL, or exerting the known luteolytic function from mid CL on (Miyamoto *et al*., 2010; Shirasuna *et al*., 2010).

Also with interest for functional luteolysis is the role of E_2 on the CL. As mentioned before, the CL of several species produces E_2. Although its role on luteolysis has not been well elucidated, pigs *in vivo* treatments with $PGF_{2\alpha}$ increased luteal E_2 production, in association with an increase in aromatase mRNA transcription (Diaz & Wiltbank, 2004). The finding of elevated E_2 production and increased ERß expression in regressing CL suggests its role on luteolysis in pigs (Diaz & Wiltbank, 2004). Moreover, in primates ERß protein expression changes throughout the luteal phase increasing during the time of luteolysis (Duffy *et al*., 2000). Nevertheless, opposite roles have been ascribed to E_2 on luteal function. As examples we have the critical role of E_2 on P_4 production and luteal maintenance in rabbits (Bill & Keyes, 1983) and the greater CL development in pigs with E_2 implants (Conley & Ford, 1989). What also seems controversial is that in pigs, the E_2 is produced by the conceptus is the signal for maternal recognition of pregnancy in this species (Bazer & Thatcher, 1977). In the study carried out by Beg *et al*. (2005) in the mare, $PGF_{2\alpha}$ reduced almost significantly aromatase mRNA transcription, showing a similar behavior to the mouse (Stocco, 2004), but further studies are indicated to objectively understand E_2 participation on luteolysis.

2.4.2 $PGF2_{\alpha}$ does not act alone

Up to now this discussion has focused on luteolysis triggering. However, besides its very complex regulation, knowledge in different species has shown that $PGF_{2\alpha}$ induces functional luteolysis by acting on multiple sites of the steroidogenic pathway. The antisteroidogenic actions are evidenced in the disruption of cholesterol transport to the mitochondria, as sterol carrier protein-2 (involved in transporting cholesterol to mitochondria) (McLean *et al*., 1995) and StAR protein (Juengel *et al*., 1995b), or on side chain cleavage enzyme (P-450scc) in the inner mitochondrial membrane (Watson *et al*., 2005).

Luteolysis does not consist exclusively of the demise in P_4 secretion by luteal cells. Briefly, it comprises also vascular changes (Miyamoto *et al*., 2005), infiltration of leucocytes (Gaytan *et*

al., 1998), and death of steroidogenic and endothelial cells by apoptotic (Juengel *et al*., 1993; McCormack *et al*., 1998) and non apoptotic mechanisms (Fraser *et al*., 1999; Gaytan *et al*., 2002). In general, the dichotomy of luteal loss of function and/or involution is species specific (Davis & Rueda, 2002). As an unusual endocrine gland, the CL possesses a transient nature. After a rapid growth and function for 10 to 14 days in cyclic animal, the CL involutes to form the corpus albicans, rich in collagen and composed by connective tissue. The referred involution is named by structural involution. In most species, both functional and structural involution are not clearly documented, being difficult to entirely separate the events.

A wide range of agents have been documented to participate on structural regression. Here, immune system and cytokines loom large, driving events like cell death and apoptosis (Davis & Rueda, 2002), tissue reorganization and extracellular matrix remodeling (McCracken *et al*., 1999). Many reports stated the importance of macrophages and other immune cells, through the release of TNF and other cytokines, in CL regression, after the triggering action of $PGF_{2\alpha}$ (Benyo & Pate, 1992). Also phagocytosis of luteal cells and cell remnants has been ascribed to macrophages (Paavola, 1979). In early eighties, several studies confirmed the presence of lymphocytes and macrophages in the CL throughout the luteal phase (Hume *et al*., 1984; Kirsch *et al*., 1981). Also in the mare, it was reported an increase in immune cells at luteolysis time (Lawler *et al*., 1999). Recruitment of macrophages into the CL is probably regulated by the expression of monocyte chemoattractant protein 1 (MCP-1; Bowen *et al*., 1999; Townson *et al*., 1996), being MCP-1 expression increased at the time of luteolysis (Bowen *et al*., 1999). As macrophages and lymphocytes become activated in the CL, they secrete cytokines (Pate & Keyes, 2001). Cytokines may play either a positive or a negative role on endocrine regulation of the CL. In pigs, the modulatory signal of TNF on $PGF_{2\alpha}$ effects, swapping from luteoprotective to luteolytic action was described (Wuttke *et al*., 1997). Moreover, cytokines may mediate $PGF_{2\alpha}$ actions during luteolysis, as the increase in phospholipase A2, formation of reactive oxygen species (ROS) and apoptosis triggering (Wu & Carlson, 1990). The action of cytokines may be considered in two perspectives: a specific role on failure of steroidogenesis and an active participation on cell death. Considering the particular example of TNF, its anti steroidogenic action was evidenced by a loss of StAR and LH-R mRNA (Chen *et al*., 1999). Progesterone production by luteal cells is also inhibited by interleukin 1 (IL-1) and IFNG (Fairchild & Pate, 1991; Nothnick & Pate, 1990). Regarding cell death, TNF promotes apoptosis in different cell types, including luteal cells (Petroff *et al*., 1999). Several examples showed the synergic action of TNF and IFNG on cytotoxicity of luteal cells (Benyo & Pate, 1992). Participation of ROS, mainly produced by nonsteroidogenic cells (Aten *et al*.,

1998), usually concerns the mediation of downstream events in the CL that lead to damaged luteal cells and demise of the luteal endocrine organ (Kato *et al*., 1997). The harmful role of ROS can be suppressed by P_4, possibly protecting luteal cells from cellular damage before the decline of steroidogenic capacity (Sugino *et al*., 1996). Finally, NO, oxidative species, (Benyo & Pate, 1992) has also been implicated in cell death and luteolysis (Korzekwa *et al*., 2006; Olson *et al*., 1996). Inhibition of NO synthase (NOS) avoided the decrease in P_4 and prolonged luteal phase in cow (Jaroszewski & Hansel, 2000). In the mare, it was also demonstrated the increased expression of endothelial NOS in CL during late LP (Ferreira-Dias *et al*., 2011).

Apoptosis as been reported in the CL of several species like mouse (Hasumoto *et al*., 1997), woman (Shikone *et al*., 1996), cow (Zheng *et al*., 1994) or pig (Bacci *et al*., 1996). Apoptosis is a physiological form of death, distinguishable from necrosis by different morphological and biochemical parameters (Davis & Rueda, 2002). It is regulated by organic proteins that can originate an intrinsic and extrinsic signaling cascade (Kliem *et al*., 2009). The intrinsic pathway is thought to be regulated by apoptotic stimuli caused within a cell, in response to drugs, radiation or growth factors withdrawal. Firstly, changes in mitochondrial permeability are mediated, through an unbalance on ratio pro-apoptotic to anti-apoptotic Bcl-2 family members (Adams & Cory, 1998). The extrinsic apoptotic pathway, in turn, is activated by extracellular factors, as TNF and FASL after acting on their receptors TNFRI and FAS, known as death receptors (Nagata, 1997). Both pathways activate a cascade of intracellular proteases, the caspases. In the mare, apoptosis has been related to luteolysis, after natural or induced regression with PGF2α (Al-zi'abi *et al*., 2002). Analyzing more deeply the apoptotic event, after being activated, apoptosis is controlled by the expression of a series of regulatory genes, such as Bcl-2 family members (Tilly, 1996). Proteins of Bcl-2 family are caspase activators, resulting on the mitochondrial release of apoptogenic factors (cytochrome c, etc.) (Davis & Rueda, 2002; Gross *et al*., 1999). The mechanism of apoptosis is known to occur as a cascade of sequential activation of initiator and effector caspases (Salvesen & Renatus, 2002). During luteal regression, besides apoptotic DNA degradation, occurs also the cleavage of putative caspase-3 substrate poly(ADP)ribose polymerase (PARP) (Davis & Rueda, 2002). Among other proteins, BAX - another member of Bcl-2 family - is also increased during CL regression (Sugino *et al*., 2000b), and can ultimately lead to disruption of steroidogenesis and cell death (Davis & Rueda, 2002). As shown by Ferreira-Dias *et al*. (2007), expression of the apoptosis effector enzyme caspase-3 was already increased in mare mid CL, when P_4 was still increased in the plasma. The same was seen in human and monkey (Peluffo *et al*., 2006;

Vaskivuo *et al*., 2002), raising the question about P_4 role on apoptosis regulation. In the mare, besides the evidences of its role on apoptosis and structural regression (Aguilar *et al*., 2006; Ferreira-Dias *et al*., 2007), caspase-3 is not able to regulate steroidogenesis (Carambula *et al*., 2002), thus its relationship with P_4 remains ambiguous.

Apoptosis in CL can also be initiated by FASL/FAS system (Benyo & Pate, 1992). Both ligand and receptor are expressed in CL of different species like cow (Taniguchi *et al*., 2002), rat (Roughton *et al*., 1999) or human (Kondo *et al*., 1996). Moreover the complex interaction between cytokines during luteolysis is worth mentioning. As demonstrated by Quirk *et al*. (2000), the association TNF+IFNG was able to upregulate FAS mRNA in murine luteal cells. These cytokines can also increase the expression of the major histocompatibility complex (MHC) (Benyo & Pate, 1992). With a critical role in the recognition of self and non self structures, MHC molecules present small peptides of exogenous or endogenous origin to the T lymphocytes, which results in cytokine release (Pate & Keyes, 2001). Generally MHC is expressed by specialized cells, as macrophages or B lymphocytes, known as antigen-presenting cells (Austyn, 2000). Nonetheless, somatic cells can also express class II MHC, a usual feature nearly in all autoimmune diseases (Bellgrau & Eisenbarth, 1999). Both class I and II of MHC molecules may present self-peptides. As reported in several species, luteal cells also express both class I and class II MHC molecules (Fairchild & Pate, 1989; Kenny *et al*., 1991). However, Lawler *et al.* (1999) failed to demonstrate the expression of class II MHC in steroidogenic equine luteal cells. Expression of class I MHC molecules was shown to be stable throughout the luteal phase. However, class II MHC expression increases from early to late CL and after treatment with $PGF_{2\alpha}$ in the cow (Fairchild & Pate, 1989). Thus, it was suggested that luteal cells could serve as antigen presenting cells, instigating a transitory immune response during luteolysis (Petroff *et al*., 1997). These findings raise the hypothesis that luteal failure may involve local autoimmune response mechanisms facilitated by increased expression of class II MHC molecules at the time of luteolysis (Benyo *et al*., 1991).

3. ANGIOGENESIS IN THE REPRODUCTIVE TRACT

Angiogenesis can be classified as the process of new blood vessel formation from pre-existing vasculature. Briefly, starting from a primary capillary plexus already shaped, which undergoes a remodeling process by the sprouting and branching of new vessels from pre-existing ones defines the process of angiogenesis (Papetti & Herman, 2002). Mechanistically, angiogenesis can be summarized in three steps: 1) breakdown of the basement membrane of the existing blood vessel, 2) migration of the endothelial cells toward an angiogenic stimulus, and 3) proliferation of the endothelial cells in the new place to establish a new blood vessel sprout (Folkman & Klagsbrun, 1987). The angiogenesis process plays an essential role during embryo development (Folkman, 1995) and in normal tissue growth in the female reproductive cycle (placental development, ovulation, corpus luteum) (Klagsbrun & D'Amore, 1991). Besides, angigenesis is essential for wound healing and also plays a major role in various diseases and tumorigenesis (Dvorak *et al*., 1995; Papetti & Herman, 2002).

An established vasculature consists of an inner lining of endothelial cells, associated mural cells such as pericytes and vascular smooth muscle cells. These vessels remain quiescent until there is an angiogenic stimulus, such as hypoxia or wounding. Key angiogenic regulators start disrupting endothelial and mural cellular contacts, simultaneously with extracellular matrix degradation by numerous proteases. Endothelial cells, then, migrate towards the angiogenic stimuli and proliferate under the influence of proangiogenic factors (Gerhardt & Betsholtz, 2003; Robinson *et al*., 2009). Once connected and aligned, the endothelial cells form a lumen and the recently formed vessel is stabilized by the recruitment of pericytes (Gerhardt & Betsholtz, 2003). Thus, angiogenesis is a highly regulated process involving a balance between pro- and anti-angiogenic factors.

3.1 Angiogenesis in the ovary

Different studies indicate that the corpus luteum is one of the most vascularized organs in the body (Bruce & Moor, 1976; Reynolds *et al*., 1994). For luteal establishment and formation the development of a microvasculature for the delivery of adequate levels of hormones and lipoprotein bound cholesterol is required (Davis *et al*., 2003). Quantitative reports in ruminants showed that in early luteal phase more than 85% of proliferating cells are endothelial cells, and in mature CL more than 50% (Reynolds *et al*., 1994).

Examples of proangiogenic factors include fibroblast growth factor 2 (FGF2), vascular endothelial growth factor A (VEGF), platelet-derived growth factor (PDGF) family and angiopoietin (Ang). In the CL, angiogenesis seems to be tightly controlled by stimulating and inhibiting factors (Redmer *et al*., 1996) that might regulate its vascularization and function (Ferreira-Dias *et al*., 2006b; Redmer *et al*., 1996).

Angiogenesis is determinant even during follicular growth, on the phase of pre-antral follicle. Around 40% of proliferating cells in the theca are of endothelial origin (Martelli *et al*., 2009). The neo vascularization is crucial for follicle growth, dominance and pre-ovulatory development. Treatments with anti-angiogenic compounds (VEGF trap) comprised follicular development (Fraser & Duncan, 2009). In mares, dominant follicles showed an increase in the blood flow prior to deviation, when compared with the subordinates (Acosta *et al*., 2004). This follicular vascular bed provides the basis on which luteal vasculature will be formed (Robinson *et al*., 2009). In the developing follicle has been noticed that granulosa and theca cells produce angiogenic factors (Redmer *et al*., 1985). The LH surge induces several cellular and biochemical changes that lead to ovulation (Reynolds & Redmer, 1999). A number of these processes, like break down of the basement membrane and immune-like response, are determinant for angiogenesis promotion (Dickson & Fraser, 2000; Shweiki *et al*., 1993). Despite several other growth factors that can regulate luteal angiogenesis (Fraser & Lunn, 2000), VEGF has been described as the main angiogenic factor produced by luteal cells (Redmer *et al*., 1996; Reynolds *et al*., 2002; Schweigerer *et al*., 1987). The mRNA and protein VEGF have been described in the CL of several species, like cow (Redmer *et al*., 1996; Reynolds *et al*., 2002), rat (Kashida *et al*., 2001), macaque (Hazzard *et al*., 2000), human (Wulff *et al*., 2000) and mare (Al-zi'abi *et al*., 2003).

Naturally, since the main product of luteal cells is P_4, and its auto-, paracrine action is recognized during luteal establishment (Ferreira-Dias *et al*., 2006b; Skarzynski & Okuda, 1999), luteal angiogenesis regulation by P_4 should be mentioned. A positive correlation between VEGF and P_4 concentration was evident in follicular fluid and media from granulosa cell culture of primates (Lee *et al*., 1997). However, blockade of steroidogenesis either *in vivo* (Hazzard *et al*., 1999) or *in vitro* (Lee *et al*., 1997) situations did not alter VEGF mRNA and protein expression in primates. This may suggest that other steroid hormones (like E_2) may influence the production, and hence the action of angiogenic factors in the ovary (Hazzard & Stouffer, 2000).

Also lypophosphatidic acid (LPA), a biologically active phospholipid that plays critical roles in physiological and pathological processes like inflammation, cell proliferation or angiogenesis (Moolenaar, 1995) has been reported to interact with cytokines on angiogenesis regulation (Chen *et al*., 2008) during luteinization process.

Shortly, the angiogenic process in the CL will be described in sequential steps.

3.1.1 Follicular vasculature

The complexity of angiogenesis regulation justifies the need for further investigations. Increased blood flow and hyperemia seen during the periovulatory period (Acosta *et al*., 2004) is probably due to an increase in nitric oxide (NO) production (Ferreira-Dias *et al*., 2006b), following upregulation of endothelial NO synthase (eNOS) (Zackrisson *et al*., 1996). Estradiol (E_2) is considered the main upregulator of eNOS, rather than LH, since E_2 is a rapid, potent stimulator of eNOS in endothelial cells (Kim *et al*., 2008). Another important trigger for the increased blood flow is the hypoxia-induced factor 1α (HIF1A), whose expression is upregulated in the collapsed follicle of pigs (Boonyaprakob *et al*., 2005), suggesting that the tissue is hypoxic. The relationship between LH, VEGF and HIF1A is not clear, for the period of follicular-luteal transition, but it is possible that VEGF increase following LH surge is mediated by HIF1A (Duncan *et al*., 2008).

3.1.2 Breakdown of blood vessels basement membrane

Breakdown and reorganization of the blood vessel basement membrane is necessary for vascular proliferation and involves a plethora of proteases, including matrix metalloproteinase (MMP) family, such as collagenases, gelatinases and membrane type MMP. Several MMPs (MMP9, MMP13, MT-MMP1) are upregulated by the LH surge (Kliem *et al*., 2007). It should be noticed that some of these MMP also participate on the ovulatory process (Riley *et al*., 2001). This process removes the physical block to vacularization of the granulosa layer and fragment and spread extracellular matrix (ECM) components, creating a more spacious environment, facilitating the motility and migration of endothelial and other cells. Another important consequence is the release of angiogenic factors sequestered in the basement membrane. One protease that appears to be critical for angiogenesis following ovulation is a disintegrin and metalloproteinase with a TSP type 1 motif (ADAMTS1; Shozu *et al*., 2005). The ADAMTS1 cleaves the matrix proeoglycans, being expressed in the periovulatory

follicle. Besides, ADAMTS1 is increased by gonadotrophin stimulation (Madan *et al*., 2003), possibly mediated by HIF1A pathway (Kim *et al*., 2009), and might be important for endothelial cell invasion, since it is upregulated when these cells invade into collagen matrix, following VEGF/FGF2 stimulation (Su *et al*., 2008).

3.1.3 Cell migration

Endothelial cell migration involves its polarization towards an angiogenic stimulus, protrusion through filopodia-like structures, traction and then retraction. Traction requires the protruding cell to adhere through integrins to the surface (e.g. ECM), over which is moving. Integrins act as " linker molecules" between ECM and cytoskeleton of the endothelial cell. Meanwhile, the organization of ECM components, such as fibronectin, create a platform where endothelial cells can migrate (Hughes, 2008). It is recognized that, in bovine CL, fibronectin forms a network of fibrils orientated along the axis of the capillary sprout (Amselgruber *et al*., 1999), acting as a "pre-patterned" guideline for endothelial cell migration. Fibronectin showed also a stimulatory effect on luteal-derived endothelial cell proliferation (Christenson & Stouffer, 1996) and formation of endothelial cell networks *in vitro* (Robinson *et al*., 2009). Still considering the endothelial cell migration, it is believed that recently formed steroidogenic luteal cells can secrete chemo-attractants VEGF and FGF2, working on endothelial cells migration towards themselves (Robinson *et al*., 2009). Alternatively, there could be other migratory stimuli. Blood clot formed during ovulation might stimulate migration. Indeed, platelets were a better stimulant for endothelial cells migration than granulosa cells (Furukawa *et al*., 2007).

3.1.4 Endothelial cell proliferation

Majority of proliferating cells in the collapsed follicle are of vascular origin (Reynolds & Redmer, 1999). Definitely, VEGF plays a central role, since its blockade abolished endothelial cell proliferation, luteal vascularization and P_4 production in the rat (Ferrara *et al*., 1998), primate (Hazzard *et al*., 2002) and mouse CL (Kuhnert *et al*., 2008). Studies in the cow have demonstrated that immunoneutralization of VEGF reduced luteal development and steroidogenesis (Yamashita *et al*., 2008) and *in vitro* VEGF inhibition suppressed endothelial networks formation (Woad *et al*., 2009). However, total vascularization inhibition was not achieved, suggesting the importance of other interveniets. The FGF2 should be also critical to

endothelial network formation, since suppression of its receptor almost completely inhibited angiogenesis, by decreasing both the number of endothelial clusters and their size (Robinson *et al*., 2009). This occurred even in the presence of VEGF, emphasizing the importance of FGF2. It is suggested that these factors may have complementary rather than redundant actions on luteal angiogenesis (Robinson *et al*., 2009).

3.1.5 A mature vascular system

Endothelial cells need structural support. Mural cells such as pericytes vascular smooth muscular cells ensure the shape and regulate blood flow through their contractile properties. The final step of angiogenesis is vessel stabilization, achieved by secretion of platelet derived growth factor beta (PDGFB), which acts on a paracrine fashion on pericyte recruitment (Gerhardt & Betsholtz, 2003). For many years pericytes role on angiogenesis was neglected. However there is a growing evidence of their importance for angiogenesis initiation accomplishment. During the ovulation time, pericytes are located at what appears to be the forefront of the endothelial migratory path (Amselgruber *et al*., 1999; Redmer *et al*., 2001), whilst in mature CL they are closely associated with endothelial cells. Furthermore, pericytes represent a large number of proliferating cells in the early ovine CL (Redmer *et al*., 2001). Pericytes act, firstly, as guiding structures aiding the outgrowth of endothelial cells. They produce MMPs and may promote endothelial cell invasion, by destroying ECM. Secondly, perycites are recruited for vessel stabilization (Robinson *et al*., 2009). Activation of pericytes was associated with PDGF system. Pre-ovulatory treatment of mice with soluble ectodomain of PDGFR prevented the recruitment of perycites and reduced the staining of vascular area in CL immunohistochemistry (Kuhnert *et al*., 2008). Recently, *in vitro* studies showed that inhibition of PDGFR domain decreased the vascular network formation in bovine CL (Woad *et al*., 2009).

A couple of words should be dedicated to Ang. In general, Ang1 is needed to stabilize formed vasculature. In contrast, Ang2 acts as a natural antagonist of Ang1 and is required for active remodeling and destabilization of vascular structure (Yancopoulos *et al*., 2000). Both angiopoietins share the same receptor, the tyrosine-kinase 2 (Tie2) (Hanahan, 1997). A relative low level of Ang1 to Ang2 in the microenvironment induces destabilization of vessels, which is required for both angiogenesis or angioregression. The presence of factors like VEGF or FGF drives the process towards new vasculature proliferation (Hanahan, 1997).

3.1.6 Luteolysis demands vascular regression

A fundamental question concerning regression of the CL is whether regression of vasculature plays a role on functional and structural luteolysis. It was reported in sheep (Sawyer *et al*., 1990) and guinea pig (Azmi & O'Shea, 1984) that apoptosis of endothelial cells presumably originated the occlusion of blood vessels with cellular debris. This could result in subsequent apoptosis of more endothelial cells followed by apoptosis of steroidogenic cells (Fraser & Wulff, 2003). A pitfall determining the importance of endothelial cells apoptosis on luteolysis may be the fact that the temporal relation between them diverges among species. The evidence in the sheep and cow that $PGF_{2\alpha}$ induces apoptosis of endothelial cells, resulting in luteolytic cascade (Acosta *et al*., 2002), is not that obvious in primates (Young *et al*., 2000). But, undoubtedly death of vascular cells lead to a reduction in the supply of oxygen and nutrients to hormonal producing cells, and perhaps contributing for their death.

The main luteolytic agent, the uterine $PGF_{2\alpha}$ has been associated with *in vivo* changes on vasculature. It has been proposed that the main consequence of $PGF_{2\alpha}$ is the decrease in luteal blood flow (Nett *et al*., 1976). However, following $PGF_{2\alpha}$ administration, different responses are seen among species. In the cow, an acute increase on luteal blood flow was verified after 30 minutes to 2h following administration of $PGF_{2\alpha}$. Besides, the blood flow increase was associated with a peak levels of 13,14 dihydro 15 keto $PGF_{2\alpha}$ (PGFM; a product of the metabolism of the $PGF_{2\alpha}$), on the day 17-18 of the estrous cycle, prior to the decline of plasma P_4 in spontaneous luteolysis in the cow (Ginther *et al*., 2007; Miyamoto *et al*., 2005). The same increase in the blood flow at the beginning of luteolysis was not confirmed for the mare (Ginther *et al*., 2008b), whose luteal blood flow decreases from the mid CL, some days before the decline in plasma P_4 (Bollwein *et al*., 2002). Several studies have related the variations in blood flow seen in the cow with the potent vasorelaxant NO. More than the direct contribution for luteolysis, after inhibiting bovine luteal cells secretion of P_4 and inducing their apoptosis (Skarzynski *et al*., 2000a; Skarzynski *et al*., 2003b), NO increases luteal blood flow as $PGF_{2\alpha}$. These findings strengthen the role of NO on luteal blood flow regulation in the cow. In the mare, eNOS was increased in the late CL, suggesting a role for NO on luteolysis (Ferreira-Dias *et al*., 2011). However, further studies are needed to better elucidate its role on vascular regression in equine luteal tissue. Changes in PGs and blood flow are considered necessary for local release of endothelin-1 (ET-1) and angiotensin II (ANG II), which further induce vasoconstriction and blood flow reduction (Ohtani *et al*.,

1998). Besides showing other biological functions, ET-1, mainly produced by endothelial cells, is considered a potent vasoconstrictor after acting on its receptor A (ETA) (Yanagisawa *et al*., 1988; Meidan & Levy, 2002). Concerning ANG II, it regulates several biological processes besides angiogenesis, including vascular tone and cellular growth. In bovines, production of ANG II in the CL was associated to renine-angiotensin system (Berisha *et al*., 2002). Both ET-1 and ANG II can reduce luteal steroidogenesis and are considered vasoactive factors determinant for the luteolytic pathway and vascular regression (Miyamoto *et al*., 1997). Supporting the present hypothesis is the fact that the local release of ET-1 (Ohtani *et al*., 1998) and ANG II (Hayashi *et al*., 2001) in the mid CL increased at 2–4h after a *in vivo* $PGF_{2\alpha}$ injection following the phase at which the acute increase in intraluteal blood flow was the highest. Moreover, ET-1 inhibited P_4 release in *in vitro* microdialyzed CL (Miyamoto *et al*., 1997). Similarly, ANG II also inhibited the local secretion of P_4 from mid CL (Hayashi & Miyamoto, 1999), but showed a stimulatory effect in early CL (Kobayashi *et al*., 2001). Cytokines, like TNF or IFNG, might also play a role in the regulation of luteal endothelial cells (Korzekwa *et al*., 2011) and interact with ET-1 and $PGF_{2\alpha,}$ inhibiting luteal steroidogenesis (Ohtani *et al*., 2004). Cytokines TNF and IFNG can directly incite MCP-1 secretion and contribute for apoptosis of endothelial cells (Cavicchio *et al*., 2002), suggesting that a cross-talk between immune and endothelial cells accounts for the increase in MCP-1 and cell death during $PGF_{2\alpha}$ induced luteal regression (Townson, 2006).

3.1.7 Vascular regulation in the equine corpus luteum

In the previous description, a chronological order of the events regulating luteal vascular dynamics was given. Since considerable differences are seen in the histology of the CL among species, pattern of luteal vascularization should diverge. So far, few studies described angiogenesis regulation in equine CL have been published. Al´zi-abi *et al*. (2003) showed the peak in the transcription of mRNA and protein of VEGF in early and mid CL. A direct temporal relation with angiogenesis, vessel proliferation and capillary density was established in this study. Thus, VEGF was indicated as the main regulator of angiogenesis in the equine CL (Al´zi-abi *et al*., 2003). More recently, Müller and coworkers (Müller *et al*., 2009) characterized the presence of VEGF, VEGF B, Ang1, Ang2 and the receptors VEGFR1, VEGFR2 and Tie 2 in follicular and luteal cells from equine ovary. A distinctive staining was seen for VEGF, VEGFR2 and Ang2, factors associated with angiogenesis promotion as mentioned before, during periovulatory period (including the tertiary, graafian follicles and

early CL) (Müller *et al*., 2009). This showed their participation on equine luteal angiogenesis initiation. The Ang 1 staining was more intensively associated with arterioles/venules/arteries/veines, compared with capillaries, suggesting a role on stabilization of this vaculature (Hanahan, 1997). Independently from luteal development, VEGFR1 was associated with mild expression intensity and the complex VEGFB/VEGFR1 was not associated with pro-angiogenic events in the equine CL (Müller *et al*., 2009). The referred study also showed that, in mature CL (mid luteal phase CL); more intense staining of studied pro-angiogenic factors could be observed specifically in the array of the vascular septa and in the luteal periphery. These findings are in agreement with those from Al´zi-abi *et al*. (2003). In mid luteal phase, capillary endothelial cells showed a less intense staining, mainly regarding VEGFR2 and Tie2, when compared with early luteal phase. Also, luteal cells were characterized by a weaker immunolabeling for VEGFR2 in the mid luteal phase (Müller *et al*., 2009).

Involvement of NO on equine CL regulation, specifically on angiogenesis modulation, was recently described (Ferreira-Dias *et al*., 2011). The eNOS protein was shown to be highly expressed in the early CL, when NO stimulated luteal tissue angiogenic factors production and induced bovine aortic endothelial cells (BAEC) proliferation (used as a model to assess angiogenic factors production by luteal cells). Expression of eNOS was reduced in mid luteal phase CL (mid CL) and NO no longer increased BAEC mitogenic activity. Nevertheless, participation of NO on regulation of vascular changes should be true also for the mare, since eNOS expression was again increased in late luteal phase CL (late CL) (Ferreira-Dias *et al*., 2011). In the same study, TNF, used as positive control, also caused BAEC proliferation suggesting a role also for cytokines on angiogenic factors stimulation in the early luteal phase CL (early CL).

In Ferreira-Dias *et al*. (2006a) the dynamics of vascular area in the mare CL is described throughout different luteal stages. A marked increase in vascular area was observed in the early and mid CL. However, vessel number was the highest in the mid and late CL. The raise in DNA content seen from early to mid CL was associated not only with hyperplasia and luteal cell proliferation, but also with endothelial cells proliferation. Besides, the decrease in vascular area in the late CL might have been associated with the decrease in blood vessel lumen by vessel contraction. This decrease in capillary diameter is considered determinant for blood flow fall and can initiate or accelerate luteal regression (Gaytan *et al*., 1999). IT should be also mentioned that, media from mare late CL equine incubation with $PGF_{2\alpha}$ reduced

mitogenic activity of BAEC, when compared with the controls (Ferreira-Dias *et al*., 2006a). Also in the late CL, expression of pro-angiogenic factors was reduced and anti-angiogenic factors production increased, after $PGF_{2\alpha}$ triggering (Al´zi-abi *et al*., 2002). After 12h of induced regression with $PGF_{2\alpha}$, on the day 10 of the luteal phase, signs of swelling in equine luteal endothelial cells and detachment from the blood vessels were observed, with TUNEL and ultrastructural examination (Al´zi-abi *et al*., 2002). Sings of apoptosis in luteal endothelial cells were depicted later. Active caspase-3 was also identified in large luteal cells and endothelial cells (Ferreira-Dias *et al*., 2007). Also, Aguilar *et al*. (2006) confirmed that active caspase-3 mediated apoptosis takes place in both cell types during luteolysis. In both steroidogenic and endothelial cells, the increase in caspase-3 expression was on day 14 of the luteal phase or 36h after $PGF_{2\alpha}$ administration (Aguilar *et al*., 2006). Another important finding is the relationship between the onset of caspase-3 expression in endothelial cells on day 14 of luteal phase, or after luteolysis induction, synchronized with the decrease in mRNA and protein expression of VEGF in steroidogenic cells (Al´zi-abi, 2003). This supports the hypothesis of another role for VEGF on equine luteal function regulation, besides angiogenesis modulation, representing a pro survival factor for endothelial cells (Al´zi-abi, 2003). Nevertheless, in the mare there are no evidences that luteal endothelial cell death is the trigger for luteolysis, since death of endothelial cells is temporarily associated with death of steroidogenic cells.

4. THE ENDOMETRIUM

In mammals, the physiologic regulation of a fertile reproductive cycle depends on the interaction between hypothalamus-pituitary gonadal axis. The anatomical relationship, evidenced in several species, between the affluent and effluent lymph and vascular drainage from blood and lymphatic vessels of uterine and ovarian structures (Alexander *et al*., 1998) is one example of how close uterus and ovaries are related. Others are the role of endometrial $PGF_{2\alpha}$ on non primates CL regression, in the absence of pregnancy (ensuring the normal cyclicity), as well as the importance of luteal P_4 for pregnancy establishment, in case of gestation (Niswender *et al*., 2000). The present review is an attempt to address the main molecular changes undergoing in the endometrium throughout the estrous cycle, concerning the mechanisms involved in the cyclic regulation of $PGF_{2\alpha}$ secretion for luteolysis achievement or, in case of pregnancy, the pathways ensuring the abolishment of the luteolytic action, preventing CL regression.

The profound recurrent changes that occur in the cyclic endometrium are mainly ruled by ovarian steroids theough their receptors. During estrus or following E_2 administration, cellular activity is increased in the equine endometrium and edema is promoted by an increase in vascularity and congestion (Ginther, 1992). The number of uterine glands per unit area is minimal, but many secretory cells are present in this phase. During diestrus, edema subsides and endometrial glands become tortuous, under the action of P_4. Likewise, the number of secretory cells decrease rapidly during this phase of the estrous cycle (Ginther, 1992). Electron microscopy studies indicated that ciliated cells are abundant on the surface of the endometrium during diestrus but its number varies during estrus (Samuel *et al*., 1979). Classically, steroid hormones bind to their receptors, autoregulate the production of their own receptors (E_2 receptor – ER and P_4 receptor - PR) (Xiao & Goff, 1999) in target tissues and increase the rate of transcription in the response gene (Mangelsdorf *et al*., 1995).

It seems pertinent to give a brief description of these receptors, facing their importance for endometrial physiologic regulation. Both ER and PR are members of steroid hormone receptor family and they can act as hormone-dependent activators of transcription. Two ERs are described, the ERα and the ERβ (Kuiper *et al*., 1996). The progesterone receptor exists as two isotypes, PRA and PRB (Lim *et al*., 1999). After ligand binding to the receptors, a classical genomic pathway induce conformational changes that lead to dimerization of receptors and their activation, usually by phosphorylation. Further on, ligand and receptor

dimer are relocated to the nucleus via different cofactors and transcription of target genes is activated (Hewitt & Korach, 2003). This classical model of steroids action demands a considerable latency period before response is triggered. Over the last years, it has become evident that steroid hormones mediate rapid responses within few minutes, incompatible with the classical model of action (Falkenstein *et al*., 2000). Indeed, it has been proposed that steroid hormones can mediate non genomic actions in two ways: (a) non specifically, by altering membrane fluidity (absence of any specific receptor); or (b) via ligand-specific steroid receptors including modified classical nuclear receptors and non classical membrane-associate receptor (Meyer *et al*., 1996). An example of the described way of action is the P_4 induced acrossome reaction in mammalian sperm within seconds of binding to a specific cell membrane receptor (Cheng *et al*., 1998), without activation of DNA transcription (Gilbert, 1997). In several cell types has been identified the membrane bound P_4 receptor (mPR; Meyer *et al*., 1996). Existence of membrane bound E_2 has been proposed (Toran-Allerand *et al*., 2002), but so far not identified.

