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NEW INSIGHTS ON THE PHYSIOLOGIC REGULATION OF THE EQUINE CORPUS LUTEUM BY METABOLIC AND ENDOCRINE FACTORS

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It is better to know useless things
than to know nothing at all

Seneca

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SUMMARY

Assisted reproduction and reproductive problems as infertility are becoming progressively more relevant in human medicine. Indeed, around one in seven human couples are infertile and in 70% of the cases this is ascribed to ovarian dysfunction. Research with human subjects is limited in many aspects, such as those of ethical nature. Consequently, animal models have considerable utility by providing knowledge on follicular development, oocyte maturation, corpus luteum (CL) function, reproductive aging and also therapeutic treatments. Thus, the use of the mare, as a monovulatory species, might be a valuable model for understanding human ovarian function due to the resemblance between these species in many aspects of ovarian dynamics, even though the mare has received limited attention in comparison to other species. This has also led to an equally scarce understanding of some physiological and pathological reproductive aspects of the equine species.

The gonadal function is based on a complex regulatory network system, based on endocrine hormones and locally-produced (autocrine/paracrine) factors. In recent decades a growing interest by the scientific community towards many factors involved on energetic balance regulation and metabolism, which have shown a crucial role on the modulation of gonadal axis function ensuring the fertility. Among these, leptin, ghrelin, Growth Hormone (GH) and Insuline-like growth factor-I (IGF-I) seem to have a regulation role on the reproductive efficiency of mammalian species, humans including. To the best of our knowledge, no studies have been performed in this field in the mare. This thesis was created with the intent to understand how these factors may regulate the luteal function in mares. After a detailed introduction (general section) to this vast field of research, the thesis was structured into two experimental studies (experimental section), in which *in vitro* models were used, such as luteal tissue culture, and molecular biology techniques, that allowed for an accurate control of

experimental conditions, while it also avoided animal suffering.

Both experiments focussed on the role of metabolic and endocrine factors such as leptin and ghrelin, in the first study, and GH and IGF-I, in the second one, on CL angiogenic activity that could modulate *in vitro* bovine endothelial cell proliferation (BAEC) and nitric oxide (NO) production. Besides, luteal secretory capacity (progesterone and prostaglandins) by the equine CL in the presence of leptin and ghrelin was also evaluated. Expression of ghrelin, GH, IGF-I mRNA and their receptors, as well as leptin receptor, in the CL throughout the luteal phase, was shown. In light of these results, it is suggested that leptin from extra-luteal source might influence mare CL, which might justify the presence of its receptor. The hypothesis of a possible involvement of these metabolic hormones in the regulation of the equine CL, arose not only by expression of their genes and/or receptors, but also by their capacity, in a dose dependent manner, to stimulate NO production (leptin, ghrelin, IGF-I associated with GH or LH) and BAEC proliferation (ghrelin, leptin) or to inhibit it (IGF associated with LH or GH), as indices of angiogenic activity. In addition, it was shown that leptin and ghrelin influence secretory capacity of the CL by promoting the production of luteotrophic prostaglandin E_2 or luteolytic prostaglandin $F_{2\alpha}$, respectively.

The knowledge of metabolic and endocrine factors that modulate regulatory mechanisms of luteal endocrine function, angiogenesis and non-vascular development during normal luteal tissue growth might bring about new approaches to fight infertility. Nevertheless, further studies should be carried out to deepen luteal physiological mechanisms in the mare, as well as the use of metabolic hormones, and growth and angiogenic factors that might be considered as luteotrophic.

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RIASSUNTO

La procreazione medicalmente assistita ed i problemi riproduttivi, come l'infertilità, stanno diventando progressivamente più rilevanti in medicina umana. Infatti, circa una coppia su sette presenta problemi di fertilità e nel 70% dei casi questi vengono attribuiti ad una disfunzione ovarica. La ricerca scientifica, su questi aspetti, nei pazienti umani incontra ovviamente molte limitazioni per diverse ragioni, tra cui quelle di natura etica. Conseguentemente i modelli animali sono considerati di estrema utilità. Infatti, tali modelli hanno fornito numerose informazioni sullo sviluppo follicolare, la maturazione degli ovociti, la funzione del corpo luteo, l'invecchiamento riproduttivo ed anche in merito agli aspetti terapeutici. La cavalla, specie monovulatoria con dinamiche ovariche per molti aspetti somiglianti a quelle umane, potrebbe costituire un valido modello per lo studio della fisiologia riproduttiva; nonostante ciò, sino a questo momento ha ricevuto scarsa attenzione rispetto ad altri modelli animali. Questa scarsa considerazione ha anche portato ad una incompleta conoscenza di alcuni aspetti fisiologici e patologici della riproduzione equina.

La funzione delle gonadi è governata da un complesso sistema di regolazione in cui agiscono sia ormoni prodotti a livello sistemico sia fattori ormonali secreti a livello locale (in modo autocrino e paracrino). Negli ultimi decenni è nato un crescente interesse da parte della comunità scientifica nei confronti di molti fattori endocrini e metabolici coinvolti nella regolazione del bilancio e del metabolismo energetico, che sembrano avere un ruolo cruciale nella fisiologia riproduttiva assicurando la fertilità. Tra questi la leptina, ghrelina, l'ormone della crescita (GH) ed il fattore di crescita insulino-simile (IGF-I) sembrano influenzare la funzione riproduttiva nei mammiferi, uomo compreso. Per quanto noto all'autore, a tutt'oggi nella specie equina non esistono studi in questo campo.

Questa tesi è stata creata con l'intento di capire come questi fattori, già investigati nella donna e in altre specie animali, possano regolare il ciclo estrale della cavalla, ed in particolare la funzione luteale. Dopo una dettagliata introduzione (sezione generale) a questo vasto settore di ricerca, la tesi è stata strutturata in due studi sperimentali (sezione sperimentale), in cui abbiamo avuto modo di avvalerci di modelli *in vitro*, come le colture tissutali, e di tecniche di biologia molecolare che ci hanno permesso, non solo di avere un accurato controllo delle condizioni sperimentali, ma anche di evitare sofferenze inutili agli animali.

In entrambe gli esperimenti abbiamo focalizzato l'attenzione sul ruolo di questi fattori metabolici ed endocrini, come la leptina e la ghrelina nel caso del primo studio e dell'GH e dell'IGF-I nel secondo, sull'attività angiogenetica del corpo luteo equino attraverso la possibile modulazione *in vitro* della proliferazione delle cellule endoteliali di aorta bovina (BAEC) e, la produzione di ossido nitrico (NO). Inoltre abbiamo valutato la capacità secretoria luteale (pregesterone e prostaglandine) in presenza di leptina e ghrelina e l'espressione genica di tutti i fattori sopracitati, recettori compresi, durante l'intera fase lutale. Alla luce di questi risultati, si può ipotizzare che la leptina, proveniente da una fonte extra-luteale, possa influenzare il corpo luteo della cavalla, giustificando in questo modo la presenza dei suoi recettori. L'ipotesi di un possibile coinvolgimento di questi fattori metabolici nella regolazione del corpo luteo, non nasce solo dall'espressione dei loro geni e/o recettori, ma anche dalla capacità dose-dipendente, di stimolare la produzione di NO (leptina, ghrelina, IGF-I associato al GH oppure all'LH) e la proliferazione delle BAEC (ghrelina, leptina) oppure di inibirla (IGF associato con GH oppure con LH). Produzione di NO e proliferazione delle BAEC sono considerati indici di attività angiogenetica. Inoltre abbiamo dimostrato che leptina a ghrelina influenzano la capacità secretoria del corpo luteo, promuovendo rispettivamente la produzione di PGE_2, prostaglandina luteotropica, e di $PGF_{2\alpha}$, prostaglandina luteolitica.

La comprensione dei meccanismi di regolazione da parte di fattori metabolici ed endocrini sulla funzione endocrina del corpo luteo, sull'angiogenesi e sullo sviluppo non vascolare durante il normale sviluppo del tessuto, potrebbe portare a nuovi approcci per combattere l’infertilità. Tuttavia, altri studi dovrebbero essere condotti in futuro per approfondire i meccanismi fisiologici nella cavalla, così come l'utilizzo di ormoni e fattori angiogenetici e di crescita, che potrebbero essere considerati luteotropici.

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Strange but important for me.... to say thank to my animals: Charlie, Zack, Lana and Nina who are no longer here....

Finally to my parents and my brother Enzo... Thank you so much for your patience and love.

Angela

LIST OF ABBREVIATIONS

AA: arachidonic acid

ACE: angiotensin-converting enzyme

aFGF (or FGF-1): acidic Fibroblast Growth Factor

Angs (or ANPTs): Angiopoietins

Ang-1 (or ANPT-1): Angiopoietin-1

Ang-2 (or ANPT-2): Angiopoietin-2

Ang I: angiotensin I

Ang II: angiotensin II

ASL: acid-labile 85-kDa subunit

BAEC: bovine aortic endothelial cell

bFGF (or FGF-2): basic Fibroblast Growth Factor

3β-HSD: 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase

Ca^{2+}: calcium

cAMP: cyclic adenosine monophosphate

CL: corpus luteum

CNS: central nervous system

Cox-: Cyclooxygenase (Cox-1, Cox-2, Cox-3)

cPLA2: cytosolic phospholipase A2

DD: Death Domain

DED: Death Effector Domain

DISC: death inducing signalling complex

E_2 : 17β-oestradiol

EC: endotheilial cell

ECE-1: endothelin converting enzyme-1

eCG: equine Chorionic Gonadotrophin

ECM: extracellular matrix

EGF: epidermal growth factor

eNOS (or NOS-3): endothelial nitric oxide synthase

EP: PGE_2 Receptor (EP1, EP2, EP3, EP4)

ER: Endoplasmatic Reticulum

ETs (or EDNs): Endothelins (ET-1, ET-2, ET-3)

ETR-A: Endothelin receptor Type A

ETR-B: Endothelin receptor Type B

FAS: Fas Ligand receptor

FasL: Fas Ligand

FGF: Fibroblastic growth factor (FGF-1, FGF-2, FGF-4)

FGFR: Fibroblastic growth factor receptor -1(flg), -2 (bek) , -3, -4.

FP: $PGF_{2\alpha}$ Receptor (FP_A and FP_B)

FSH: Follicle Stimulating Hormone

GH: Growth Hormone

GHS: growth hormone secretagogue

GHSR: growth hormone secretagogue receptor

GH-R: growth hormone-receptor

GHRH: Growth Hormone-releasing hormone

GnRH: Gonadotrophin Releasing Hormone

GRLN-R: ghrelin receptor

GPCR: G protein-coupled receptor superfamily

hCG: human Chorionic Gonadotrophin

HDL: high-density lipoprotein

HIF-1α: Hypoxia-Inducible Factor 1α

HPG: hypothalamus-pituitary-gonadal axis

HUVECs: Human Umbilical Vein Endothelial Cells

IFN-: interferon-β (IFNβ), interferon-γ (IFNγ)

IFNR: interferon receptor

Ig: Immunoglobulin

IGF: Insulin-like Growth Factors (IGF-I and IGF-II)

IGF-II/M6P: IGF-II/Mannose-6-Phosphate receptor

IGFBPs: Insuline-like Growth factor binding proteins (-1, -2, -3, -4, -5, -6)

IGFBP-rPs: Insuline-like Growth factor binding proteins-related proteins

IGFR: Insulin-like Growth Factors Receptors (IGFR-I and IGFR-2)