Studies on ER and PR concentration in equine endometrium showed their increase during estrus and early diestrus and a decrease by the mid and late diestrus (Watson *et al*., 1992). Moreover, studies in cow (Vesanen *et al*., 1992) or ewe (Miller, 1977) drew similar conclusions. Regarding molecular and cellular action of the receptors on the endometrium, before ovulation, E_2 binding to its receptors promotes endometrial regeneration and growth, preparing the tissue to responds to P_4 (Groothuis *et al*., 2007). Studies in knockout mice demonstrated that ERα is essential to mediate E_2 actions in endometrium (Hewitt *et al*., 2003). Several works proved that early response (4-6h) to E_2 includes an increase in RNA and protein synthesis, as well as water imbibition. Later response (12-16h) to estrogen induces DNA synthesis and epithelial cell mitosis (Groothuis, *et al*., 2007). After E_2 induction of endometrial cell proliferation, during follicular phase, P_4 acts as a differentiation factor (Cummings & Yochim, 1984), inhibiting mitosis in the endometrium (Padykula *et al*., 1989). Ablation of both PRA and PRB isoforms in PR knockout mice resulted in lumen and glandular epithelial tissue hyperplasia (Lydon *et al*., 1995). Concerning PRs, PRA has been shown to exert a negative effect on PRB, being considered its repressor (Vegeto *et al*., 1993). But this receptor does not regulate exclusively P_4, since PRA can inhibit target gene activation of both PRB and ERα, down regulating overall response of E_2 and P_4 (Kraus *et al*., 1995). Another action of the main product of luteal cells is the stimulation of glandular secretions, modulating the pattern of proteins secreted by endometrial cells (Strinden & Shapiro, 1983). Also important for pregnancy is the induction of endometrium quiescence

(Parkington, 1983), achieved with a decrease in the uptake of extracellular calcium, required for myometrial contractions (Batra, 1986). Moreover, P_4 blocks myometrial contractions by blocking the ability of E_2 to induce α-adrenergic-receptors activation, which provokes contractions (Bottari, 1983).

4.1 Luteal-endometrial cross talk

During diestrus, the endometrium is prepared for two opposite events: (a) luteolysis, in the absence of a viable conceptus; or (b) luteal maintenance in case of pregnancy. When gestation happens, besides the existence of adequate conditions in the uterine lumen, which are ensured by the precedent action of steroid hormones and other factors during previous phases of the estrous cycle, the conceptus should produce a signal determant for luteal maintenance (Bazer & Thacther, 1977). This process is known as maternal recognition of pregnancy (MRP). In the mare, initial signals of pregnancy recognition take place in the oviduct, with secretion of PGE_2 by the conceptus (Weber *et al*., 1991), allowing for its selective entrance in the uterus approximately at day 6 after ovulation (Battut *et al*., 1997). Thus, pregnancy is ensured by a successful transport of conceptus through the oviduct, luteal function maintenance and uterine secretion of the histotrophe necessary for conceptus nutrition and development (McDowell & Sharp, 2011). Besides the extensive work carried out during the last four decades, the precise signal for MRP is not fully characterized in the mare. A crucial species-specific characteristic for pregnancy assumption in mares is the conceptus mobility. Equine conceptus is mobile within the uterine lumen from around day 8 after ovulation until nearly day 15 (Leith & Ginther, 1984), migrating from one uterine horn to the other 12 to 14 times per day on days 12-18 of pregnancy (Ginther, 1983). Further experiments pointed out the necessity for extensive conceptus mobility through the uterus for the adjustment on $PGF_{2\alpha}$ production during MRP (McDowell *et al*., 1988). Moreover, movement throughout the uterine lumen, propelled by myometrial contractions stimulated by conceptus PGs production (Gastal *et al*., 1998; Stout & Allen, 2001), also allows for a better access to uterine histotrophe.

For MRP different hypotheses were raised, like alteration of $PGF_{2\alpha}$ distribution towards endometrial lumen, as suggested by Vernon *et al*. (1981) [similar process is seen in pigs (Bazer *et al*., 1989)], or secretion of a substance that could counteract the effects of $PGF_{2\alpha}$. Nevertheless, evidences revealed that probably it consists in preventing the secretion of $PGF_{2\alpha}$. Different *in vivo* (Douglas & Ginther 1976; Zavy *et al*., 1984a) and *in vitro* (Sharp & McDowell, 1985; Sharp *et al*., 1989) works showed that, in the presence of conceptus,

endometrial secretion of $PGF_{2\alpha}$ was reduced. But the specific signal modulating endometrial $PGF_{2\alpha}$ secretion is unknown. Further studies considering the nature of this signal clarified that its molecular weight should be greater than 1000, but lower 6000 daltons (Sharp *et al*., 1989). Comparing with ruminants, its size is much smaller than interferon tau (IFNt), the acidic protein with molecular weight around 17000 daltons (ewe) (Godkin *et al*., 1982) or 21000 daltons (bovine) (Roberts, 1989) mainly responsible for MRP in these animals. Also studies on genomics and proteomics stated the existence of discrepancies between INFt and equine signal for MRP (Baker *et al*., 1991; McDowell *et al*., 1990).

The last years of search for the unrevealed signal for maternal recognition of pregnancy in the mare showed also that insulin growth factor I, presumably a candidate for that visible mission, failed to suppress the endometrium's ability to produce luteolytic $PGF_{2\alpha.}$ Administration of IGF I to cyclic mares, from day 7 until 17 after ovulation (time that conceptus travels in through the uterus) did not cause any luteotrophic effect and even caused lower concentrations of circulating P_4 in treated animals (Rambags *et al*., 2008).

5. THE CYTOKINES

Immune cells participate in several physiological events in reproductive tract and their effects are largely mediated by cytokines (Adashi, 1990). Cytokines are intracellular signaling peptides (between 8 and 30 kDa mass) that can act on a auto/, paracrine or endocrine fashion in the organism (Cannon, 2000). Nearly all nucleated cells are able to synthesize these proteins and, in turn, to respond to them (Dinarello, 2000). Cytokines primarily participate in host responses to disease or infection, but are also involved in homeostatic mechanisms. An analogy to hormones would be a less accurate comparison, since hormones tend to be synthesized by highly specialized tissues and cytokines are produced by almost every cell (Dinarello, 2000). Whereas hormones are the primary product of a cell, cytokines account for a rather small amount of the cellular output. Moreover, hormones are expressed in response to homeostatic control signals, while cytokine genes are not expressed unless specifically stimulated (Dinarello, 2000). But the physiological function of the systems is dependent on a cross talk between them, where modified hormonal signals determine cytokines action directly on endocrine organs to bring about adaptations in cellular function (Cannon, 2000). Examples of this kind of interaction are the involvement of cytokines in ovarian and uterine cyclic transition from follicular to luteal phase and on early embryonic development stages (Cannon, 2000).

Cytokines identification diverges, depending on the biological function, if they either promote inflammation (proinflammatory cytokines) or suppress the activity of other proinflammatory cytokines (anti-inflammatory cytokines) (Dinarello, 2000). A balanced action between different cytokines is thought to determine the outcome of the disease, whether in short or long term. Thus, proinflammatory cytokines in nonpathological states can modify the release of normal feedback control hormones that regulate homeostasis. Reproductive processes, like follicle rupture, invasion of maternal decidua during implantation in primates, and relaxation of birth canal at term involve cytokine mediated events (Cannon, 1998). The cytokines can induce changes in cellular growth or function, antioxidative effects, heat-shock proteins, sodium excretion, hematopoiesis, and other responses (Cannon, 2000). The importance of cytokines on physiological baseline and homeostasis is unmistakable.

Cytokine signaling transduction appears to be taking place generally within 2-5 minutes (O'Neill, 1995) after binding to cells. Cytokine receptors lack intrinsic catalytic activity. Rather, they are associated with a structural unique class of kinases, the Janus Kinase (JaKs)

(Ihle, 1995): Jak1, Jak2, Jak3 and Tyr2. Cytokine binding to receptor subunits induces homo-heterodimerization resulting in apposition of Jaks, which, in turn, are bound to the receptors. Activation of Jaks by transphosphorylation leads to cytokine receptor subunit phosphorylation, providing a docking site for transcription factors. This docking site is recognized by several proteins (STAT family), which after dimerization are translocated to the nucleus and bind DNA, regulating gene expression (Yan *et al*., 1996).

Among all cytokines, a particular group should be mentioned, the tumor necrosis factor superfamily. This superfamily comprises an increasing number of structurally related ligand-receptor pairs, including tumor necrosis factor alpha, its receptors TNFR1 and TNFR2, Fas Ligand and its receptor FAS (Gravestein & Borst, 1998; Tansey & Szymkowski, 2009).

The present revision will focus on three conserved cytokines: the Tumor necrosis factor alpha (TNF); the interferon gamma (IFNG) and the Fas Ligand (FASL) and their receptors.

5.1 Tumor necrosis factor alpha

The TNF is known as a pleiotropic factor, acting as a major mediator of immune regulation and inflammatory response (Fiers, 1991). The activities of TNF are signaled by two distinct cell surface receptors (Smith *et al*., 1990). The TNFRI can induce apoptosis and activate the transcription factor nuclear factor-kappa B (NF-κB), while TNFRII can directly signal certain activities in lymphocytes such as NF-κB activation, and also plays an auxiliary role by helping deliver TNF to TNFRI (Tartaglia *et al*., 1993).

Generally, the TNFRII signaling complex involves the activation of two intracellular proteins, the TNF receptor-associated factor 1 (TRAF1) and the TNF receptor-associated factor 2 (TRAF2), originating a complex associated with apoptosis inhibition (Rothe *et al*., 1995). Conversely, the TNFRI contains a death domain in its intracellular domain that signals two important activities: apoptosis and NF-κB activation (Hsu *et al*., 1995). For instance, TNF receptor-associated death domain (TRADD), an intracellular protein that interacts with TNFRI, is over expressed inducing apoptosis and activation NF-κB (Hsu *et al*., 1995). Besides, TRADD, Fas associated death domain (FADD) and the receptor interacting protein (RIP) are proteins involved in the TNFRI pathway to apoptosis (Hsu *et al*., 1996). TRAF2 plays a role on NF-κB activation by both TNF receptors, but it is probably not involved in the signaling of apoptosis. The death domains of TRADD and FADD interact efficiently, being FADD required for TNF-mediated apoptosis (Boldin *et al*., 1995).

5.2 Interferon gamma

The IFNG exerts its effects on cells by interacting with the specific IFN-gamma receptor (IFNR), composed of two subunits, IFNRI and IFNRII (Bach *et al*., 1997). Binding of IFNG to its receptor first promotes oligomerization of the receptor and then activation of the receptor-associated Jak1 and Jak2, also involved in TNF intracellular signal (Ramana *et al*., 2002). The activated Jaks phosphorylate the intracellular domain of the receptor that serves as a docking site for the signal transducer and activator of transcription 1 (STAT1). The STAT1, after phosphorylization is translocated to the nucleus and regulates gene expression (Ramana *et al*., 2002). There are many known STAT1 targets in IFNG mediated signaling, specifically the SMAD family member 7 (SMAD7), the interferon regulatory factor 1 (IRF1) and proteins involved in cell cycle regulation (Chin *et al*., 1996).

5.3 Fas Ligand

The FASL is a transmembrane protein, member of TNF superfamily and one of the major effectors of CD8+ cytotoxic T lymphocytes and natural killer (NK) cells, usually associated with apoptosis (Nagata, 1999). Metalloproteinases are thought to be involved in the proteolytic cleavage of membrane-bound FASL, producing its soluble form (Tanaka *et al*., 1999). Interaction with its receptor FAS triggers the formation of the death-inducing signaling complex (DISC) by recruiting the adaptor molecule FADD. The N-terminal region of death effector domains (DED) of FADD is determinant for procaspase 8 recruitment. After recruitment, procaspase 8 is proteolytically activated (Budihardjo *et al*., 1999) and at this point, two pathways can be followed, depending on cell type. In type I cells (mitochondria independent, Bcl-2 insensitive) apoptosis is induced through the death-receptor initiated pathway where caspase 8 activates procaspase 3 (Budihardjo *et al*., 1999). In type II cells, caspase 8 instead of activating procaspase 3, cleaves Bid (a cytoplasm protein) to activate the mitochondrial pathway with the release of cytochrome C (Budihardjo *et al*., 1999). In this pathway, Fas-induced apoptosis can be blocked by pro-survival factors such as Bcl-2. Upon release, cytochrome C forms the apoptosome together with Apaf-1 and procaspase 9. Finally, this complex triggers the caspase 3 activation and the cleavage of several substrates, including DNA repair enzymes, structural proteins and endonucleases (Nagata, 1999).

CHAPTER II. EXPERIMENTAL WORK

1. IS FAS/FAS LIGAND SYSTEM INVOLVED IN EQUINE CORPUS LUTEUM FUNCTIONAL REGRESSION?

(Published in *Biology of Reproduction*)

1.1 Abstract

Pro-apoptotic factor Fas Ligand (FASL), and its cell surface receptor FAS are Tumor necrosis factor Superfamily members that trigger apoptosis in different cell types. However, their influence on luteal steroidogenesis is not clearly understood. The aim of the present work was to determine (i) the presence of the cytokine FASL and its receptor FAS in the mare's corpus luteum (CL) throughout the luteal phase, as well as (ii) the influence of FASL alone, or together with the cytokines tumor necrosis factor alpha (TNF) and interferon gamma (IFNG), on equine luteal cells production of luteotrophic and luteolytic factors, cell viability and apoptosis. FASL and FAS protein expression and mRNA transcription were evaluated in different luteal stages of the equine CL by Western Blotting and real time PCR assays, respectively. Protein expression and *FASL* mRNA transcription increased in the Late CL. Also, FAS and FASL proteins were present in large steroidogenic and endothelial CL cells throughout the luteal phase, by immunohistochemistry. Equine luteal cells isolated from Mid luteal phase CL were stimulated without (Control) or with exogenous cytokines: FASL (10 ng/ml); TNF+IFNG (10 ng/ml each) (positive control) or FASL+TNF+IFNG (10 ng/ml each). FASL clearly inhibited *in vitro* P_4 and PGE_2 production by equine luteal cells but increased $PGF_{2\alpha}$. Furthermore, FASL effect on equine luteal cell viability depended on the presence of cytokines TNF and IFNG. In conclusion, this study shows the presence of FASL and FAS in the equine CL and suggests their importance on functional luteolysis.

1.2 Introduction

During its lifespan, the corpus luteum (CL) goes through a rapid sequence of events, starting with its growth and establishment as an active endocrine organ. Its main function, progesterone (P_4) production, allows for the maintenance of pregnancy (Peluffo *et al*., 2006). In the absence of gestation, CL regression, which is characterized by changes and degeneration of both vascular and steroidogenic cells, is essential for normal ovarian cyclicity and resumption of a new estrous cycle (Ferreira-Dias *et al*., 2006a; Roberto da Costa *et al*., 2005; Stouffer *et al*., 2001).

The luteolytic process requires the participation of different triggering factors, very often acting synergistically in a complex way. The onset of luteal regression depends on luteolytic factors action, such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), nitric oxide (NO) (Ferreira-Dias *et al*., 2006a; Vega *et al*., 2000) and cytokines (Friedman *et al*., 2000; Petroff *et al*., 2001). Interferon gamma (IFNG) was shown as deleterious for bovine luteal cells, mainly cooperating with tumor necrosis factor alpha (TNF) on cell death (Petroff *et al*., 2001). As a result, a fall in P_4 production (functional luteolysis) and changes in luteal tissue morphology (structural luteolysis), occur during CL regression (Liszewska *et al*., 2005; McCracken *et al*., 1972; Roberto da Costa *et al*., 2005).

Programmed cell death or apoptosis, as a conserved mechanism among species, is essential for cell number and tissue size control and homeostasis maintenance. It is determinant for normal reproductive function and development in the ovary (Slot *et al*., 2006). This process has been reported in CL involution in several species, such as mouse (Hasumoto *et al*., 1997; Quirk *et al*., 2000), rat (Gaytan *et al*., 2001; Guo *et al*., 1998), rabbit (Dharmarajan *et al*., 1999; Goodman *et al*., 1998), sheep (O'Shea *et al*., 1997; Rueda *et al*., 1995), cow (Juengel *et al*., 1993; Zheng *et al*., 1994), pig (Bacci *et al*., 1996), woman (Shikone *et al*., 1996) and mare (AL-zi'abi *et al*., 2002; Ferreira-Dias *et al*., 2006b).

Two distinct signaling cascades can lead to apoptosis. In apoptosis intrinsic pathway, internal stimuli originated within the cell, promote changes in mitochondrial permeability (Adams & Cory, 1998). However, in the extrinsic pathway, apoptosis is triggered when cytokine receptors, including tumor necrosis factor super family receptors (TNFRs) are activated (Nagata, 1997). TNF and Fas ligand (FASL) are cytokines mainly produced by immune cells (Suda *et al*., 1993), which bind to cell surface receptors TNFR1 and FAS respectively, and trigger apoptosis. This process starts with cytoplasm proteins recruitment, which bind to Death Domain (DD) and to Death Effector Domain (DED) (Locksley *et al*., 2001).

Consecutively it promotes the formation of death inducing signalling complex (DISC), which propagates the signalling cascade (Budihardjo *et al*., 1999). The formation of this complex activates downstream pro-caspase zymogens spreading the apoptotic signal, ending with the activation of effectors caspases (Ferreira-Dias *et al*., 2006b; Slot *et al*., 2006).

In vivo studies have demonstrated the stimulatory action of FASL on structural regression in the mouse (Sakamaki *et al*., 1997) and buffalo CL (Yadav *et al*., 2005). Also *in vitro* research made clear the increase in FASL expression in cow luteolysis (Taniguchi *et al*., 2002). These data suggest an important role for FASL and FAS on structural involution and luteolysis. Nevertheless, the influence of FASL on steroidogenic capacity and functional luteolysis is not fully understood. Thus, the aim of the present work was to determine (i) the presence of the cytokine FASL and its receptor FAS in the mare´s CL throughout the luteal phase, as well as (ii) the influence of FASL alone, or together with the cytokines TNF and interferon gamma (IFNG), on equine luteal cells production of luteotrophic and luteolytic factors and (iii) on cell viability and apoptosis mediation.

1.3 Materials and methods

1.3.1 Collection of equine corpora lutea

Luteal tissue and venous blood from jugular vein were collected *post mortem* at the local abattoir from randomly designated cyclic Lusitano mares age ranging from 3 to 8 years old, from March (Vernal equinox) until the end of August. The mares were euthanized after stunning according the European Legislation concerning welfare aspects of animal stunning and killing methods (EFSA, AHAW/04-027) and to the Portuguese legislation (DL 98/96, Art. 1º), and approved by the Faculty of Veterinary Medicine Ethics Committee.

Luteal structures were classified based on plasma progesterone (P_4) levels, on follicle size and on the morphological appearance of the CL (Roberto da Costa *et al*., 2008). Briefly, luteal tissue was considered as: early luteal phase CL, (presence of corpus hemorrhagicum, P4>1ng/ml, Early CL; n=9), mid luteal phase CL, (CL associated with follicles 15 to 20 mm in diameter and P_4>6 ng/ml, Mid CL; n=15) and late-luteal phase CL (CL associated with preovulatory follicle 30-35mm in diameter and P4 between 1 and 2 ng/ml, Late CL; n=9). Immediately after collection, luteal samples were included in specific solutions: (i) RNAlater (AM7020, Ambion, Applied Biosystems, CA, USA) for gene and protein expression quantification; (ii) transport media – M199 (M2154; Sigma-Aldrich, St. Louis, MO, USA)

with 0.1% bovine serum albumin (BSA), 20 µg/ml gentamicin (G1397, Sigma, USA) and 250 µg/ml amphotericin (A2942, Sigma, USA) for *in vitro* studies; or (iii) in buffered formaldehyde, for immunostaining studies.

In order to study protein and gene expression of cytokines FASL and the receptor FAS in the equine CL, immunohistochemistry, Western blotting and Real time PCR were carried out (Early CL, n =5; Mid CL, n =5; Late CL, n =5).

1.3.2 Immunohistochemistry analysis

As previously described immunostaining studies were performed on consecutive 4 µm histological sections, for the determination FASL and FAS localization in luteal tissue (Ferreira-Dias *et al*., 2006b). The primary mouse monoclonal antibodies raised against FASL and the receptor FAS (Ref. 556387 and Ref. 610197 respectively, BD Bioscience; Franklin Lakes, NJ, USA) were diluted at 1:50 and 1:100 in 0.1 M PBS (pH 7.4), respectively. Negative control was performed by replacing the primary antibody by 0.1 M PBS (pH 7.4). Immunostaining was assessed as a characteristic brown staining of the cytoplasm and cell membrane, with a light microscope (Olympus BX51, Tokyo, Japan), on 10 random fields, at 400 magnification. Microscopic fields were photographed (DP11 Olympus, Tokyo, Japan).

1.3.3 Western blotting

FAS and FASL protein expression on equine luteal tissue was assessed by Western blotting. After total protein concentration was determined, 100 µg of protein was loaded for FASL and 40 µg for FAS and for Beta-actin (ß actin) on a 12% Acrylamide gel (ref. 161-0155, Bio-Rad, Hercules, CA, USA) and further transfer to nitrocellulose membranes (Ref. 1620116, Bio-Rad, USA) (Rodrigues *et al*., 2002). Level of active FASL was evaluated by using a polyclonal antibody raised in rabbit (sc-834, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and diluted 1:200, while for FAS quantification the same specific antibody for immunohistochemistry was used, but diluted at 1:1000. For ß actin (A5441, Sigma, USA) a mouse monoclonal antibody was diluted 1:10,000. Membranes were incubated overnight at 4ºC. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit (P0448, Dakocytomation, Carpinteria, CA, USA), at 1:15,000 for FASL and HRP-conjugated goat anti-mouse (A2554, Sigma, USA) for FAS at 1:100,000, and incubated for 2 h at room temperature. Protein expression was visualized by means of luminol-enhanced

chemiluminescence (SuperSignal® West Pico, 34077, Thermo Scientific, Waltham, MA, USA) and exposure of the membrane to a blue light-sensitive autoradiography film (Kodak BioMax Light Film; Kodak-Industrie, Chalon-sur-Saone, France). Equal protein loading per lane was confirmed with ß actin. Target protein expression was normalized by dividing units of arbitrary densimetry for FASL and FAS by ß actin for each band. Signals were densitometrically assessed using the ImageMaster 1D Elite densitometric analysis program (Amersham Biosciences, Piscataway, NJ, USA).

1.3.4 Real Time PCR

Real time PCR relative quantification was performed in order to assess *FASL* and *FAS* gene transcription in corpora lutea throughout the luteal phase. RNA was extracted from luteal tissue (Qiagen's Kit for Total RNA Extraction and Purification; ref. 28704, Qiagen, Hilden, Germany) and DNA digested (RNase-free DNase Set; ref.50979254, Qiagen, Germany), according to manufacturer's instructions. Later on, evaluation of RNA concentration was done spectrophotometrically (260 nm and 280 nm) and RNA quality assessed by visualization of 28S and 18S rRNA bands, after electrophoresis through a 1.5% gel agarose and ethidium bromide staining. Reverse Transcription was carried out using Reverse Transcriptase Superscript III enzyme (ref.18080093, Invitrogene, Gibco, Carlsbad, USA), from 1 μg total RNA in a 20 μl reaction volume, using oligo (dT) primer (27-7858-01, GE Healthcare, Buckinghamshire, UK). Primer design was determined due to the lack of previously sequenced mRNA sequence for *FASL* and *FAS* genes in the horse, in Gene Bank Sequence Data Base (Gene DB). Degenerate primers (Table 1) were designed based on other species sequences deposited on Gene DB. Briefly, using CLC Free Workbench 3.2.3 software (CLC bio, Aarhus, Denmark) (Persson *et al*., 2007), conserved regions among species were identified after sequence alignment, and degenerate primers designed using different internet-based interfaces, such as Primer-3 (Rozen & Skaletsky, 2000) and Primer Premier software (Premier Biosoft Int., Palo Alto, CA, USA) (Feng *et al*., 1993). Several conventional PCR reactions, using FideliTaq DNA polymerase master mix (71180, USB, Cleveland, USA), were carried out using a default thermocycler (Applied Biosysthems, California, USA) as follows: 2 min at 94ºC for denaturation; 35 cycles of 15 sec at 94ºC for enzyme activation, 45 sec at 44º-55ºC for annealing (depending on the gene) and 45 sec at 68ºC for extension; and 5 min at 68ºC for finalization. Agarose (1%) (BIO-41025, Bioline, Luckenwalde, Germany) electrophoresis gel and ethidium bromide (17896, Thermo, USA) staining showed a specific

and single product. After purification using GFX PCR DNA and Gel Band Purification Kit (28903470, GE Healthcare, Buckinghamshire, UK), cDNA samples in duplicate were sent for sequenciation (Stabvida, Lisbon, Portugal). Sequences homology was confirmed and submitted to the GenBank at the National Center for Biotechnology Information (NCBI) (Table 2). Specific primers were then chosen for these target genes and housekeeping gene (Table 2).

Table 1: List of degenerate primers (bp-basepair).

Gene	Sequence 5′→ 3′	Length (bp)
FASL	For: TGCAGCAGCCCWTSAATTAC	760
	Rev: TGRTCAGCACTGGTWAGATTGAA	
FAS	For: GACCCRGAATACCAAGTGC	450
	Rev: GSACYTTCTGYTCMGCTGTKTC	

To avoid genomic DNA amplification, primers were designed for two different exons and all primers design followed specific guidelines (Wang & Seed, 2006). To determine the most stable internal control gene, under our experimental conditions, three potential housekeeping genes (HKG) were initially considered (hypoxanthine phosphoribosyltransferase-1, β-actin and β2-microglobulin-*B2MG*). *B2MG* gene transcription was unaffected by our experimental conditions, and therefore, elected as HKG.

Table 2: Specific primers sequences used for quantitative real time PCR (bp-basepair)

Gene	Accession number	Sequence 5′→ 3′	Length (bp)
FASL	GQ429291	For: GCTGGTTGTTGCAGGACTGA	69
		Rev: TCAATGACACCGGGCTGTAC	
FAS	GQ429290	For: TTACGTGCAAACATGGGATCA	71
		Rev: TCCGGATCCTTCTCTGCATT	
B2MG	X69083	For: CGGGCTACTCTCCCTGACTG	92
		Rev: TTGGCTTTCCATTCTCTGCTG	

Real-time PCR assays were performed in a 7300 Real Time PCR System (Applied Biosystems™, Warrington, UK), using the default thermocycler program for all genes: a 10 min pre-incubation at 95ºC was followed by 40 cycles for 15 sec at 95ºC and 1 min at 60ºC. A further dissociation step (15 sec at 95ºC, 30 sec at 60ºC and 15 sec at 95ºC) ensured the presence of a single product. In each real time assay, both target gene and HKG were run simultaneously. All reactions were carried out in duplicate wells on a 96 well optical reaction plate (Applied Biosystems, ref. 4306737, UK) in 25 µl reaction volume: 6.5 µl water; 2 µl forward primer (160 nM); 2 µL reverse primer (160 nM); 12.5 µl Power SYBER® Green Master Mix (Applied Biosystems, Ref. 4367659, UK) and 2 µl of cDNA. After analysing the melting curves, PCR products were run through a 2.5% agarose gel to confirm specificity. Relative mRNA quantification data were then analyzed with the Real-time PCR Miner algorithm (Zhao & Fernald, 2005). Primer concentration was previously optimized to the ratio minimum concentration/lowest cycle threshold.

1.3.5 In vitro studies – cell culture model validation

In order to evaluate cytokines effect on luteal function, a luteal cell culture model with cells isolated from fresh CL was developed and optimized.

1.3.5.1 Luteal Cell isolation

Connective tissue and blood clot (whenever present) were removed and CL minced into small pieces. Enzymatic digestion was preformed for 50 min with M-199 (M2520, Sigma, St. Louis, USA) with 0.1% (w/v) collagenase (C-0130; Sigma, USA), 0.01% DNase I (D-5025; Sigma, USA) and 0.1% (w/v) BSA (#735078; Roche Diagnostics GmbH Mannheim, Germany) in Petri boxes at 37ºC. Every 10 min, dissociated cells were removed, and fresh medium containing enzymes was added. At the end, all collected cells were filtered twice with a cell dissociation Sieve - Tissue grinder Kit (cd1-1kt, Sigma, USA) through metal wire meshes [mesh 80, 190 µm opening size (S3770-5AE, Sigma, USA) and mesh size 200, 73.7 µm opening size (S4145-5AE, Sigma, USA)] to remove non dissociated tissue fragments. The filtrate was washed twice by centrifugation for 10min at 180 g with M-199 with 0.1% BSA. Supernatant was discarded. Later, erythrocyte lyses was accomplished by washing the pellet with Red blood lyses buffer (R7757, Sigma, USA) and two further washing steps were

performed. Cells were resuspended in Dulbecco's modified eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 [v/v], D-8900; Sigma, USA) containing 10% FBS (26140-079, Gibco, USA) and gentamicin [20 µg/ml]. Cell viability, determined by trypan blue exclusion dye (T8154, Sigma, USA), was higher than 85%.

Dispersed luteal cells ($2.0x10^5$/ml) were then cultured in 1 ml of D/F medium with 10% FCS, amphotericine [250 µg/ml] and gentamicin [20 µg/ml], in 24-well culture plates (142475, Nunc, Kamestrupvej, Denmark), at 37ºC with 5% CO_2. In order to validate the cell culture model, luteal cells isolated from different luteal stages (Early CL, n=4; Mid CL, n=4; and Late CL, n=4) were incubated for 24 h, with different concentrations of equine luteotrophic hormone (eLH, L9773; Sigma, USA). This dose and time course study was performed 8 h after cell plating, when cells adherent in a monolayer were 90-95% confluent. Cells were washed with M-199 with 0.1% BSA and phenol red free DMEM/F_{12} medium (1:1) (11039, Gibco, USA) with 0.1% BSA, gentamicin [20 µg/ml] and transferrin (T1428, Sigma, USA) [5 µg/ml] added. After a 30 min stabilization period, cells were incubated with 1, 10, 50 or 100 ng/ml eLH for 8 or 24 h. Afterwards, conditioned media from negative control (no LH added) and eLH treatment groups were stored at -30°C until P_4 concentration assessment.

1.3.5.2 Cytokines stimulation

To assess the adequate FASL (F0427, Sigma), TNF (T6674, Sigma, USA) or IFNG (407306, Calbiochem, San Diego, CA, USA) dose stimulation, luteal cells from Mid CL were cultured as described above and stimulated for 24 h as follows: no exogenous factor (Control); or with each one of the individual cytokines (TNF, IFNG, FASL) at three different concentrations (1 ng/ml; 10 ng/ml; 50 ng/ml). Conditioned media by luteal cells were stored at -30°C for further analysis.

1.3.5.3 Cytokine effect on progesterone and prostaglandins release

In order to study cytokines effect on prostaglandins and progesterone release from the equine CL, luteal cell cultures from Mid CL (n = 6) were stimulated for 24 h, in a 5% CO_2 chamber with: (i) no exogenous cytokines (negative control); (ii) FASL (10 ng/ml); (iii) TNF+IFNG (10 ng/ml) (as a positive control); and (vi) FASL+TNF+IFNG (10 ng/ml). Conditioned media were stored at -70°C until evaluation of P_4, PGE_2 and $PGF_{2\alpha}$ concentrations.

1.3.5.4 Cytokines influence on cell viability and apoptosis

Luteal cells (n=6) were plated in a 96-well cell culture plates (Corning, Corning, NY, USA), as described in Experiment 3.1 (experiments carried out simultaneously), at the concentration of $2.0x10^4$/ml and incubated for 24 h, at 37ºC in a humidified, 5% CO_2 atmosphere. Cell viability was determined with Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (G3581, Promega, Madison, WI, USA), according to the manufacturer manual. Absorbance (A) was read at 490 nm using a microplate reader (Model 450, BIO-RAD, Hercules, CA, USA).

Poly (ADP-ribose) polymerase-1 (PARP-1) assay kit (4677-096-k, R&D Systems, Abingdon, United Kingdom) was used in order to assess apoptosis. After a 24 h stimulation with cytokines (task performed after Experiment 3), Mid CL cells were scraped from the wells (541070, Greiner bio-one, Monroe, NC, USA) and kept in RNA later at -70ºC, until PARP-1 activity quantification. Protein was extracted from the cells and quantified as described before (Osnes *et al*., 1993). According to the manufacturer manual, a minimum of 20 µg of protein was used. PARP standard curve consisted on serial dilutions of PARP HAS-enzyme and the negative control was PARP buffer without enzyme or cells. Absorbance was measured at 630 nm with a plate reader (KHB ST-360; Shanghai Kehua Laboratory System, Shanghai, China) at room temperature.

1.3.6 Hormone determinations

Concentrations of P_4, PGE_2 and $PGF_{2\alpha}$ were determined directly from the cell culture media by direct enzyme immunoassay (EIA). As described previously (Korzekwa *et al*., 2004) for P_4 concentration assessment, antiserum was used at a final dilution of 1:100,000. Horseradish peroxidase (HRP)-labeled P_4 was used at a final concentration of 1:75,000. The standard curve ranged from 0.39 to 100 ng/ml and the concentration of P_4 at 50% binding (ED50) was 4.1 ng/ml. The intra- and inter-assay coefficients of variation (CV) were 5.5% and 8.5%, respectively.

Assessment of PGE_2 and $PGF_{2\alpha}$ concentrations followed the methodology previously described (Skarzynski & Okuda, 1999). The PGE2 standard curve ranged from 0.39 ng/mL to 100 ng/mL, and the concentration of 50% binding (ED50) was 6.25 ng/mL. The intra- and interassay coefficients of variation were 1.6% and 11.0%, respectively. The $PGF_{2\alpha}$ standard curve ranged from 0.016 ng/mL to 4 ng/mL, and the concentration of 50% binding (ED50)

was 0.25 ng/mL. The intra- and interassay coefficients of variation were on average 7.1% and 11.3%, respectively.

1.3.7 Statistical analysis

Data regarding western blotting analysis and real time PCR in the luteal tissue, as well as P_4, PGE_2 and $PGF_{2\alpha}$ concentrations in culture medium and cell viability were analyzed using one way analysis of variance followed by Bonferroni's Multiple Comparison Test (ANOVA; GraphPAD PRISM, Version 4.00, GraphPad Software, San Diego, CA, USA). Statistical differences in mRNA and protein were compared by paired student's *t*-test (GraphPAD PRISM, USA). Significance was defined as values of $p<0.05$.

1.4 Results

1.4.1 FASL and FAS in the equine CL

Immunohistochemistry analyses for active FASL and FAS showed that both the cytokine and its receptor were present in large luteal cells (LLC) and endothelial luteal (ELC) cells throughout the luteal phase (Fig. 5). No staining was present both in small luteal cells (SLC) (Fig. 5f) and in negative controls (Fig. 5a).

When comparing the different stages of the luteal phase, expression of FASL and FAS protein by western blotting analysis showed that immunoreactive bands intensity for FASL increased from Early to Late CL ($p < 0.05$; Fig. 6a), while FAS protein expression did not change throughout the luteal phase (Fig. 6. b).

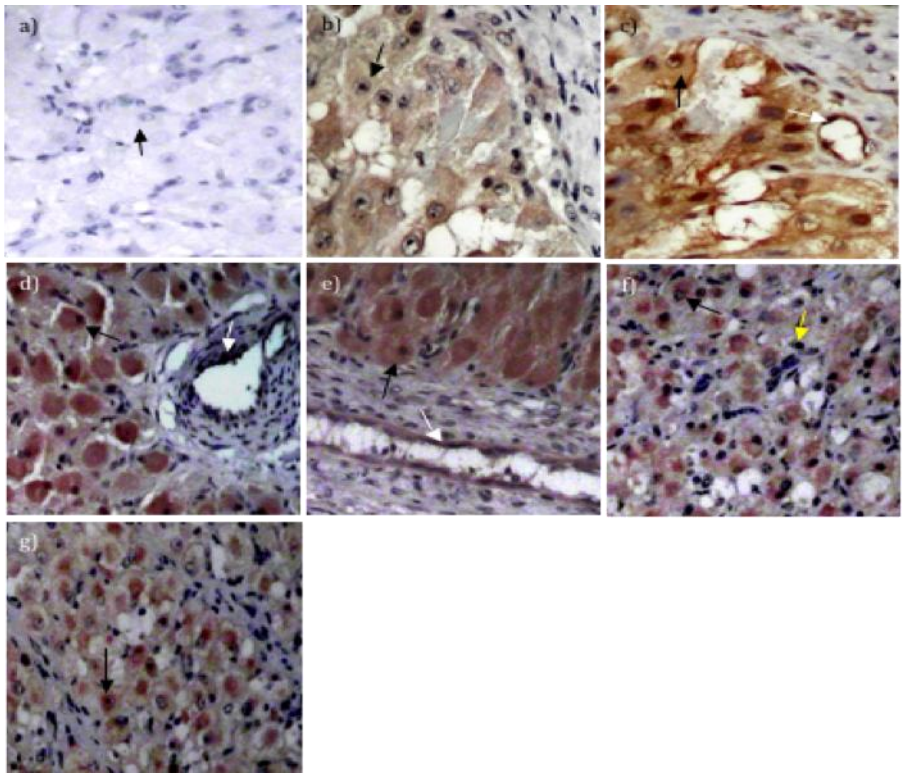


Figure 5: FASL and FAS immunostaining in 4 µm histological sections of equine CL. Black arrow indicates LLC, yellow arrow indicates SLC and white arrow ELC. a) negative control; b) Early CL FASL; c) Early CL FAS; d) Mid CL FASL; e) Mid CL FAS; f) Late CL FASL; g) Late CL FAS. Photographed at the magnification 400x.

Assessment of *FASL* and *FAS* gene transcription in the mare's CL was possible after degenerate primers (Table 1) design and specific primers choice for these target genes and HKG respectively (Table 2). In the mare's Early and Mid CL, *FASL* mRNA transcription was reduced with respect to Late CL ($p<0.05$; Fig. 7a), whereas its receptor *FAS* showed no changes (Fig. 7b).

1.4.2 In vitro studies – cell culture model validation

Different cellular components of the CL (small and large luteal cells, fibroblasts, endothelial cells, among others) were isolated and cultured together, in order to mimic *in vivo* luteal environment, as much as possible. After luteal cell isolation, about 25% of the isolated cells were LLC in the early CL, while 70% were SLC. Mid and Late CL consisted of 70% LLC and 25% of SLC. All eLH doses used to test the luteal cell culture model showed luteal cell

viability and P_4 responsiveness. After a 24 h eLH Mid CL luteal cell stimulation, P_4 release was higher at the concentration of 10 ng/ml ($p<0.001$; Fig. 8).

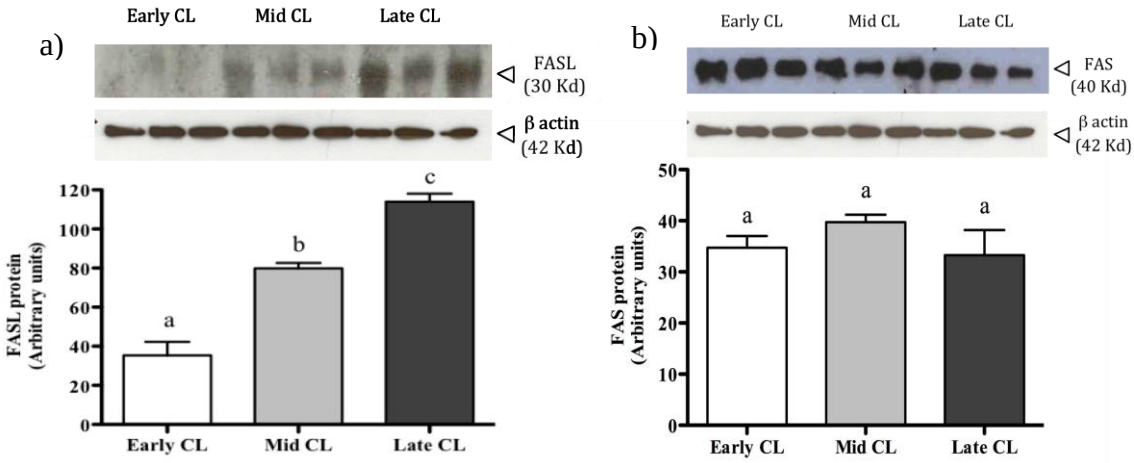


Figure 6: FASL (a) and FAS (b) protein expression in equine luteal tissue. Upper panels depict Western blotting for FASL and FAS. Data were normalized against β actin density values. Bars present mean±SEM. Different superscripts indicate significant differences ($p < 0.05$).

1.4.3 Cytokines stimulation

1.4.3.1 Cytokines stimulation dose assessment

This preliminary experiment allowed for the assessment of the most adequate cytokine stimulation dose. The concentration of 10 ng/ml for all cytokines studied was chosen since it showed the most consistent results (data not shown).

1.4.3.2 Cytokines effect on progesterone and prostaglandins release

The evaluation of FASL effect on Mid CL luteal steroidogenesis showed that FASL inhibited *in vitro* P_4 release ($p<0.05$; Fig. 9a). Nevertheless, when FASL was associated with other cytokines, no effect was observed. Regarding PGE_2 output from luteal cells, all treatments reduced its production, when compared to negative control ($p<0.05$; Fig. 9b). In contrast to this inhibitory effect, FASL stimulated $PGF_{2\alpha}$ output with respect to control ($p<0.001$; Fig. 9c). The association of three cytokines (FASL+TNF+IFNG) was also capable of stimulating

$PGF_{2\alpha}$ release, even though its effect was not as strong as when FASL was used alone ($p<0.05$; Fig. 9c).

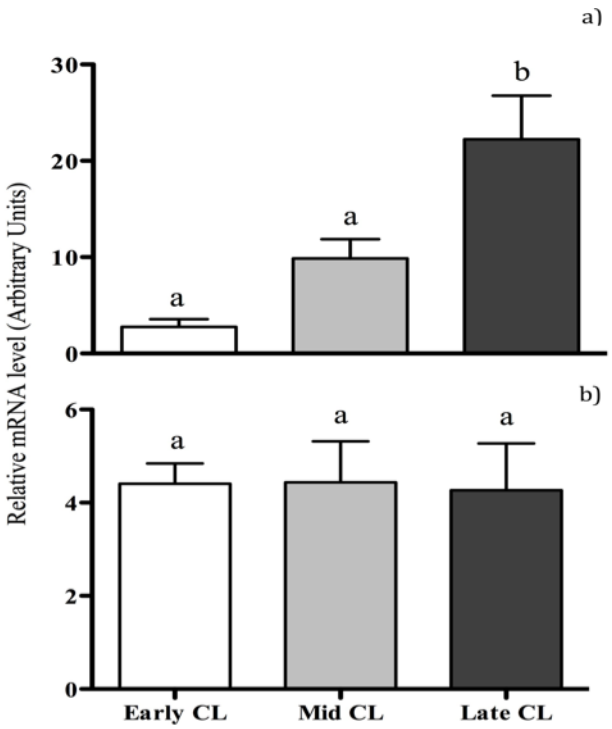


Figure 7: Relative quantification of gene transcription by real time PCR, throughout the luteal phase. Expression normalized with the housekeeping gene – B2MG. Bars represent mean±SEM. Different letters indicate significant differences ($p<0.05$). a) *FASL* gene; b) *FAS* gene.

1.4.3.3 Cytokines influence on cell viability and apoptosis

A reduction in luteal cell viability occurred when cells were exposed to the association of cytokines FASL+IFNG+TNF, with respect to negative control ($p<0.01$), or to positive control TNF+IFNG ($p<0.05$; Fig. 10a). Apoptosis, evaluated by PARP 1 activity, showed it was significantly increased in cells stimulated with TNF+IFNG ($p<0.01$) and the cytokines association FASL+IFNG+TNF ($p<0.001$; Fig. 10b).

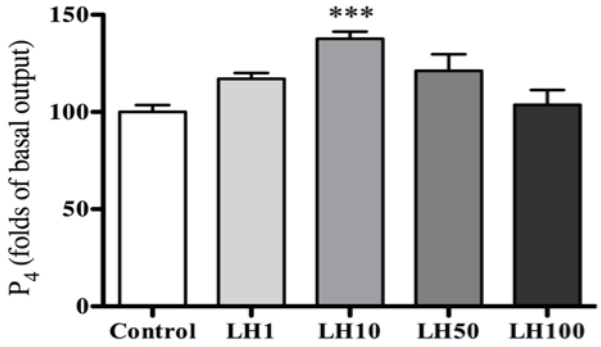


Figure 8: Effects of LH on P_4 production by Mid CL cells after a 24 h stimulation. Stimulation dose - ng/ml. Bars represent mean±SEM. Asterisks indicate significant differences ($p<0.001$).

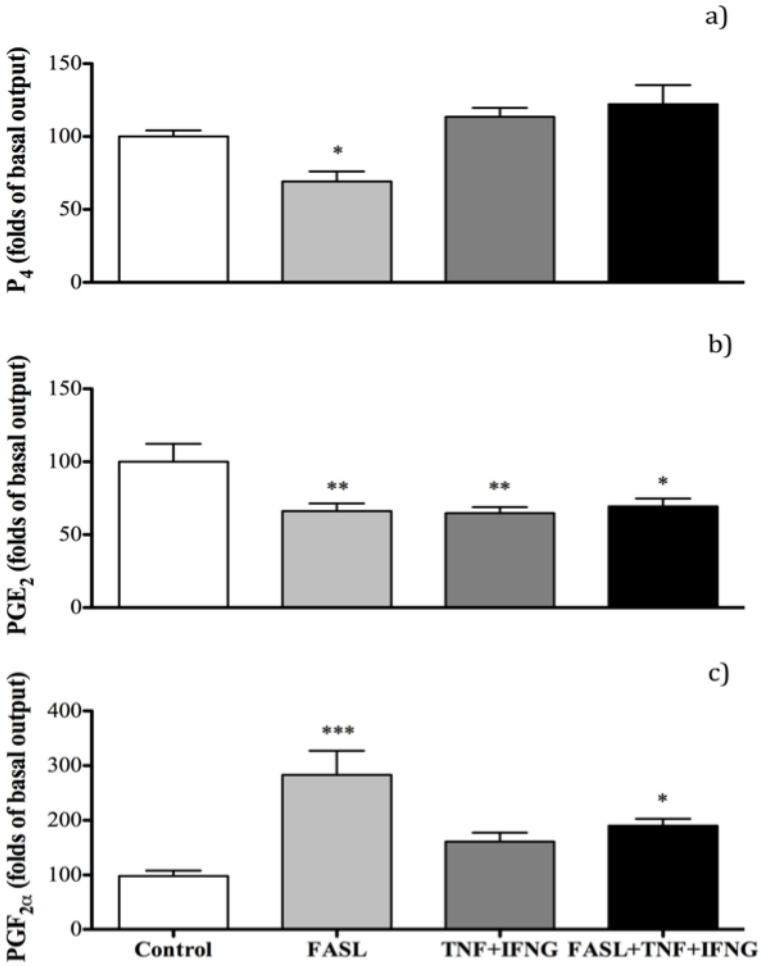


Figure 9: Mid CL luteal cells *in vitro* production of: a) P_4; b) PGE_2, and c) $PGF_{2\alpha}$, after 24 h stimulation by no exogenous cytokines - Control; or by FASL 10 ng/ml; TNF + IFNG 10 ng/ml of each, and FASL + IFNG + TNF 10 ng/ml of each. Bars represent mean±SEM. Asterisks indicate significant differences (*$p<0.05$; **$p<0.01$; *** $p<0.001$).

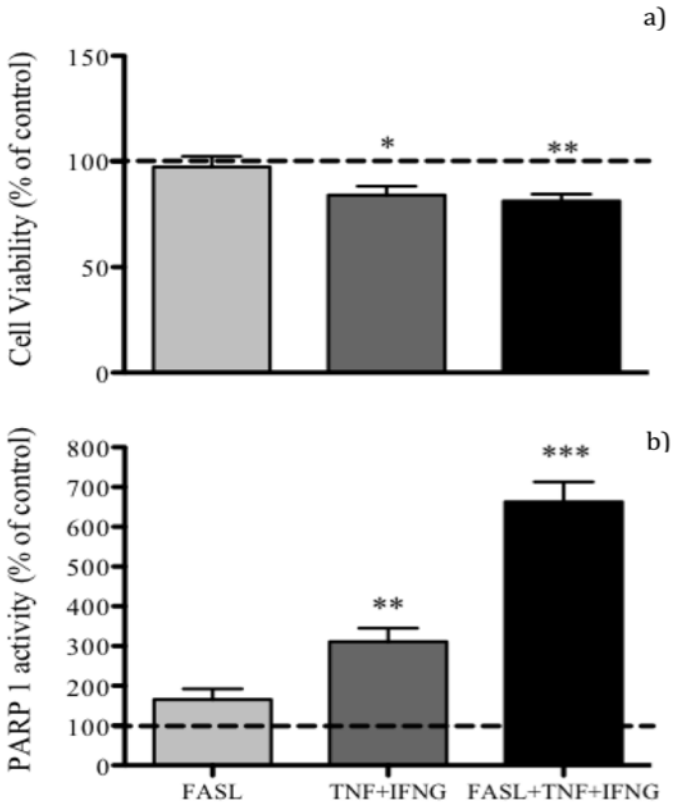


Figure 10: Equine Mid CL luteal cells viability (a) and PARP-1 activity (b) after a 24 h stimulation with cytokines at the concentration of 10 ng/ml. Control indicated by dashed line. Bars represent mean±SEM. Asterisks indicate significant differences (*p<0.05; **p<0.01;*** p<0.001).

1.5 Discussion

To the best of our knowledge, this is the first study showing the presence of FASL and its receptor FAS in the equine CL, describing the cell types where they are expressed, as well as their mRNA transcription and protein expression profiles throughout the luteal phase. Moreover, the study of FASL effect, alone or together with other cytokines, on prostaglandins and P_4 production by Mid CL luteal cells, helped to clarify the role of cytokine-receptor complex on mares CL regression. Their influence on luteal cell viability and apoptosis was also addressed. Like in other species, such as mouse, cow or rat (Sakamaki *et al*., 1997; Slot *et al*., 2006), the cytokine FASL and its receptor FAS were expressed throughout the luteal phase in the mare. Their presence might indicate a physiologic role during the luteal phase, since biological activities of these molecules are dependent on their expression profiles. Okuda and Sakumoto (2003) described the importance of TNF Superfamily members on luteal events, including establishment, maintenance and regression of the CL. Other studies suggested that FAS-mediated apoptosis plays an important role in structural regression of the

CL in a number of species (Roughton *et al*., 1999; Sakamaki *et al*., 1997; Taniguchi *et al*., 2002). Specifically, in the mare, both *FASL* mRNA transcription and protein expression increased towards the end of the luteal phase, while no significant changes were observed for the receptor *FAS*. This dramatic increase in FASL expression in the Late CL suggests its involvement on luteolysis in this species. Even though the *FAS* gene was transcripted and its protein expressed, no changes were present throughout the luteal phase.

Although FAS/FASL system has been related with CL structural regression (Roughton *et al*., 1999; Taniguchi *et al*., 2002), its precise role on luteal steroidogenic capacity during physiologic CL regression is lacking. In a study undertaken by Pru *et al*. (2002) FASL stimulation of bovine steroidogenic luteal cells had no effect on P_4 production. On the contrary, in the present study, equine Mid CL cell stimulation with FASL alone significantly reduced P_4 concentration in the culture medium. Also, in pseudopregnant mice, the *in vivo* administration of FAS-activating antibody decreased P_4 production (Sakamaki *et al*., 1997). In our study, after *in vitro* luteal cell stimulation with FASL alone, both luteotrophic factors P_4 (Roberto da Costa *et al*., 2005) and PGE_2 (Schams *et al*., 1995) decreased, while the luteolytic hormone $PGF_{2\alpha}$ production increased. This is the first evidence that FASL alone can influence luteal steroidogenesis and eicosanoids production in the mare, suggesting that FASL can work as an important regulator of functional luteolysis in the mare. The cytokine association TNF+IFNG, used as a positive control, has been tested in *in vitro* cytotoxicity studies (Pate, 1995; Petroff *et al*., 2001). Also, cytokines synergic action on luteal $PGF_{2\alpha}$ stimulation has been shown in different species (Kurusu *et al*., 2007; Petroff *et al*., 2001). However, in the present study in the mare, TNF+IFNG only inhibited PGE_2 output, with no effect on $PGF_{2\alpha}$ production by the CL. When FASL was used together with the other cytokines (FASL+TNF+IFNG) a significant production of $PGF_{2\alpha}$ was seen, as well as a PGE_2 reduction. Nevertheless, further studies should be performed in order to clarify the specific role of FASL on intraluteal synthesis of prostaglandins and P_4 and also FASL interactions with other factors involved in CL regulation. Luteotrophic factors, like P_4 and PGE_2 might have a protective role by preventing luteal cell vulnerability to apoptotic mechanisms, possibly regulating the ratio of pro/anti-apoptotic gene translation and protein expression. Thus, equine luteal cell susceptibility to FASL action might depend on the absence of P_4 protective role, as shown before (Feng *et al*., 1995; Liszewska *et al*., 2005; Rotello *et al*., 1992). In the mare, the coordination between steroidogenic impairment and apoptosis during CL regression is complex, and might depend on the temporal relationship among different factors/hormones and their concerted action during luteolysis.

In the present study, equine luteal cell stimulation with more than one cytokine always markedly reduced cellular viability. This synergic effect between cytokines on luteal cell viability reduction was also reported when TNF and IFNG increased FASL cytotoxic effect in cow (Taniguchi *et al*., 2002), and murine luteal cells (Ginther *et al*., 2005). Nevertheless, in the cow, in disagreement with the present study, the deleterious effect of FASL alone on cell viability and the increase in apoptosis, after a 24 h stimulation, was shown (Pru *et al*., 2002). In fact, in the present work no agreement between luteal fuctional modulation and structural regulation was found. Cytokine FASL alone, after a 24 h incubation, was able to inhibit P_4 and PGE_2 output and reduce $PGF_{2\alpha}$, but had no effect on cell viability or apoptosis. We may speculate if cells were submitted to FASL for longer incubation periods (48h, 72h) it would significantly affect cell viability and apoptosis. Therefore, this emphasizes the need for further studies. When comparing plasma P_4 pattern level throughout the luteal phase between both species it is evident that the luteolytic process in the mare starts earlier than in the cow. In the mare, P_4 starts dropping around day 11-12 of the cycle, after ovulation (Ginther *et al*., 2005), resulting in a long luteolytic process, as well as a long follicular development period, prior to ovulation. According to Ginther *et al*. (2005), in the mare, luteolysis is related to follicles estradiol production, which increase might be needed for structural regression to occur. Thus, association of these specific physiologic aspects in the mare, might explain the different chronologic susceptibility of steroidogenic luteal cells to FASL apoptosis induction, with respect to the cow. It was evident that the association between three cytokines (FASL+TNF+IFNG) was highly effective on triggering apoptosis, in the mare. As shown in other species, such as cow (Miyamoto & Shirasuna, 2009; Yadav *et al*., 1995) or woman (Duncan *et al*., 2009; Kondo *et al*., 1996; Quirk *et al*., 1995), the joint action of these cytokines seem to be determinant for structural involution and luteolysis accomplishment.

In conclusion, the present work shows the presence of FASL and FAS in the equine CL and points out, for the first time, the important role of FASL on both functional and structural luteolysis. FASL alone can inhibit steroidogenesis and modulate $PGF_{2\alpha}$ and PGE_2 production in the equine corpus luteum. The physiologic luteal functional involution in the mare is an intricate process with multiple intervenient factors/hormones and actions that deserve to be further studied.

2. THE CYTOKINES TUMOR NECROSIS FACTOR-α AND INTERFERON-γ PARTICIPATE IN THE MODULATION OF THE EQUINE CORPUS LUTEUM AS AUTO-, PARACRINE FACTORS

(Published in *Journal of Reproductive Immunology*)

1.1 Abstract

Knowledge on the regulation of corpus luteum (CL) function in the mare is scarce. In this study, presence of cytokines tumor necrosis factor alpha (TNF) and interferon gamma (IFNG), and their receptors (TNFRI, TNFRII and IFNRI), were investigated in equine CL throughout the luteal phase (LP). Further on, TNF and IFNG effects on luteal *in vitro* secretory function and viability were demonstrated. Ligands and receptors were present in large luteal steroidogenic and endothelial cells. Protein expression for TNF was greater in mid and late LP, for TNFRI was increased during late LP and TNFRII did not change. IFNG and IFNRI showed the highest expression in late LP. Transcription of mRNA for *TNF* was greater in early and late LP, *TNFRI* was the greatest in the early CL, less in late than in mid LP, and *TNFRII* was greater in early and mid than late LP. Transcription of mRNA for *IFNG* was less in CL from early than mid or late LP, and for *IFNRI* was not changed. In the early LP, TNF increased P_4 and PGE_2 but decreased $PGF_{2\alpha}$ secretion; in the mid LP, TNF increased $PGF_{2\alpha}$ secretion and TNF+IFNG decreased PGE_2 secretion; in the late LP, TNF, IFNG and TNF+IFNG decreased P_4 and PGE_2 secretion, but TNF and TNF+IFNG increased $PGF_{2\alpha}$ secretion by luteal cells. Cell viability was reduced by TNF+IFNG in late LP. These data show the presence of cytokines TNF and IFNG, and their receptors, in the equine CL and their potential involvement in luteal function regulation.

2.2 Introduction

It has been known for decades that an immuno-endocrine crosstalk is essential for normal ovarian function in the non-pregnant female (Pepperell *et al*., 1992). Inflammatory-like mechanisms are involved in ovarian function including follicle development, oocyte maturation and ovulation (Espey *et al*., 1994). Ovulation process requires extracellular matrix reorganization, microvascular changes, chemokines expression, and recruitment of leukocytes from circulating blood (Brannstrom & Enskog, 2002). Moreover, hormones like progesterone (P_4) or prostaglandins (PG), and factors including nitric oxide (NO), and growth and angiogenic factors are involved in regulation of corpus luteum (CL) function (Ferreira-Dias *et al*., 2006a; Ferreira-Dias *et al*., 2011). Also cytokines, such as tumor necrosis factor alpha (TNF) and interferon gamma (IFNG), mainly produced by immune cells in the CL (Pate, 1995), play specific roles in luteal establishment (Skarzynski *et al.*, 2008).

In the absence of pregnancy, luteolysis is crucial for resumption of a new ovarian cycle. In a number of species, the presence of different inflammatory cells like mast cells (Mori, 1990), macrophages and lymphocytes (Brannstrom & Norman, 1993) has been demonstrated in the ovary throughout the estrous cycle. Particularly in the mare, $CD4^+$ and $CD8^+$ cytotoxic T cells are present in the CL during luteolysis (Lawler *et al*., 1999). Other than T-lymphocytes, neutrophils and macrophages predominate in the CL around the time of luteolysis and may be directly involved in luteal cells destruction and subsequent P_4 secretion loss (Brannstrom *et al*., 1994). Besides, cytokines TNF and IFNG were shown to be involved in bovine CL regression (Fairchild & Pate, 1989; Fairchild & Pate, 1992; Pate, 1995; Skarzynski *et al*., 2007). Also, cytokine FasL was shown to regulate secretory function during luteal regression in mare (Galvão *et al*., 2010).

However, in spite of some research on other species, to the best of our knowledge, the precise role of cytokines TNF and IFNG in regulation of equine CL growth, differentiation and regression has not been clarified yet. Thus, the working hypothesis was that local factors such as cytokines TNF and IFNG might be involved in regulation of equine CL growth, differentiation and regression. Therefore, this study was carried out to evaluate (i) protein and mRNA transcription for cytokines TNF and IFNG and their receptors (TNFRI, TNFRII, IFNRI) in equine CL, and (ii) *in vitro* effects of TNF and IFNG on P_4, PGE_2 and $PGF_{2\alpha}$ secretion by luteal cells and cell viability throughout the estrous cycle in the mare.

2.3 Materials and Methods

2.3.1 Collection of equine corpora lutea

Blood samples and CL were collected *post mortem* at the abattoir, as by-products, from randomly designated cyclic Lusitano mares (3 to 8 years old) from early April to late September. After stunning, mares were euthanized, according to Portuguese legislation (DL 98/96, Art. 1º) and European Legislation concerning welfare aspects of animal stunning and euthanasia methods (EFSA, AHAW/04-027), and approved by the Faculty of Veterinary Medicine Ethics Committee.

Luteal structures were classified based on plasma P_4 levels, follicle size and morphological appearance of the CL, as described previously (Galvão *et al*., 2010). The CL was categorized as: early luteal phase CL (early CL; n=11), mid-luteal phase CL (mid CL; n=11) and late-luteal phase CL (late CL, n=11). Immediately after collection, CL were prepared as described (Galvão *et al*., 2010). Blood samples were collected into heparinized tubes (Monovettes®-Sarstedt, Numbrecht, Germany) for estrous cycle confirmation based on plasma P_4 concentration.

2.3.2 Immunohistochemistry

The presence of the cytokines and their receptors in the CL throughout the luteal phase was determined as described previously (Ferreira-Dias *et al*., 2006b). Immunohistochemistry studies were performed on consecutive 4 µm histological sections, for TNF, IFNG and the receptors TNFRI, TNFRII and IFNRI protein identification in the CL (early CL, n=6; mid CL, n=6; late CL, n=6). Tissue sections were incubated for 3 hours at room temperature with primary rabbit polyclonal antibodies against TNF (anti-equine TNF alpha, 1:500, AHP853Z, AbD Serotec, Oxford, UK), IFNG (anti-human IFN gamma, 1:200, sc-8308, Santa Cruz Biotechnology, Santa Cruz, CA, USA), TNFRI (anti-human TNF receptor 1, 1:1000, ab19139, Abcam, Cambridge, UK), TNFRII (anti-mouse TNF receptor 2, 1:50, ab15563, Abcam) or IFNRI (anti-human IFN receptor 1, 1:100, ab61179, Abcam). Primary antibody was detected using biotinylated secondary antibody (Histostain Plus; Zymed Laboratories Inc., Ref. 85-9043) for 30 minutes at room temperature. Sections were incubated 30 minutes with streptavidin-peroxidase (Histostain Plus; Zymed Laboratories Inc., Ref. 85-9043) and for colour development was used 3,3´- diaminobenzidine for 5 minutes. Negative control was performed by replacing the primary antibody by 0.1 M PBS (pH 7.4). Expression of a specific

antigen was assessed as a characteristic nuclear and cytoplasm brown staining using a light microscope (Olympus BX51, Tokyo, Japan) at 400x magnification. Tissue areas were photographed (DP11 Olympus, Tokyo, Japan). Histologic identification of different cell types was based in previous work in the mare CL (Al-zi'abi *et al*., 2003; Roberto da Costa *et al*., 2005).

2.3.3 Western blot

Protein expression of TNF, IFNG and receptors TNFRI, TNFRII and IFNRI on equine luteal tissue was assessed by Western blot, throughout the luteal phase. Luteal tissue (n=4/stage) was processed as described (Ferreira-Dias *et al*., 2006b). Level of active studied proteins was evaluated with the same antibodies used for immunohistochemistry, but diluted at 1:300 for TNF, 1:100 for IFNG, 1:300 for TNFRI, 1:200 for TNFRII and 1:200 for IFNRI. To normalize loaded protein, a mouse monoclonal antibody against ß actin (A5441, Sigma, USA) was used at the dilution 1:10,000. For all proteins, except for ß actin, the secondary antibody used was horseradish peroxidase (HRP)-conjugated anti-rabbit (P0448, Dakocytomation, Carpinteria, CA, USA), at 1:10,000. For ß actin HRP-conjugated goat anti-mouse (A2554, Sigma, USA) was used at 1:100,000.

2.3.4 Real Time PCR analysis

Extraction, quantification and quality assessment of RNA from equine CL (n=6/stage) was performed as described (Galvão *et al*., 2010). For *TNFRI* and *IFNRI* genes, degenerate primers were designed, due to the lack of previously sequenced mRNA sequence for horse in Gene Bank (National Center for Biotechnology Information - NCBI, National Institutes of Health, Bethesda, MD; http://www.ncbi.nlm.nih.gov/) (Table 3) (Galvão *et al*., 2010). Specific primers for the Housekeeping gene, after optimization *(β2 microglobulin - B2MG*) (Galvão *et al*., 2010), and target genes were then chosen (Table 4). Real time PCR was run and data analyzed as described (Galvão *et al*., 2010).

Table 3: List of degenerate primers (bp-basepair).

Gene	Sequence 5′→ 3′	Length (bp)
TNFRI	For: TGCTGYACSAAGTGCCACAAA	890
	Rev: CTGTGBCACACYCCGAC	
IFNRI	For: GTTAAAGCYARGGTTGGACA	557
	Rev: ACAGAGAKCAAGGACTTRGGTAA	

Table 4: Specific primers used for quantitative real time PCR (bp-basepair).

Gene	Accession number	Sequence 5′→ 3′	Length (bp)
TNF	AB035735	For: ACCGAATGCCTTCCAGTCAA	143
		Rev: CATTTGCACGCCCACTCA	
TNFRI	GU166822	For: TCAACGGCACAGTGCATCT	98
		Rev: CAGGACATGCTCTCTT	
TNFRII	XM_001489844.2	For: TGCATACTTCCAAGGCAGGAG	108
		Rev: GCACACCACGTTTGATGTCG	
IFNG	NM_001081949.1	For: TAACAGCAGCACCAGCAAGC	165
		Rev:CGAAATGGATTCTGACTCCTCTTC	
IFNRI	GU166821	For: CCGAAGAACTCTGCCTGACC	113
		Rev: ACAACACGCAACCAGCAGAAT	
B2MG	X69083	For: CGGGCTACTCTCCCTGACTG	92
		Rev: TTGGCTTTCCATTCTCTGCTG	

2.3.5 In vitro studies

2.3.5.1 Luteal Cell isolation

Equine luteal cell isolation followed the method described by Galvão *et al*. (2010). Cell viability, determined by trypan blue exclusion dye (T8154, Sigma), was greater than 88%.

Dispersed luteal cells ($2.0x10^5$/mL) were cultured with different treatment (as described below) in 1 mL of D/F medium with 10% FBS, amphotericin (250 µg/mL) and gentamicin

(20µg/mL), in 24-well culture plates (142475, Nunc, Kamestrupvej, Denmark), at 37ºC in humidified atmosphere (5% CO_2, 95% air).

2.3.5.2 Cytokine stimulation dose assessment

Optimal TNF (T6674, Sigma) and IFNG (407306, Calbiochem, San Diego, CA, USA) human recombinant cytokines dose for luteal cell stimulation was determined using luteal cells from mid CL. Cells were cultured as described (Galvão *et al*., 2010). After 30 to 45 min of stabilization, cells were incubated with: 0 (Control); 1; 10 or 50 ng/mL TNF or IFNG for 24 h at 37ºC in humidified atmosphere (5% CO_2 and 95% air). Conditioned media by luteal cells were stored at -70°C.

2.3.5.3 Cytokine effect on progesterone and prostaglandins secretion

Luteal cells from early, mid and late CL (n=5/stage) were incubated for 24 h with: (i) no exogenous cytokines (Control); (ii) TNF (10 ng/mL); (iii) IFNG (10 ng/mL) and (vi) TNF+IFNG (10 ng/mL each). Conditioned media were stored at -70°C until P_4, PGE_2 and $PGF_{2\alpha}$ concentration assessment.

2.3.5.4 Cytokines influence on cell viability

Luteal cells from all luteal stages were plated in a 96 well (1.0 x 10^4cells /mL) cell culture plates (Corning, Corning, NY, USA), and cultured for 24 h with or without TNF or IFNG, as described in 2.5.2 (experiments performed simultaneously). Cell viability was determined with Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega, Ref.G3581, Madison, WI, USA), according to manufacturer manual.

2.3.6 Hormone determinations

Concentrations of P_4, PGE_2 and $PGF_{2\alpha}$ luteal conditioned media and P_4 in plasma were determined by direct enzyme immunoassay (EIA). Assay conditions followed previously described method (Galvão *et al*., 2010). Hormones concentration in culture media was normalized for the number of live cells, after viability assessment (base line corresponded to control level).

2.3.7 Statistical analysis

Data were analyzed using one way analysis of variance followed by Bonferroni's Multiple Comparison Test (ANOVA; GraphPAD PRISM, Version 5.00, GraphPad Software, San Diego, CA, USA). Significance was defined as $p<0.05$.

2.4 Results

2.4.1 Cytokines and their receptors protein expression

Immunohistochemistry demonstrated the presence of both ligands TNF, IFNG, and their specific receptors TNFRI, TNFRII and IFNRI in large luteal luteal cells (LLC) and endothelial luteal cells (ELC), but not in small luteal cells (SLC). No staining was present in negative control (Fig. 11f) and SLC (yellow arrow) (Fig. 11a). In early and mid CL, an evident staining was present in LLC cytoplasm (Fig. 11a, b and c - black arrow), as well as in endothelial cells (Fig. 11a, b - white arrow). In late CL, LLC depicted some stain among vacuoles while connective tissue did not stain (Fig. 11f). Protein expression analysis by western blot showed that TNF immunoreactive bands intensity increased from early to mid and late CL ($p<0.05$) (Fig. 12a), while TNFRI had the highest expression in late CL ($p<0.05$) (Fig. 12b) and TNFRII expression did not change throughout the luteal phase (Fig. 12c). For INF and IFNRI, protein expression was not detected in early CL, being the greatest in late CL ($p<0.05$) (Fig. 13a; Fig. 13b).