IL-: Interleukin- (IL-1α, IL-1β, IL-6, IL-8)

iNOS or (NOS-2): cytokine- inducible nitric oxide synthase

JAK: Janus Khinasi

KGF/FGF-7: Keratinocyte Growth Factor

LDL: low-density lipoprotein

LH: Luteinising Hormone

LHR: luteinising hormone receptor

LLC: large luteal cells

L-NAME: NG-nitro-L-arginin methyl ester

LT: Leucotrienes

MCP-: monocyte chemotactic protein-

MHC: major histocompatibility complex

MMPs: matrix metalloproteinases

mPGES-: microsomal PGES-1

mRNA: messenger Ribonucleic Acid

NA: noradrenaline

NO: nitric oxide

$NO2^{-}$: nitrite

$NO3^{-}$: nitrate

NOS: nitric oxide synthase

nNOS (or NOS-1): neuronal nitric oxide synthase

NRPs-: neuropilin-1 and neuropilin-2 (NRP-1, NRP-2)

ob: obesity gene

OB-Ra (and -Rc, -Rd, -Rf) : short leptin receptors

OB-Rb: long leptin receptor

OB-Re: soluble leptin receptor

OT: oxitocin

P_4: progesterone

P450scc: cytochrome P450 side chain cleavage

PACAP: pituitary adenylate cyclase activating polypeptide

PAI-1: plasminogen activator inhibitor 1

PCNA: proliferating cell nuclear antigen

PDGF: Platelet-Derived Growth Factor

PGs: prostaglandins

PGDH:15-Hydroxyprostaglandin Dehydrogenase

PGE_2: Prostaglandin E_2

PGE2-9-ketoreductase

PGEM: PGE_2 metabolite

PGES: PGE_2 Synthase

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

PGFM: $PGF_{2\alpha}$ metabolite

PGFS: PGF Synthase

PGG_2: Prostaglandin G_2

PGH_2: Prostaglandin H_2

PGI_2: Prostacyclin I_2

PGIS: PGI-synthase

PKA: Protein Kinase A

PKC: Protein Kinase C

PlGF: Placental Growth Factor

PMN: polymorphonuclear neutrophils

ppET-1: pre-propeptide endothelin-1

PR: progesterone receptor

PRL: prolactin

proET-1: big endothelin-1

PT: Permeability Transition pore

RAS: renin-angiotensin system

RT-PCR : Real Time-Polymerase Chain Reaction

SER: smooth endoplasmic reticulum

SLC: small luteal cells

SRIF: Somatotropin release-inhibiting factor or Somatostatin

SREBP-1: regulatory element binding protein 1

StAR: Steroidogenic Acute Regulatory Protein

STAT**:** Signal Transducers and Activators of Transcription

TGF: Transforming Growth Factor (TGFα and TGFβ)

Tie1: angiopoietin 1 tyrosine kinase receptor 1

Tie2 : angiopoietin 2 tyrosine kinase receptor 2

TIMPs: Tissue Inhibitors of metalloproteinases (TIMP-1, -2, -3, -4)

TNF-: tumour necrosis factor-α and -β (TNFα and TNFβ)

TNFR: tumour necrosis factor receptors (TNFRI and TNFRII)

TRH: thyrotropin releasing hormone

uPA: urokinase-type Plasminogen Activator

VEGFs: Vascular Endothelial Growth Factors (-A, -B, -C, -D, -E)

VEGFR-1: Vascular Endothelial Growth Factor Receptor-1 (or Flt-1)

VEGFR-2: Vascular Endothelial Growth Factor Receptor-2 (Flk-1/KDR)

VEGFR-3: Vascular Endothelial Growth Factor Receptor-3 (Flt-4)

1. INTRODUCTION

The ovarian cycle is characterized by repeated patterns of cellular proliferation, remodeling of exracellular matrix and cell differentiation that accompany follicular development as well as the formation and regression of the corpus luteum (CL). The CL lifespan is described by a rapid growth, differentiation and controlled regression of the luteal tissue, accompanied by an intense angiogenesis and a vascular regression.

Ovulation is the critical event that initiates the transformation of the fluid-filled preovulatory follicle into the solid CL. After ovulation, profound morphological and biochemical changes occur in the theca and granulosa layers, which enable CL formation and maturation. Just before follicular rupture, granulosa cells in the follicular fluid, which contains anticoagulant substances (Shimada *et al*., 2001), proceed to luteinization and shift their main products from estrogen (E_2) to progesterone (P_4). After ovulation, these granulosa cells undergo hypertrophy to differentiate into large luteal cells, are in contact with migrating endothelial cells (Corner, 1956) and produce extracellular matrix (ECM) around the newly formed luteal cells (Fujiwara *et al*., 1997; Yamada *et al*,. 1999). Furthermore, ECM produced by luteinizing granulosa cells is considered to modulate the migration and outgrowth of endothelial cells (Davis and Senger, 2005).

Dramatic centripetal angiogenesis also occurs from the vascular network surrounding the follicle, although follicular fluid contains anti-angiogenetic factors (Gruemmer *et al.,* 2005).

After ovulation, the basement membrane between the granulosa and theca layers undergoes dissolution and thecal capillaries expand by sprouting into the avascular granulosum to form a dense network of sinusoidal capillaries surrounding the luteinized granulosa cells. Thus, this rapid growth and regression of ovarian tissues are accompanied by equally rapid changes in their vascular beds (Kaczmarek *et al.*, 2005). Microvascular development of the follicular wall becomes even more extensive after ovulation, in association with vascularization of the CL (Redmer and Reynolds, 1996; Reynolds and Redmer, 1998). As a matter of fact, the CL becomes so vascular, in fact, that the majority of the parenchymal (steroidogenic) cells of the mature CL are in contact with one or more capillaries (Dharmarajan *et al.*, 1985; Reynolds *et al*., 1992; Redmer and Reynolds, 1996-1998). In addition, the mature CL also receives most of the ovarian blood supply, and ovarian blood flow is highly correlated with the rate of P_4 secretion (Reynolds, 1986; Niswender and Nett, 1988; Reynolds *et al.*, 1994).

The formation of a dense capillary network is defined as angiogenesis: the generation of new blood vessels through sprouting from already existing blood vessels in a process involving the migration and proliferation of endothelial cells from pre-existing vessels. An adequate blood flow is necessary in all tissues to supply oxygen and nutrients, to remove CO_2 and other metabolic by-products and to transfer hormones from endocrine glands to target cells. Physiologic angiogenesis rarely occurs in the adult with exceptions such as the female reproductive system and wound healing (Reynolds and Redmer, 1998). Aside from luteal and uterine tissue, wound healing and some pathologic processes, including neoplasia, the vascular system in the adult is generally quiescent. Over 85% of proliferating cells in the CL are endothelial cells and they make up around 50% of all cells in the mature ovarian gland (Fraser and Duncan, 2005). Endothelial cells in the CL undergo apoptosis each ovarian cycle (Armstrong and Bornestein, 2003; Ferreira-Dias *et al.*, 2007). In contrast, in non-reproductive

tissues, endothelial cells, once differentiated, normally remain functional for 2-3 years (Fraser and Duncan, 2005).

Since it is almost certain that some forms of ovarian dysfunction are associated with abnormalities of the angiogenic process, the ovary therefore represents an exceptional and highly relevant tissue in which to study the physiological and pathological control of blood vessel development (Fraser and Duncan, 2005). On the contrary to that observed during pathological tissue growth (*e.g*. tumor growth), the angiogenic process in female reproductive tissues is limited and, therefore, must be tightly regulated (Reynolds *et al*., 1992).

The mechanism controlling angiogenesis (complex interactions among stimulatory, modulatory and inhibitory factors) and development, maintenance and secretory function of the CL may involve factors that are produced both within the CL and outside the ovary (Fig. 1).

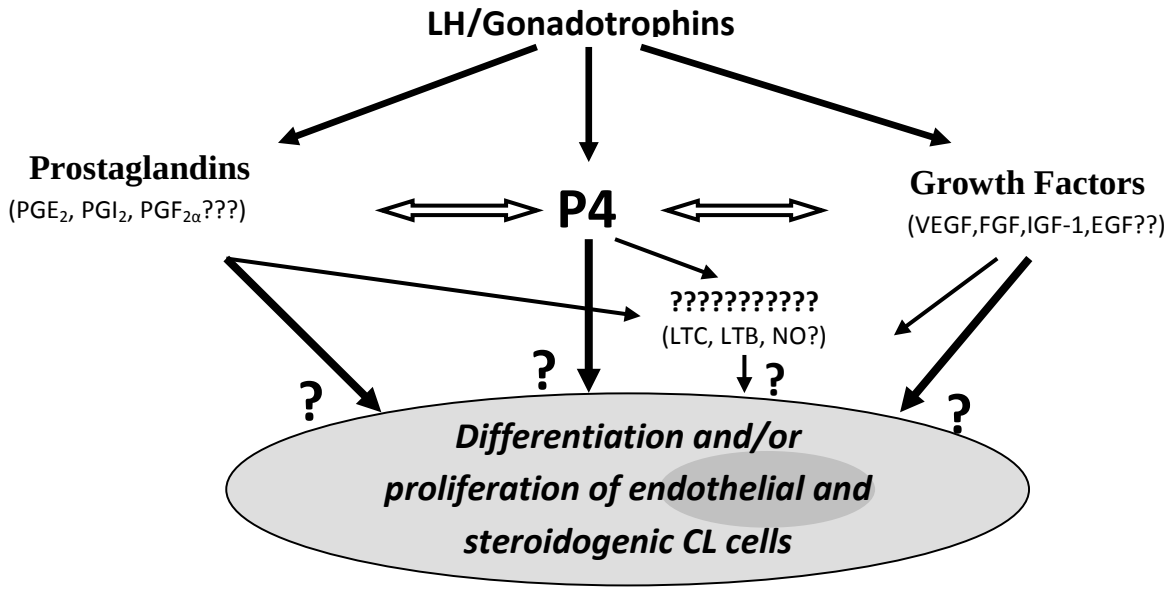

Fig. 1. *From Ferreira-Dias and Skarzynski (2008) modified.* Hypothetical model of the regulation of the CL development.

Some of these regulators, that act as autocrine and/or paracrine factors, seem to be prostaglandins (PGs) (Douglas and Ginther, 1972-1976; Marsh, and LeMaire, 1974; Henderson

and McNatty, 1975; Boiti *et al*., 2001; Weems *et al*., 2002) and other arachidonic acid metabolites [PGE_2, $PGF_{2\alpha}$, leucotrienes (LT)], neuropeptides [noradrenaline (NA)], peptide hormones [*i.e*. oxytocin (OT)], nitric oxide (NO)(Yamauchi e*t al*., 1997), growth factors and hormones such as vascular endothelial growth factors (VEGFs) (Kamat *et al*., 1995; Endo *et al*., 2001; Mattioli *et al*., 2001; Boonyaprakob *et al*., 2003), fibroblastic growth factors (FGFs)(Reynolds *et al*., 1994; Redmer and Reynolds, 1996; Grazul-Bilska *et al*., 1993; Grazul-Bilska *et al*., 1995a), epidermal growth factor (EGF), insulin-like growth factors (IGFs)(Apa *et al*., 1996; Yuan and Lucy, 1996), angiopoietins (Angs)(Goede *et al*., 1998), endothelial-1 (ET-1 or EDN-1) (Flores, 2000; Berisha *et al*., 2002), transforming growth factors (TGFs), nerve growth factors, tumor necrosis factors (TNFs), growth hormone (GH)(Spicer *et al*., 1992; Spicer *et al*., 1993; Lucy *et al*., 1994), Luteinising Hormone (LH)(Hoyer *et al*., 1984; Ohara *et al*., 1987; Alila *et al*., 1988; Tekpetey and Armstrong, 1991), prolactin (PRL), leptin (Ruiz-Cortés *et al*., 2000; Löffler *et al*., 2001; Nicklin *at al*., 2007), ghrelin (Caminos *et al*., 2003; Gaytan *et al*., 2003; Miller *et al*., 2005) and steroids (P_4 and E_2) (Rothchild, 1981; Chappell *et al*., 1997; Kotwica *et al*., 2004).

Because of the potential benefits in being able to therapeutically manipulate angiogenesis, inhibitors are being developed to prevent angiogenesis action for treatment of several pathologies among numerous types of tumors (Kim *et al*., 1993; Baringa, 1997). Tissue growth depends on vascular development. Pro-angiogenic strategies are being developed to stimulate the process where it is lacking, such as in ischemic heart disease. These molecules may also be employed to elucidate the processes involved in physiological angiogenesis in the female reproductive tract, and to explore possible new approaches to the regulation of fertility, the treatment of infertility and reproductive tract pathologies (Fraser and Duncan, 2005).

As a monovulatory species, the mare, in contrast to laboratory animals, is a better study model to understand angiogenesis and ovarian kinetics in general. In the mare, a cyclic coordinated vascular and non-vascular luteal growth and regression, involves both cell proliferation and active caspase-3 mediated apoptosis (Roberto da Costa *et al*., 2005; Ferreira-Dias *et al*., 2006a-2006b; Ferreira-Dias *et al.*, 2007). Several evidences confirme the hormonal regulation of angiogenesis in the CL by LH (Shweiki *et al*., 1993; Laitinen *et al.*, 1997; Dickson and Fraser, 2000). Nevertheless, other factors like hormones, vasoactive peptides and cytokines might control luteal angiogenesis (Davis *et al.*, 2003). In spite of well established evidence that angiogenesis and inflammation are co-dependent processes in various diseases, their precise cross-talk on the physiologic regulation of the reproductive function in the mare, is yet to be known. Besides, they are yet to be understood the mechanisms by which many hormones and metabolic factors such as leptin, ghrelin, GH and IGF-I involved on energetic balance regulation and metabolism modulate gonadal axis function. Ghrelin and leptin, besides their importance on food intake and appetite, contribute for gonadal regulation, in different species. Ghrelin, a hormone mainly produced by the stomach under the influence of metabolic, nutritional and hormonal factors (Ven der Lely *et al*., 2004), is the ligand of the growth hormone secretagogue receptor, stimulating growth hormone (GH) secretion. Ghrelin mRNA expression in ovary, placenta and testes has been reported (Gualillo *et al*., 2001; Tena-Sempere *et al*., 2002). The existence of local ghrelin-GH axis in the ovary, where GH increase locally produced ghrelin secretion, seems to be evident. Ghrelin, in association with GH, stimulated estradiol synthesis and secretion by ovarian follicles and prevented apoptosis in porcine ovary (Rak and Gregoraszczuk, 2008). Leptin also regulates nutritional status, contributing for reproductive efficiency (Karlsson *et al.*, 1997). A maturation-dependent action of leptin on GH and IGF-I stimulated follicular steroidogenesis (Gregoraszczuk *et al.*, 2004). Moreover, leptin acts sinergistically with GH and IGF-I in luteinization in different species. When combined with

IGF-I, anti-apoptotic effect is evident. This mechanism is essential for maintenance and development of the CL as an endocrine organ (Gregoraszczuk *et al.*, 2004), even though it is not yet known in the mare.

Regulatory role of cytokines on luteal function has been proven in different species like human (Yan *et al*., 1993; Chae *et al*., 2007), mouse (Yamamoto *et al.*, 1995), cow (Korzekwa *et al*., 2008) or mare (Galvão *et al*., 2010). During equine luteal development, a number of growth/angiogenic factors, P_4, the cytokine Tumor necrosis factor (TNF_α), and NO mediated prostaglandin E_2 (PGE_2) production might also be involved (Roberto da Costa *et al*., 2005; Ferreira-Dias *et al*., 2006a). TNF_α and INFγ mainly produced by non-steroidogenic immune cells in the CL (Taniguchi *et al.*, 2002), together with steroid and non steroid hormones, growth factors and eicosanoids, play specific roles in luteal establishment (Reynolds and Redmer, 1999; Berisha and Schams, 2005; Skarzynski *et al.*, 2008; Ferreira-Dias *et al*., 2010). Thus, there is a strong evidence that the immunoendocrine crosstalk is essential for normal ovarian function in the non-pregnant female. It also appears that CL secretory function in the mare is highly dependent on *in situ* angiogenesis and cytokine mediated role. In the absence of gestation, among other factors, P_4, TNFα and NO might contribute for luteal regression (Ferreira-Dias *et al.*, 2006a). Regarding another cytokine that influences equine CL functional and structural regulation, the Fas Ligand (FasL) (Galvão *et al.*, 2010), its involvement on angiogenesis coordination is reported in several organs (Biancone *et al*., 1997; Kim *et al*., 2007), but only slightly studied in CL (Pru *et al.*, 2003).

Even though a lot of research has been done on the luteal function in the mare, knowledge on regulatory mechanisms of the CL is lacking. Therefore, further studies are needed to elucidate the mechanisms of luteal formation and the relationship between the dichotomy of loss of function and/or luteal involution in the mare. Thus, the objectives of the present study were to evaluate the role of the hormones leptin, ghrelin, GH and IGF-I on NO and angiogenic

activity by the equine corpus luteum (CL) during its establishment and regression. The effect of leptin and ghrelin on CL secretory function (P_4 and PG) was also studied. Furthermore, gene identification of ghrelin, leptin, GH and IGF-I in luteal tissue was also accomplished.

2. CORPUS LUTEUM

2.1. CORPUS LUTEUM' S STRUCTURE

The CL is a transient endocrine gland, whose primary secretory product – progesterone - is required for establishment and maintenance of early pregnancy. In the presence of the conceptus, the lifespan of the CL is further prolonged. Therefore, within a relatively short period, the CL must undergo a few days period of intense angiogenesis, followed either by regression of the vasculature in the non-fertile cycle during luteolysis (Ferreira-Dias *et al.*, 2006b), or by stabilization and maturation of the vasculature during luteal rescue in the fertile cycle. Inadequate P_4 production is a major cause of infertility and embryonic loss since this hormone is a necessary requirement for both endometrium development and embryo survival. (Stouffer, 1999).

The lifespan and function of the CL is regulated by complex interactions between stimulatory (luteotrophic) and inhibitory (luteolytic) mediators (Bachelot and Binart, 2005) that lead to fast physiological growth followed by a regression process characterized by changes and degeneration (apoptosis) of both vascular and steroidogenic cells (Reynolds *et al.*, 1992; Ginther 1992a; Stouffer *et al.*, 2001; Davis *et al.*, 2003). These mechanisms vary among species. In humans and other primates, the lifespan of the CL is absolutely dependent on the support from pituitary-derived LH (Devoto *et al.*, 2002). In domestic animals, ruminants and horses, the primary luteotrophic hormone is LH (Ginther 1992b, Watson *et al.*, 1995; Berisha and Schams, 2005), while the termination of luteal activity is caused by uterine prostaglandin factor $PGF_{2\alpha}$.

Luteal development is associated with a dramatic increase in the number of blood vessels. LH and the other luteotrophic hormones modulate luteal synthesis of growth factors, cytokines, and other factors that in turn influence luteal cell function.

Luteal tissue is very complex and is composed by a heterogeneous mixture of cell types. The CL consists of not only steroidogenic luteal cells but also non-steroidogenic cells, i.e. vascular endothelial cells which are a major component of the CL accounting for approximately half of all cell types at mid-cycle (Reynold and Redmer,. 1999), fibroblasts, pericytes and immune cells such as lymphocytes, leucocytes and macrophages (Lei *et al*., 1991). There are at least two types of steroidogenic cells, large (LLC) and small (SLC) luteal cells. In the mare, it appears that large luteal cells might play an important role on most regulation or synthesis of P_4 in luteal structures (Roberto da Costa *et al*., 2005). Synthesis of P_4 starts at a very early stage of the luteal structure and was accompanied by an increase in P_4 receptors and proliferation of large luteal cells, until mid luteal phase (Roberto da Costa *et al*., 2005). Macrophages and lymphocytes T production IL-1β and $TNF_{\alpha,}$ respectively (Brannstrom *et al*., 1999; Kohen *et al*., 1999), cytokines can modulate luteal steroidogenesis. Macrophages and endothelial cells infiltrate into the newly formed CL concomitant with vascular angiogenesis (Reynolds and Redmer, 1999), establishing close contact with other luteal cells, which facilitate luteal cell regulation by paracrine mechanisms. The most membranes of luteal cells are either directly adjacent to capillaries (59%) or adjacent to the interstitial space (37%) in close proximity to capillaries. Such juxtapositioning of luteal cells to capillaries provides for the high metabolic demands of corpora lutea, which consume two to six times more oxygen per unit weight than does the liver, kidney, or heart. All these cells together funcion like a network to secrete a variety of encocrine, paracrine and autocrine products to establish and maintain pregancy.

In many species, including the human, the smaller steroidogenic cells are thought to be derived from the theca interna, and the large steroidogenic cells are thought to be derived from

the granulosa cells lining the follicle wall (Alila and Hansel, 1984). There is also evidence that at least some SLC may be transformed into LLC as the corpus luteum matures. (Alila and Hansel, 1984; Farin *et al.*, 1986). The SLC are highly responsive to LH/hCG and $PGF_{2\alpha}$ but appear to produce low basal levels of progesterone and few, if any, regulatory peptides. The LLC are unresponsive to LH/hCG but highly responsive to $PGF_{2\alpha}$, produce high basal levels of progesterone and several growth factors (such as oxitocin) and cytokines (Koos and Hansel, 1981; Fitz *et al.*, 1993). In addition, LLC express both estrogen and progestin receptor. When comparing quantitative cell composition between human and cow CL it was noted that both had a greater number of small luteal cells than large luteal cells at any stage of the cycle, except for the late luteal stage in the cow where a greater of large luteal cells was observed (Lei *et al*., 1991). There are also morphological distinctions such as a spherical nuclei in large cells but irregular nuclei in small cells and presence of rough endoplasmic reticulum in large but not small luteal cells (Farin *et al.*, 1986; O'Shea *et al.*, 1989; Sawyer *et al.*, 1992; Wiltbank, 1994).

In the mare, in which ovulation occurs into an ovulation fossa and which develops secondary corpora lutea during pregnancy, it appears that only the granulosa lutein cells contribute to the formation of the mature corpus luteum (Broadley *et al.*, 1994). In contrast to the other species, small luteal cells are not thecal origin (van Niekerk *et al*., 1975; Ginther, 1992). In the mare, LLC are light in colour while small luteal cells are dark, as described previously in humans and cows (van Niekerk *et al.*, 1975; Lei *et al*., 1991). In this species the presence of progesterone receptores (PR) in LLC from all stages of luteal phase suggests the role predominant of this cells in the regulation or synthesis of P_4 . Moreover, during mid- luteal phase (when P_4 concentration is high) there is a correspondence to an increases number of large luteal cells and PR expression in these cells, but not in size (da Costa *et al.*, 2005). Some authors (Farin *et al.*, 1986; Lei *et al.*, 1991; Gredoraszczuk, 1996) suggested that small cells are actually large luteal precursors, which are still small in size at the ovulation and begin increasing in size

as the cells avance to mid-lutel phase.

At the end of the luteal phase, the release of $PGF_{2\alpha}$ causes a dramatic decrease in plasma progesterone, which is defined as functional luteolysis and precedes structural regression of the CL (Douglas and Ginther, 1972-1976; Henderson and McNatty, 1975). A distinct sequence of events is associated with the demise of the CL including changes in blood supply (Miyamoto *et al.*, 2005), infiltration of leucocytes (Bukovsky *et al.*, 1995; Gaytan *et al.*, 1998), and death of steroidogenic and endothelial cells by both apoptotic (Juengel *et al.*, 1993; McCormack *et al.*, 1998) and non-apoptotic mechanisms (Fraser *et al.*, 1999; Morales *et al.*, 2000; Gaytan *et al.*, 2002).