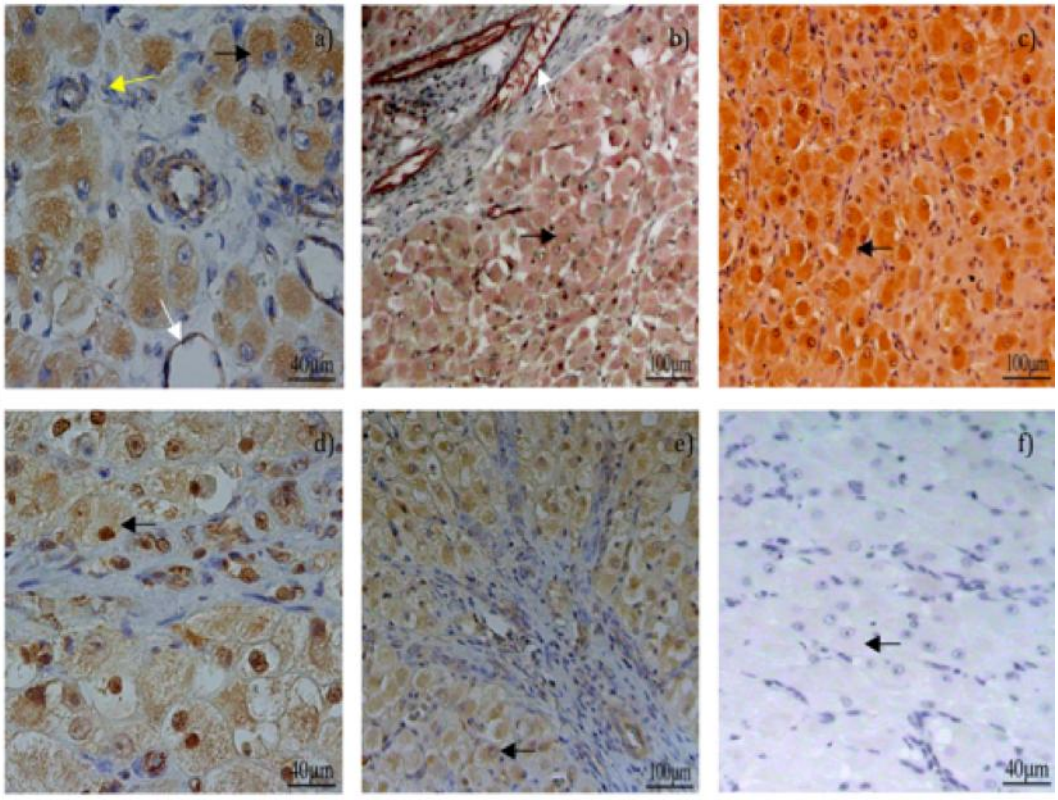


Figure 11: Representative images of equine CL immunostained for the presence of TNF in early CL (a), TNFRI in mid CL (b), IFNG in mid CL (c), TNF in late CL (d) and IFNRI in late CL (e). Negative control (f). Black arrow indicates LLC, yellow arrow indicates SLC and white arrow indicates ELC. Since all cytokines/receptors stained equally throughout the estrous cycle, pictures from each luteal phase were randomly assigned.

2.4.2 Cytokines and their receptors mRNA transcription

The *TNF* mRNA transcription in mare´s CL was reduced in mid CL, with respect to early CL ($p<0.05$) and increased in late CL ($p<0.05$) (Fig. 14a), whereas for *TNFRI* and *TNFRII* transcription decreased from early to late CL ($p<0.05$) (Fig. 14b; 14c). Concerning *IFNG* mRNA transcription was less in early CL, increasing afterwards, from early to late CL ($p\leq0.05$) (Fig. 15), while receptor *IFNRI* did not change in any luteal stage (data not shown)**.**

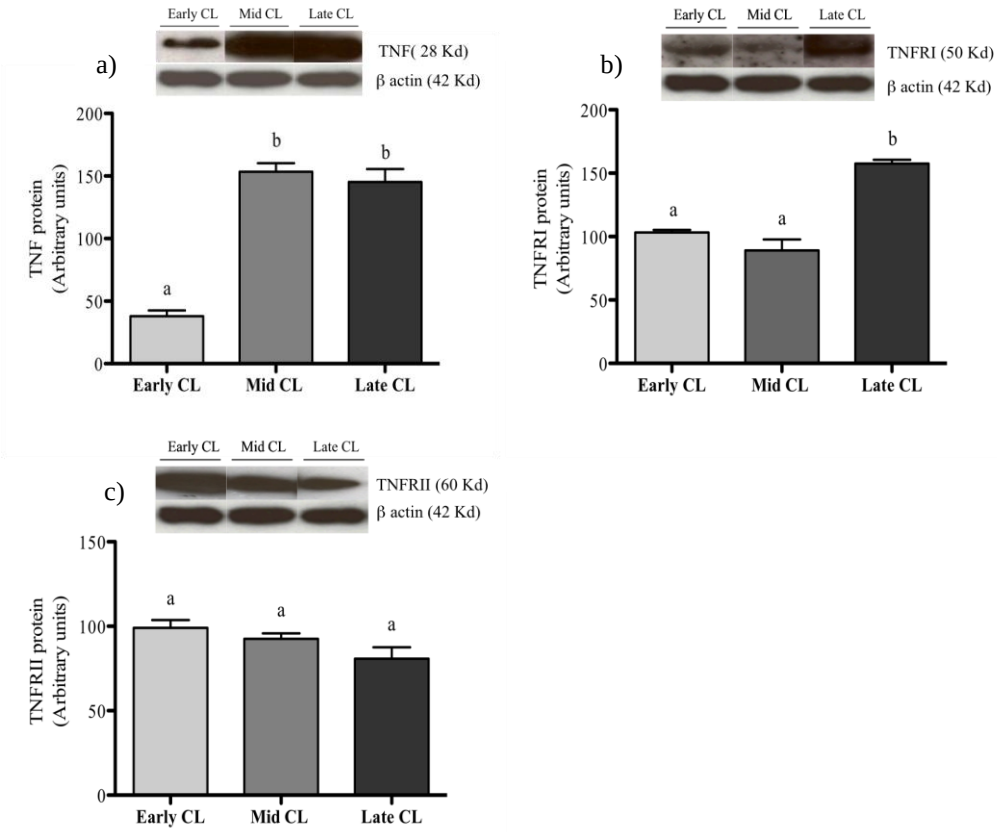


Figure 12: TNF (a), TNFRI (b) and TNFRII (c) protein expression in equine luteal tissue. Upper panels depict representative western blot (n=4). Data normalized against β actin density values. Bars represent mean±SEM. Different superscripts indicate significant differences ($p < 0.05$).

2.4.3 Cytokines stimulation dose assessment

Different cellular components of the CL (small and large luteal cells, fibroblasts, endothelial cells, among others) were isolated and cultured together. The concentration of 10 ng/mL for both cytokines studied was chosen since it showed the most consistent results ($P<0.05$) in the present work and other studies performed (data not shown).

2.4.4 Cytokines effect on progesterone and prostaglandins release

In early CL, TNF increased P_4 and PGE_2 but decreased $PGF_{2\alpha}$ secretion ($p<0.05$) (Fig 16a); in mid CL, TNF increased $PGF_{2\alpha}$ secretion and TNF+IFNG decreased PGE_2 secretion ($p<0.01$) (Fig 16b); in late CL, TNF, IFNG and TNF+IFNG decreased P_4 and PGE_2 secretion ($p<0.001$), but TNF and TNF+IFNG increased $PGF_{2\alpha}$ secretion by luteal cells ($p<0.05$) (Fig 16c).

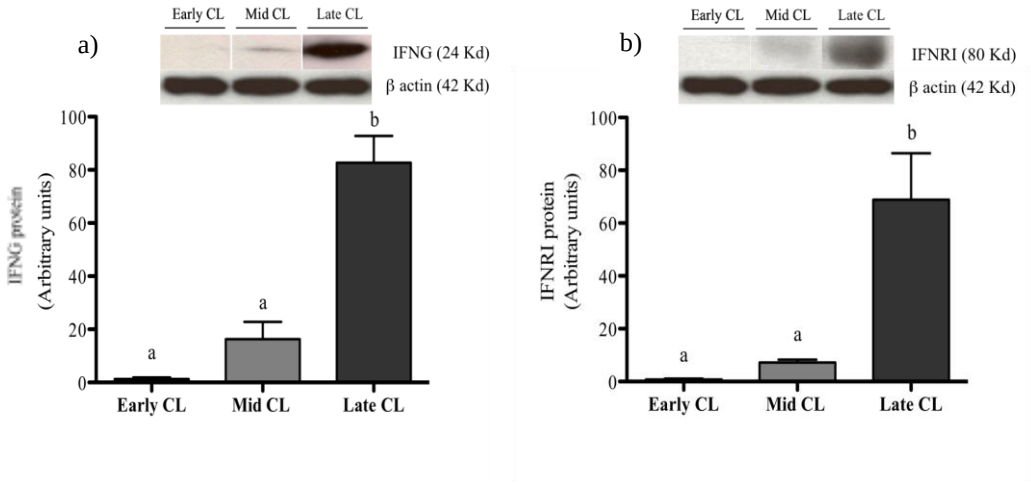


Figure 13: IFNG (a) and IFNRI (b) protein expression in equine luteal tissue. Upper panels depict representative western blot (n=4). Data normalized against β actin density values. Bars represent mean±SEM. Different superscripts indicate significant differences ($p < 0.05$).

2.4.5 Cytokines influence on cell viability

Both cytokines tested, either alone or combined, had no effect on *in vitro* luteal cell viability from early CL, but combined cytokines decreased ($P<0.05$) cell viability in mid (to 84±4.6%) and late (to 55±3.8%) CL.

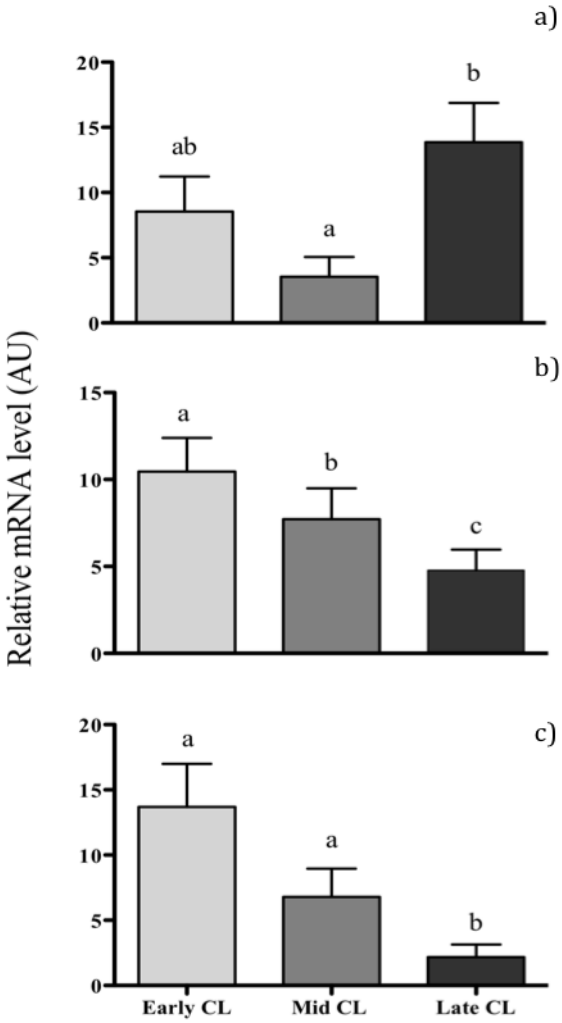


Figure 14: Transcription of mRNA for *TNF* (a), *TNFRI* (b) and *TNFRII* (c) throughout the luteal phase. Expression was normalized to the housekeeping gene - *B2MG*. Different letters indicate significant differences ($p<0.05$). AU – arbitrary units.

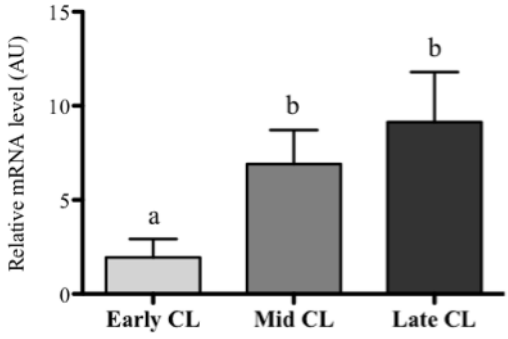


Figure 15: Transcription of mRNA for *IFNG* throughout the luteal phase. Expression was normalized to the housekeeping gene - *B2MG*. Different letters indicate significant differences ($p<0.05$). AU – arbitrary units.

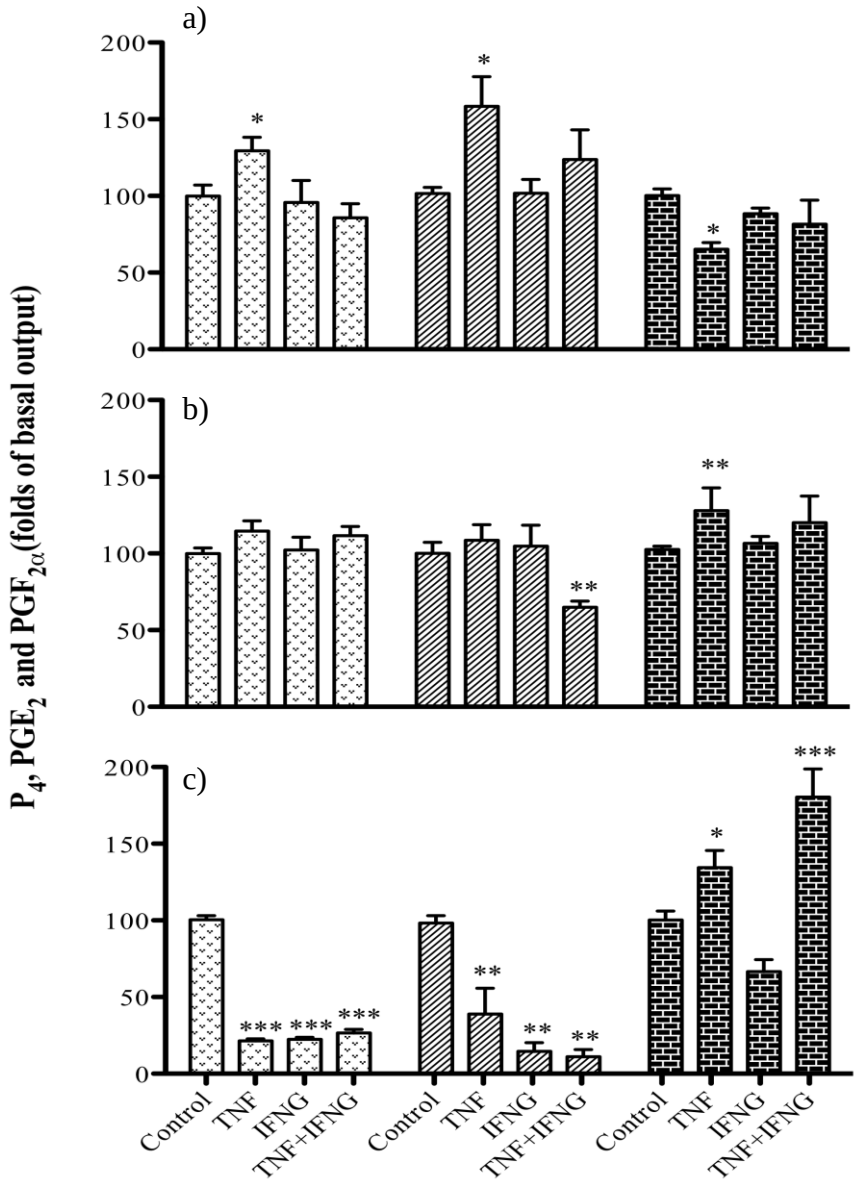


Figure 16: Effects of TNF and IFNG on P_4 , PGE_2 and $PGF_{2\alpha}$ production by luteal cells from the early (a), mid (b), and late (c) luteal phases. Asterisks indicate significant differences from a control (100%) value (*p<0.05; **p<0.01; *** p<0.001). The correspondent control values for hormones production (Mean ± SEM): for P_4 early CL (29.74± 3.39 ng/2.0x10^5cells), for P_4 mid/late CL (224.20± 30.00 ng/2.0x10^5cells) and for PGE_2 (8.95±2.11 ng/2.0x10^5cells) and $PGF_{2\alpha}$ (0.73±0.03 ng/2.0x10^5cells) throughout luteal phase.

2.5 Discussion

To the best of our knowledge, the expression of cytokines TNF and IFNG and their receptors was reported for the first time in equine CL, across the luteal phase. Moreover, cytokines were able to modulate luteal secretory capacity (P_4, PGE_2, $PGF_{2\alpha}$) and to influence luteal cell viability throughout the luteal phase. These data show that TNF may be involved on luteal growth and differentiation, while both cytokines TNF and IFNG may play a role on luteal regression in the mare.

Like in other species as cow (Petroff *et al*., 1999; Sakumoto *et al*., 2000), pig (Wuttke *et al*., 1997), or human (Vaskivuo *et al*., 2002), TNF, IFNG and their receptors protein was shown by immunohistochemistry in steroidogenic LLC and endothelial cells throughout the luteal phase. Auto-, paracrine regulation of luteal function by TNF and its receptors might be considered, since the pattern of their expression significantly changed throughout the luteal phase. Transcription of mRNA for *TNF* was greater in early and late CL than in mid CL, while TNF protein had the highest expression in mid and late CL. Apparently, the increase in this protein in mid CL, when mRNA transcription is decreased, seems less consistent. However, gene expression regulation is a complex process that may be influenced by different biological factors (Gry *et al*., 2009). Indeed, post-transcriptional modifications, like mRNA retention in the nucleus, do not originate immediate comparable levels of protein (Gorivodsky *et al*., 1998; Vannya *et al*., 2004). Similar results were seen for TNF expression in rat placenta (Gorivodsky *et al*., 1998), showing the intricacies of TNF expression regulation. Besides, increased protein expression in mid and late CL is in agreement with the hypothetical participation of TNF in luteal establishment and function, as well as regression. In addition, TNF can induce both cell proliferation and death, depending on which receptor it binds to (TNFRI or TNFRII) (Boldin *et al*., 1995; Hsu *et al*., 1995). Thus, also in the mare, its action might be differentially modulated depending on the receptor it binds to and the specific luteal stage. Moreover, cytokines effect depends on cell differentiation, as well as the presence of growth factors and other regulatory factors (Pate, 1995). Since in early and mid CL, TNFRII expression was markedly increased, this suggests that TNF interaction with its receptor II protein can promote luteal growth at this luteal stage. In contrast, TNFRI has an intracellular death domain required for signaling pathways associated with apoptosis via death domain (Bodmer *et al*., 2002). In the present study, the increased protein expression of TNFRI in late luteal phase CL suggests that, at that stage, TNF might differently drive its action towards luteolysis. In early CL in the mare *TNFRI* high mRNA transcription is in agreement with previous findings of Sakumoto *et al*. (2000) in the cow. However, contradictory results were

reported (Friedman *et al*., 2000; Korzekwa *et al*., 2008). In fact, post-transcriptional modifications might determine the absence of a positive relationship between mRNA transcription and protein translation for TNFRI observed in the mare CL.

Enhanced IFNG and IFNRI protein expression in late CL suggests the participation of these cytokines in luteolysis in the mare. Increased mRNA transcription for *IFNG* in mid CL could affect protein translation on the following luteal stage. Nevertheless, luteal mRNA transcription profile of *IFNG* in other species such as dog (Engel *et al*., 2005) and cow (Petroff *et al*., 1999) differed from this one in the mare, indicating species differences in luteolysis regulation.

The influence of cytokines TNF and IFNG on luteal secretory function coordination has been demonstrated (Petroff *et al*., 2001; Okuda & Sakumoto, 2003). Cytokine TNF is a potent stimulator of luteal PG production, such as PGE_2 and PGI_2 (Benyo & Pate, 1992; Sakumoto *et al*., 2000), suggesting a luteotrophic action during CL growth and differentiation (Okuda *et al*., 1998; Ferreira-Dias *et al*., 2011). In the present study, the stimulatory effect of TNF on P_4 and PGE_2 production by early luteal phase luteal cells, as well as the inhibitory effect of TNF on $PGF_{2\alpha}$ secretion supports previous conclusions. In addition, these results show that TNF might control intraluteal pathways involving not only stimulation of P_4 and PGE_2 secretion, but also may differently modulate $PGF_{2\alpha}$ synthesis, throughout the luteal phase. The inhibitory effect seen on $PGF_{2\alpha}$ production in early luteal phase CL was reverted in the following luteal stages. Further studies are needed to better understand how TNF modulates $PGF_{2\alpha}$ luteal synthesis in the mare. Apparently, from mid luteal phase onward, the enzymatic mechanisms involved in $PGF_{2\alpha}$ synthesis, which are responsive to TNF, are present in the mare CL. Low IFNG protein expression in early CL and the absence of IFNG effects on *in vitro* P_4, PGE_2 and $PGF_{2\alpha}$ production at the same luteal phase, suggest that IFNG may not be involved on secretory function regulation in early CL.

The role of TNF in luteolysis regulation, together with IFNG, has been previously described for the cow (Davis & Rueda, 2002). In addition, the inhibitory effect of TNF and IFNG on luteal steroidogenesis (P_4) during luteal regression was shown in rat and pig (Gorospe *et al*., 1988; Pitzel *et al*., 1993). During luteolysis, it appears that these two cytokines are particularly determinant for ending P_4 and PGE_2 action in equine CL, since these cytokines, alone or in combination, inhibited P_4 and PGE_2 production in late CL. Moreover, intraluteal $PGF_{2\alpha}$ synthesis seems to be determinant for luteolysis accomplishment and is particularly

regulated, at the end of luteal phase, by exogenous or uterine $PGF_{2\alpha}$ in the mare (Beg *et al*., 2005) and other species (Tsai & Wiltbank, 1997). Thus, we speculate about the auto-, paracrine role of TNF on $PGF_{2\alpha}$ intraluteal synthesis pathway in the mare. As mentioned before, the present study demonstrated TNF stimulatory effects on $PGF_{2\alpha}$ secretion by luteal cells from mid and late CL, and synergistic effects of TNF and IFNG on $PGF_{2\alpha}$ secretion in late CL. Similar effects have been described for cow and rat (Benyo & Pate, 1992). The IFNG alone inhibited PGE_2 secretion by late CL, which has not been demonstrated before in the mare. It is important to point out that, even though luteal steroidogenesis has been thoroughly studied, only very few studies have described the interaction between TNF and IFNG in luteal PGE_2 production (Weems *et al*., 1988).

The number of viable luteal cells from mid and late CL was decreased by synergistic action of TNF and IFNG, but not by any cytokine alone. In cow, the negative effect of these cytokines on cell viability was also observed (Benyo & Pate, 1992). Thus, we hypothesize that these cytokines may reduce luteal cell viability, only when they act synergistically. Even though apoptosis was not assessed in the present work, in a similar study by Galvão *et al*. (2010), TNF+IFNG increased apoptosis of *in vitro* cultured mare luteal cells from mid luteal stage after 24 h. Apoptosis was also increased in cow CL by synergistic action of TNF and IFNG (Taniguchi *et al*., 2002).

In conclusion, this study showed the presence of cytokines TNF and IFNG and their receptors, at protein and mRNA level, in equine CL throughout the luteal phase. During early luteal phase, TNF might be involved in the regulation of CL growth and secretory function. On the contrary, later in the luteal phase, TNF alone or together with IFNG appears to contribute for eicosanoid $PGF_{2\alpha}$ synthesis and reduction in luteal cell viability during luteolysis. Thus, one might conclude that cytokines synergic action may be determinant for CL structural regression at luteolysis. Nevertheless, further studies are needed to better elucidate the role of these cytokines on luteal growth, secretion, and luteolysis induction in the mare.

3. EQUINE LUTEAL FUNCTION REGULATION MAY DEPEND ON THE INTERACTION BETWEEN CYTOKINES AND VEGF

(Submitted to *Biology of Reproduction*)

3.1 Abstract

We hypothesized that in mare, luteal cytokines influence angiogenesis, and, in turn, angiogenic factors can also regulate luteal secretory capacity. Therefore, the goal was to evaluate: the role of cytokines tumor necrosis factor alpha (TNF), interferon gamma (IFNG) and Fas ligand (FASL) in mare corpus luteum (CL) throughout the luteal phase, on (i) modulation of angiogenic activity; and (ii) mRNA transcription of VEGF, its receptor VEGFR2, thrombospondin 1 (TSP1) and its receptor CD36. Besides, in mid luteal phase CL cells, (iii) VEGF protein expression after cytokine incubation; and (iv) the effect of VEGF on secretory capacity (Progesterone - P_4 and prostaglandin E_2 – PGE_2) and mRNA transcription of 3-Beta-hydroxysteroid dehydrogenase - 3βHSD, and PGE synthase - PGES were assessed. Angiogenic activity increased in early luteal phase CL by TNF and reduced in mid and late CL by TNF, IFNG and TNF+IFNG+FASL. In early CL, TNF increased *VEGF* and *VEGFR2* mRNA transcription and reduced *CD36*, which was also lowered by IFNG, FASL and TNF+IFNG+FASL. In mid CL, TNF increased VEGF mRNA transcription and protein and reduced *CD36* mRNA transcription. In the same stage, FASL and TNF+IFNG+FASL decreased VEGF protein expression. In the late CL, TNF and TNF+IFNG+FASL reduced mRNA transcription of *VEGFR2* while TNF+IFNG+FASL increased *TSP1* and *CD36*. VEGF cell stimulation increased mRNA transcription of 3βHSD and PGES, as well as P_4 and PGE_2 secretion from lueta cells. Interaction between cytokines, VEGF, P_4 and PGE_2 can determine luteal function in the mare.

3.2 Introduction

After ovulation, luteinization process leads to corpus luteum (CL) formation. Vascular development is a crucial step for proliferation and establishment of this transient luteal endocrine organ. As shown before for equine CL, a cyclic coordinated vascular and non-vascular growth and regression, involves both cell proliferation and active caspase-3 mediated apoptosis (Ferreira-Dias *et al*., 2006a; Ferreira-Dias *et al*., 2006b; Ferreira-Dias *et al*., 2007; Roberto da Costa *et al*., 2008) and a balance between luteotrophic and luteolytic actions regulate CL growth, establishement and regression (Niswender & Nett, 1994; Pate, 1996). Besides being distinctly coordinated and well characterized in different organic departments, molecular regulation of angiogenesis in equine the CL is a mechanism not yet fully understood. Large evidences confirm the hormonal regulation of angiogenesis in the CL by luteinizing hormone (LH) (Dickson & Fraser, 2000; Laitinen *et al*., 1997; Shweiki *et al*., 1993). Nevertheless, since not all cell types in the CL expresses LH receptors (Duncan *et al*., 2009; Robinson *et al*., 2009), other factors like vasoactive peptides, growth factors and cytokines might control luteal angiogenesis (Davis *et al*., 2003).

Angiogenesis and inflammation are co-dependent processes well characterized in different physiologic and pathogenic organic events (Voronov *et al*., 2007), but their precise cross-talk in physiologic regulation of luteal establishment, function and regression in the mare is yet to be known. The endothelial cells are the major targets of cytokines during the immune process, particularly inflammation, in most tissues. Since a large number of microvascular endothelial cells form the CL (Dickson & Fraser, 2000; Gaytan *et al*., 1999; O'Shea *et al*., 1989), their responsivness to cytokines are thought to be important in the process during luteal function (Cherry *et al*., 2008). Regulatory role of cytokines on luteal function has been confirmed before in different species like human (Chae *et al*., 2007; Yan *et al*., 1993), cow (Nishimura *et al*., 2004; Sakumoto *et al*., 2000; Skarzynski *et al*., 2005) or mare (Galvão *et al*., 2010; Galvão *et al*., 2011). Concerning luteal angiogenesis regulation, cytokines like tumor necrosis factor alpha (TNF) or interferon gamma (IFNG) were shown to regulate bovine luteal endothelial cells (Fenyves *et al*., 1993; Okuda *et al*., 1999; Pru *et al*., 2003). The way these cytokines may regulate angiogenesis in the equine CL is still unknown.

Among several other factors, vascular endothelial growth factor A (VEGF) is recognized as the main promoter of angiogenesis in the CL of different species (Al-zi'abi *et al*., 2003; Fraser *et al*., 2000; Kashida *et al*., 2001; Reynolds & Redmer, 1998; Sugino *et al*., 2000a). Its

high expression during early and mid CL luteal phases in the mare is associated with endothelial cell proliferation (Al-zi'abi *et al.*, 2003). Specifically, VEGF interaction with its receptor 2 (VEGFR2) triggers the signaling transduction for angiogenesis stimulation (Ferrara *et al*., 2003). Conversely, thrombospondin 1 (TSP1) and its receptor CD36 have been described as putative anti-angiogenic factors (Greenaway *et al*., 2007; Jimenez *et al*., 2000; Tamanini & Amori, 2004), beeing associated with inhibition of follicular angiogenesis (Armstrong & Bornstein, 2003; Greenaway *et al*., 2005). Litle is known about TSP1 on luteal function regulation, but its participation on angiogenesis regulation together with VEGF was demonstrated (Petrik *et al*., 2002).

Vascular and non-vascular cell dynamics during CL establishment culminate with luteal strucutural consolidation and progesterone (P_4) secretion. Thus, the relationship between events like angiogenesis regulation and immune response should not be dissociated from secretory functionality. Previous *in vivo* studies in primates reported VEGF modulatory effects on supression of P_4 secretion in mid luteal phase CL (Fraser *et al*., 2005). Thus, we hypothesized that in the mare, cytokines may influence luteal angiogenesis, and that angiogenic factors themselves also regulate luteal secretory capacity. Therefore, the goal of this study was to evaluate: the role of cytokines TNF, IFNG and Fas ligand (FASL) in mare CL throughout the luteal phase, on (i) modulation of angiogenic activity; and (ii) mRNA transcription of VEGF, its receptor VEGFR2, TSP1 and its receptor CD36. Besides, cells isolated from mid luteal phase CL, when vascular and non-vascular growth has reached its maximum (Ferreira-Dias *et al*., 2006a), (iii) VEGF protein expression after cytokine incubation; and (iv) the effect of VEGF on secretory capacity (P_4 and prostaglandin E_2 – PGE_2) and mRNA transcription of 3 Beta hydroxysteroid dehydrogenase - 3βHSD, and PGE synthase - PGES were assessed.

3.3 Materials and methods

3.3.1 Collection of equine corpora lutea

Luteal tissue and venous blood from jugular vein were collected *post mortem* at the local abattoir from randomly designated cyclic Lusitano mares age ranging from 3 to 8 years old, from May until the end of August. The mares were euthanized after stunning according the European Legislation concerning welfare aspects of animal stunning and killing methods

(EFSA, AHAW/04-027) and to the Portuguese legislation (DL 98/96, Art. 1º), and approved by the Faculty of Veterinary Medicine Ethics Committee.

As previously described (Roberto da Costa *et al*., 2007a; Galvão *et al*., 2010), luteal structures were classified in early luteal phase CL, (presence of corpus hemorrhagicum, P_4>1ng/ml, early CL; n=6), mid luteal phase CL, (CL associated with follicles 15 to 20 mm in diameter and P_4>6 ng/ml, mid CL; n=11) and late-luteal phase CL (CL associated with preovulatory follicle 30-35mm in diameter and P_4 between 1 and 2 ng/ml, Late CL; n=6). After collection, luteal samples were placed in culture medium – M199 (M2154; Sigma-Aldrich, St. Louis, MO, USA) with 0.1% bovine serum albumin (BSA) (#735078; Roche Diagnostics GmbH Mannheim, Germany), 20µg/ml gentamicin (G1397, Sigma, USA) and 250 µg/ml amphotericin (A2942, Sigma, USA) and transported to the laboratory for *in vitro* studies.

3.3.2 In vitro luteal cells isolation and culture

Luteal cell isolation followed the methodology described before (Galvão *et al*., 2010). Briefly, connective tissue and blood clot were removed and CL minced into small pieces and enzymatic digestion was performed for 50 min with M-199 (M2520, Sigma, St. Louis, USA) with 0.1% (w/v) collagenase (C-0130; Sigma, USA), 0.01% DNase I (D-5025; Sigma, USA) and 0.1% (w/v) BSA (#735078; Roche Diagnostics GmbH Mannheim, Germany)**.** After the digestion process, all collected cells were filtered twice with a cell dissociation Sieve - Tissue grinder Kit (cd1-1kt, Sigma, USA) through metal wire meshes (Galvão *et al*., 2010). After several washing steps (180 g with M-199 with 0.1% BSA), erythrocytes were lysed with Red blood lyses buffer (R7757, Sigma, USA). Cells were then resuspended in Dulbecco's modified eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 [v/v], D-8900; Sigma, USA) containing 10% fetal bovine serum (FBS) (26140-079, Gibco, USA) and gentamicin [20 µg/ml]. Cell viability, which ranged between 83-89% live cells, was assessed by trypan blue exclusion dye (T8154, Sigma, USA). Dispersed luteal cells were then cultured in 1 ml of D/F medium in 24-well culture plates (142475, Nunc, Kamestrupvej, Denmark) at the concentration of $2.0x10^5$/ml, or in 5 ml D/F medium in T25 culture flasks (136196, Nunc) at the concentration of $5.0x10^6$/ml, with 10% FBS, amphotericine [250 µg/ml] and gentamicin [20 µg/ml], at 37ºC with 5% CO_2.). In early CL, around 40% of the cells were LLC and about 50% were SLC. Mid CL consisted of about 65% LLC and 20% SLC, while in late CL 45% were LLC and 35% SLC. ELC and fibroblasts corresponded to approximately 10% of the cells. When cells were adherent in a monolayer and 90-95% confluent, they were washed

with M-199 with 0.1% BSA, phenol red free DMEM/F_{12} medium (1:1) (11039, Gibco, USA) with 0.1% BSA, gentamicin [20 μg/ml] and transferrin (T1428, Sigma, USA) [5 μg/ml] were added.

After cells stimulation (Experiments 3.3.5.1 and 3.3.6.1) conditioned media was stored at -70°C. For further experiments cells were scraped (179693, Nunc), washed in sterile phosphate-buffered saline (PBS) 0.1 M (pH 7.4) and then ressuspended in TRIZOL reagent (Invitrogen, Carlsbad, CA, USA; No. 15596-026) for mRNA transcription analysis or ice cold homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, Complete (protease inhibitor cocktail; Roche, No. 1697498, Indianapolis, USA), pH 7.4) for western blotting analysis and stored at -80ºC.