2.2. LUTEAL STEROIDOGENIC PATHWAY

A critical feature of ovarian function in mammalian species is the further differentiation of the ovulatory follicle into the CL and its subsequent role in producing hormones (notably P_4) that are essential for the initiation and maintenance of intra-uterine pregnancy by inducing a state of quiescence in the myometrium and by suppressing the maternal immune response to fetal antigens (Csapo and Pulkkinen, 1978). In addition to providing a uterine environment suitable for the development of the embryo, P_4 also reduces the cyclic ovarian activity during pregnancy in most mammals and is responsible, in part, for mammary development. The CL also plays a key role in regulating the length of the ovarian cycle in most cyclic mammals, and the extension of the life span of the corpus luteum and P_4 secretion is necessary in most species to maintain gestation in its early stages (Szekeres-bartho, 1992). In some species the placenta is source of P_4 and in these, such as sheep, horse, and human the placenta usually becomes the dominant source of P_4 during the later stages of pregnancy (McCracken *et al.*, 1999). In the goat, pig, rabbit, and mouse, the corpus luteum (or in polyovulatory species, corpora lutea) remains the major source of P_4 throughout pregnancy. Regulation of steroid production by the CL varies remarkably for

different species. In humans, monkeys and ruminants the CL is largely dependent on pituitary-derived LH acting through the cAMP/protein kinase A pathway (Niswender *et al*., 2000). Several studies have examined the role of LH pulse amplitude and frequency in the maintenance of CL. Conversely, in rodents and rabbits, it is well established that prolactin and E_2 are critical luteotrophic hormones (Stormshak *et al.*, 1987). LH acts both directly on the luteal cells via interaction with their as luteotrophic hormone, and indirectly modulating luteal synthesis of growth factors, cytokines, and other factors that in turn influence luteal cell function (Devoto *et al.*, 2000). The luteinizing hormone binds to and activates a specific glycoprotein hormone receptor on the membrane of the steroidogenic cells, initiating a cAMP second messenger signaling system that regulates processes essential for steroid synthesis encompassing the uptake of lipoprotein-carried cholesterol, cholesterol translocation to the inner mitochondrial membrane and the expression of several steroidogenic enzymes, including cytochrome P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Wiltbank *et al*., 1993).

All steroids are derived from the common precursor cholesterol, a hydrophobic molecule synthesized in the liver and transported to steroidogenic tissues (such as the adrenal cortex, follicle, corpus luteum, and testis), in the circulatory system, in form of lipoproteins or within the cell is dependent upon transport proteins. The cholesterol for steroidogenesis can come from multiple sources within a cell including: cellular membranes, cholesterol ester stores, circulating lipoproteins, or de novo cholesterol biosynthesis. Cholesterol exists in two forms in cells and plasma lipoproteins, namely free cholesterol and cholesterol esters. Free cholesterol is the precursor substrate for steroidogenesis. The major mechanisms for obtaining cholesterol are either the endocytosis of cholesterol rich low-density lipoprotein (LDL) or the selective uptake of cholesterol esters from high-density lipoprotein (HDL). The uptake of LDL by luteal cells occurs by receptor-mediated endocytosis (Brown and Golstein, 1986). Once internalized, the endosomes combine with lysosomes where the LDL dissociates from the receptor and is broken

down making free cholesterol available to the cell. The LDL receptor is recycled or degraded (Grummer and Carroll, 1988). Uptake of extracellular HDL occurs after binding to a plasma membrane-bound HDL binding protein, and cholesterol is transported into the cell by an undefined mechanism that does not appear to be receptor-mediated endocytosis (Lestavel and Fruchart, 1994). Once free cholesterol is present in the cytosol of the cell, it can be used for steroidogenesis or formation of cell membranes, or it can be esterified with fatty acids to form cholesterol esters by cholesterol ester synthetase and stored (Johnson *et al*., 1997). The cholesterol esters often form lipid droplets that have long been used as a morphological characteristic of steroidogenic cell types. Cholesterol esterase, activated when phosphorylated by protein kinase A (PKA)(Caffrey *et al.,* 1979), hydrolyzes the stored cholesterol esters and provides free cholesterol for use by the cell. This is one of the first steps in steroidogenesis that is acutely controlled by second messenger pathways.

The biosynthesis of P_4 requires two enzymatic steps. First, translocation of cholesterol from the outer to the inner mitochondrial membrane takes place, and then cholesterol is conversted to pregnenolone, catalyzed by P450 side chain cleavage (P450scc) located on the inner mitochondrial membrane (Belin *et al.,* 2000). Pregnenolone has two hydrophilic residues that make it less stable in cellular membranes and more readily mobile through the cell. It diffuses out of the mitochondria to the smooth endoplasmic reticulum (SER) where it is converted to P_4 by 3βHSD. P_4 then diffuses out of the luteal cell and into the bloodstream to be transported to target tissues.

The steroidogenic acute regulatory protein (StAR) governs the rate-limiting step in steroidogenesis, which is the tissue specificity for StAR appears to vary among species. In human StAR is not highly expressed in granulosa cells of the preovulatory follicle (Kiriakidou *et al.*, 1996). Therefore, the process of luteinization is associated with a dramatic up-regulation of StAR in granulosa-lutein cells. In the CL or luteal cells, StAR protein is induced by many factors

that also increase progesterone production including IGFs (Devoto *et al*., 1999), LH (Pon and Orme-Johnson, 1988), and E_2 (Townson *et al*., 1996). StAR mRNA is most abundant during early and mid-luteal phase and declines significantly in the late luteal phase (Devoto *et al*., 2002) where it is expressed in both theca-lutein cells (greatest in the early luteal phase, moderate in the mid and least in the late luteal phase) and granulosa-lutein cells (moderate expression in early luteal tissue, increased levels in mid-luteal phase CL and declining expression in late luteal phase) (Pollack *et al*., 1997). In the bovine Graafian follicle, StAR appears to be present exclusively in the theca interna (Soumano and Price, 1997; Bao *et al*., 1998), although an earlier study reported its presence in both granulosa and theca cells (Pescador *et al.*, 1996). Treatment with eCG increases accumulation of StAR mRNAin bovine follicles and it has been suggested that this reflects granulosa cell expression (Soumano and Price, 1997).

In equine preovulatory follicles, mRNA StAR protein was confined to theca cells and was undetectable in granulosa cells before treatment with hCG. StAR expression increased markedly 30 h after hCG treatment. In the equine CL, immunostaining for StAR was confined to the LLC, which are presumed to be luteinized granulosa cells (Harrison, 1946; Van Niekerk *et al*., 1975), and was not detectable in the trabeculae which contain the small cells. No differences in distribution of immunostaining for StAR were noted in corpora lutea from non-pregnant mares (Watson *et al.*, 2000).

Once the CL has obtained it mature size and reached its maximal potential for secretion of progesterone, luteal function is maintained for a few to several days depending on the species, and then if the animal does not become pregnant, luteal regression must occur to allow for another ovulation and another chance for pregnancy to occur. Concentrations of progesterone in serum are dependent on the amount of steroidogenic tissue, blood flow, and capacity of the steroidogenic tissue to synthesize P_4.

In the mare, aside from the traditional P_4 target tissues such as vagina, cervix and

endometrium where receptors for this hormone have been detected (Tomelli *et al*., 1991; Watson *et al.*, 1992; Re *et al*., 1995; McDowell *et al.*, 1999), the CL may be a site a P_4 action acting as luteotrophic factors, influencing steroidogenesis and inhibiting apoptosis in the ovary.

2.3. PROGESTERONE IN THE MARE

In the horses, GnRH pulsatile secretion and modulation of pulse frequency by hypothalamus, is signal for the release of gonadotrophins (LH and FSH). In late diestrus, FSH activity caused the selection of a dominant follicle which under the influence of increasing E_2 levels early in estrus will develop to reach ovulatory status approximately 21 days after the previous ovulation occurs. E_2 is produced by the interrelationship between theca and granulosa cells within the developing follicle. The theca cells convert androgen precursors to P_4, which diffuses across to the neighbouring granulosa cells where it is converted to 17β- oestradiol by aromatase, a FSH-dependent enzyme. It begins to increase 6 to 8 days before ovulation, rising to peak around 2 days before ovulation. High levels of E_2 in concert with GnRh influence circulating LH (Alexander and Irvine, 1984).

The pulsatile release of LH from the pituitary gland represents the signal for follicle rupture and conversion of the mature follicle into the CL. Its increase in episodic pulse frequency and amplitude is responsible for the LH peak concentration which provides more P_4 to diffuse across the granulosa cells for conversion to E_2. In this way the synchronization of ovulation and oestrous behaviour is ensured. The ovulatory LH surge of the mare is unique in that it continues for several days after ovulation. In cyclic mares, LH release increases gradually during the first 4–5 days of oestrus, reaches a peak 1 or 2 days after ovulation and then falls slowly to dioestrus concentrations (Foster *et al*., 1979; Ginther, 1992). During dioestrus, LH pulse frequency is low and LH pulse amplitude declines steadily between day 4 and 10 after ovulation (Irvine *et al.*, 1998). It is thought that this prolonged increase in LH is important for establishing the CL

(Ginther, 1992). Unlike LH, FSH shows a biphasic profile. One surge occurs at or just after ovulation and a second one during mid- to late-luteal phase, with a 10 to 12 day intervals, respectiving in this way an an interval of 21 days between ovulation and the other one (Alexander and Irvine, 1993; Ginther and Bergfeldt, 1993).

Therefore, 24-48 hours post-ovulation P_4 levels begins to rise. Maximum concentration is reached on approximately 5-6 days after ovulation and it continues until 14-15 days after ovulation, in the absence of gestation. Luteolysis normally begins around days 14–16 post-ovulation in mares and is controlled by an oxytocin-dependent release of $PGF_{2\alpha}$ from the endometrium (Sharp, 2000)(Fig. 2).

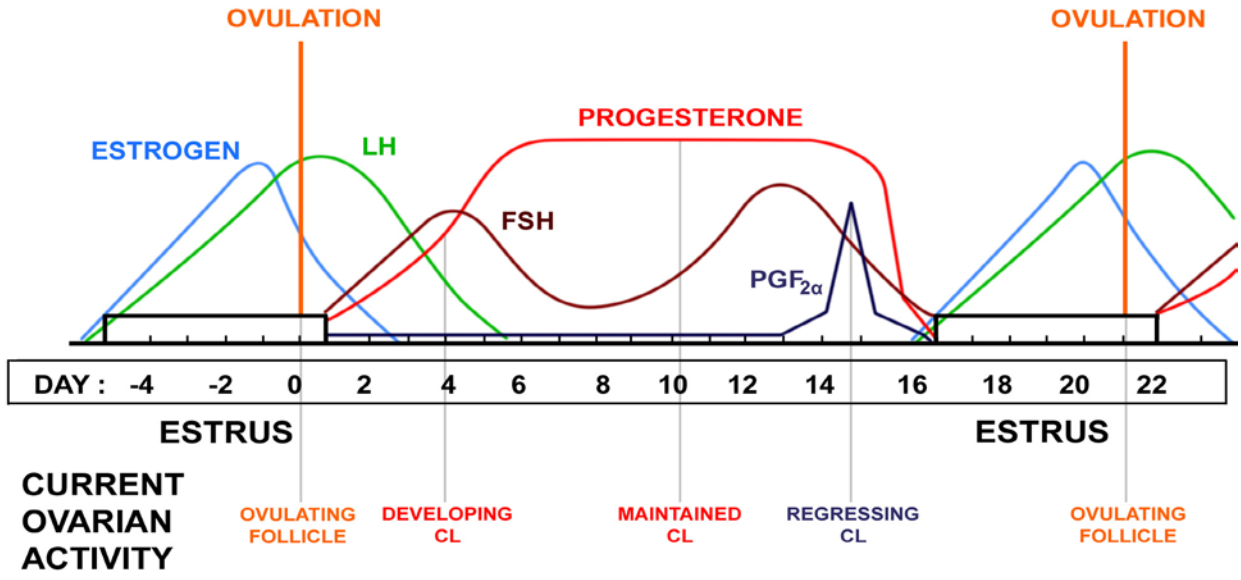

Fig. 2. *From Mottershead J. and Equine-Reproduction.com* (2001) *modified.* Sequential overview of the regular estrous cycle in the mare.

Numerous studies have shown that high P_4 concentrations act at the hypothalamus and lead to reduced LH pulse frequency (Goodman and Karsch, 1980; Soûles *et al*, 1984). Plasma P_4 increases more rapidly after ovulation in mares than in ewes (Ginther, 1992), being significantly above oestrous values between 12 h and 24 h after ovulation. It may then be that the hypothalamus in mares responds relatively slowly to the rising progesterone negative feedback.

2.4. LUTEAL PROSTAGLANDIN PRODUCTION

Usually, prostaglandins (PGs) are producted and secreted by the uterine endometrium, even though intraluteal production of PGs has been reported in horses, cattle, sheep, pigs, rats and primates (Olofsson and Leung, 1994).

The CL has a rich source of arachidonic acid (AA), an essential fatty acid stored in membrane phospholipids and the primary precursor of all PGs. The firs step in the PGs biosynthesis is the release of arachidonic acid from the phospholipids membrane catalyzed by the hormone-responsive enzyme cytosolic phospholipase A2 (cPLA2) (Clark *et al.*, 1991). The cPLA2 belongs to larger family of enzymes organized into 11 groups (I-XI) that also include various secreted forms of PLA2 (Six and Dennis, 2000). Activation of cPLA2 leads an increase in free intracellular calcium that binds to an amino-terminal domain causing translocation to cellular membranes, particularly to the nuclear envelope and endoplasmic reticulum, where it can hydrolyze arachidonic acid from membrane phospholipids (Gijon and Leslie, 1999).

There are two enzymatic steps in the conversion of free arachidonic acid to PGH_2, considered the rate-limiting step to the PGs synthesis pathway. First, a cyclooxygenase step that catalyzes the conversion of arachidonic acid to PGG_2 and a second peroxidase step that reduces PGG_2 to PGH_2, the common intermediate metabolite for different forms of PGs. The enzymes Cox-1 and Cox-2 catalyze the conversion of arachidonic acid to PGH_2, through a similar catalytic site and mechanism. Cox-3, third Cox (isoform derived from the Cox-1) has been isolated and characterized (Chandrasekharan *et al.*, 2002). Cox-1 is characterized by a constitutive expression in many tissues and may regulate various homeostatic functions such as arterial blood pressure and gastric epithelium function. Cox-2 is inducible in many tissues and has been found to regulate PG production during many acute responses such as inflammation. The subcellular localization of Cox-1 (endoplasmic reticulum and nuclear membrane) and Cox-2 (nuclear membrane) differ and this may be important for the differential use of substrate by these

enzymes. $cPLA_2$ also localizes to perinuclear membranes. This may explain why cPLA2-released arachidonic acid is utilized by Cox-2 while exogenously-added arachidonic acid is utilized by Cox-1 (Gijon and Leslie, 1999).

Finally, the conversion from PGH_2 to PGE_2, $PGF_{2\alpha}$ and prostacyclin (PGI_2) is catalyzed by PGE_2 synthase (PGES), PGF synthase (PGFS) and PGI-synthase (PGIS), respectively (Smith and Dewitt, 1996). Current evidence suggests three forms of PGES; among them microsomal PGES-1 (mPGES-1) is highly inducible along with Cox-2 (Thoren *et al.*, 2003). Intraluteal metabolism of PGs could also be a physiologically regulated event. In fact, PGE_2-9-ketoreductase that can convert $PGF_{2\alpha}$ to PGE_2 or alternatively PGE_2 to $PGF_{2\alpha}$ (Watson *et al*., 1979).

The enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH) ensures PGs catabolism by converting PGE_2 and $PGF_{2\alpha}$ into inactive forms: PGEM (PGE_2 metabolite) and PGFM ($PGF_{2\alpha}$ metabolite) (Tai *et al*., 2002). PGDH mRNA was abundant in the pig CL (Diaz *et al*., 2000). In the sheep CL, PGDH activity is greatest in the early CL and during maternal recognition of pregnancy, both times when the CL is relatively resistant to $PGF_{2\alpha}$ action (Silva *et al*., 2000).

3. LUTEAL ANGIOGENESIS

The transition from pre-ovulatory follicle to CL is a dynamic process involving a series of biochemical and morphological changes following the LH surge that causes differentiation of follicular cells into luteal cells (luteinization). Luteinization is characterized by increased steroid production and a switch from producing estradiol (E_2) to progesterone (P_4) and of enzymes responsible for these changes. Hemodynamic changes are involved in the cyclic remodelling of ovarian tissue that occurs during final follicular growth, ovulation and new CL development (Acosta *et al.,* 2002). This increase reflects normal CL development and underlines the

importance of angiogenesis.

Angiogenesis is defined as the generation of new blood vessels through sprouting from already existing blood vessels in a process involving the migration and proliferation of endothelial cells (EC) from pre-existing vessels. Angiogenesis occurs rarely in the adult with exceptions such as the female reproductive system, wound healing and cancer (Reynolds and Redmer, 1998). The development of new blood vessels in the ovary is essential to guarantee the necessary supply of nutrients and hormones to promote follicular growth and corpus luteum formation. The angiogenic process begins with capillary sprouting and culminates in formation of a new microcirculatory bed composed of arterioles, capillaries and venules. The vessels remain quiescent until there is an angiogenic stimulus such as hypoxia or wounding, which then upregulates proangiogenic factors. Physiological angiogenesis is basically characterised by a gradually progressing process : 1) dilatation of pre-existing vessels, increase of vascular permeability and degradation of extracellular matrix (ECM) and basal lamina; 2) proliferation and migration (so-called sprouting) of endothelial cells from the existing vessels towards an angiogenic stimulus, and 3) proliferation of endothelial cells under the influence of proangiogenic factors (Klagsbrun and D'Amore, 1991). Once connected and aligned, the endothelial cells form a lumen and the newly formed vessel is then stabilised by the recruitment of pericytes (Gerhardt and Betsholtz, 2003). New blood vessel development is completed by formation of capillary basal lamina and differentiation of new capillaries into arterioles and venules.

How mentioned in the introduction, the angiogenic process in the developing CL begin with dissolution of tha basal membrane between granulosa e theca interna layers. Following, the expansion of theca capillaries is initiated by sprouting into the avascular granulosa layer to form a dense network of capillaries surrounding the granulosa cells. Hyperpermeability plays a

fundamental role in both normal and abnormal tissue growth and remodelling. It allows fibrin and other blood components to enter the extravascular compartment, thereby creating a temporary environment for optimal cell growth and migration (Senger *et al.*, 1993). Therefore, the fibrin deposited in the residual cavity after ovulation likely contributes to the induction of angiogenesis in newly forming CL.

The high density of capillaries is demonstrated by the fact that microvascular endothelial cells are the most abundant cell type in the CL, with each luteal cell in apparent contact with at least one neighbouring capillary (Gaytan *et al.*, 1999). Endothelial cells proliferation is 4- to 20-fold more intense than in some of the most malignant human tumours (Christenson and Stouffer, 1996). This intense blood vessel formation in the newly forming CL enables mature CL to receive one of the greatest rates of blood flow of any tissue in the body (Kaczmarek *et al*., 2005). Endothelial cells of arterioles and venules recruit smooth muscle cells to stabilise the vessels and control their vasotonia; whereas endothelial cells in microvessels recruit pericytes to ensheath the capillaries and influence vessel function (Carmeliet, 2000). Some reports have indicated that up to 60% of microvessels in the mature CL contain pericytes, these represent a population of cells fist migrating into hypoxic granulosa layer after ovulation and, therefore, may play critical role in angiogenensis during luteinization. In women, luteal vascularization is maximally developed in the mid-luteal phase (Wulff *et al*., 2001b), as in the mare (Al-ziabi *et al*., 2003; Ferreira-Dias *et al.*, 2006b), when the proliferation of endothelial cells and pericytes peaks.

Angiogenesis is a complex process in which a delicate balance between promoters and inhibitors is maintained. Therefore, the precise control of angiogenesis in the ovary is critical for normal luteal function. Among the numerous ANGIOGENIC FACTORS that have been identified, the most important ones appear to be vascular endothelial growth factor A (VEGF-A), acidic and basic fibroblast growth factor (FGF-1 and FGF-2), insulin-like growth factors

(IGF-I and IGF-II) and angiopoietins (ANPT-1 and ANPT-2). Several other possible VASOACTIVE FACTORS have been identified, including members of transforming growth factors (TGF) family, tumour necrosis factor (TNF), vasoactive peptides like angiotensin II (Ang II) and endothelin-1 (ET-1), nitric oxide (NO) and proteins of the extracellular matrix (ECM).

3.1. ANGIOGENIC FACTORS

Vascular Endothelial Growth Factor (VEGF) system

VEGF is the main angiogenic factor; promotes proliferation, migration and survival of endothelial cells as well as stimulates microvascular leakage which allows tissue infiltration of plasma proteins (hence the alternative name vascular permeability factor - VPF; Connolly *et al*., 1989; Keck *et al*., 1989). In addition to increasing EC cytoplasmic (Ca^{2+}) and permeability to macromolecules, VEGF causes ECs from several different sources to assume an elongated shape and stimulates their replication. VEGF increases the permeability of microvessels, primarily postcapillary venules and small veins, to circulating macromolecules. It acting as a survival factor by expression of antiapoptotic proteins such as Bcl-2 (Gerber *et al*., 1998).

The VEGF family currently comprises several members, including the first identified molecule VEGF-A (also referred as VEGF), Placental Growth Factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E (a viral homologue of VEGF). PlGF is restricted to the placenta, while VEGF-B is particularly abundant in heart (coronary growth and vascularity) and skeletal muscle. VEGF-C and VEGF-D are involved in lymphangiogenesis and maintenance of the lymphatic vessels (Robinson and Stringer, 2001; Ferrara *et al*., 2003b). VEGF-A is not only an angiogenic factor, but also a survival factor for endothelial cells in certain types of microvessels. In addition, VEGF isoforms are generated by alternative splicing of a single VEGF mRNA.

The VEGF proteins exert their biological functions almost exclusively through a family of closely related receptor tyrosine kinase: fms-like tyrosine kinase (Flt-1 or VEGFR-1), fetal

liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR or VEGFR-2), and VEGFR-3 (Flt-4). They contain an extracellular region with seven immunoglobulin (Ig)-like loops, a single transmembrane region and an intracellular tyrosine kinase domain interrupted by a kinase-insert sequence (Robinson and Stringer, 2001; Cross *et al*., 2003). The receptor VEGFR-1 (or Flt-1) is thought to be critical for VEGF-induced formation of vascular capillary tubes (Boonyaprakob *et al*., 2003) and is expressed in both proliferating and quiescent endothelial cells (Berisha *et al*., 2000). The 180-KDa glycoprotein VEGFR-1 has the highest affinity for VEGF (de Vries *et al*., 1992; Quinn *et al.*, 1993). VEGFR-1 is also shared by the related growth factors PlGF and VEGF-B. However, VEGFR-1 has been implicated in upregulated endothelial expression of tissue factor, urokinase-type Plasminogen Activator (uPA) and plasminogen activator inhibitor 1 (PAI-1) (Clauss *et al*., 1996; Olofsson *et al.*, 1998). In other cell types VEGFR-1 has different roles, such as tissue factor induction and chemotaxis in monocytes, and enhancing matrix metalloproteinase expression by vascular smooth muscle cells (Barleon *et al*., 1996; Wang and Keiser, 1998). VEGFR-2 (Flk-1/KDR) is a 200-230-KDa high-affinity receptor for VEGF (Terman *et al.*, 1992; Quinn *et al*., 1993), as well as, for VEGF-C, VEGF-D and VEGF-E. VEGFR-2 is expressed by endothelial angiogenic cells, haematopoietic stem cells and umbilical cord stroma. However, in quiescent adult vasculature, VEGFR-2 mRNA appears to be downregulated (Millauer *et al*., 1993; Quinn *et al.*, 1993). Although VEGFR-1 has the greater affinity for VEGF, VEGFR-2 is tyrosine phosphorylated much more efficiently upon ligand binding and in endothelial cells leads to mitogenesis, chemotaxis and changes in cell morphology (Quinn *et al*., 1993; Waltenberger *et al*., 1994). VEGFR-3 (Flt-4) expression is restricted mainly to the lymphatic endothelium of adult tissues (Pajusola *et al.*, 1992; Kaipainen *et al*., 1995). VEGFR-3 binds VEGF-C and VEGF-D, but not VEGF, and is though to control lymphangiogenesis.