3.3.3 RNA isolation and cDNA synthesis

Total RNA was extracted from luteal cells with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA; No. 15596-026) following the manufacturer's instructions. As described before (Galvão *et al*., 2010), after RNA concentration assessment, 1 μg of total RNA was reverse transcribed using Reverse Transcriptase Superscript III enzyme (ref.18080093, Invitrogene, Gibco, Carlsbad, USA) and oligo (dT) primer (27-7858-01, GE Healthcare, Buckinghamshire, UK).

3.3.4 Real time polymerase chain reaction (PCR)

Semi quantitative real-time PCR assays were performed in a 7300 Real Time PCR System (Applied Biosystems^{TM}, Warrington, UK), using a default thermocycler program for all genes: a 10 min pre-incubation at 95ºC was followed by 40 cycles for 15 sec at 95ºC and 1 min at 60ºC. A further dissociation step (15 sec at 95ºC, 30 sec at 60ºC and 15 sec at 95ºC) ensured the presence of a single product. In each real time assay, both target gene and housekeeping gene (HKG) β2-microglobulin (*B2MG*) were run simultaneously and reactions were carried out in duplicate wells on a 96 well optical reaction plate (Applied Biosystems, ref. 4306737, UK) in 25 μl reaction volume. Primers for *B2MG* were previously designed (Table 5) (Galvão *et al*., 2010). After the run, PCR product specificity was confirmed in a 2.5% agarose (BIO-41025, Bioline, Luckenwalde, Germany) gel. Real time results were analyzed with the Real-time PCR Miner algorithm (Zhao & Fernald, 2005).

Table 5: Specific primers sequences used for quantitative real time PCR (bp-basepair).

Gene	Accession number	Sequence 5´- 3´	Length (bp)
VEGF	NM_001081821	For: ATGCGGATCAAACCTCACCA	117
		Rev: AGGCCCACAGGGATTTTCTT	
VEGFR2	XM_001916946.1	For: CTTCCAGTGGGCTGATGACC	100
		Rev: AGCTTCCACCGAAGATTCCA	
TSP1	XM_001503599.2	For: GCTCCAGCTCTACCAATGTCCT	91
		Rev: TTGTGGCCGATGTAGTTAGTGC	
CD36	XM_001487907.1	For: AACCACACCGTCTCCTTCGT	107
		Rev: GCCGCTACAGCCAGATTGAG	
3β-HSD	AF031665	For: CAGACCAGAATTGCGGGAAG	179
		Rev: TCATGGTGACCTGTGGGTTA	
PGES	AY057096	For: CACGCTGCTGGTCATCAAGA	151
		Rev: CTCTCAGGCAACGCTCCAC	
B2MG	X69083	For: CGGGCTACTCTCCCTGACTG	92
		Rev: TTGGCTTTCCATTCTCTGCTG	

3.3.5 Cytokine effect on angiogenic and antiangiogenic factors production

3.3.5.1 Cell culture stimulation with cytokines

Dose assessment for the cytokines was determined before (Galvão *et al*., 2010; Galvão *et al*., 2011). Following the stabilization period, cells from different luteal stages (early CL, n=6; mid CL, n=6; and late CL, n=6) were incubated in T25 for 24 h in a 5% CO_2 chamber with: (i) no exogenous cytokines (negative control); (ii) TNF (10 ng/ml); (iii) IFNG (10 ng/ml); (iv) FASL (10 ng/ml); or (v) TNF+IFNG+FASL (10 ng/ml). After incubation, cell conditioned media were frozen at -80ºC for further endothelial cell proliferation. Cells were scraped and kept frozen for evaluation of angiogenic and antiangiogenic mRNA transcription analysis.

3.3.5.2 Endothelial cell proliferation assay

The effects of different cell stimulations (*i.e.* control, TNF, IFNG, FASL or TNF+IFNG+FASL) on angiogenic activity in early, mid and late luteal phase conditioned media were assessed after evaluation of bovine aortic endothelial cells (BAEC) proliferation using alamarblue reagent (Serotec, Oxford, UK). The reagent was developed for quantitative assessment of animal cells proliferation. Medium reduction was evaluated by cellular incorporation of the fluorimetric/colourimetric growth indicatior (Lancaster & Fields, 1996). Protocol optimization for BAEC was performed before (Ferreira-Dias *et al*., 2006b), being optimal reading time after 5 h incubation of BAEC with the conditioned media from equine luteal cell cultures, since this time-point represented the point at which the linear correlation between the percentage reduction of the indicator and cell density was the highest (R^2 =0.9507), after calculation according to the manufacturer's instruction (Ferreira-Dias *et al*., 2006b). Briefly, BAEC (2×10^4 cells/ml) were incubated in 24- well plates at 37ºC in a humidified, 5% CO_2 atmosphere for 14 h, until cells adhered to the wells. Further on, samples of luteal conditioned media (30% concentration) were added in triplicate to the wells and cells were incubated for a further 48 h. Conditioned media were then removed and fresh phenol red free DMEM/F_{12} medium containing 10% alamarBlue was added. The plates were incubated for a further 5h and absorbance (abs) read at 570 and 600 nm (SpectrMax 340 PC; Molecular Devices; Biocitek SA, Lisbon, Portugal). Proliferation response of BAEC to luteal conditioned culture media was evaluated by comparing the percentage reduction by these media with that produced by the negative controls (whitout luteal cells), being cell proliferation or mitogenesis in response to negative control considered to be 100% (Redmer *et al*., 1988; Ferreira-Dias *et al*., 2006b). Alamarblue percentage reduction using abs was determined according to alamarblue technical datasheet.

3.3.5.3 Cytokine effect on VEGF, VEGFR2, TSP1 and CD36 mRNA transcription

Mare CL cells obtained at the different phases of the luteal phase of the estrous cycle (early-CL, n=5; mid-CL, n=5; late-CL, n=5) and collected after cytokine stimulation were used in this experiment. For *VEGF*, *VEGFR2*, *TSP1* and *CD36* genes, specific primers were designed (Table 5), following the guidelines for real time PCR (Wang & Seed, 2006).

3.3.5.4 Western blotting analysis for VEGF

VEGF protein expression quantification on mid CL equine luteal cells, incubated with cytokines, was assessed by Western blotting after protein extraction from cells cultured in T25 culture flasks. Radio-immunoprecipitation Assay (RIPA) buffer (250 µl) (R0278, Sigma, USA) with a protease inhibitor (Complete, Roche) (pH 7.4) was added to collected cells. Concentration was determined and 100 µg of protein was loaded for VEGF and beta-actin (ß actin) on a 12% Acrylamide gel (ref. 161-0155, Bio-Rad, Hercules, CA, USA) and further transfered to nitrocellulose membranes (Ref. 1620116, Bio-Rad, USA) (Galvão *et al*., 2010; Rodrigues *et al*., 2002). Level of active VEGF was evaluated by using a rabbit polyclonal antibody (sc-152, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200. For ß actin (A5441, Sigma, USA) a mouse monoclonal antibody was diluted 1:10,000. Membranes were incubated overnight at 4ºC. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit (P0448, Dakocytomation, Carpinteria, CA, USA*)*, at 1:5,000 for VEGF and HRP-conjugated goat anti-mouse (A2554, Sigma, USA) for and ß actin at 1:100,000, both incubated for 2.5 h at room temperature. Protein expression was visualized by means of luminol-enhanced chemiluminescence (SuperSignal® West Pico, 34077, Thermo Scientific, Waltham, MA, USA) and exposure of the membrane to a blue light-sensitive autoradiography film (Kodak BioMax Light Film; Kodak-Industrie, Chalon-sur-Saone, France). Equal protein loading per lane was confirmed with ß actin. For protein expression quantification, target protein expression was normalized after dividing units of arbitrary densimetry for VEGF by ß actin for each band. Signals were densitometrically assessed using the ImageMaster 1D Elite densitometric analysis program (Amersham Biosciences, Piscataway, NJ, USA).

3.3.6 Evaluation of VEGF effect on luteal cells secretory capacity

3.3.6.1 Cell culture stimulation with VEGF-dose assessmen

To determine the adequate stimulation dose for VEGF, mid CL cells cultured as described, were stimulated in 24 well plates for 24 h as follows: no exogenous factor (Control); or with VEGF (recombinant human VEGF, V7259, Sigma, USA), at three different concentrations (1 ng/ml; 10 ng/ml; 50 ng/ml). Equine LH (LH) (L9773; Sigma, USA) (10 ng/ml) was used as positive control (Galvão *et al*., 2010). Conditioned media were stored at -80°C for further analysis.

3.3.6.2 VEGF effect on P_4 and PGE_2 secretion

Cell cultures from mid luteal phase CL (n=5) were stimulated for 24 h at 37ºC in a 5% CO_2 atmosphere with: (i) no exogenous factor (Control), (ii) VEGF (50 ng/ml), and (iii) LH (positive control; 10 ng/mL) (Galvão *et al*., 2010). Conditioned media were stored at -80°C until evaluation of P_4 and PGE_2 concentration, and cells were treated as described before and frozen in -80ºC for mRNA transcription quantification.

3.3.6.3 VEGF effect on 3βHSD and PGES mRNA transcription

Equine luteal cells from the previous experiment (2.2) were used for *3βHSD* and *PGES* mRNA transcription quantification. Specific primers were designed (Table 5), following the guidelines for real time PCR (Wang & Seed, 2006). Real time PCR was run and data were analyzed as previously described (Galvão *et al*., 2010).

3.3.7 Luteal cell viability and proliferation assessment

In all *in vitro* studies, luteal cells were plated in 96-well cell culture plates (Corning, Corning, NY, USA) in parallel, and stimulated with the same factors as described before (Galvão *et al*., 2010). Briefly, cells at the concentration of $1.0x10^4$/ml were incubated for 24 h, at 37ºC in a humidified, 5% CO_2 atmosphere. Cell viability was determined with Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (G3581, Promega, Madison, WI, USA), according to the manufacturer manual, at 490 nm.

3.3.8 Hormone determinations

Concentrations of P_4 and PGE_2 luteal conditioned media and P_4 in plasma were determined by direct enzyme immunoassay (EIA). For P_4 evaluation, antiserum was used at a final dilution of 1:100,000, as described previously (Korzekwa *et al.*, 2004). Horseradish peroxidase (HRP)-labeled P_4 was used at a final concentration of 1:75,000. The standard curve ranged from 0.39 to 100 ng/mL and the concentration of P_4 at 50% binding (ED50) was 4.1 ng/mL. The intra- and inter-assay coefficients of variation (CV) were 5.5% and 8.5%, respectively.

Determination of PGE_2 concentrations followed the methodology described before (Skarzynski & Okuda 1999). The PGE_2 standard curve ranged from 0.39 ng/mL to 100 ng/mL, and the concentration of 50% binding (ED50) was 6.25 ng/mL. The intra- and interassay CV were 1.6% and 11.0%, respectively. Concentration of hormones in culture media was normalized for the number of live cells, after viability assessment. On viability assessment, assay base line corresponded to control level.

3.3.9 Statistical analysis

Data were analyzed using one way analysis of variance followed by Bonferroni's Multiple Comparison Test (ANOVA; GraphPAD PRISM, Version 5.00, GraphPad Software, San Diego, CA, USA). Significance was defined as values of $p<0.05$.

3.4 Results

3.4.1 Cytokines effect on angiogenic and anti-angiogenic factors metabolites production

The assessment of cytokines effect on luteal cells secretion of angiogenic and anti-angiogenic factors showed that TNF increased BAEC proliferation ($p<0.05$) (Fig. 17a) in early CL. In the mid CL, cytokine association TNF+IFNG+FASL reduced BAEC proliferation ($p<0.05$) (Fig. 17b), while in the late luteal phase CL the same effect was verified for TNF and IFNG alone ($p<0.05$) (Fig. 17c) or TNF+IFNG+FASL ($p<0.01$) (Fig. 17c).

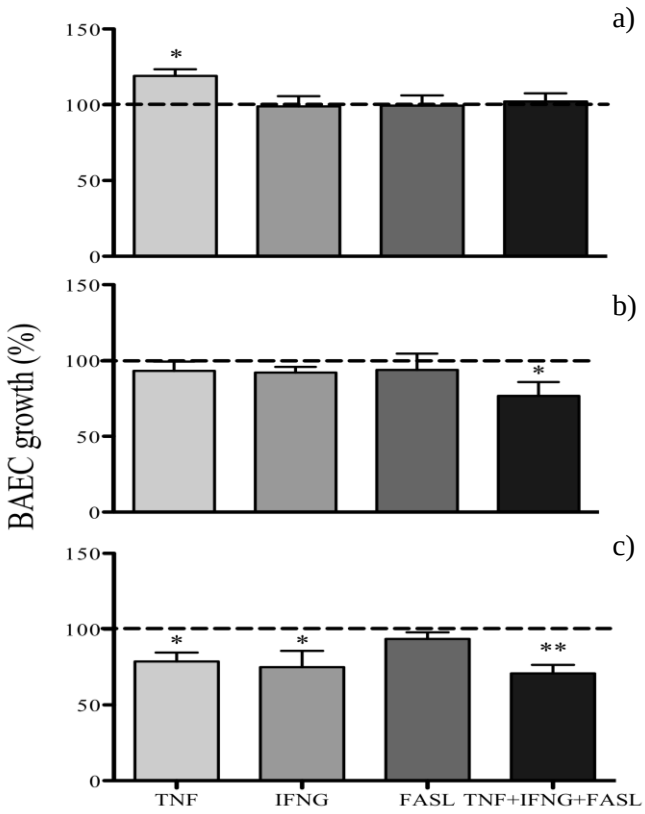


Figure 17: Bovine Aortic Endothelial Cell (BAEC) proliferation rate, after incubation with conditioned media from cells obtained from early CL (a), mid CL (b) and late luteal phase CL and stimulated for 24 h with cytokines. Asterisks indicate significant differences (*p<0.05; **p<0.01).

3.4.2 Cytokine effect on mRNA transcription of VEGF, VEGFR2, TSP1 and CD36

Cells isolated form early luteal phase CL and incubated with TNF showed an increased transcription on *VEGF* and *VEGFR2* mRNA transcription (p<0.05) (Fig. 18a), while all stimulations drastically reduced *CD36* mRNA transcription (p<0.001) (Fig. 18a). Luteal cells obtained from mid CL and incubated with TNF also presented a reduction in mRNA transcription for *CD36* (p<0.05) (Fig. 18b), as seen in the previous stage. In late CL isolated cells, stimulations with TNF or TNF+IFNG+FASL were responsible for a reduction in *VEGFR2* mRNA transcription (p<0.05) (Fig. 18c), whereas TNF+IFNG+FASL increased mRNA transcription of both *TSP1* and *CD36* (p<0.05) (Fig 18c).

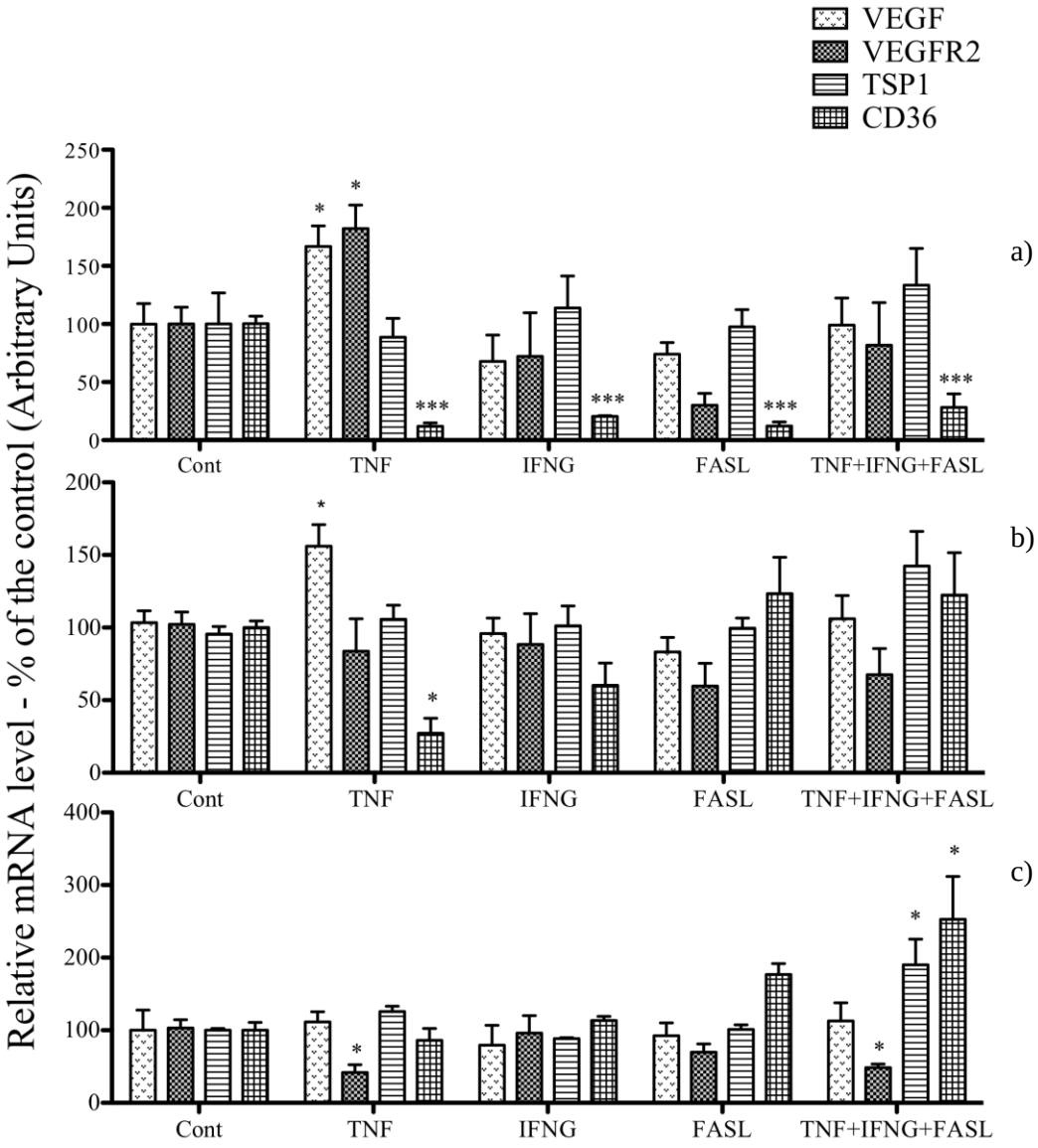


Figure 18: Relative quantification of *VEGF*, *VEGFR2*, *TSP1* and *CD36* mRNA transcription by real time PCR, in cytokines stimulated (24 h) luteal cells obtained from early CL (a), mid CL (b) and late CL (c). Expression normalized with the housekeeping gene – *B2MG*. Bars represent mean±SEM. Different letters indicate significant differences (*p<0.05; ***p<0.001).

3.4.3 Protein expression for VEGF

Analysis of the immunoreactive bands of protein expression for VEGF showed an increase on band intensity for TNF treatment, while for FASL and TNF+IFNG+FASL less intense bands were obtained, when comparing with control ($p<0.05$) (Fig. 19).

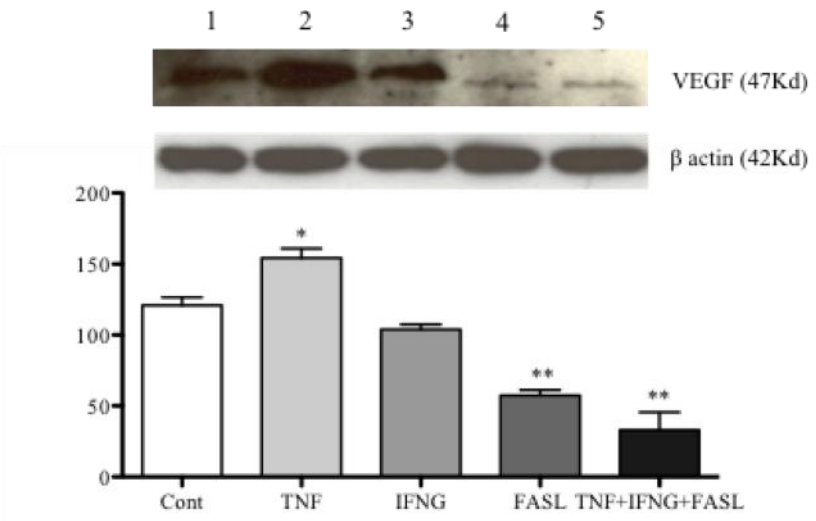


Figure 19: VEGF protein expression in equine mid CL stimulated luteal cells. Upper panels depict representative western blot (n=4). Lanes: 1- control; 2- TNF; 3- IFNG; 4- FASL and 5- TNF+IFNG+FASL. Data normalized against β actin density values. Bars represent mean±SEM. Asterisks indicate significant differences (*p<0.05; **p<0.01).

3.4.4 In vitro VEGF-dose assessment

The concentration of 50 ng/ml was chosen since it showed the most consistent results ($p<0.05$) (Fig. 20).

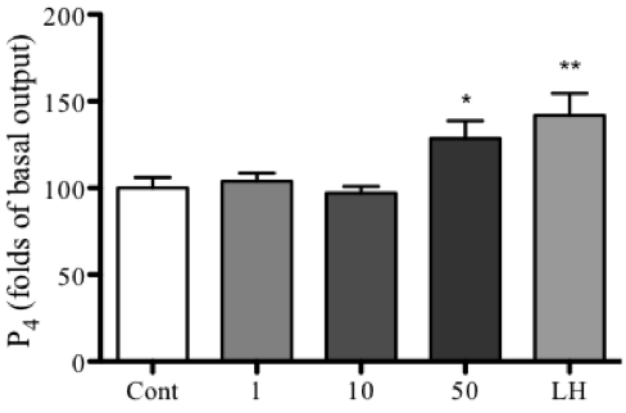


Figure 20: VEGF and LH (positive control) action on P_4 production by mid CL cells, after a 24h stimulation. Stimulation dose - ng/ml. Bars represent mean±SEM. Asterisks indicate significant differences (*p<0.05; **p<0.01).

3.4.5 VEGF effect on P_4 and PGE_2 secretion

A 24 h incubation of mid CL luteal cells with VEGF (50 ng/ml) caused an increase in *in vitro* P_4 secretion ($p<0.05$) (Fig. 21). The same response was seen for the positive control (LH) ($p<0.05$) (Fig. 21), what accounts for the presence of viable cells in culture. The secretion of PGE_2 was increased also by VEGF and LH ($p<0.01$) (Fig. 21).

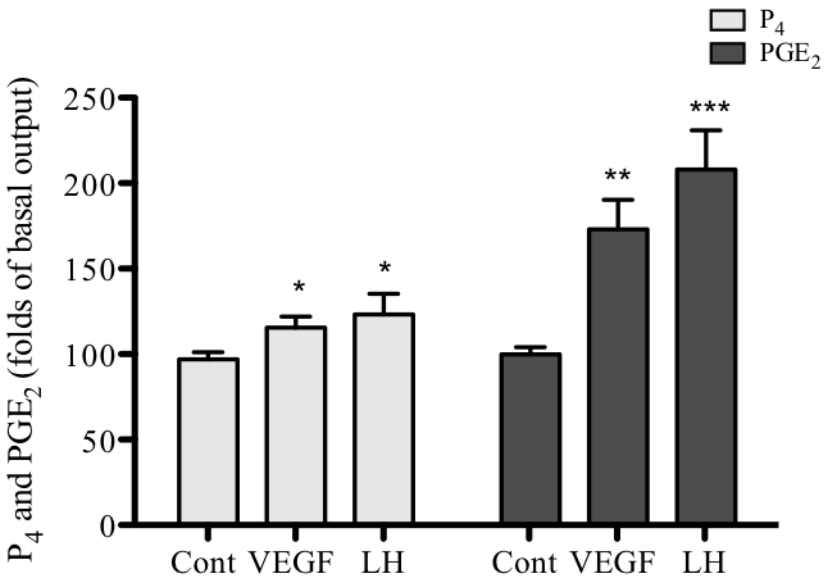


Figure 21: Mid CL cells *in vitro* production of: a) P_4 and b) PGE_2 after 24 h stimulation by no exogenous factor - Control; or by VEGF (50 ng/ml) or LH (10 ng/ml). Bars represent mean±SEM. Asterisks indicate significant differences (*$p<0.05$; **$p<0.01$; *** $p<0.001$).

3.4.6 VEGF effect on 3βHSD and PGES mRNA transcription

After a 24 h incubation of mid CL isolated cells, with VEGF (50 ng/ml) or LH (positive control, 10 ng/ml), an increase in mRNA transcription of both *3βHSD* ($p<0.01$) (Fig. 22) and *PGES* ($p<0.05$) (Fig. 22) was seen.

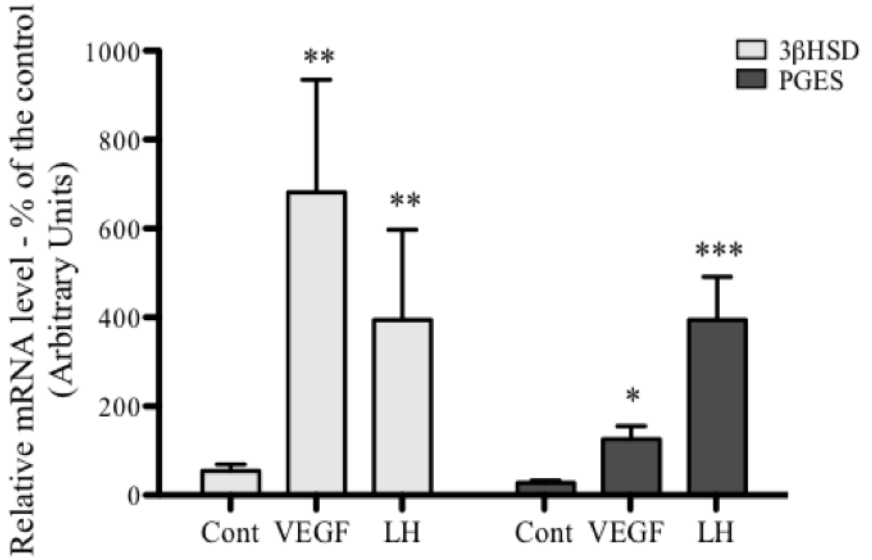


Figure 22: Relative quantification of *3β-HSD* and *PGES* mRNA transcription by real time PCR, in VEGF and LH stimulated mic CL cells (24 h). Expression normalized with the housekeeping gene – *B2MG*. Bars represent mean±SEM. Different letters indicate significant differences ($p<0.05$).

3.4.7 Luteal cell viability and proliferation assessment

Tested cytokines, either alone or combined, had no effect on *in vitro* viability and proliferation of cells obtained from early luteal phase mare CL. Cytokine association TNF+IFNG+FASL reduced luteal cell viability and proliferation in mid (to 77.3±3.1%) and late (to 57.8 ±5.2%) luteal phase CL ($p<0.05$). Concerning VEGF study on mid CL isolated cells, viability and proliferation were increased for VEGF (to 150.7±12.7%) and LH (to 154.1±20.2%) ($p<0.05$).

3.5 Discussion

The present study evidenced the existent interaction between cytokines (TNF, IFNG and FASL) and vascular dynamics in the equine CL. Moreover, since the complexity of luteal function regulation determines the interaction of factors from different nature, the interrelationship between the conserved angiogenic factor VEGF and secretory function (P_4 and PGE_2) was desmonstrated.

Soon after ovulation, several cellular and biochemical processes triggered by LH, like extracellular matrix remodeling or immune-like response, lead to angiogenesis (Robinson *et al*., 2009). Among other factors as fibroblast growth factor or epidermal growth factor not presently investigated, the VEGF has been described as the main angiogenic factor produced by luteal cells (Redmer *et al*., 1996; Reynolds *et al*., 2002). The exact effect of LH on VEGF production is not clear, since its in vitro stimulatory effect on VEGF secretion by granulosa cells seen in cows (Schams *et al*., 2001) and primates (van den Driesche *et al*., 2008) was less conclusive after in vivo studies in primates (Stouffer *et al*., 2001), mice (Kim *et al*., 2009) or cow (Robinson *et al*., 2007). These findings substantiate the complexity of luteal angiogenic process regulation. The ability of cytokines to modulate angiogenic and/or antiangiogenic factors production by equine luteal cells throughout the luteal phase was shown in the present work, by assessing BAEC proliferation. Previous studies have also evidenced the importance of immune-like responses on angiogenesis regulation in the CL (Webb *et al*., 2002) and other tissues (Numasaki *et al*., 2004; Zhang, 2001). Besides, angiogenic function has also been ascribed to human macrophages (Malaguarnera *et al*., 2005). Particularly, in a previous study on equine CL, the cytokine TNF stimulated angiogenic activity on luteal tissue from early luteal phase, when used as positive control (Ferreira-Dias *et al*., 2011). In the present work similar results were seen, since media from early CL luteal cells stimulation with TNF increased BAEC proliferation. Moreover, mRNA transcription of VEGF was also increased by TNF in the early and mid CL, as well as its receptor VEGFR2 in early CL luteal phase. Protein analysis also showed an increase on VEGF expression on mid CL luteal cells, when comparing with control. When the inhibitory effect of TNF on mRNA transcription of the anti angiogenic receptor CD36 in the early CL is taken into consideration, the present findings suggest that TNF might participate on angiogenesis promotion in the equine CL, specifically upregulating VEGF expression, at the time of luteal establishment. Downregulation of CD36 (TSP1 receptor) mRNA transcription may be as well indicative of the proangiogenic action exerted. Very few studies reported the action of TNF and other cytokines on luteal VEGF production and angiogenesis. Schams *et al*. (2001) also demonstrated the stimulatory effect of TNF on VEGF secretion in cultured bovine granulosa cells. In another study in porcine luteal cells, TNF stimulated VEGF secretion from mid and late CL and inhibited mRNA transcription of VEGFR2 (Kowalczyk *et al*., 2008). As shown by the present data, TNF stimulatory effect on VEGF mRNA transcription during early and mid CL was lost in the subsequent luteal phase, what may indicate the demise of the proangiogenic effect towards the end of the luteal phase, in the mare. These findings are in disagreement with others reported

for the sow CL (Kowalczyk *et al*., 2008), what may indicate differences between species. Indeed, in the present study in the late CL, TNF, either alone or combined with the other cytokines reduced BAEC proliferation and *VEGFR2* mRNA transcription. The ability of TNF to diversely modulate angiogenesis was shown before in different organic deparments (Klagsbrun & D'Amore, 1991), suggesting that TNF can act on diferent angiogenic signaling pathways, depending on the biological system (Patterson *et al*., 1996). Additionally, modulation of luteal VEGF secretion by TNF should be considered. Paracrine actions of TNF have been demonstrated on regulation of luteal secretory function, like P_4 (Galvão *et al*., 2011; Sakumoto *et al*., 2000; Skarzynski *et al*., 2003a; Szostek *et al*., 2011), PGE_2 and $PGF_{2\alpha}$ (Benyo & Pate 1992; Galvão *et al*., 2011), as well as on vasoactive nitric oxide (Skarzynski *et al*., 2003b; Ferreira-Dias *et al*., 2011), endothelin 1 (ET-1) (Miyamoto *et al*., 2009) and growth factors (Webb *et al*., 2002), among others. Moreover, different effects of TNF on luteal function could depend on its concentration, type of receptor (receptor I or receptor II) expressed and stage of the estrous cycle (Sakumoto & Okuda, 2004). As shown by Skarzynski *et al*. (2003a, 2007), this cytokine modulates the lifespan of bovine CL *in vivo* in a dose-dependent manner. In fact, proinflammatory pluripotent cytokines such as TNF and INF present a broad spectrum of biological actions. Besides, the direct action of TNF on luteal endothelial cells should also be considered, since cytokine receptors were shown in bovine luteal endothelial cells (Okuda *et al*., 1999), acting alone or together with other cytokines on endothelial cells viability (Pru *et al*., 2003). Concurrent action of TNF on the secretion of those factors might indirectly determine its effect on VEGF signaling pathway system, since they can modulate angiogenesis as well.

Another studied cytokine, the IFNG, has been described as an important regulator of luteal function (Davis & Rueda, 2002; Fairchild & Pate, 1989; Galvão *et al*., 2011; Pate *et al*., 2001). Concerning angiogenesis control in the mare, IFNG did not affect proangiogenic factors production (*VEGF* and *VEGFR2*) in the early CL, but mRNA transcription of the antiangiogenic receptor *CD36* was reduced. Conversely, in the late CL, IFNG alone increased the production of antiangiogenic factors by equine luteal cells, since luteal conditioned media reduced BAEC proliferation, but no changes were observed on VEGF protein expression or mRNA transcription of studied factors (*TSP1* and *CD36*). This cytokine was associated with cellular senescense of cultured bovine luteal endethelial cells (Fenyves *et al*., 1993), being considered that IFNG may support cytotoxic effect of TNF on luteal steroidogenic (Petroff *et al*., 2001; Taniguchi *et al*., 2002) and endothelial cells during bovine CL regression

(Friedman *et al*., 2000; Pru *et al*., 2003). Further studies are needed to better elucidate how IFNG can influence angiogenesis and endothelial cells function, in the equine CL.

Another interesting finding on this study was the inhibition of VEGF protein expression by FASL. A relevant role on luteal regression and secretory capacity modulation was recently ascribed to FASL in equine CL (Galvão *et al*., 2010). Other than inducing $PGF_{2\alpha}$ and inhibiting P_4 and PGE_2 secretion at the time of luteolysis, it seems that FASL may also suppress angiogenesis, by reducing VEGF protein expression and in this way guarantees the demise of the equine CL. This cytokine has been decribed as a downregulator of angiogenesis in different organs (Lee *et al*., 2003; Roychoudhury *et al*., 2010). However, to the best of our knowledge, this is the first report of luteal angiogenesis (VEGF) modulation by FASL.