PlGF, VEGF-B, VEGF-E and certain isoforms of VEGF-A bind selectively to two

neuropilins (NRP-1 and NRP-2), which are transmembrane non–tyrosine kinase receptors, identified firstly on neuronal growth cones as mediators of semaphoring/collapsing control of axonal guidance (Gluzman-Poltorak *et al*., 2000; Robinson and Stringer, 2001; Stouffer *et al*., 2001).

The receptor VEGF is substantially overexpressed at both mRNA and protein levels in a high percentage of malignant and benign animal and human tumors, non-neoplastic pathological states (i.e. healing cutaneous wounds, psoriasis, ischemic myocardium), placenta, many fetal tissues, and a number of normal adult tissues undergoing physiological angiogenesis eg, in the proliferating endometrium, and in the corpus luteum.VEGF is also expressed at low levels in a wide variety of normal adult human and animal tissues and at higher levels in a few select sites, namely, podocytes of the renal glomerulus, cardiac myocytes, prostatic epithelium and semen, and certain epithelial cells of the adrenal cortex and lung (Berse *et al.*, 1992; Brown, *et al.*, 1992; Shweiki *et al*., 1993).

The importance of VEGF throughout CL lifespan has been demonstrated in several species. In the newly forming CL, VEGF mRNA and protein expression are observed both in the granulosa- and theca-derived luteal cells. VEGF expression levels were higher in granulosa-derived than in theca-derived luteal cells (Kamat *et al.*, 1995; Endo *et al*., 2001; Boonyaprakob *et al*., 2003). Highly expressed VEGF in granulosa-derived luteal cells may act as a chemoattractant for sprouting endothelial cells in order to initiate the invasion of avascular granulosa layer, establishing an extensive capillary network that nourishes the developing CL and maintains the luteal function throughout its lifespan (Mattioli *et al*., 2001). VEGF is also a modulator of the vascular permeability in the CL (Dickson *et al.*, 2001), may serve as a factor for increasing the uptake of cholesterol to luteal cells (Otani *et al.*, 1999). Treatment with hCG *in vivo*, which is associated with increased VEGF concentrations, reduces the expression of endothelial and steroidogenic cell tight junctional proteins (Groten *et al.*, 2006). This suggests

that one endothelial function, in the form of permeability, is regulated by VEGF in the mature CL and this may be associated with P_4 secretion into the circulation.

The VEGF protein was present in luteal connective tissue cells in the luteal capsule and connective tissue tracts, and in cells within the luteal parenchymal lobule. VEGF-expressing cells are indeed capillary pericytes by co-localization of VEGF and smooth muscle α-actin, which is a specific marker of cells of the smooth muscle lineage, including pericytes (Hirschi and D'Amore, 1996). Pericytes represent a population of cells first migrating into the hypoxic granulosa layer after ovulation. Under hypoxic conditions, VEGF has been shown to be mitogenic for pericytes (Yamagishi *et al*., 1999). The primary role of perivascular cells, and especially capillary pericytes, is probably to regulate endothelial cell function and angiogenesis during tissue growth and development. VEGF has been shown to be hemotactic for leukocytes (Barleon *et al*.,1996; Clauss *et al.*, 1996) and leukocyte number increases inside and around the follicle prior to ovulation (Espey and Lipner, 1994). Serum, platelets and leukocytes could all be a source of additional growth factors essential for rapid tissue repair and remodeling in developing CL.

The duration of the intense angiogenic phase in CL varies among species, but appears to be completed by day 8 of the luteal phase in caprine (Kawate *et al*., 2003) and in bovine (Acosta *et al*., 2003), and by day 6 in primates when capillaries surround most luteal cells and capillary dilation is evident (Lei *et al*., 1991; Christenson and Stouffer, 1996). In general, VEGF expression is higher during this period of intense angiogenesis (Redmer *et al*., 1996; Berisha *et al*., 2000; Ribeiro *et al*., 2007). A higher mRNA expression was also found during the early luteal phase in cow CL by RT-PCR (Goede *et al.*, 1998). The targets for VEGF localized in luteal cells are EC where both receptors (VEGFR-1 and VEGFR-2) are found. It is possible that co-expression of VEGFR-1 and VEGFR-2 in EC leads to formation of heterodimers in response to VEGF. However, in human (Otani *et al*., 1999; Endo *et al*., 2001) and equine CL (Al-zi'abi *et*

al., 2003), high levels of VEGF mRNA and protein are found to be maintained in the mid-luteal phase.

In the mare, VEGF mRNA and protein were expressed mainly in the luteal cells during the early (3-4 days) and mid-luteal (10 days) phases and expression declined at early regression (day 14) (Al-zi'abi *et al.*, 2003). In the mare, VEGF A, VEGFR- 2 (also Ang-2 and Tie2) seem to play a significant role in follicular and luteal vascularisation. This not only assures the optimal follicular and luteal supply of oxygen, nutrients, substrates, gonadotrophins to the follicles, P_4 precursors to the CL, but also supports the delivery of follicular/luteal hormones into the circulation (Watson and Al-zi'abi, 2002; Al-zi'abi *et al.*, 2003). During the early and mid-luteal phases, the peak expression of VEGF mRNA and protein indicate a temporal association with high endothelial cell proliferation and the presence of a dense capillary network (Al-zi'abi *et al.*, 2003). In the mature CL, VEGF was present in cells bordering the trabeculae where the blood vessels enter the luteal tissue (Al-zi'abi *et al.*, 2003). During the mid-luteal phase, the high expression of VEGF mRNA is compatible with its established role in stimulating and maintaining the newly formed vasculature in the corpus luteum (Dickson *et al*., 2001).

Non-luteal cells, which were identified as macrophages, showed expression of VEGF mRNA and protein in the cavity of the corpus luteum throughout the luteal phase. The number of these macrophages expressing VEGF mRNA increased during regression, whereas there was a decrease in VEGF expression in luteal cells within the same corpus luteum. It is possible that macrophages trapped in the central clot experienced hypoxia which was responsible for increasing the expression of VEGF mRNA (Al-zi'abi *et al*., 2003).

Many cytokines and growth factors upregulate VEGF mRNA or induce VEGF release. These include PDGF, TNFα, TGFα, TGFβ, FGF-4, keratinocyte growth factor (KGF/FGF-7), epidermal growth factor (EGF), IL-1α, IL-1β, IL-6 and IGF-I. TNFα is produced mainly by macrophages and endothelial cells infiltrate into newly formed CL concomitantly with vascular

angiogenesis, stimulated PGE_2 secretion by bovine cultured endothelial cells (Okuda *et al.*, 1999). TNFα and PGE_2 may play a role in inducing the proliferation of endothelial cells and vascular angiogenesis as autocrine and/or paracrine regulators. Moreover, PGE_2 and VEGF mRNA expression was stimulated from VEGF in luteal cells. An increase in VEGF expression may induce Cox-II and mPGES (membrane-associated prostaglandin E synthase) mRNA expression, which together stimulate PGE_2 production. Both VEGF and PGE_2 are produced at high levels around the time of ovulation and luteinization in the ovary. This autocrine feedback loop may contribute to the increase in the levels of these factors and thus may be associated with angiogenesis and blood vessel permeability in the development of the CL (Sakurai *et al*., 2004). In addition, the cytokine TNFα is pro-angiogenic factor released by activated polymorphonuclear neutrophils (PMN)(Cacalcano *et al*., 1994), and it has been shown to induce IL-8, VEGF, and bFGF expression in microvascular endothelial cells (Yoshida *et al*., 1997). Proangiogenic capacity of PMN consists of at least two components: de novo synthesis of IL-8 and release of VEGF from preformed stores. IL-8 stimulates angiogenesis in the rat cornea model by promoting proliferation and chemotaxis of endothelial cells (Koch *et al*., 1992). Besides, IL-8 inhibits endothelial cell apoptosis and induces the upregulation of endothelial matrix metalloproteinase-2 and -9, which play an important role in angiogenesis (Li *et al*., 2003).

Hormones, such as LH can induce VEGF mRNA expression in preovulatory rat follicles and in cultured bovine luteal cells (Garrido, 1993; Koos, 1995). If VEGF is the major luteal angiogenic factor, its regulation by LH would make sense because LH is an important luteotrophic factor and is critical for normal luteal development and function. In humans, LH, FSH and hCG do not exert a direct effect on VEGF production, but they rather act in combination with other factors such as insulin or insulin-like growth factors (IGFs) (Stouffer *et al.*, 2001). One unresolved issue is whether LH or hypoxia is the prime regulator of luteal VEGF expression across a number of cell and tissue types, which is consistent with the concept that

metabolic demand is the primary factor regulating vascular development in all tissues (Adair *et al.*, 1990; Neufeld *et al.*, 1999). It may be that there is a role for both hypoxia and LH in the regulation of luteal VEGF expression. These processes are thought to occur under hypoxic conditions.

It is known that hypoxia is a potent stimulator of VEGF synthesis and secretion in many tissues, and there is a hypoxia inducible factor (HIF)-1α response on VEGF stimulation. There is a relationship between HIF-1α expression and LH. Nuclear HIF-1α immunostaining is markedly up-regulated in primate granulosa cells at the time of ovulation (Duncan *et al.*, 2008). It is therefore possible that HIF-1α expression is regulated by LH/hCG as well as hypoxia. In cell culture hCG increased the expression of both HIF-1α and VEGF (van den Driesche *et al.*, 2008). Indeed the hCG-dependent increase in VEGF expression also occurred under hypoxic conditions. It is therefore likely that the regulation of VEGF in the CL has both ligand-induced and hypoxic elements that may be differentially expressed according to the stages of the luteal phase. A recent study in cows indicates that HIF-1α is essential for the VEGF-induced angiogenesis during luteal development, and suggests that formation of luteal vasculature is regulated by hypoxic conditions following follicle rupture (Nishimura *et al.*, 2008).

Moreover, nitric oxide (NO), which is primarily an endothelial product and a potent vasodilator, can stimulate VEGF production and angiogenesis (Chin *et al*, 1997; Frank *et al*., 1999). Similarly, VEGF, present in luteal perivascular cells, can stimulate endothelial NO synthase (eNOS) expression and thus NO production (Van der Zee *et al*., 1997; Bouloumie *et al*., 1999). Paracrine loop exists between luteal endothelial cells release of NO, which stimulates perivascular VEGF production, which in turn stimulates endothelial expression of eNOS. This paracrine loop would thereby serve as a feed-forward system to maximize vasodilation and angiogenesis during luteal development (Fig. 3). In support of this finding, it was recently found in the mare CL that eNOS is expressed in endothelial cells of luteal arterioles and capillaries

early in the luteal phase but that its expression is greatly reduced by mid cycle (Ferreira-Dias *et al.*, 2011).

Fibroblast growth factor (FGFs) family

Fibroblast growth factors (FGFs) make up a large family of polypeptide growth factors that are found in organisms ranging from nematodes to humans. During embryonic development, FGFs have diverse roles in regulating cell proliferation, migration and differentiation. In the adult organism, FGFs are homeostatic factors and function in tissue repair and response to injury. When inappropriately expressed, some FGFs can contribute to the pathogenesis of cancer.

FGFs with the ligans FGF-1 (acid-FGF or aFGF) and FGF-2 (basic-FGF or bFGF) are probably involved not only in luteal angiogenesis (bFGF but not aFGF is probably a major FGF involved in luteal angiogenensis) (Grazul-Bilska *et al.*, 1993; Reynolds *et al*., 1994; Grazul-Bilska *et al*., 1995a; Redmer and Reynolds, 1996), which occurs primarily early in the estrous cycle, but are playing additional roles in luteal function as well. FGFs have been shown affect luteal P_4 production (Miyamoto *et al*., 1992) and protect luteal cells from undergoing cell death, as it has been reported for other cell types.