When all cytokines were tested together (TNF+IFNG+FASL), angiogenesis restriction was very efective. In fact, inhibition of BAEC proliferation was evident in the mid and late CL. In the late luteal phase CL, this cytokine association increased both *TSP1* and *CD36* and reduced *VEGFR2* mRNA transcription. Although no changes were seen in mRNA transcription, the same cytokine association also reduced VEGF protein expression in the mid CL. Furthermore, cytokines association appears to be determinant for angio-regression also in bovine CL (Davis *et al*., 2003). In another study in the mare, early regression after *in vivo* $PGF_{2\alpha}$ administration induced luteolysis, which was associated with a decrease in mRNA and protein expressions for VEGF, as well as a reduction in endothelial cell proliferation in the CL (Al-zi'abi *et al.*, 2003). More recent *in vitro* studies on mare CL showed that in late CL, during luteal regression, $PGF_{2\alpha}$ also decreased endothelial cell proliferation, suggesting that this hormone may be involved on vascular regression (Ferreira-Dias *et al*., 2006a). In the present study we have demonstrated the importance of cytokines TNF, IFNG and FASL on angiogenesis inhibition, through VEGF downregulation for further luteolysis. Considering that 12 h to 36 h hours after administration of $PGF_{2\alpha}$ a massive infiltration of neutrophils was present in mare luteal tissue (Al-zi'abi *et al.*, 2002), and that cytokines are their main product (Minegishi *et al*., 2002), one may speculate that $PGF_{2\alpha}$ mediated cytokines production may downregulate vasoproliferation and VEGF angiogenic pathway and increase antiangiogenic factors mRNA transcription in the mare. Also in ruminants it is suggested that members of TNF superfamily may mediate the action of $PGF_{2\alpha}$ on *in vivo* regression of capillary beds during luteal structural regression (Davis *et al*., 2003; Pru *et al*., 2003). The interaction between neutrophils, cytokines and angiogenesis has been recently characterized in bovine CL (Jiemtaweeboon *et al*., 2011). As a matter of fact, neutrophils migrate into cow early CL

partially due to its major chemoattractant interleukin 8 (IL-8), produced at high levels in the CL (Jiemtaweeboon *et al*., 2011). Neutrophils act as potential regulators of angiogenesis together with IL-8 in cow developing CL (Jiemtaweeboon *et al*., 2011). Evidences uphold the importance of the immune system, secretory activity and angiogenesis interactions on luteal function regulation.

The knowledge gathered in pthe present study might contribute for the understanding of cytokines action on vascular dynamics in pathologic conditions. Tumor progression is closely related with microvascular proliferation. Besides, inflammation is essential for tumor microenvironment adequacy and interaction between cytokines TNF and IFNG with VEGF was recently demonstrated on tumor angiogenesis regulation (Liu *et al*., 2011). It has been ascribed to TNF an important role on tumor microenvironment to further promote cancer development (Balkwill, 2009) by enhancing tumoral angiogenesis (Wu & Zhou, 2010). Thus, a better knowledge of the cross talk between cytokines and angiogenic factors on molecular coordination of luteal angiogenesis my help to clarify similar events on tumoral development. From this standpoint, CL represents a magnific tool to better understand angiogenesis, since intense dynamics and celerity of the process manifest similarities between physiologic and tumor angiogenesis (Redmer *et al*., 2001).

The hypothetical role of VEGF on secretory capacity of equine CL was also addressed. Since VEGF expression remains high until day 10 of luteal phase in the mare (Al-zi'abi *et al*., 2003), when vasculature is fully developed (Christenson, 1996; Wulff *et al*., 2000), another role on luteal regulation is suggested beyond angiogenic function. It was previously shown that *in vivo* VEGF trap administration in marmosets, at the time of luteal establishement, resulted on lower plasma levels of P_4 (Fraser *et al*., 2005; Fraser *et al*., 2010). Fraser *et al*. (2005) also evidenced that plasma P_4 drop after VEGF inhibition during mid luteal phase should not be related with angiogenesis, once the microvascular tree was already complete. Thus, the same author suggested for VEGF a role on vessels survival and permeability maintenance, needed for the access of LH and LDL cholesterol to luteal steroidogenic cells and P_4 synthesis (Fraser *et al*., 2005). Moreover, Kobayashi *et al*. (2001) showed that infusion of VEGF stimulated *in vitro* P_4 secretion in early bovine CL. Also, *in vivo* data showed that VEGF can directly stimulate bovine CL development and P_4 secretion during the early luteal phase (Yamashita *et al*., 2008). In the present study we confirmed that VEGF could effectively modulate luteal secretory function. To the best of our knowledge, it was shown for the first time that VEGF increases P_4 and PGE_2 secretion from mid CL luteal cells, as well as

mRNA transcription of 3β HSD and PGES. Hence, it would be interesting to consider the participation of P_4 and PGE_2 on angiogenesis regulation as well. The role of P_4 and PGE_2 on VEGF stimulation was not shown for the mare up to now, even though it was evidenced in other species. One of the effects of PGs *in vivo* is their influence on vascular system, being evident their vasoactive properties and the ability to regulate angiogenesis (Form & Auerbach, 1983; Ziche *et al*., 1982). In porcine cultured luteal cells, PGE_2 increased VEGF in a dose dependent maner (Kowalczyk *et al*., 2008). The same response was seen for human granulosa luteal cells (Laitinen, 1997), rat luteal cells (Sakurai, 2004) or swine endometrial cells (Kaczmarek *et al*., 2008). Concerning P_4, it increased VEGF secretion from bovine granulosa cells (Shimizu & Miyamoto, 2007) and from uterus (Hyder, 2000). Thus, it is suggested that VEGF can stimulate secretion of P_4 and PGE_2, which in an auto-, paracrine way will support proangiogenic factors and VEGF secretion in an intraluteal feedback loop.

In conclusion, the recent findings allow us to propose a schematic interaction of luteal establishement that involves the auto-, paracrine action of cytokines, specifically TNF, on the upregulation of VEGF for angiogenesis promotion. In addition, it should be considered that TNF also increases P_4 and PGE_2 secretion in the early CL (Galvao *et al*., 2011). As mentioned before, VEGF itself, besides working on vessels proliferation (Al-zi'abi *et al*., 2003; Redmer *et al*., 1996), induces P_4 and PGE_2 production, supporting luteal structure and function. The hormones P_4 and PGE_2, triggered by TNF and VEGF, may promote other proangiogenic factors and VEGF production, establishing an auto-, paracrine intraluteal feedback loop, crucial for luteal establishement (Fig. 23a). In contrast, at the time of luteolysis, following the uterine trigger of $PGF_{2\alpha}$ (Douglas & Ginther, 1972), neutrophil number dramatically increases in the CL (Al-zi'abi *et al.*, 2003). It is suggested that at that time, the increase in cytokines production, namely TNF+IFNG+FASL in association will contribute for angiogenesis inhibition, downregulating VEGF protein expression and promoting antiangiogenic factors (as TSP1) secretion. In a coordinated fashion, luteal P_4 and PGE_2 secretion is reduced and $PGF_{2\alpha}$ increased (Galvao *et al*., 2011) what may amplify the luteal chemoattractive signal for immune cells and augment cytokines production, reducing luteal cell viability and increasing apoptosis, determinant for equine CL demise (Fig. 23b) (Galvão *et al*., 2010). A short frame of the complex mechanism rulling luteal function has been proposed here. However, further studies are needed for a better understanding these regulatory mechanisms, namely concerning the specific role of equine luteal endothelial cells

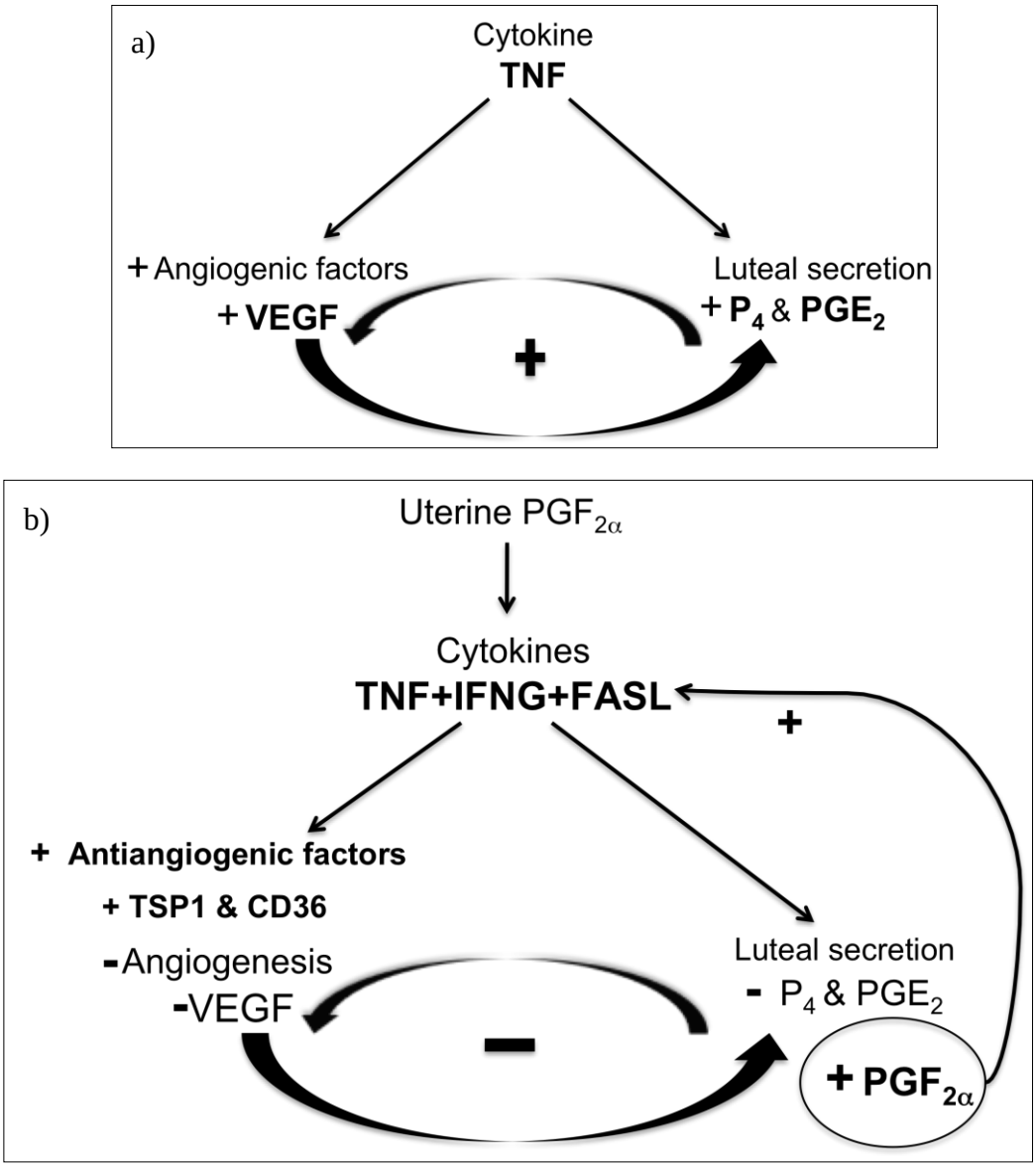


Figure 23: Schematic interrelationship among cytokines, angiogenic factors, prostaglandins and progesterone in equine CL: (a) luteotrophic loop triggered by TNF, between VEGF and P_4 and PGE_2; (b) luteolytic role of cytokines by inhibiting VEGF/P_4 and PGE_2 loop and stimulating antiangiogenic factors and $PGF_{2\alpha}$.

4. EFFECT OF CYTOKINES AND OVARIAN STEROIDS ON EQUINE ENDOMETRIUM FUNCTION: AN *IN VITRO* STUDY

(Under review in *Reproduction, Fertility and Develpment*)

4.1 Abstract

In mare, endometrial physiology regulation is not fully understood. The aim was to (i) evaluate the presence of cytokines tumor necrosis factor alpha (TNF), interferon gamma (IFNG) and Fas ligand (FASL), and their receptors in mare endometrium throughout the estrous cycle; (ii) establish an equine endometrium *in vitro* cell culture model; (iii) and assess endometrium secretory function (PGE_2 and $PGF_{2\alpha}$), angiogenic activity and cell viability in response to TNF, estradiol (E_2), progesterone (P_4) and oxytocin (OXT). Cytokine ligands and receptors were present in surface and glandular epithelium, and stroma. TNF and TNFRI protein expression increased in follicular phase (FP) and mid luteal phase (LP), while TNFRII did not change. IFNG was expressed in mid LP and IFNRI in mid and late LP. The highest expression of FASL and FAS was in late LP. *TNF* and *FASL* mRNA transcription increased in early and late LP, while *IFNG* mRNA rose in late LP. Receptors *TNFRI* and *TNFRII* mRNA transcription was the highest in mid LP, and *FAS* and *IFNRI* were constant. OXT increased FP and mid LP prostaglandin (PG)E_2 and $PGF_{2\alpha}$ production. TNF action was similar, but it did not stimulate $PGF_{2\alpha}$ in mid LP. E_2 and P_4+E_2 augmented $PGF_{2\alpha}$ in mid LP. TNF and P_4 increased cell viability in mid LP. All treatments increased NO and angiogenic activity (except P_4). Cytokines differential expression throughout the estrous cycle might suggest a role on endometrial function. Besides, cytokines coordinated action with ovarian hormones may regulate secretory, angiogenic and proliferative functions in equine endometrium.

4.2 Introduction

Physiologic changes experienced by the endometrium throughout the estrous cycle are required for pregnancy establishment and maintenance. The endometrium is a complex tissue whose cyclic regulation is mainly driven by the changing pattern of the ovarian steroids estradiol (E_2) and progesterone (P_4) (Raw *et al.*, 1995; Riley & Poyser, 1990; Roberto da Costa *et al*., 2007a). These hormones are known as crucial regulators of cell differentiation, angiogenesis, morphogenesis and endometrial physiology (Ferreira-Dias *et al.*, 2001; Mangelsdorf *et al*., 1995; Roberto da Costa *et al*., 2007a). Several factors other than ovarian steroids, such as cytokines and nitric oxide (NO) have also been shown to participate in endometrium function regulation, throughout the estrous cycle (Murakami *et al*., 2001; Pitzel *et al.*, 1993; Roberto da Costa *et al*., 2007a; Roberto da Costa *et al*., 2008; Spencer *et al*., 2004; Skarzynski *et al.*, 2005). In the mare, NO is involved in prostaglandins (PG) production modulation, namely PGE_2 and $PGF_{2\alpha}$, which play an important role in utero-ovarian physiology (Roberto da Costa *et al*., 2008). In the absence of pregnancy, the influence of the endometrium on ovarian function regulation, specifically during luteolysis, is also well evidenced (Poyser, 1995). In the seventies, the temporal association between uterine $PGF_{2\alpha}$ and the onset of luteolysis in nonpregnant cow (Shemesh & Hansel, 1975), ewe (Wilson *et al.*, 1972), gilt (Moeljono *et al.*, 1977), woman (Singh *et al.*, 1975) and mare (Douglas & Ginther, 1976; Zavy *et al.*, 1978) was first referred. Later on, the importance of oxytocin (OXT) on triggering the luteolytic $PGF_{2\alpha}$ was also addressed for species like ewe (Silvia *et al*., 1991), pig (Uzumcu *et al.* 2000) and mare (Stout & Allen, 1999). In particular for the mare, during the last decades, several attempts have been made for a better understanding of the regulation of endometrium secretory function during both cyclicity and early pregnancy (Boerboom *et al*., 2004; Eroh & Ealy, 2007; Goff *et al*., 1987; Sharp & McDowell, 1985; Sharp *et al*., 1989; Sharp *et al*., 1997). Complexity of the events coordinating equine endometrial dynamics throughout the estrous cycle is evidenced by the interdependence between different physiologic pathways involved in cell proliferation and active caspase-3 mediated apoptosis (Roberto da Costa *et al*., 2007b), vascular and non-vascular tissue growth and secretory function (Ealy *et al*., 2010; Ferreira-Dias *et al*., 2001; Roberto da Costa *et al*., 2007b; Roberto da Costa *et al*., 2008). The auto-, paracrine action of cytokines, such as tumor necrosis factor alpha (TNF), interferon gamma (IFNG) and Fas ligand (FASL) in the reproductive tract has been associated with physiologic processes (Galvão *et al*., 2010; Miyamoto *et al*., 2000; Pitzel *et al*., 1993; Skarzynski *et al*., 2007). Despite the well-established role of cytokines on endometrial function in the cow (Miyamoto *et al*., 2000;

Skarzynski *et al*., 2000a) and woman (Lea & Sandra, 2007), cytokine mediated regulatory mechanisms in the equine endometrium are yet to be fully understood. Recently, in vitro explant or cell culture models have been successfully used for the study of endometrial function in a number of species, either under physiologic or pathologic conditions (Davis & Blair, 1993; Miyamoto *et al*., 2000; Roberto da Costa *et al*., 2007a; Schatz *et al*., 1987; Skarzynski *et al*., 1999a; Uzumcu *et al*., 2000). Significant advances in our understanding of maternal recognition of pregnancy or interaction between uterus and embryo have been possible due to in vitro model systems (Ealy *et al*., 2010; Mardon *et al*., 2007). Also in the mare, these methodologies have been commonly accepted as a powerful tool to investigate the mechanisms regulating endometrial physiology (estrous cycle and pregnancy) and pathology (Ealy *et al*., 2010; Ferreira-Dias *et al*., 2001; Nash *et al*., 2008; Theuβ *et al*., 2010; Watson *et al*., 1992). The advantages over other techniques are worth noting. Animal suffering is avoided, and the accurate control of experimental conditions is feasible. The present work was carried out to address the hypothesis that cytokines TNF, IFNG and FASL may interact with ovarian steroid hormones (P_4 and E_2) and OXT in the mare endometrium, and thus regulate endometrial environment (secretory function and angiogenesis) in different stages of the estrous cycle. Besides, these interactions may be responsible for physiologic changes needed either for luteolysis, in the absence of pregnancy, or for luteal maintenance in case of fertilization. Thus, the aim of the present study was (i) to evaluate the presence and expression of the cytokines TNF, IFNG, FASL, and their receptors in the mare endometrium; (ii) to develop and validate an in vitro cell culture model for equine endometrium; (iii) to assess endometrium secretory function (PGE2 and PGF2α), angiogenic activity and cellular dynamics (cell viability and proliferation) in response to TNF, ovarian hormones (P_4 and E_2) and OXT stimulations.

4.3 Materials and methods

4.3.1 Uteri collection

Equine internal genitalia and venous blood from jugular vein were collected *post mortem* at the local abattoir from randomly designated cyclic Lusitano mares, age ranging from 3 to 8 years old, from May until the beginning of September. Mares were euthanized after stunning according to the European Legislation concerning welfare aspects of animal stunning and killing methods (EFSA, AHAW/04-027) and to the Portuguese legislation (DL 98/96, Art. 1º), and approved by the Faculty of Veterinary Medicine Ethics Committee. Since the

reproductive status of the animals was unknown, blood samples were collected at the time of exsanguination into heparinized tubes (Monovettes®- Sarstedt, Numbrecht, Germany), for further estrous cycle confirmation based on plasma P_4 concentration. Thus, estrous cycle phase of the mares was assessed based on macroscopic evaluation of structures present in the ovary and on plasma P_4 levels (RIA) (Coat-a-Count Progesterone, Diagnostic Produtc Corp., Los Angeles, CA, USA) as described before (Roberto da Costa *at al*. 2007a; Roberto da Costa *at al*. 2008). Collected uteri were classified as belonging to follicular phase (FP; n=15), early luteal phase (early LP; n=10), mid luteal phase (mid LP; n=15) and late luteal phase (late LP; n=10). Follicular phase was characterized by the absence of an active corpus luteum (CL) and presence of follicles with different sizes, but diameter always > 35 mm, in the ovary together with a concentration of plasma P_4<1 ng/ml. In the early LP, the ovary presented a corpus hemorrhagicum and plasma P_4>1 ng/ml, while in mid LP a developed CL was associated with follicles 15 to 20 mm in diameter and P_4>6 ng/ml. In the late LP, a CL undergoing regression was present, follicles with diameter between 30 and 35 mm, and P_4>1 ng/ml and P_4<2.5 ng/ml (Roberto da Costa *at al*. 2007a; Roberto da Costa *at al*. 2008). Immediately after uteri collection, endometrium samples from the uterine horn ipsilateral to the ovary (presenting either a growing follicle or a CL), were isolated from the underlying myometrium and washed in sterile phosphate-buffered saline solution (PBS). Samples were then placed in: (i) RNAlater (AM7020, Ambion, Applied Biosystems, CA, USA) for mRNA and protein expression quantification; or (ii) buffered formaldehyde, for immunohistochemistry. Remaining material (rest of the uterine horns) was washed in PBS and kept intact in chilled (4ºC) culture medium M-199 (M2154; Sigma-Aldrich, St. Louis, MO, USA) with streptomycin (100 µg mL−1; S9137, Sigma), penicillin (100 IU mL−1; P3032, Sigma) and 2mg/mL amphopthericin (A2942, Sigma) for *in vitro* cell culture studies. After collection, endometrial samples, uterine horns and blood were transported to the laboratory within 1 h.

4.3.2 Immunohistochemistry analysis - cytokines localization in the equine endometrium

The presence of the cytokines TNF, IFNG and FASL and the receptors TNFRI, TNFRII, IFNRI and FAS in the endometrium at the different phases of the estrous cycle was determined following the methodology described before (Ferreira-Dias *et al*., 2006a). Immunohistochemistry was performed on consecutive 4 µm histological sections of the endometrium (FP, n=6; early LP, n=6; mid LP, n=6 and late LP, n=6). Tissue sections were incubated for 3 h at room temperature (RT) with primary rabbit monoclonal antibody against

TNF (anti-equine 1:500, AHP853Z, AbD Serotec, Oxford, UK), FASL (anti-human 1:50, Ref. 556387, BD Bioscience; Franklin Lakes, NJ, USA) or mouse monoclonal antibody FAS (anti-human 1:100, Ref. 610197, BD Bioscience), and rabbit polyclonal antibodies IFNG (anti-human 1:200, sc-8308, Santa Cruz Biotechnology, Santa Cruz, CA, USA), TNFRI (anti-human 1:1000, ab19139, Abcam, Cambridge, UK), TNFRII (anti-mouse 1:50, ab15563, Abcam) and IFNRI (anti-human 1:100, ab61179, Abcam), diluted in 0.1 M PBS (pH 7.4). Primary antibody was detected using biotinylated anti-mouse secondary antibody (Histostain Plus; Zymed Laboratories Inc., Ref. 85-9043) for 30 min at RT. Sections were incubated 30 min with streptavidin-peroxidase (Histostain Plus; Zymed Laboratories Inc., Ref. 85-9043) and for color development was used 3,3´- diaminobenzidine for 5 minutes. Negative control was performed by replacing the primary antibody by the rabbit polyclonal IgG (ab27478, Abcam), for antobodies developed in rabbit, and the mouse IgG (550878, BD Bioscience) for antibodies developed in mouse, in the same dilution and incubation time as the primary antibody. Immunohistochemistry staining was assessed as a characteristic brown staining, with a light microscope (Olympus BX51, Tokyo, Japan) at 400x magnification. Tissue areas were photographed (DP11 Olympus, Tokyo, Japan).

4.3.3 Western blot

Protein expression of cytokines and receptors on equine endometrial tissue was assessed by Western blot, throughout the estrous cycle. Endometrial tissue (n=3/stage) was minced and placed in ice-cold RIPA buffer (50 mM Tris-Hcl, pH 7.4; 50 mM EDTA, 150 mM NaCl, 1% Triton X-100) with protease inhibitor (Roche No.11697498 001, Roche Diagnostics Poland, Warsaw, Poland) and homogenized on ice. After protein extraction and concentration assessment with Bradford reagent (ref. 500-0006, Bio-Rad, Hercules, CA, USA) (Ferreira-Dias *et al*., 2006a), 120 µg of protein was loaded on a 12% acrylamide gel (ref. 161-0155, Bio-Rad,) for analysis of studied factors. Further on, protein was transfered to a nitrocellulose membrane (Ref. 1620116, Bio-Rad) (Rodrigues *et al*., 2002). Level of active studied proteins was evaluated with the same antibodies used for immunohistochemistry, but diluted at 1:250 for TNF, 1:100 for IFNG, 1:200 for FASL, 1:300 for TNFRI, 1:200 for TNFRSF2B, 1:200 for IFNRI and 1:1000 for FAS. To normalize loaded protein, a mouse monoclonal antibody against ß actin (A5441, Sigma) was used at the dilution 1:10,000. Membranes were incubated with the primary antibody for 1h at 37ºC. For all proteins (except for FAS and ß actin), the secondary antibody used was horseradish peroxidase (HRP)-conjugated anti-rabbit (P0448,

Dakocytomation, Carpinteria, CA, USA), at 1:10,000 incubated 2.5 h at RT. For FAS and ß actin, was used HRP-conjugated goat anti-mouse (A2554, Sigma) at 1:100,000 for 2.5 h at RT. Protein expression was visualized by means of luminol-enhanced chemiluminescence (SuperSignal® West Pico, 34077, Thermo Scientific, Waltham, MA, USA) and exposure of the membrane to a blue light-sensitive autoradiography film (Kodak BioMax Light Film; Kodak-Industrie, Chalon-sur-Saone, France). Target proteins were normalized dividing units of the arbitrary densimetry by ß actin for each band. Signals were densitometrically assessed using the ImageMaster 1D Elite densitometric analysis program (Amersham Biosciences, Piscataway, NJ, USA).

4.3.4 Real Time PCR

As described before (Galvão *et al*., 2010), RNA was extracted from the endometrium (FP, n=5; early LP, n=5; mid LP, n=5 and late LP, n=5) using Qiagen Kit for Total RNA Extraction and Purification (ref. 28704, Qiagen, Hilden, Germany), including the DNA digestion step with RNase-free DNase Set (ref.50979254, Qiagen, Germany), according to manufacturer's instructions. Further on, quantification and quality assessment of the RNA were performed and reverse transcription was done with a Reverse Transcriptase Superscript III enzyme (ref.18080093, Invitrogene, Gibco, Carlsbad, USA), from 1 μg total RNA in 20 μl reaction volume, using oligo (dT) primer (27-7858-01, GE Healthcare, Buckinghamshire, UK). Specific primers for target genes TNF, IFNG, FASL, TNFRI, TNFRSF2B, IFNRI and FAS and for housekeeping gene were designed (Table 6) using different internet-based interfaces like Primer-3 (http://frodo.wi.mit.edu/primer3/) and Primer Premier software (Premier Biosoft Int., Palo Alto, CA, USA) (Feng *et al*., 1993). Before running the assay, the housekeeping gene (HKG) validation was performed. To determine the most stable internal control gene four potential HKG were initially considered: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase A (*SDHA*), β-actin and β2-microglobulin (*B2MG*). As described by Dheda *et al*. (2004) mRNA transcription of a suitable HKG should not vary more than 2-folds between different biological conditions. During the validation process, samples (n=4) from different stages of the estrous cycle (FP, early LP, mid LP and late LP) were run in parallel for tested genes. The mRNA transcription of *B2MG* was the most stable and unaffected by the experimental conditions (less than 2-folds changes between stages). Primer concentration was optimized to the ratio minimum concentration/lowest cycle threshold. Assay was performed in a 7300 Real Time PCR System (Applied BiosystemsTM,

Warrington, UK), using the default thermocycler program for all genes: a 10 min pre-incubation at 95ºC was followed by 40 cycles for 15 sec at 95ºC and 1 min at 60ºC, followed by a dissociation step (15 sec at 95ºC, 30 sec at 60ºC and 15 sec at 95ºC). Both target gene and HKG were run simultaneously and all reactions were carried out in duplicate wells on a 96 well optical reaction plate (Applied Biosystems, ref. 4306737, UK) in 25 μl reaction volume: 6.5 μl water; 2 μl forward primer; 2 μL reverse primer; 12.5 μl Power SYBER® Green Master M9 (Applied Biosystems, Ref. 4367659, UK) and 2 μl of cDNA. All the PCR products were run through a 2.5% agarose (BIO-41025, Bioline, Luckenwalde, Germany) gel in order to confirm specificity. Relative mRNA quantification data were then analyzed with the Real-time PCR Miner algorithm (Zhao & Fernald, 2005).

Table 6: Specific primers for quantitative real time PCR (bp-basepair).

Gene	Accession number	Sequence 5′- 3′	Length (bp)
TNF	AB035735	For: ACCGAATGCCTTCCAGTCAA	143
		Rev: CATTTGCACGCCCACTCA	
TNFRI	GU166822	For: TCAACGGCACAGTGCATCT	98
		Rev: CAGGACATGCTCTCTT	
TNFRII	XM_001489844.2	For: TGCATACTTCCAAGGCAGGAG	108
		Rev: GCACACCACGTTTGATGTCG	
IFNG	NM_001081949.1	For: TAACAGCAGCACCAGCAAGC	165
		Rev:CGAAATGGATTCTGACTCCTCTTC	
IFNRI	GU166821	For: CCGAAGAACTCTGCCTGACC	113
		Rev: ACAACACGCAACCAGCAGAAT	
FASL	GQ429291	For: GCTGGTTGTTGCAGGACTGA	69
		Rev: TCAATGACACCGGGCTGTAC	
FAS	GQ429290	For: TTACGTGCAAACATGGGATCA	71
		Rev: TCCGGATCCTTCTCTGCATT	
B2MG	X69083	For: CGGGCTACTCTCCCTGACTG	92
		Rev: TTGGCTTTCCATTCTCTGCTG	

4.3.5 In vitro endometrial cell experiments

4.3.5.1 Equine endometrial cells isolation

Uteri from FP (n=5) and mid LP (n=5) were used for endometrial cell isolation and cell culture validation. Isolated uterine horn was washed three times with sterile Hank´s balanced salt solution (HBSS) (55021C, Sigma) with 40 µg/ml gentamicin (G1397, Sigma). The tubular structure was cut transversely in rectangular pieces of approximately 3x6 cm, and incubated in M-199 medium supplemented with 0.48% Dispase (17105, Gibco, NY, USA) and 0.1% (w/v) bovine aortic serum (BSA) (#735078; Roche Diagnostics GmbH Mannheim, Germany), in a petri dish, for 40 min at 37ºC in humidified atmosphere (Biosafe Eco- Integra Biosciences, Chur, Switzerland; 37ºC, 5% CO_2, 95% air), with gentle shaking (Titertek; Huntsville, AL, USA; 150 rpm). Every 10 min, dissociated cells were removed, and fresh medium containing enzymes was added. Collected cells were washed by centrifugation (10 min at 120 g) with M-199 plus 0.1% BSA and gentamicin (20 µg/ml) and kept at 8ºC until the end of the digestion process. After the first digestion step, endometrial strips were dissected from the myometrium, washed in HBSS with gentamicin (20 µg/ml), and minced into small fragments (1 mm^3) with a scalpel and isolated, as described previously for bovine endometrial cells (Skarzynski *et al.*, 1999b). Minced tissue (8-10 g) was then incubated with M-199 supplemented with 0.1% (w/v) collagenase (C-0130; Sigma), and 0.1% BSA for further 50 min, under the same conditions described above. Dissociated cells from both digestions were filtered through a metal wire mesh (100 µm) with a cell dissociation Sieve - Tissue grinder Kit (cd1-1kt, Sigma), to remove the undissociated tissue fragments. The filtrate was washed twice by centrifugation (10 min at 120 g) with M-199 plus 0.1% BSA and gentamicin (20µg/ml). Supernatant was discarded and erythrocyte lysis was accomplished after treating the pellet with Red blood lyses buffer (R7757, Sigma). Another washing step was performed. Cells were resuspended in Dulbecco's modified eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 [v/v], D-8900; Sigma) containing 10% fetal bovine serum (FBS) (26140-079, Gibco, USA), gentamicin [20 µg/ml] and amphotericin [250 µg/ml] (A2942, Sigma). Cell viability, determined by trypan blue exclusion dye (T8154, Sigma), was higher than 89%. Dispersed endometrial cells ($2.0x10^5$/ml) were then cultured in 1 ml of D/F medium with 10% FBS, amphotericin (250 µg/ml) and gentamicin (20 µg/ml), in 24-well culture plates (142475, Nunc, Kamestrupvej, Denmark) for Experiment 2.2 and in 100 µL ($2.0x10^4$/ml) in a 96-well cell culture plates (Corning, Corning, NY, USA) for Experiment 2.3, at 37ºC in humidified atmosphere (5% CO_2 and 95% air). Confluence of 80-90% was achieved approximately 36 h to 48 h after, being mainly identified two cell types – epithelial

like cells (ELEC, both surface and glandular) and stromal like cells (SLEC) based on their morphology. Cell composition from FP was represented by 70% SLEC and 15% ELEC while in the mid LP endometrial cell culture 50% were SLEC and 35% ELEC.

4.3.5.2 Immunofluoresce of endometrial cells

Stromal and epithelial cells identification was assessed using immunofluorescent staining for specific markers of epithelial (cytokeratin) and stromal (vimentin) cells, as described before (Malayer & Woods, 1998). Briefly, immunocytochemistry started with platting the cells in 8 well chamber slides (354108, BD Biosciences), at the final volume of 300 uL ($1.0x10^5$/ml). After attachment, cells were washed 5 min with PBS (3 times) and fixed with 2% paraformaldehyd for 10 min. After washing for 5 min with PBS (3 times), blocking was performed with donkey serum (D9663, Sigma), for 1 h at RT. Incubation with primary antibody against cytokeratin (mouse monoclonal anti-cytokeratin; C9687 Sigma; diluted 1:25) or vimentin (mouse monoclonal anti-vimentin; V6630 Sigma; diluted 1:37) in PBS containing 0.5% BSA was carried out overnight at 4ºC. Slides were washed 3 times with PBS (5 min each) and then incubated with secondary antibody (anti-mouse IgG, A21203, Invitrogene, Oregon, USA; diluted 1:300) diluted in PBS with 0.5% BSA, for 1 h at RT. Slides were washed 3 times with PBS (5 min each) and mounted in Vectashiled with DAPI (Vectashield with DAPI, H-1200;Vector Laboratories, Inc. Burlingame, CA 94010). Images were captured with a digital camera (DC 350F, Leica, Germany) and visualized with a fluorescence microscope (DM5000B, Leica).

4.3.5.3 Cell culture stimulation

In order to validate the cell culture model and to study secretory function modulation by factors under study, cultures of endometrial cells isolated from FP (n=5) and mid LP endometria (n=5) were stimulated. After reaching 80-90% confluency, cells were washed with M-199 with 0.1% BSA and phenol red free DMEM/F_{12} medium (1:1) (11039, Gibco, USA) with 0.1% BSA, gentamicin (20 µg/ml) and transferrin (5 µg/ml) (T1428, Sigma) added. After 30 min stabilization period, cells were incubated for 24 h as follows: without factors (control); with TNF (10ng/ml; T6674, Sigma); OXT as a positive control (10^{-7} M; O3252, Sigma); P_4 (10^{-7} M; P0130, Sigma); E_2 (10^{-9}M; E8875, Sigma) or with P_4 + E_2 (10^{-}

^{7}M+10^{-9}M). Afterwards, conditioned media from negative control and treatment groups were stored at -70°C for further analysis.

4.3.5.4 Cell viability and proliferation assessment

Endometrial cells from FP (n=5) or mid LP (n=5) were plated in a 96-well cell culture plates (Corning, Corning, NY, USA) as described before, at the concentration of 1.0x10^{4}/ml and incubated for 24 h, with the same factors as in Experiment 2.2 (experiments run in parallel). Cell viability was determined with Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (G3581, Promega, Madison, WI, USA), according to the manufacturer manual. Absorbance (A) was read at 490 nm using a microplate reader (Model 450, BIO-RAD, Hercules, CA, USA).