The mammalian FGF family has 4 members FGFR-1 (flg), FGFR-2 (bek), FGFR-3 and FGFR-4. The FGFRs consist of three extracellular immunoglobulin-type domains (D1-D3), a single-span trans-membrane domain and an intracellular split tyrosine kinasi domain.

Expression of both FGFR-1 and FGFR-2 proteins increased with the age of the CL; that is, their expression was least in the early luteal phase and greatest in the late cycle corpora lutea (Doraiswamy *et al*., 1998). In ruminants, FGFR-1 was present at high concentration in luteal parenchymal cells during early to mid-cycle and was dramatically reduced during luteal regression, whereas in luteal endothelial cells, FGFR-1 was present at all stages, especially in those of the larger luteal microvessels late in the estrous cycle (Doraiswamy *et al*., 1998).

Instead, FGFR-2 protein was present in parenchymal cells at all stages, but was present in the vasculature, primarily the larger microvessels, only late in the estrous cycle (Doraiswamy *et al*., 1998). In this way, the increase in FGFR in the larger luteal microvessels during luteal regression, could explain the selective avoid cell death while the rest of the luteal tissue is resorbed (Redmer and Reynolds, 1996). Therefore, FGFs may affect not only luteal cell proliferation but also luteal cell function and turnover (Redmer and Reynolds, 1996).

The proteins aFGF and bFGF are present in bovine and ovine corpora lutea (Grazul-Bilska *et al*., 1992; Jablonka *et al.*, 1997). Even though aFGF and bFGF stimulate proliferation of ovine luteal cells from several stages of the luteal phase, their effect is especially noted in the early CL (Grazul-Bilska et al, 1995b). Moreover, the pattern of expression of bFGF mRNA by bovine CL closely follows that of angiogenic activity production and also is stimulated by luteinizing hormone (LH) as angiogenic activity (Stirling *et al*., 1991). The mRNA expression of FGF-1 in bovine CL increased during mid-luteal stage (Schams and Berisha, 2002a). In contrast, FGF-2 expression is highest during very early luteal phase (days 1–2) in cytoplasm of capillary EC and in smooth muscle cells of arteries. The dominant localization of FGF-2 in EC at the early stage suggests that FGF-2 is a dominating factor for endothelial growth in bovine CL. FGF-2 produced a self-upregulation and led to greater mRNA levels for its receptor FGFR and both receptors of VEGF (VEGF-1 and VEGF-2), while VEGF only promotes up-regulation of FGFR mRNA (Galber *et al.*, 2004). Could exists synergistic effects maybe on the other factors such as ECM components, important for angiogenensis (Gabler *et al.*, 2004). Moreover, VEGF and FGF-2 are critical factors for regulation of P_4 production.

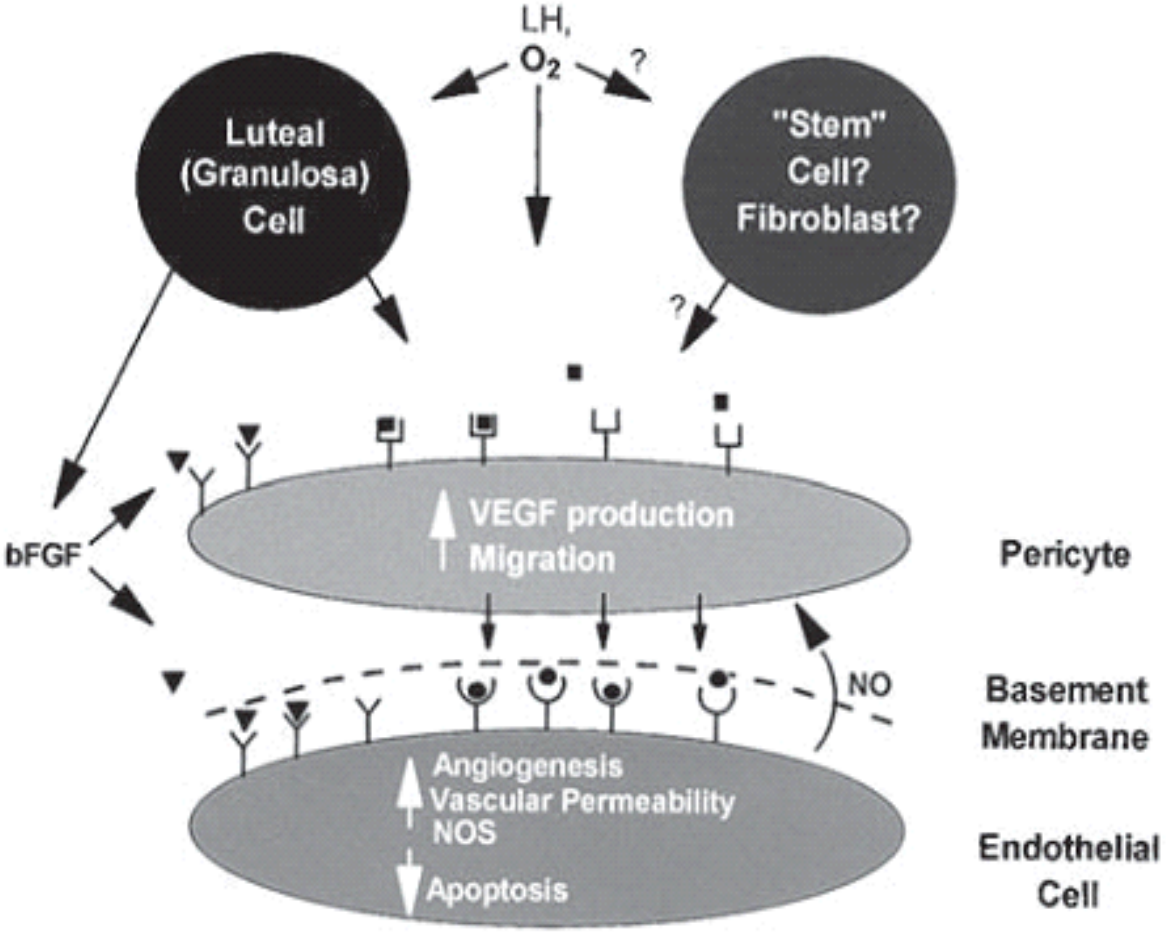

Fig. 3. *From Reynolds and Redmer (1998) modified. Hypothetical model for luteal vascularization.* Granulosa cells and the luteal cells derived from them are able of producing bFGF, which may regulate both migration of pericytes and endothelial cell survival via inhibition of cell death. VEGF derived from the perivascular cells may stimulate luteal angiogenesis and luteal vascular permeability and blood flow by via increasing NOS production. NO produced by the luteal endothelial cells can act in a paracrine loop to stimulate relaxation of arteriolar smooth muscle, and thus vasodilation, as well as increase VEGF production by capillary pericytes, and thus angiogenesis. Although LH stimulates production of angiogenic factors by luteal tissues, whether this effect is direct or mediated by luteal parenchymal or other cells is unknown. Oxygen tension seems to be a major regulator of luteal VEGF production; again, whether this effect is direct or indirect is not known.

Angiopoietins (Ang or ANPTs) system

Angiopoietins (Angs or ANPT) are another family of specific factors for endothelial growth. Co-ordination of blood vessel formation, maintenance, stabilisation and regression also involve other factors than VEGF (Fraser and Duncan, 2005). The angiopoietins influence the

stabilisation of newly formed vasculature, as well as the destabilisation of existing vascular network. These include the angiopoietins Ang-1 (o ANPT-1) and Ang-2 (ANPT-2), which act via the tyrosine kinase receptor Tie1 and Tie2. They may have an important role for the modulation of angiogenesis and angiolysis in the CL during the oestrous cycle (Goede *et al*., 1998). Specifically, Ang-1 activation of Tie2 enhances the maturation and stabilisation of newly formed blood vessels promoting the interaction between endothelial cells and surrounding matrix. It could therefore be hypothesized that Ang-1 would be increased in the fully functioning CL of the mid-luteal phase with its mature vascular network. Its expression in the human CL across the luteal phase did not really change (Wulff *et al.*, 2000; Sugino *et al.*, 2005), while in non-human primates it tended to be higher in the late-luteal phase (Hazzard *et al.*, 2000), and in bovine CL was low in the early-luteal phase. Ang-2 also binds to Tie2, but can act as an endogenous antagonist, blocking Ang-1 mediated receptor phosphorylation. Ang-2 is thought to be involved in the destabilization and remodeling of blood vessels loosing the supporting cell matrix and thus allowing angiogenic factors, such ae VEGF to stimulate endothelial cell proliferation and migration. It tends to be higher in the late luteal phase of women (Wulff *et al.*, 2000; Sugino *et al.*, 2005) and non-human primates (Hazzard *et al.*, 2000).

As Ang-1 and Ang-2 bind to the same receptor Tie2, the ratio Ang-2/Ang-1 appears to play a crucial role for vascular stability. The high ratio in microenvironment induces destabilization of blood vessels, which is a prerequisite for vascular formation and regression. In such conditions, the presence of angiogenic factor such as VEGF could determine the fate of destabilized blood vessel.

In the presence of VEGF, increased autocrine expression of Ang-2 by the vascular endothelium is associated with angiogenesis, while in the absence of VEGF or other pro-angiogenic factors, its expression is associated with degenerative changes in the vasculature (Maisonpierre *et al*., 1997). At luteolysis, VEGF mRNA decreased and the Ang2:Ang1 ratio

increased. It was proposed that this change would destabilise vessels in the CL and lead to vascular regression via apoptosis (Maisonpierre *et al.*, 1997).

Insulin-like growth factor family

The insulin-like growth factors (IGF-I and -II) are homologous polypeptide growth factors with widespread roles in growth and development. In some species, the insulin-like growth factor, has been shown as an important autocrine/paracrine system on ovarian and luteal functions. Two receptors mediate the activities of IGFs. Type I receptor mediates most of the somatomedin-like actions of both IGF-I and IGF-II. It is a α2β2 tetramer structurally and functionally related to the insulin receptor. The affinity of this receptor for IGF-I is slightly higher than for IGF-II, and much higher than for insulin. The type II receptor, or IGF-II/Mannose-6-Phosphate (IGF-II/M6P) receptor, binds IGF-II and molecules which possess a Mannose-6-Phosphate residue such as lysosomal enzymes and TGF-β1. This receptor would also play a role in degradation of IGF-II after internalization and degradation (Jones and Clemmons, 1995).

IGF action is also regulated by specific interactions with IGF binding proteins (IGFBPs) which bind IGF-I and IGF-II with high affinity (Rajaram *et al*., 1997). Six high affinity IGFBPs have been identified and characterised, and several related proteins comprising a family of low affinity IGFBPs, designated IGFBP-related proteins (IGFBP-rPs) (Hwa *et al*., 1999). IGFBPs are present in all biological fluids. IGFBPs increase IGFs' half-life and maintain a stable pool of IGFs in many compartments of the organism. They can be arbitrarily classified in two groups:

a) IGFBP-1, -2, -4, -5 and -6, which are present in the serum and in other fluids in a so-called 'small complex'. In serum, their levels are either negatively regulated (IGFBP-1 and -2) or unaffected by Growth Hormone (GH).

b) IGFBP-3 is the predominant IGFBP in serum, where it is found primarily in a 150-kDa form

('large complex'), composed of IGF-I or IGF-II, and an acid-labile 85-kDa subunit (ALS). The concentration of IGFBP-3 is positively regulated by GH and IGF-I.

IGF-I and -II may regulate luteal steroidogenesis, angiogenesis and apoptosis. Even if VEGF and FGFs are main regulators of ovarian angiogenesis (Reynolds and Redmer, 1998), IGF-I has also been implicated in neovascularization occurring in response to injury (Hansson *et al.*, 1989), in some disease (Vialettes *et al.*, 1994) and in the angiogenic model of the rabbit cornea (Grant *et al*., 1993).