4.3.6 Angiogenic factors and NO production

4.3.6.1 Endothelial cells proliferation assay

Effect of treatments (*i.e.* control, TNF, OXT, P_4, E_2 and P_4 +E_2) on angiogenic activity in follicular P and mid LP endometrial cells was assessed. Those media collected after endometrial cells treatment were further incubated with bovine aortic endothelial cells (BAEC; kindly donated by Dr. D. A. Redmer). Thus, angiogenic activity was indirectly assessed based on BAEC proliferation determined by alamarblue reagent method (Serotec, Oxford, UK). Protocol optimization for BAEC was performed before (Ferreira-Dias *et al*., 2006b). Thus, the optimal reading time was at 5 h incubation of BAEC with the conditioned media from equine luteal cell cultures, since this time-point represented the point at which the linear correlation between the percentage reduction of the indicator and cell density was the highest (R2 =0.9507), after calculation according to the manufacturer's instruction (Ferreira-Dias *et al*., 2006b). Briefly, BAEC (2×10^{4} cells/ml) were incubated in 24-well plates at 37ºC in humidified atmosphere (5% CO_2 and 95% air) for 14 h, until cells adhered to the wells. Further on, samples of endometrial cells conditioned media (obtained from experiment 2.2) (30% concentration) were added in triplicate to the wells and cells were incubated for further 48 h. Conditioned media were then removed and fresh phenol red free DMEM/F12 medium containing 10% alamarBlue was added. The plates were incubated for further 5 h and absorbance (abs) read at 570 and 600 nm (SpectrMax 340 PC; Molecular Devices; Biocitek

SA, Lisbon, Portugal). The BAEC proliferation response to endometrial cells conditioned culture media was evaluated by comparing the percentage reduction by these media with that produced by the negative controls (whitout endometrial cells), being cell proliferation or mitogenesis in response to negative control considered to be 100% (Redmer *et al*., 1988; Ferreira-Dias *et al*., 2006b). Alamarblue percentage reduction was determined according to alamarblue technical datasheet.

4.3.6.2 Assessment of NO metabolites production

Quantification of *in vitro* NO production by cultured endometrial cells followed the methodology previously described (Jaroszewski *et al*., 2003) Briefly, after thawing the media originating from experiment 4.3.5.3, 50 µl of each sample were added to a 96-well plate and incubated with the reagents provided by the kit for Griess Reagent System (G2930, Promega, Madisson, USA), following manufacturer´s instructions. The amount of NO produced was determined spectrophotometrically as a formed nitrite (NO_2) and absorbance measured at 540 nm using a microplate reader (Model 450, BIO-RAD). The nitrite content was calculated based on a standard curve made with $NaNO_2$ (0-100 M).

4.3.7 Hormone determinations

Assessment of PGE_2 and $PGF_{2\alpha}$ concentrations followed the methodology previously described (Skarzynski & Okuda, 1999). The PGE_2 standard curve ranged from 0.039 ng/ml to 10 ng/ml and the concentration of 50% binding (ED50) was 0.625 ng/ml. The intra- and interassay coefficients of variation were 5.9% and 11.0%, respectively. The $PGF_{2\alpha}$ standard curve ranged from 0.016 ng/ml to 4 ng/ml, and the concentration of 50% binding (ED50) was 0.25 ng/ml. The intra- and interassay coefficients of variation were on average 10.1% and 11.3%, respectively.

4.3.8 Statistical analysis

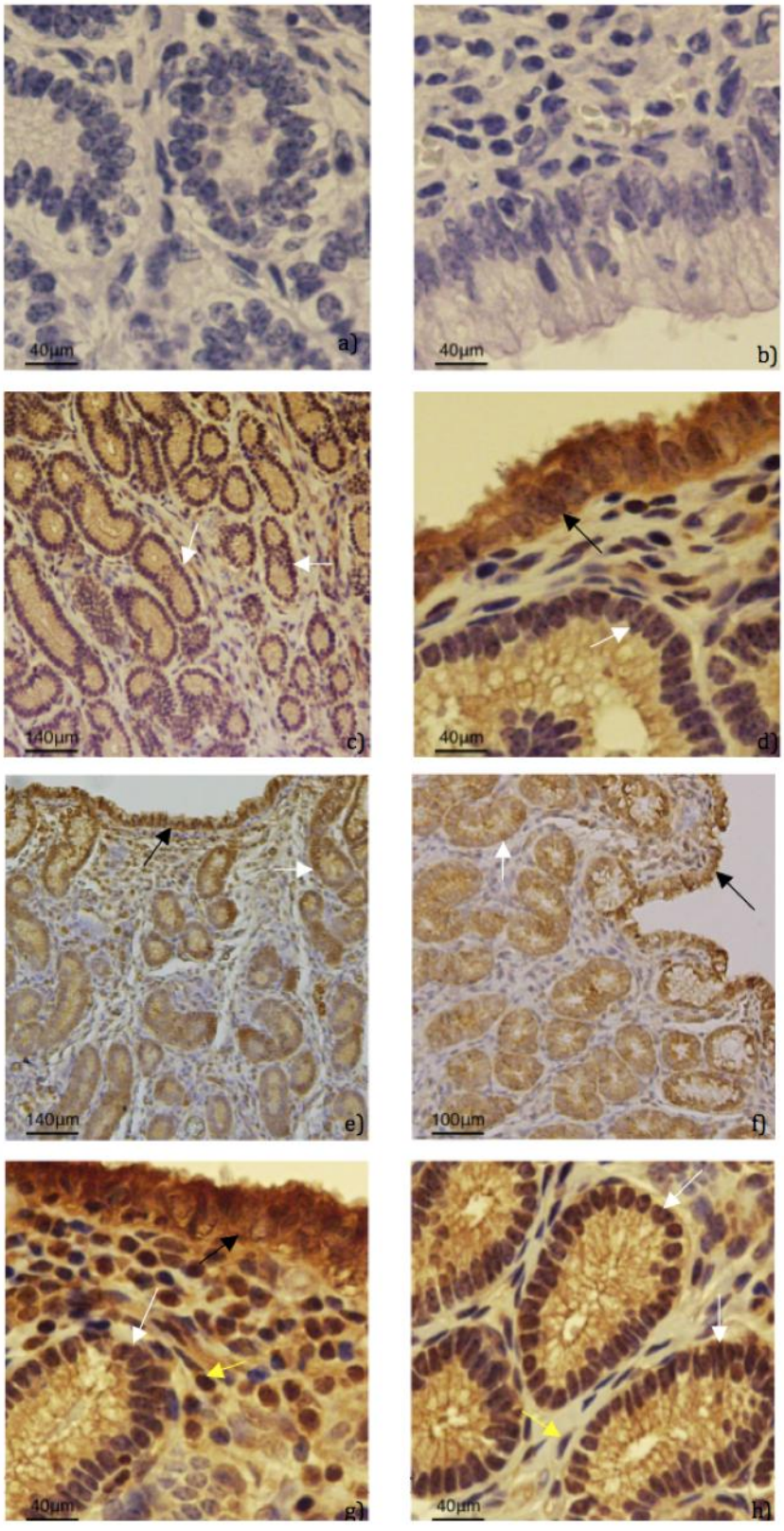
Data concerning real time PCR in the endometrial tissue, PGE_2, $PGF_{2\alpha}$ and NO metabolites quantification concentrations in culture medium, as well as BAEC proliferation and cell viability were analyzed using one way analysis of variance followed by Bonferroni's Multiple

Comparison Test (ANOVA; GraphPAD PRISM, Version 4.00, GraphPad Software, San Diego, CA, USA). Significance was defined as values of $p<0.05$.

4.4 Results

4.4.1 Cytokines and receptors protein expression

Immunohistochemistry showed the presence of ligands TNF, IFNG, and FASL, as well as their specific receptors TNFRI, TNFRSF2B, IFNRI and FAS, in the cells of the surface epithelium and glandular epithelium. Also, in the lamina propria, cytoplasm of stromal cells was slightly stained for all cytokines and receptors (Fig. 24g). No staining was present in negative controls (Fig. 24a, 24b). Western blot for protein expression analysis showed that immunoreactive bands intensity for TNF was the highest in FP, decreased to the lowest in early LP ($p=0.000003$), increased again in mid LP ($p=0.00003$) and decreased in late LP ($p=0.00002$) (Fig. 25a). The TNFRI showed the highest expression in FP, followed by a decrease in early LP ($p=0.00001$), while it increased in mid LP ($p=0.000001$) and in late LP was the lowest ($p=0.000008$) (Fig. 25b). The TNFRII expression did not change (Fig. 25c). The INFG protein expression was detected just in mid LP ($p=0.00005$) (Fig. 25d), and expresion of IFNRI was detected in mid ($p=0.00007$) and late LP ($p=0.00003$) (Fig. 25e). The FASL protein was expressed in the late LP ($p=0.00005$) (Fig.25f), while FAS expression increased from FP to early ($p=0.00005$) and mid LP ($p=0.0005$) (Fig. 25g) and towards the late LP ($p=0.0007$), where it presented the highest expression (Fig. 25g).


40µm
a)
40µm
b)
140µm
c)
40µm
d)
140µm
e)
100µm
f)
40µm
g)
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h)

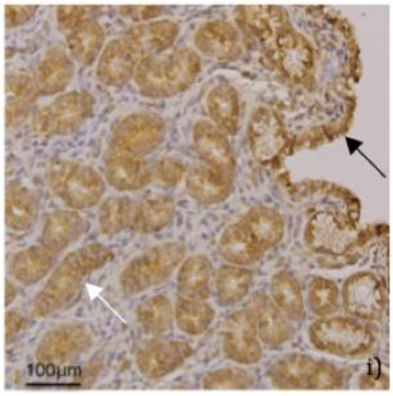
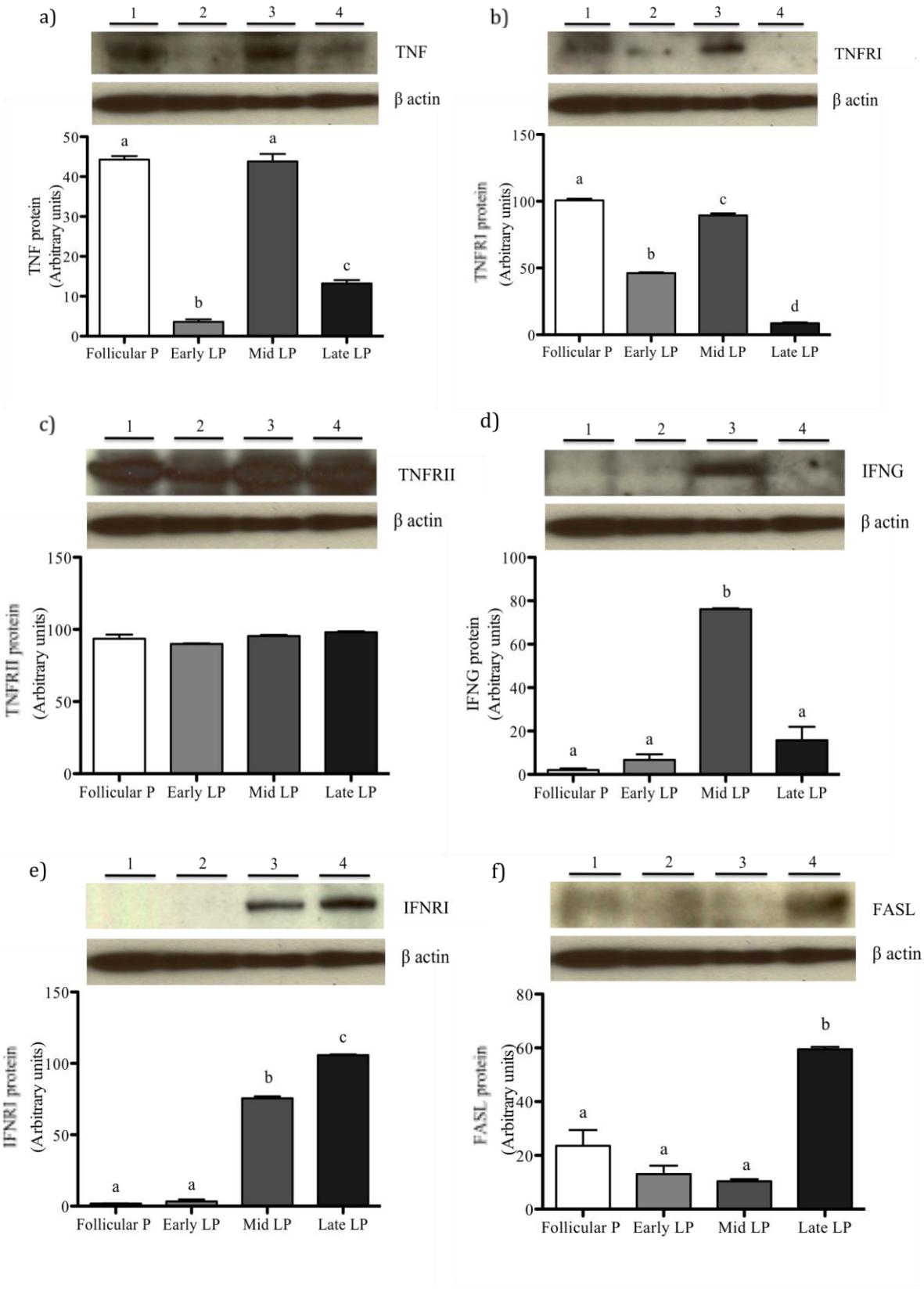


Figure 24: Representative images of equine endometrium immunostained for the presence of TNF in early LP (c), TNFRI in mid LP (d), TNFRII in FP (e), IFNG in late LP (f), IFNRI in late FP (g), FASL in FP (h) and FAS in mid LP (i). Negative control: (a) primary antibody replaced by rabbit IgG; (b) primary antibody replaced by mouse IgG. Black arrow indicates luminal epithelial cells, white arrow indicates glandular epithelial cells and yellow arrow indicates stromal cells. Since all cytokines/receptors stained equally throughout the estrous cycle, pictures from each luteal phase were randomly assigned. Immunostaining in 4 µm histological sections.

4.4.2 Cytokines and receptors mRNA transcription

The *TNF* mRNA transcription in mare´s endometrium was increased in early and late LP with respect to the FP (p=0.004) and mid LP (p=0.008) (Fig. 26a). Concerning *TNFRI*, its transcription was increased from FP to early LP (p=0.00008) and towards mid LP (p=0.006), where reached its highest level, being followed by a fall in the late LP (p=0.003) (Fig. 26b). The *TNFRII* had the highest mRNA transcription in the mid LP (p=0.005), comparing with other stages of the estrous cycle (Fig. 26c). The mRNA transcription of *IFNG* increased from FP to early LP (p=0.0007) and had the highest transcription in late LP (p=0.0005) (Fig. 26d), while *IFNRI* did not change (Fig. 26e). The *FASL* mRNA transcription was reduced in the mid LP comparing with early LP (p=0.0002) and late LP (p=0.007), but it was similar to FP endometria (Fig. 26f). *FAS* mRNA transcription did not change throughout the estrous cycle (Fig. 26g).


a)
1
2
3
4
TNF
β actin
TNF protein (Arbitrary units)
50
40
30
20
10
0
a
b
a
c
Follicular P
Early LP
Mid LP
Late LP
b)
TNFRI
β actin
TNFRI protein (Arbitrary units)
150
100
50
0
a
b
c
d
c)
TNFRII
β actin
TNFRII protein (Arbitrary units)
d)
IFNG
β actin
IFNG protein (Arbitrary units)
100
80
60
40
20
e)
IFNRI
β actin
IFNRI protein (Arbitrary units)
f)
FASL
β actin
FASL protein (Arbitrary units)

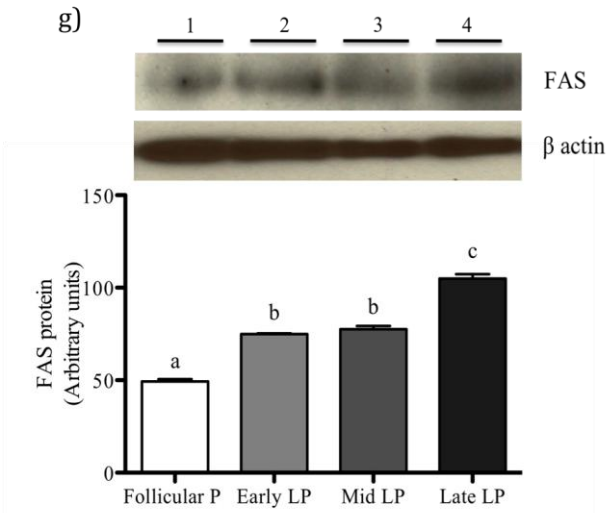
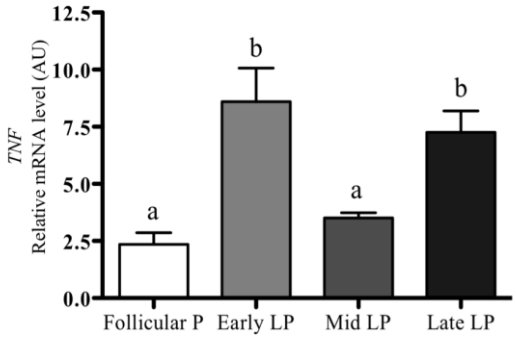


Figure 25: Protein expression quantification by western blot in equine endometrium (n=3/each phase). Upper panels depict representative western blot. Data normalized against β actin density values. Bars represent mean±SEM. Different superscripts indicate significant differences ($p < 0.05$). a) TNF protein; b) TNFRI protein; c) TNFRII protein; d) IFNG protein; e) IFNRI protein; f) FASL protein; g) FAS protein.

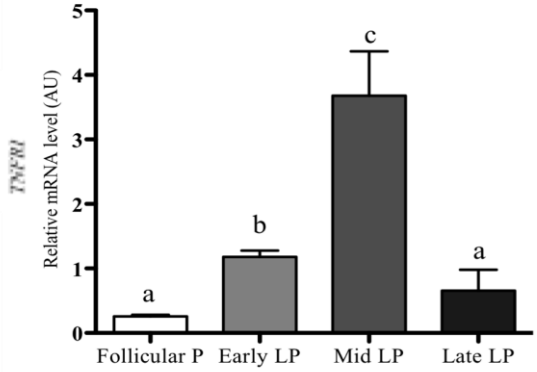
a)

	Follicular P	Early LP	Mid LP	Late LP
TNF	31,05	26,28	29,79	26,89
B2MG	17,72	17,52	16,91	17,29



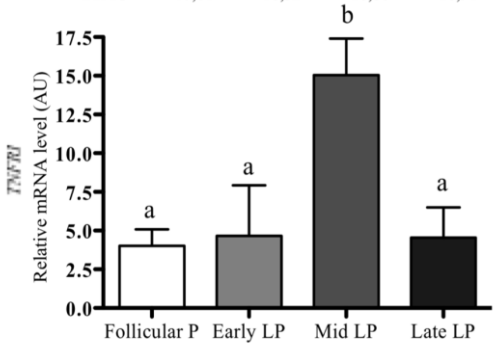
b)

	Follicular P	Early LP	Mid LP	Late LP
TNFRI	28,27	26,58	22,83	26,30
B2MG	16,38	16,16	16,56	16,15



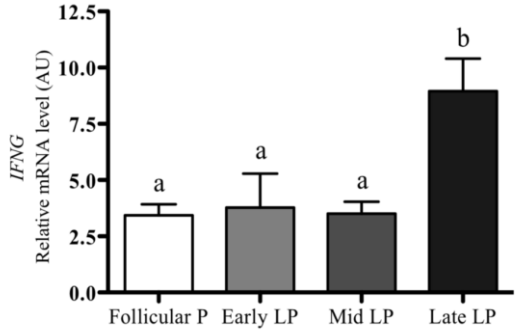
c)

	Follicular P	Early LP	Mid LP	Late LP
TNFRII	27,42	26,66	23,77	28,09
B2MG	17,01	16,32	16,66	16,91



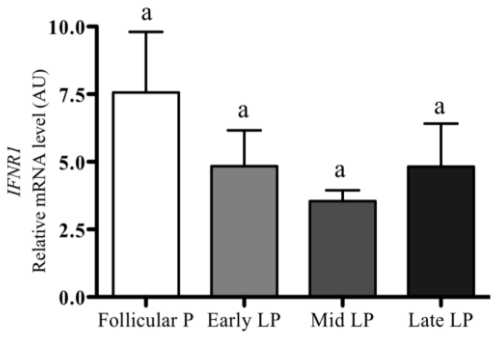
d)

	Follicular P	Early LP	Mid LP	Late LP
IFNG	30,04	29,82	29,87	26,10
B2MG	17,85	18,27	17,12	17,38



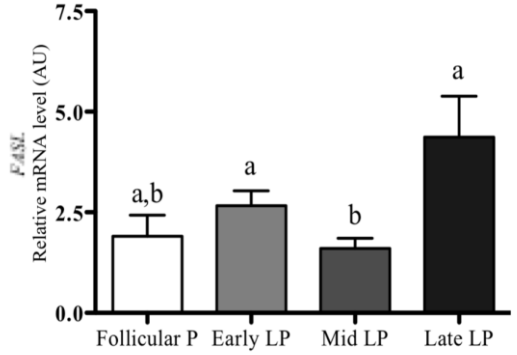
e)

	Follicular P	Early LP	Mid LP	Late LP
IFNR1	20,91	21,77	22,81	21,93
B2MG	16,42	17,28	16,70	17,01



f)

	Follicular P	Early LP	Mid LP	Late LP
FASL	30,80	29,90	31,89	28,58
B2MG	18,04	17,45	16,59	17,08



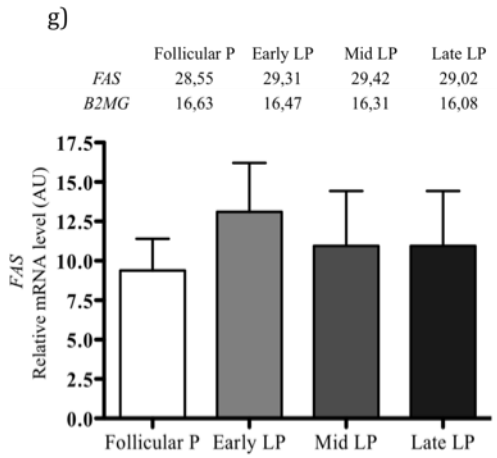


Figure 26: Relative quantification of gene transcription by real time PCR, in equine endometrium (n=5/each phase). Upper panels represent the average Ct of target and HKG in diffente phases of the estrous cycle. Expression normalized with the HKG – *B2MG*. Bars represent mean SEM. Different letters indicate significant differences ($p<0.05$). a) *TNF* gene; b) *TNFRI* gene; c) *TNFRII* gene; d) *IFNG* gene; e) *IFNRI* gene; f) *FASL* gene; g) *FAS* gene.

4.4.3 Cell culture characterization

Cultured cells presented two main characteristic morphologies: one group with cuboid-like shape and the other showed a spindle-like shape (Fig. 27). Staining with cell specific antibodies cytokeratin and vimentin allowed for the differentiation of both cell types, since spindle-like cells stained with vimentin (Fig. 28a), thus being classified as stromal cells (Fig. 27c; 27d) and cube-like cells stained with cytokeratin (Fig. 28c), being identified as epithelial cells (Fig. 27c; 27d).

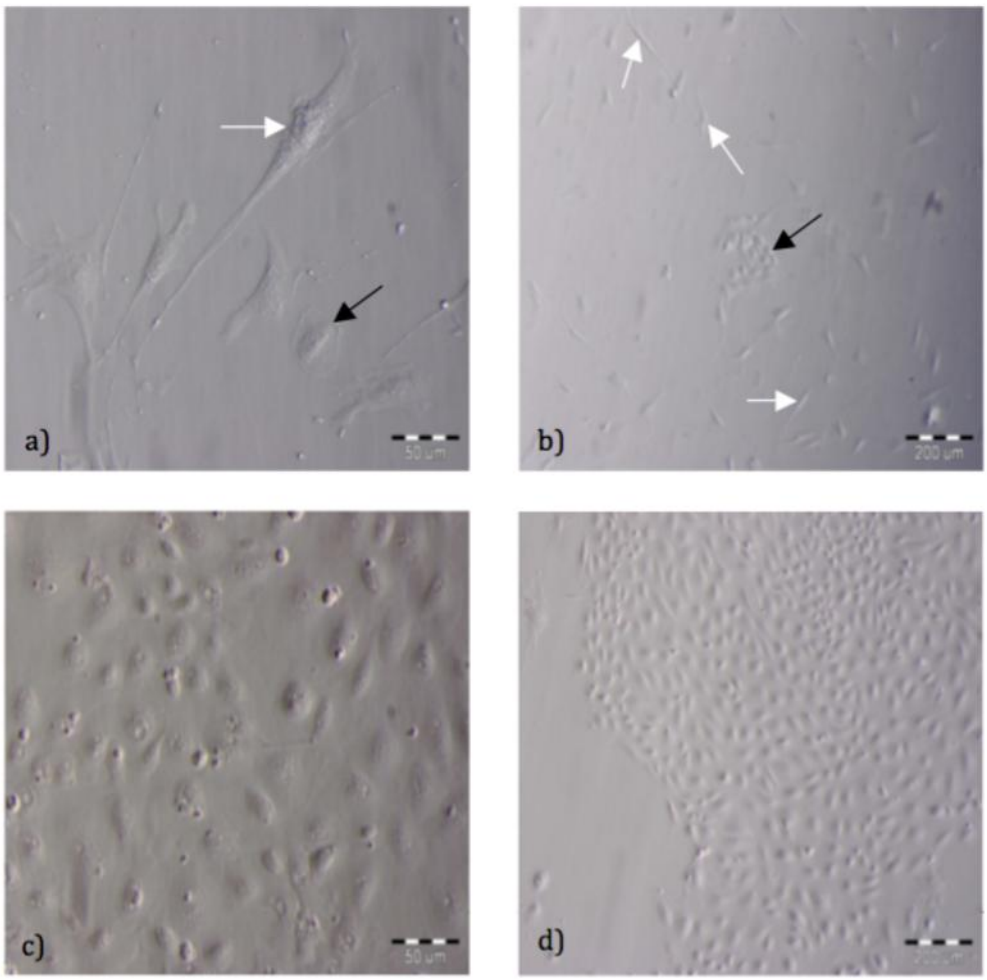


Figure 27: Cultured equine endometrial cells: (a) and (b) cells at an early stage after plating (white arrow – stromal cell; black arrow – epithelial cell); (c) and (d) 48 h hours after plating epithelial and stromal cells.

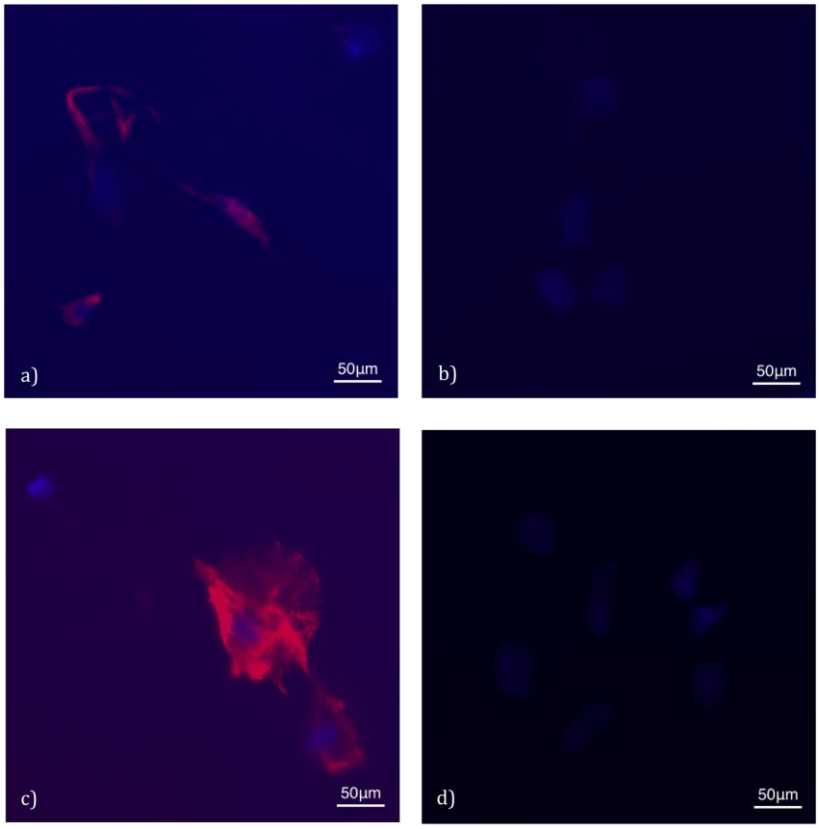


Figure 28: Immunofluorescence of stromal and epithelial cultured cells by cytokeratin and vimentin: a) cultured stromal cells stained with vimentin; b) negative control for vimentin; c) cultured epithelial cells stained with cytokeratin; d) negative control for vimentin.

4.4.4 Prostaglandins quantification in culture media

Evaluation of TNF, OXT, P_4, E_2 and $P_4 + E_2$ influence on equine endometrial cells eicosanoid production showed that PGE_2 production by equine endometrial cells from FP was increased after stimulation with TNF ($p=0.04$) and the peptide hormone OXT ($p=0.007$), as well as in cells from mid LP, by TNF ($p=0.02$) and OXT ($p=0.0003$) (Fig. 29a). Concerning $PGF_{2\alpha}$ secretion, it was increased in the FP by TNF ($p=0.03$) and OTX ($p=0.009$) and in mid LP by OXT ($p= 0.00005$), E_2 ($p= 0.00006$) and $P_4 + E_2$ ($p=0.03$) (Fig. 29b).

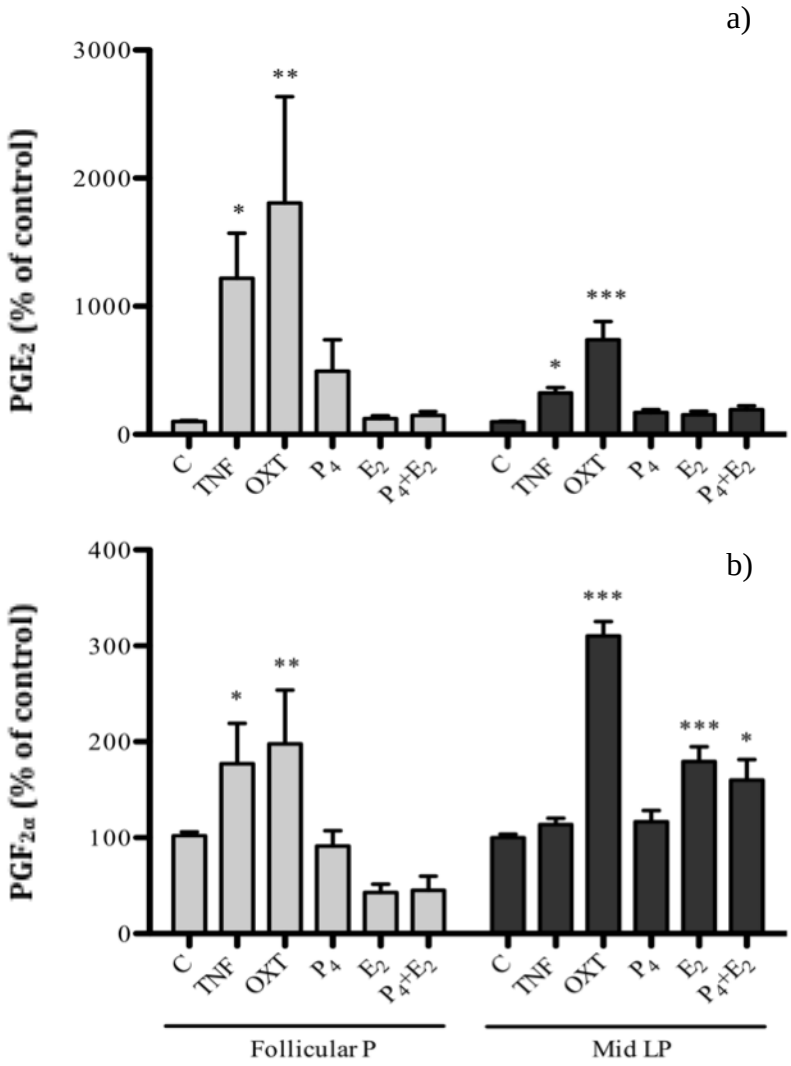


Figure 29: Effects of TNF, OXT, P_4, E_2 and P_4 + E_2 on PGE_2 (a) and $PGF_{2\alpha}$ (b) production by equine endometrial cells from follicular P (silver bars) and mid LP (dark grey bars). Asterisks indicate significant differences (*p<0.05; **p<0.01; *** p<0.001). Control values for hormones production (Mean ± SEM): PGE2 (3.93±0.41 ng/2.0x10^5cells); PGF2α (2.15±0.83 ng/2.0x10^5cells).

4.4.5 Viability and proliferation assessment

No changes on cell viability and proliferation were observed in FP (Fig. 30a). In mid LP, incubations with TNF and P_4 significantly increased cell viability (p=0.003) (Fig. 30b).

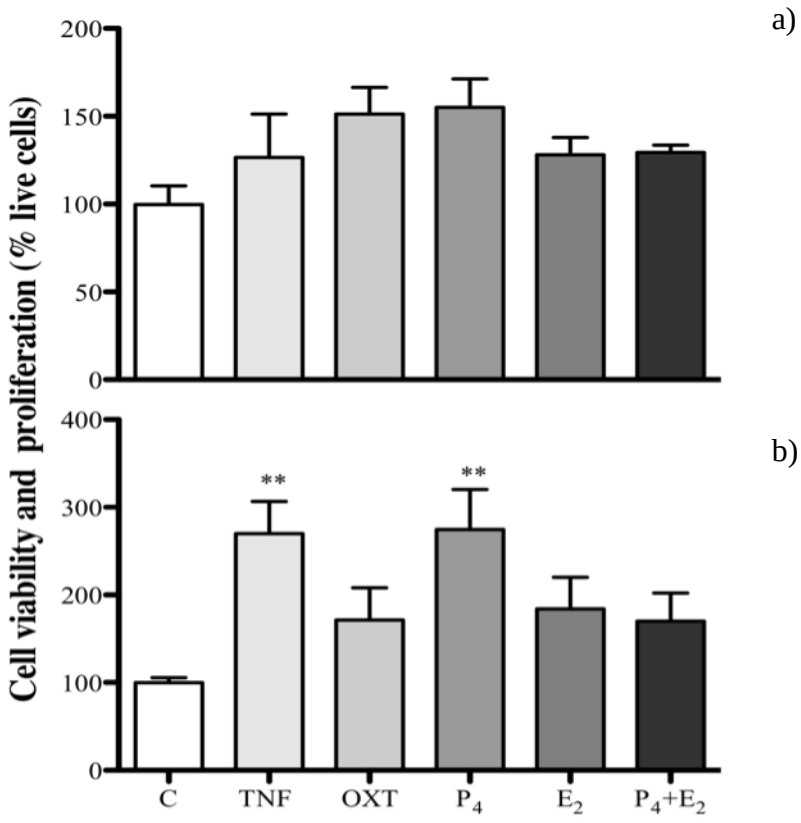


Figure 30: Equine follicular P (a) and mid LP (b) endometrial cells viability and proliferation after a 24 h incubation. Asterisks indicate significant differences (**p<0.01). Variations represent percentage changes from basal output (C).

4.4.6 Endothelial cell proliferation and NO production

Assessment of *in vitro* angiogenic activity by equine endometrial cells from FP showed an increase in BAEC proliferation when stimulated with TNF (p=0.02), E_2 (p=0.005) and $P_4 + E_2$ (p=0.009) (Fig. 31a). Conditioned media from endometrial cells isolated from mid LP, and stimulated with TNF (p=0.0007), P_4 (p=0.03), E_2 (p=0.002) and $P_4 + E_2$ (p=0.001) also caused

BAEC proliferation (Fig. 31b). Nevertheless, OXT did not cause any proliferative effect on endothelial cell, from cells collected at either stage of the estrous cycle studied.

Equine endometrial cells from FP showed a rise in *in vitro* NO production when stimulated by TNF (p=0.03) and the steroids P_4 (p=0.03) and E_2 (p=0.02), either alone or in association (P_4 + E_2) (p=0.006) (Fig. 32a). Also, an increase in NO was obtained when endometrial cells from the mid LP were stimulated with TNF (p=0.02), P_4 (p=0.03), E_2 (p=0.008) and (P_4 + E_2) (p=0.006) (Fig. 32b).

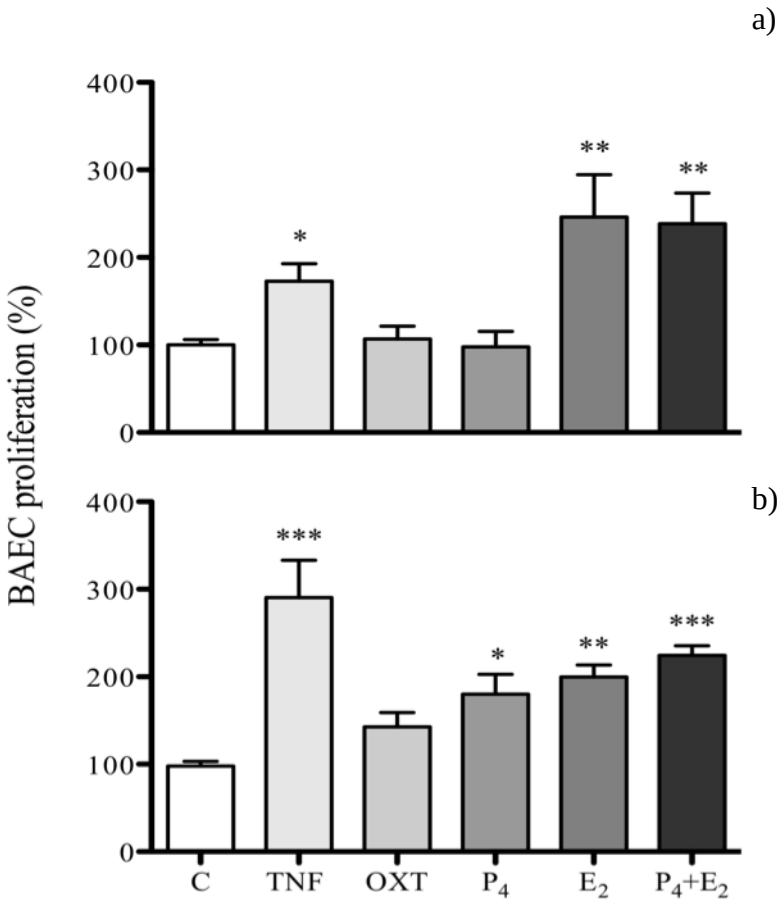


Figure 31: Bovine Aortic Endothelial Cells (BAEC) proliferation rate, after incubation with media from follicular P (a) and mid LP (b) endometrial cells stimulation (24 h). Asterisks indicate significant differences (*p<0.05; **p<0.01; *** p<0.001). Variations represent changes in percentage from basal output (C).

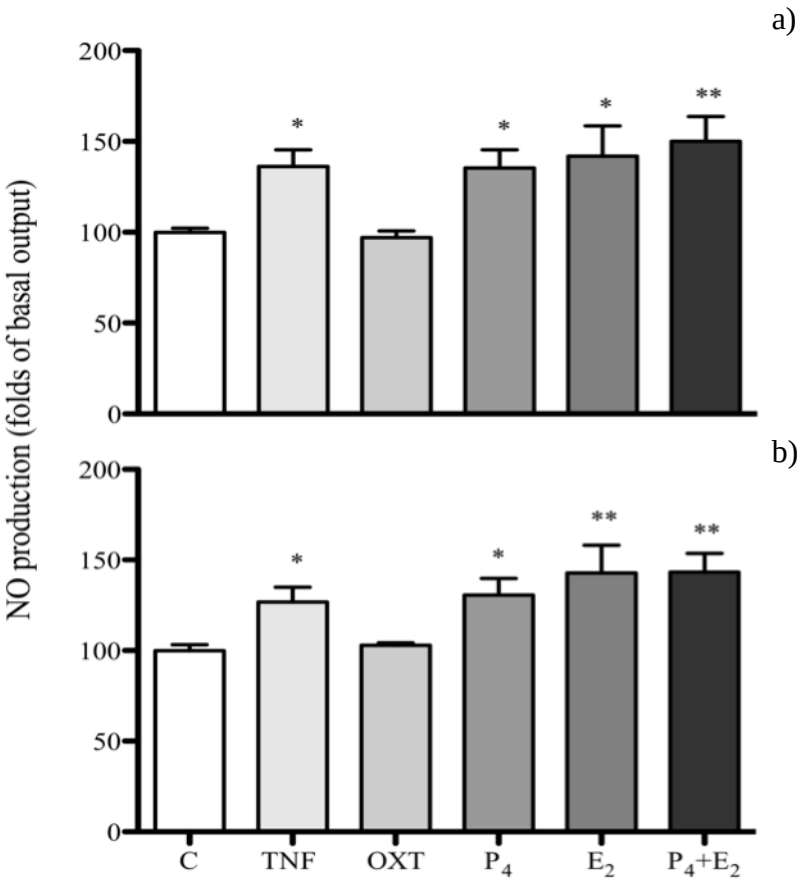


Figure 32: Follicular P (a) and mid LP (b) endometrial cells NO *in vitro* production. 24 h incubation. Asterisks indicate significant differences (*p<0.05; *** p<0.001). Variations represent changes in percentage from basal output (C). Standard curve range: 0-100µM.

4.5 Discussion

This study reported the presence of TNF, IFNG and FASL and their receptors in equine endometrium, evidencing changes in mRNA transcription and protein expression throughout the estrous cycle. Additionaly, an *in vitro* culture model with cells isolated from equine endometrium was developed and validated, in order to study the effect of cytokine TNF, peptide hormone OXT and steroid hormones P_4 and E_2 on endometrial secretory capacity (PGE_2, $PGF_{2\alpha}$), cell viability and angiogenesis activity (endothelial cell mitogenesis and NO secretion).

Paracrine regulation of reproductive function by cytokines has been broadly demonstrated (Fluhr *et al*., 2007; Murakami *et al*., 2001; Okuda *et al*., 2010; Woclawek-Potocka *et al*., 2004; Wolff *et al*., 2000). In different species like woman (Hunt *et al*., 1992), mouse (Joswig *et al*., 2003) and cow (Okuda *et al*., 2010), gene expression of TNF and receptors TNFRI and TNFRII was shown in the endometrium. As a pleiotropic factor, TNF and its receptors are

involved in several physiologic processes in cyclic endometrium (Lea & Sandra, 2007; Haider & Knöfler, 2009), as local regulation of genes involved in E_2 biosynthesis and activation in woman (Salama *et al*., 2009). In the mare, a couple of studies confirmed the involvement of cytokines on physiologic (Roberto da Costa *et al*., 2008) and pathologic equine endometrial function (Fumuso *et al*., 2003). Thus, cytokine TNF expression in the equine endometrium, throughout the estrous cycle, might indicate a role on endometrial function regulation. The IFNG is another cytokine that participates in different endometrial processes, during cyclicity in woman (Fluhr *et al*., 2007; Kawano *et al*., 2000) or early pregnancy in other species (Jokhi *et al*., 1994; Tayade *et al*., 2007; Wu *et al*., 2006). Both TNF and IFNG, acting synergically, increased FASL induced apoptosis in human endometrium (Fluhr *et al*., 2007; Joswig *et al*., 2003).

In the present study, FASL and FAS mRNA transcription and protein expression patterns in the equine endometrium were comparable. This cytokine is usually associated with apoptosis (Nagata 1999), but this event was not currently evaluated. Nevertheless, the increase in FASL and FAS gene expression in late LP might be related with endometrial tissue reorganization taking place during this phase of the estrous cycle. Thus, the reported decrease in cell proliferation in luminal epithelium and superficial stromal cells in late LP in the mare endometrium in a previous study of ours (Roberto da Costa *et al*., 2007b) might be related to this cytokine action. In contrast to FASL and FAS, both mRNA and protein expression profiles for TNF, TNFRI, TNFRII, IFNG and IFNRI were not consistent. The same discrepancy was seen also in the equine corpus luteum for these genes (unpublished data), indicating that post-transcriptional modifications might determine the absence of a positive relationship between mRNA transcription and protein translation. Even though mRNA transcription for TNF was increased in early and late LP, protein was higher in FP and mid LP. The same expression profile was seen for TNFRI, while TNFRII did not change throughout the estrous cylce. Each of these receptors determines opposite cellular responses for TNF, being TNRII associated with cell death and apoptosis, and TNFRII associated with cell growth and proliferation (Boldin *et al*., 1995; Hsu *et al*., 1995). Considering the importance of apoptotic events on endometrial homeostasis (Okano *et al*., 2007), the observed raise in TNF and TNFRI expression during FP might be explained by their putative role on the control of cell proliferation and replacement during that phase of the estrous cycle. Besides, the involvement of TNF/TNFRI apoptotic pathway should be considered, since caspase-3 expression in the equine endometrium was also higher during the FP (Roberto da Costa *et al*., 2007b). In the mid LP, increased protein expression of TNF, TNFRI, TNFRII,

IFNG and IFNRI was evident. This may suggest their role on endometrial increased protein secretory activity in this stage of the luteal phase, essential for pregnancy (Bazer *et al*., 1983; Hempstock *et al*., 2004). However, knowledge on cytokines role on equine endometrial function is scarce.

The development of an equine endometrial cell culture model, with both epithelial and stromal cells, allowed for the *in vitro* study of ovarian hormones, OXT and cytokine TNF interactions in the equine endometrium. Thus, the hypothesis that cytokines may modulate the cross talk between E_2, P_4 and OXT and prostaglandins secretion, in different phases of the estrous cycle was tested. The increase in PGE_2 and $PGF_{2\alpha}$ secretion, after equine endometrial cell culture stimulation with TNF and OXT, accounts for an appropriate response of this cell culture system as referred before (Tanikawa *et al*., 2008).

Changes undergoing every estrous cycle in the endometrium are needed for the adequacy of uterine environment at the time of maternal recognition of pregnancy (MRP) and, consequently, pregnancy establishment. In spite of the extensive research on equine endometrium from cyclic and pregnant mares carried out during the last decades, the mechanism by which the conceptus disrupts the luteolytic loop between uterine OXT and $PGF_{2\alpha}$ (Stout & Allen, 1999; Sout *et al*., 1999; Vanderwall *et al*., 2007) is yet to be fully understood. The coordinated action of steroid hormones E_2 and P_4 on endometrium was shown to be determinant for prostaglandin genes expression during different stages of the estrous cycle, influencing endometrium physiology and fertility (Goff, 2004; Tamm *et al*., 2009). Moreover, ovarian steroids affect protein content of the endometrium (McDowell *et al*., 1990; Zavy *et al*., 1979), accounting for endometrium and uterine lumen environment. The need for a sequential exposure of the endometrium to both E_2 and P_4 for triggering luteolysis has been shown in the mare (Vernon *et al*., 1981), sheep (Hixon & Flint, 1987; Homanics & Silvia, 1988) and cow (Lafrance & Goff, 1988). Also, the present results showed the stimulatory role of E_2 on $PGF_{2\alpha}$ secretion by endometrial cells from the mid LP, but not from the FP. These findings are in agreement with previous studies where just in P_4 primed-endometrium, E_2 mediated $PGF_{2\alpha}$ secretion (King & Evans, 1988; Vernon *et al*., 1981). Our data reported FP equine endometrium PG responsiveness to OXT and TNF. These findings point out that besides P_4 (Zavy *et al*., 1984a), E_2 (King & Evans, 1988; Vernon *et al*., 1981) and OXT (Goff *et al*., 1987), also TNF is able to modulate PG secretion. Thus, it might be suggested that complex pathways involved in equine endometrium secretory function during the estrous cycle, may also depend on a strong immuno-endocrine interaction.

Paracrine action of TNF on endometrium secretory function modulation is well documented in other species, like cow (Miyamoto *et al*., 2000; Skarzynski *et al*., 2000b) and pig (Blitek *et al*., 2006). In bovine endometrium, TNF increased secretion of PGE_2 in both FP and mid LP during *in vitro* (Murakami *et al*., 2001), as well as *in vivo* (Skarzynski *et al*., 2007) studies. Nevertheless, the data presently obtained are not completely in agreement with our previous work in equine endometrial tissue explants culture, where TNF effectively increased PGE_2 production just in the mid LP (Roberto da Costa *et al*., 2008). Moreover, discrepancies were also seen concerning $PGF_{2\alpha}$ output (Roberto da Costa *et al*., 2008). There are, in fact, specific features of each system that might explain the disagreement between both studies. Possibly, the proportion of both stromal and epithelial cells between systems was not the same. In spite of not completely characterized for the mare, endometrial cell specific PG production is well described for the cow (Murakami *et al*., 2001) and sow (Blitek *et al*., 2006), where stromal cells synthesize PGE_2 and epithelial cells $PGF_{2\alpha}$. In the mare, PGE_2 synthase (PGES) and $PGF_{2\alpha}$ synthase (PGFS) were localized solely in endometrium surface epithelium, and no reference was made to stromal cells (Boerboom *et al*., 2004). Thus, these data could justify the present findings, which should be further investigated. Nonetheless, in both studies TNF was able to modulate endometrial PGs secretion, specifically increasing PGE_2 output in mid LP. The relevance of these findings surely depends on PGE_2 actions in the equine reproductive tract. While in several species PGE_2 is considered anti-luteolytic/luteoprotective (Akinlosotu *et al*., 1986; Kotwica *et al*., 2003), in the mare its role is not yet well established. However, the action of PGs in uterine lumen should be also considered. Despite no changes were observed on PGE_2 synthase (PGES) gene expression between cyclic and pregnant mares (Boerboom *et al*., 2004), and the claim that PGE_2 does not protect CL lifespan (anti-luteolytic) (Stout & Allen, 2002; Watson & Sertich, 1989), intraluminal treatment of PGE2 prolonged the luteal phase in mares (Vanderwall *et al*., 1994). Moreover, a slight increase in intraluminal PGE_2 in pregnant mares, comparing with non pregnant animals, was reported (Stout & Allen, 2002). It is also well established that equine conceptus starts secreting PGE_2 as early as day 5 after ovulation (Weber *et al*., 1991), and that this secretion, and also PGE_2 receptors, increase from day 11 to day 15 of LP (Vanderwall *et al*., 1993). The claims made here support a possible role for PGE_2 during MRP in the mare, as previously suggested (Boerboom *et al*., 2004). Under this scope, TNF could promote PGE_2 secretion by the endometrium during mid LP, supporting intraluminal content of this PG when conceptus arrives in the uterus.

Another intriguing point is $PGF_{2\alpha}$ synthesis modulation, between cyclic and pregnant mares. It is believed that the signal produced by the conceptus is determinant to reduce $PGF_{2\alpha}$ in uterine venous blood (Douglas & Ginther, 1976), peripheral blood (Kindahl *et al*., 1982) and uterine lumen (Zavy *et al*., 1984a), but the nature of this signal and the physiologic responses triggered are unknown. Both OXT and OT receptor (OXTR) are key factors associated with endometrial $PGF_{2\alpha}$ synthesis (Goff *et al*., 1987; Stout & Allen, 1999), being OXTR expression regulated by P_4 and E_2 in ruminants (Beard *et al*., 1994; Wathes *et al*., 1996) and in the mare (Behrendt-Adam *et al*., 2000). During pregnancy, it was observed the absence on OXTR expression increases by day 14 after ovulation, usualy seen during cyclicity (Starbuck *et al*., 1998). Other important features to be considered are OXTR affinity, which is decreased in early pregnancy (Sharp *et al*., 1997), and the interference of conceptus signal with second messenger systems responsible for synthesis and secretion of $PGF_{2\alpha}$. Mare endometrium has been shown to secrete OXT (Behrendt-Adam *et al*., 1999; Watson *et al*., 2000b). However, from the present data it is not possible to understand how cytokines can regulate OXT secretion or OXTR expression. Nevertheless, it should be noticed that in both FP and mid LP, OXT stimulated $PGF_{2\alpha}$ secretion, but TNF stimulated $PGF_{2\alpha}$ simply during the FP. Taken together with our previous conclusion that TNF could modulate PGE_2 secretion during mid LP, we may speculate that TNF, through an unknown pathway, might drive PGs secretion towards PGE_2, specifically avoiding luteolysis in the mid LP. This might be important to allow for MRP.

For an adequate uterine environment, besides PGs secretion, changes in endometrial vasculature are absolutely necessary to fulfill the demands in nutrients and oxygen for embryo survival. Ovarian steroids and TNF influenced positively angiogenic factors production by endometrial cells, in both studied phases. These data are in agreement with previous studies in the mare (Honnens *et al*., 2010; Roberto da Costa *et al.* 2007a; Roberto da Costa *et al*., 2008). Brenner and Slayden (2004) showed the presence of E_2 receptor β (ERβ) on endothelial cells from human endometrium. In this regard, E_2 was shown to increase uterine blood flow in several species (Ford *et al*., 1982). Also important is the embryonic E_2 production in cow (Shemesh *et al*., 1979) and mare (Zavy *et al*., 1984b) that should not be dissociated from temporal vascular changes in the endometrium of those species. Moreover, PGs have been associated as well with endometrial angiogenesis in other models (Lewis, 1989; Matsumoto & Sato, 2006). Another study with uterine microvascular cells isolated from woman endometrium demonstrated that P_4, alone or in combination with E_2, induced the expression of genes involved in angiogenesis, showing once again the role of ovarian steroids on

endometrial differentiation, acting locally on the vascular bed (Yasuo & Kitaya, 2009). Consequently, it might be concluded that TNF may also participate on equine endometrium vascular changes, together with steroid hormones, in a paracrine/autocrine way. The similar responsiveness of BAEC proliferation and NO production by endometrial cells may suggest that angiogenesis regulation in the equine endometrium by TNF and ovarian steroids could be mediated by NO. As previously shown by our group, it is likewise relevant that endothelial NO synthase (eNOS) protein expression was increased in the mare endometrium during follicular and mid LP (Roberto da Costa *et al*., 2007a). Recent in vivo data in the mare reported a correlation between blood flow and mRNA transcription of eNOS, evidencing that uterine blood flow should be regulated by steroid hormone-mediated stimulation of NOS (Honnens *et al*., 2011). This indicates NO involvement on uterine blood flow regulation. Besides, as previously stated, NO production induced by TNF and steroid hormones (E_2 or P_4 + E_2) also regulates PGs secretion (Roberto da Costa *et al*., 2008; Salvemini *et al*., 1993; Skarzynski *et al*., 2000a; Skarzynski *et al*., 2007). This occurs mainly during mid LP, the time when, in case of fertilization, the embryo arrives in the uterus and MRP signal starts. Thus, actions of NO on endometrial function, mainly through the regulation of PGs secretion (Roberto da Costa *et al*., 2008) and modulation of angiogenic function, might depend on cytokine TNF and steroid hormones. Nevertheless, further studies are needed to understand the intricacies of these interactions.

Cellular viability and proliferation of endometrial cells were also evaluated in the present work. Participation of TNF together with P_4 on cell viability increase emphasizes the complexity of TNF action on endometrial regulation. Additionally, this stimulatory effect on cell viability was evident just during mid LP. This is in agreeement with physiologic requirements for early pregnancy establishment, such as endometrial receptivity for conceptus and maintenance of endocrine homeostasis (Toloubeydokhti *et al*., 2008; Xia *et al*., 2010). In fact, we have previously shown that during the mid LP, a simultaneous increase in DNA, cell proliferation and protein synthesis in the equine endometrium might suggest that cell hyperplasia occurs at the time histotroph is needed for eventual embryo nourishment (Roberto da Costa *et al*., 2007b). Thus, TNF and P_4 may be determinant for endometrial cell proliferation and function. The role of P_4 on endometrial pathways for cell proliferation and differentiation is an up-dated issue that just started to be investigated (Fu *et al*., 2011; Satterfield *et al*., 2008).

In conclusion, the observed fluctuations on the expression of cytokines TNF, IFNG, FASL and their receptors might suggest their role on endometrial function in the mare. Therefore, the role of TNF was specifically investigated. This cytokine appears to be determinant for physiologic changes in the mid LP endometrium, such as increased secretory function (PGE_2), cell changes (cell viability), and angiogenic activity and NO production. In addition, peptide hormone OXT only increased PG secretion, while ovarian steroids evidenced a broader action, participating on endometrial secretion, angiogenesis and cell viability. Temporal agreement on the observed findings encourage us to speculate that in mid LP, TNF together with OXT and steroid hormones, might cause determinant changes on endometrial milieu supportive of early pregnancy establishment.

CHAPTER III. GENERAL DISCUSSION

Reproductive system regulation depends on the coordinated interaction of different factors in the hypothalamic-pituitary-gonadal system. Considering specifically the female sexual gonads (ovaries and uterus) on reproductive function regulation, a major role is ascribed to the ovarian steroids estradiol and progesterone, and to the uterine and ovarian eicosanoids prostaglandins. The classical concept of hormone considers it as a substance that can act on distant organs, with a broad range of effects (Dinarello, 2000). Nevertheless, hormones do not play the game alone, acting just in an endocrine fashion, but their actions are modulated by a myriad of auto-, paracrine factors, as growth/angiogenic factors (Redmer *et al*., 1985; Reynolds *et al.*, 1994; Stocco *et al*., 2007), cytokines (Fairchild & Pate, 1989), neurotransmitters (Al-Zi'abi *et al*., 2009; Kotwica *et al*., 1996), fatty acid signaling molecules such as leukotrienes (Korzekwa *et al.*, 2010a), reactive oxygen species (Ferreira-Dias *et al*., 2011; Jaroszewski & Hansel, 2000; Sugino *et al*., 1996), lysophosphatidic acid (Woclawek-Potocka *et al*., 2009), among others.

The present work gathers a both descriptive and functional analysis of cytokines TNF, IFNG, FASL and their receptors role on equine CL function throughout the luteal phase, as well as in the endometrium during specific stages of the estrous cycle (follicular phase and mid luteal phase). Cytokines, considered as the main secretory product of immune cells (Adashi, 1990), have been largely demonstrated to participate on luteal (Fairchild & Pate, 1989; Taniguchi *et al*., 2002) and endometrial (Lea & Sandra, 2007) function regulation. Hence, this study focused on some aspects of the complex immuno-endocrine crosstalk regulating luteal and endometrial secretory, angiogenic and cell proliferation activity in the mare.

The overall aim for luteal experiments concerned the characterization of the expression of cytokine ligands TNF, IFNG and FASL, and also their receptors TNFRI, TNFRII, IFNRI and FAS, in the equine CL throughout the luteal phase. In addition, modulation of CL function, by assessment of *in vitro* secretion of P_4, PGE_2 and $PGF_{2\alpha}$, angiogenic activity, cell viability and proliferation, as well as apoptosis was addressed. Different studies have demonstrated the importance of these cytokines on CL regulation on species like woman (Vaskivuo *et al*., 2002), mouse (Sakamaki *et al*., 1997), rat (Slot *et al*., 2006), cow (Petroff *et al*., 1999; Taniguchi *et al*., 2002) or pig (Wuttke *et al*., 1997). In the present study, immunohistochemistry analysis depicted that large luteal cells and endothelial cells clearly express ligands and receptors of the cytokines under study. Moreover, their expression changed throughout the luteal phase, suggesting an auto-, paracrine role on the regulation of luteal function. In general, TNF action can be differentially regulated throughout the luteal

phase, based on its receptor expression. It is well known that TNFRI is associated with apoptosis and cell death (Boldin *et al.,* 1995), while TNFRII with cell proliferation (Hsu *et al.*, 1995). Thus, increased protein expression of TNFRII during the initial phases of equine CL may account for a supportive role of TNF on luteal establishment, whereas in the late luteal phase the increased protein expression of TNFRI may switch TNF action to become a luteolytic agent. The same opposing role of TNF on luteal function was evidenced in other species (Sakumoto *et al*., 2000; Vaskivuo *et al*., 2002). Considering the potential supportive action of TNF during CL growth and establishment, it increased *in vitro* secretion of P_4 and PGE_2. The luteoprotective role of P_4 was shown in several species, by maintaining its own synthesis and preventing the onset of apoptosis, a mechanism dependent on P_4 receptor itsef (Ottander *et al*., 2000; Skarzynski & Okuda, 1999). Also PGE_2, despite of not being considered as a luteotrophic agent in the mare, is well known to promote mitogenesis, immunomodulation and to act as an anti-apoptotic agent in luteal cells of other species (Bowolaksono *et al*., 2008) and in other organs in mares (Atli *et al*., 2010). Even more interesting is the differential regulation of eicosanoids synthesis pathway by TNF, as shown in this study. In the early equine CL, when PGE_2 secretion was increased, $PGF_{2\alpha}$ was reduced. Also important for luteal establishment is the development of a vascular system and the cooperative action of TNF on this process, by stimulating angiogenic factors secretion. Thus, one may conclude that during equine CL growth, the pleiotropic cytokine TNF operates as a luteotrophic agent by stimulating P_4 and PGE_2 secretion, increasing the expression of VEGF and the production of other proangiogenic factors, as well as by decreasing $PGF_{2\alpha}$ secretion (Fig. 33).

Therefore, a notorious interaction between the immune system, angiogenesis modulation and secretory function occurs during luteal establishement in the mare. Analysis of the present interactions suggests the existence of a luteotrophic intraluteal loop where TNF increases VEGF, which besides promoting angiogenesis increases P_4 and PGE_2 secretion. In turn, P_4 and PGE_2 (also augmented by TNF) might stimulate VEGF and other angiogenic factors, ensuring luteal growth and maintenance (Fig. 23a).

The interaction of studied cytokines at the time of equine CL demise also drew quite pertinent conclusions. Even though the contribution of the cytokines IFNG and FASL was not that relevant for equine CL growth, their role during luteolysis should be considered. The FASL action on its receptor FAS has been largely associated with structural involution in other species (Roughton *et al*., 1999; Taniguchi *et al*., 2002). However, the present work has

demonstrated for the first time their participation on luteal secretory function regulation in the mare. Actually, FASL alone could reduce P_4 and PGE_2 and increase $PGF_{2\alpha}$ in mid CL isolated cells, but no effect was seen in apoptosis promotion or reduction of cellular viability. Thus, increase of FASL expression in equine mid CL might be considered as a requirement for functional luteolysis triggering and impairment of luteoprotective role of P_4 in this species. This statement is even stronger if the role of FASL on VEGF protein regulation, in mid CL isolated cells is taken into account. Since TNF increased VEGF expression, the presence of FASL might downregulate its expression, a result also seen with the cytokine association TNF+IFNG+FASL. From this standpoint, it appears that FASL can disrupt the previously proposed luteotrophic intraluteal loop (TNF/VEGF/ P_4 and PGE_2) by its direct action on the decrease in P_4 and PGE_2 secretion and down regulation of VEGF expression. Cytokines TNF and IFNG were also shown to regulate secretory function during luteolysis. Already during mid luteal phase, luteal cells responded to TNF by increasing $PGF_{2\alpha}$ or to TNF+IFNG, by reducing PGE_2 secretion. Nevertheless, during late CL, both cytokines drastically reduced P_4 and PGE_2 and increased $PGF_{2\alpha}$ secretion. Regarding angiogenesis regulation in the mid CL, cytokine association TNF+IFNG+FASL decreased angiogenic factors or increased antiangiogeninc factors secretion. This effect was strongly evidenced in late CL by the same cytokine association or TNF and IFNG alone. These cytokines when acting in association (TNF+IFNG+FASL), contributed as well for the increase in mRNA expression of TSP1 and its receptor CD36, known to induce apoptosis and destabilize endothelial cells (Armstrong & Bornstein, 2003).

Discussing structural luteolysis, apoptosis promotion and reduction on cell viability and proliferation rate were only evident when cytokines were used synergically (TNF+IFNG or TNF+IFNG+FASL). It clearly indicates that luteal cytotoxity can be more effectively achieved when cytokines act together. Consequently, the present data imply the synergic action of cytokines with structural involution during luteolysis in the mare (Fig. 34).

The present work also aimed at the involvement of the cytokines under study on endometrial function regulation. The overall presented hypothesis for endometrial experiment concerned the importance of cytokines on endometrial function regulation. Firstly, the expression of TNF, IFNG, FASL, and the receptors TNFRI, TNFRII, IFNRI and FAS in the equine endometrium was evaluated during the estrous cycle. Secondly, the hypothetical interaction of TNF with steroid hormones and oxytocin on endometrial secretory activity, angiogenesis and cell proliferation during follicular and mid luteal phase was assessed. Expression of cytokine

ligands and receptors is in agreement with the speculative role of these factors on endometrial function control. This work accurately characterized the localization of cytokines and receptors in endometrial epithelium and stroma cells and their mRNA and protein expression quantification. Protein expression of cytokines TNF, IFNG and their receptors presented a consistent change during mid luteal phase, being increased for TNF, TNFRI, IFNG and IFNRI. It is important to note that those changes should not be dissociated from cyclic endometrial events, mainly regulated by steroid hormones (Ginther, 1992). Regulation of prostaglandins secretion by the equine endometrium is a complex process modulated by different factors (Goff *et al*., 1987; Vernon *et al*., 1981). It has also been demonstrated that TNF modulates prostaglandins secretion by equine endometrium, as shown in other species (Miyamoto *et al*., 2000; Blitek *et al*., 2006). Moreover, during this mid luteal phase of the estrous cycle several changes on eicosanoids secretion should adjust $PGF_{2\alpha}$ production, in case of pregnancy (reviewed in McDowell & Sharp, 2011). Thus, the expression of these cytokines with the main events regulating endometrial function, may suggest their role on the unknown process of maternal recognition of pregnancy. Clearly, TNF could stimulate PGE_2 secretion by endometrial cells during mid luteal phase, but the same did not hold true for $PGF_{2\alpha}$ production. Once again, data from the present work can raise the question whether PGE_2 is as an anti-luteolytic/luteoprotective agent in the mare. In fact, even though questionable by some authors during the last decades, more recent findings have referred the important role PGE_2 on maternal recognition of pregnancy in the mare (Boerboom *et al*. 2004; Atli *et al*., 2010).

Pluripotency of TNF actions on the endometrium is confirmed by its promotion of angiogenic factors and NO secretion by endometrial cells. The present results indicate that this cytokine might interact with steroid hormones to modulate vascular changes in the endometrium during the estrous cycle. Finally, TNF, together with P_4, are able to increase cell viability and proliferation of endometrial cells from mid luteal phase. There exist good clues to believe that TNF might be involved on the regulation of endometrial function, particularly on events that might participate on the complex process of maternal recognition of pregnancy. Cytokine stimulation of PGE_2 secretion and promotion of angiogenesis and cell viability may account for this speculation. Nevertheless, further studies are required to better elucidate cytokines role on equine endometrial function.

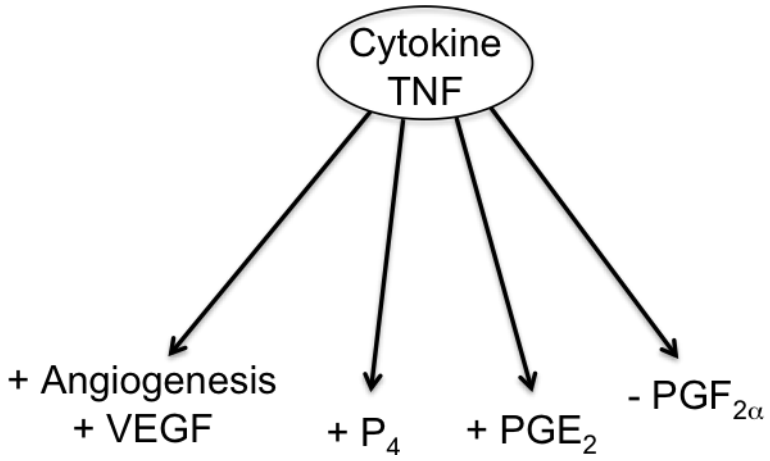


Figure 33: Schematic representation of TNF supportive role during CL establisment.

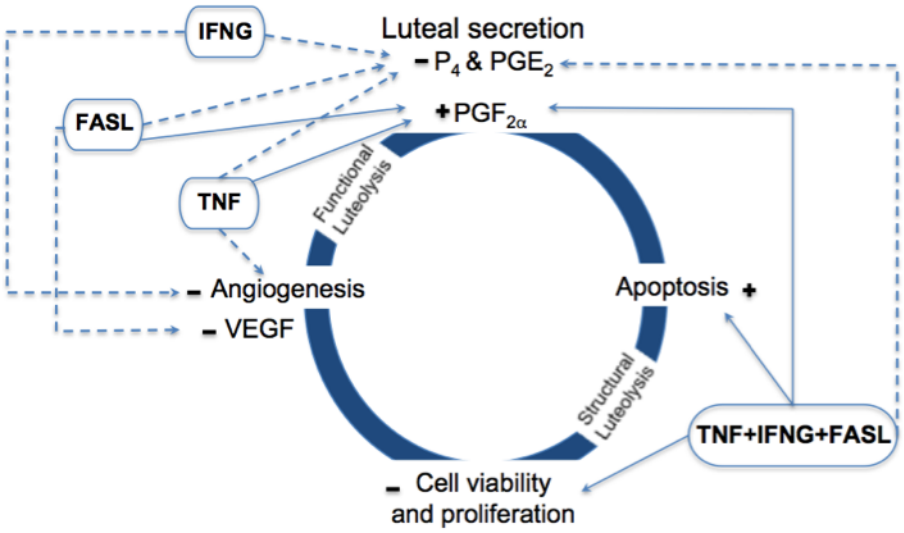


Figure 34: Schematic representation of cytokine interactions during luteolysis. Full line – stimulation; dashed line – inhibition.

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