The IGF system may have indirect or direct effects on angiogenesis by stimulating actions for VEGF production in luteal cells as well as by proliferation and differentiation of EC (Reynolds and Redmer, 1998; Berisha *et al.*, 2000). The distinct localization for IGF-II in pericytes suggests a major role in coordinating angiogenic processes, vessel maintenance and capillary stabilization (Amselgruber *et al.*, 1994). In fact, the loss of IGF- II during luteolysis may affect primarily the nourishing vascular system. Besides, the interaction of the IGF receptor with IGF-I or -II has been shown to protect a range of cell types, including ovarian cells, from apoptosis (Yang and Rajamahendran, 2000).

3.2. VASOACTIVE FACTORS

Nitric oxide (NO)

Nitric oxide (NO) is a potent biologic mediator of paracrine interactions especially within the vascular system. Endothelial-derived NO is a powerful inhibitor of platelet aggregation and a potent vasodilatator. NO is also a neurotransmitter in the central and peripheral nervous system and plays a role in cell-mediated cytotoxicity. Nitric oxide is produced following the five electron oxidation of one of the chemically equivalent guanidine nitrogens of L-arginine by the family of nitric oxide synthase (NOS) enzymes, forming the free radical NO and citrulline as byproduct. NO has a short half-life and is rapidly oxidized to the stable, inactive end-products,

nitrite and nitrate ($NO2^-$ and $NO3^-$)(Fig.4). The redox reactivity and chemical properties of this free radical gas permit it to function in many unique interactions with cellular signaling systems. NO is a hydrophobic gas, it readily diffuses across cellular membranes. Thus, cellular levels of NO are controlled by systems that synthesize and metabolize NO.

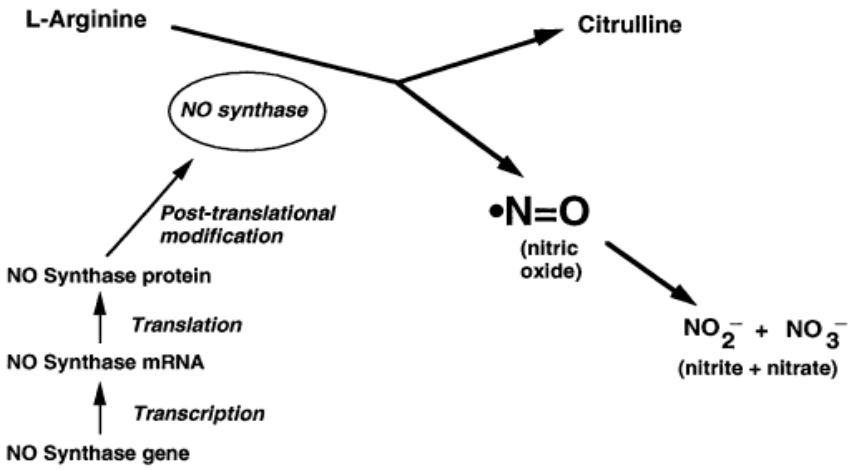

Fig. 4. *From Geller and Billiar (1998) modified.* Nitric oxide biosynthetic pathway.

There are three different isoforms of NO synthases, which can be divided into two functional classes. One group is the constitutive class, which consists of neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3). These Ca^{2+}/calmodulin-dependent enzymes produce NO in short bursts in low concentrations (nM) for physiological purposes. nNOS has also been identified in other tissues such as skeletal muscle (Nakane *et al*., 1993). The other class is formed by the inducible form (iNOS or NOS-2), which produces NO in high concentrations (μM) for as long as the enzyme is activated. Although iNOS is constitutively present in some tissues, such as lung epithelium and distal tubules of the kidney, it is mainly expressed by inflammatory cells after induction by cytokines (such as INFγ, IL-1, or TNFα), lipopolysaccharides, and other inflammatory mediators. Its activity is independent of Ca^{2+} (Andrew and Mayer, 1999). iNOS has important antimicrobial functions (Nussler and Billiar,

1993), and it is also known to inhibit the growth of viruses (Karupiah *et al.*, 1993) and parasites (Green *et al.*, 1991). In the immune system, NO reduces both neutrophil adhesion and lymphocyte activation, and has an immunoregulatory role in allograft rejection (Langrehr *et al*., 1991).

NO is an essential mediator in the regulation of angiogenesis as well as in the individual processes of endothelial cell proliferation, migration (by stimulating endothelial cell podokinesis), and differentiation. In addition, in animal models it has been shown flow-induced vessel enlargement to be diminished in the presence of the NO synthase (NOS) inhibitor NG-nitro-L-arginin methyl ester (L-NAME), implicating NO functions in arteriogenesis as well (Guzman *et al.*, 1997). A variety of studies have indicated that NO also plays a direct role in the mechanism of action of VEGF, eFGF and EGF. VEGF has been shown to trigger the production of NO from the endothelium (Wu *et al*.,1996). Endothelial cell proliferation (Ziche *et al*., 1997a), migration (Ziche *et al*., 1997a), and tube formation in response to VEGF in vitro could all be inhibited by the addition of NOS inhibitors to the cultures (Papapetropoulos *et al.*, 1997).

The dependence of bFGF activity on NOS is less clear. bFGF stimulated the production of NO by cultured endothelial. Endothelial cell tube formation in vitro in response to bFGF could be blocked by the addition of L-NAME, suggesting a role for NO in this process (Babaei *et al*., 1998). On the other hand, NOS inhibition had no effect on bFGF-induced endothelial cell migration or proliferation (Shizukuda *et al*., 1999) or rabbit cornea angiogenesis (Ziche *et al*., 1997a) in two of the studies that had documented a blockade of VEGF effects by these inhibitors. Thus, in at least some cases, bFGF and VEGF can produce similar effects via pathways that converge downstream from the point of involvement of NOS. The angiogenic factor VEGF, which has been shown to be resident in luteal perivascular cells, can stimulate endothelial NO synthase (eNOS) expression and thus NO production (Van der Zee *et al*., 1997; Bouloumie *et al*., 1999). Reynols *et al.* (2000) have proposed the existence of a paracrine loop,

whereby luteal endothelial cells release NO, which stimulates perivascular VEGF production, which in turn stimulates endothelial expression of eNOS. This paracrine loop would thereby serve as a feed-forward system to maximize vasodilation and angiogenesis during luteal development. Indeed, eNOS is expressed in endothelial cells of luteal arterioles and capillaries early in the estrous cycle but that its expression is greatly reduced by mid cycle. Rosiansky-Sultan *et al.* (2006) also showed that elevated NO levels up-regulated $PGF_{2\alpha}$ receptors in cultured luteal endothelial cells. Luteal $PGF_{2\alpha}$ receptors are induced soon after the LH surge, mainly as a result of LH/cAMP action on steroidogenic cells (Anderson *et al*., 2001). This suggests that induction of these receptors in luteal endothelial cells by NO can contribute to the increase of $PGF_{2\alpha}$ receptors in the developing CL. Moreover, $PGF_{2\alpha}$ stimulates the eNOS-NO system and thus vasodilation of blood vessels, such that the net response is an increase in luteal blood in the periphery of the mature CL (O'Shea *et al*., 1989; Shirasuna *et al*., 2008).

Since NO acts as a potent vasorelaxant and since it inhibits P_4 production by luteal endothelial cells, it has been suggested to be an important mediator of luteolysis in the cow (Skarzynski *et al*., 2000b; Korzekwa *et al*., 2004), rabbit (Boiti *et al*., 2003), rat (Motta *et al*., 1999), and human (Vega *et al*., 1998- 2000). NO not only acts directly inhibiting P_4 production, but also modulating $PGF_{2\alpha}$ action and inducing apoptosis in luteal cells by increase DNA fragmentation and expression of FAS, BAX and Caspase-3 mRNA pro-apoptotic proteins (Korzekwa *et al.*, 2006). However, in the mare, NO stimulates early CL secretory function (P_4 production and PGE_2), as well as angiogenic activity (Ferreira-Dias *et al*., 2011). Thus, in this species, NO may play a role in CL growth during early luteal development,when vascular development is more intense (Ferreira-Dias *et al.*, 2011).

Endothelin-1 (ET-1) system

Endothelin-1 (ET-1) belongs to a structurally homologous peptide family, which includes

ET-2, -3 and sarafotoxins. Although ET-1 was originally isolated from porcine aortic endothelial cells, many other cell types such as cardiac myocytes, lung, brain, intestine, stomach, kidney, spleen, adrenal zona glumerulosa cells and testicular Sertoli cells were shown to produce ET-1. Nevertheless, the endothelium remains the primary source of ET-1 as demonstrated by endothelial cell-specific ET-1 gene knockout (Kisanuki *et al.*, 2001). ET-1, has been demonstrated to stimulate VEGF and FGF-2 expression (Davis *et al.*, 2003). ET-1, the only isopeptide synthesized and secreted by vascular endothelial cells (Levin, 1995) and initially defined as a potent vasoconstrictor, is synthesised as a pre-propeptide, which is cleaved by endopeptidases to big ET-1 (proET-1). A zinc metallopeptidase, endothelin-converting enzyme (ECE-1), converts big ET-1 into an active ET-1, which acts through two receptors ETR-A and ETR-B. The ET-1 system has been shown to regulate oocyte maturation, ovulation and CL functions (Flores, 2000; Berisha *et al*., 2002) as well as to inhibit progesterone production in luteal cells after administration of $PGF_{2\alpha}$ (Girsh *et al.*, 1996a; Miyamoto *et al.*, 1997).

In bovine CL not only luteal steroidogenic cells, but also endothelial cells of the CL have ETR-A. Very little is known on the occurrence of ETR-B in the CL. However, it is likely however that luteal endothelial cells, as endothelial cells derived from other organs, contain the ET-B receptors which mediates vasodilatation through the release of factors, such as NO and prostacyclin (Schmitz-Spanke and Schipke, 2000).

The two steroidogenic luteal cell types, differed in their hormonal regulation of ETR-A; in theca-derived luteal cells, LH or forskolin down-regulated ETR-A, whereas in the granulosa-derived luteal cells IGF-I inhibited the ET-A receptor mRNA levels (Mamluk *et al*., 1999). During early stages of luteinization, when peak levels of both LH and IGF-I exist, the expression of ETR-A in the CL are suppressed. In light of the inhibitory role of ET-1 on luteal steroid production, this mechanism may function to ensure an undisturbed P_4 production (Meidan *et al.*, 1999).

Angiotensin II

The renin-angiotensin system (RAS) plays physiological roles in the female reproductive system in several mammals. Similar to Endothellin-I, Ang II functions as a vasoconstrictor. There is evidence that the biologically active form of angiotensin, angiotensin (Ang) II, regulates various ovarian functions such as oocyte maturation, follicular rupture, and steroidogenesis (Yoshimura *et al*., 1993). The vasoactive peptide Ang II is converted from Ang I by angiotensin-converting enzyme (ACE). Ang I is produced from the precursor angiotensinogen by the enzymatic activity of renin. In cattle, theca cells have been identified as a major source of ovarian prorennin (Schultze *et al*., 1989), and as the sites of Ang II binding (Brunswig-Spickenheier and Mukhopadhyay, 1992).

Matrix Metalloproteinases (MMP) system

The CL formation, maintenance and regression are cyclic events that depend upon extensive luteal tissue and ECM remodelling. Components of the ECM modulate cellular processes (including gene expression, cellular proliferation, migration and differentiation and apoptosis) via cellular surface receptors and serve as a reservoir for a number of biologically active factors. Consequently, the controlled degradation of ECM by the action of a specific class of proteolytic enzymes (encompass at least 25 related proteolytic enzymes and synthesised as preproenzymes that are secreted in an inactive form) known as matrix metalloproteinases (MMPs) and their associated endogenous inhibitors (TIMPs: Tissue Inhibitors of Metalloproteinases) may be essential for preserving a microenvironment appropriate to luteal function. The role of the MMPs and their inhibitors throughout the oestrous cycle and pregnant CLs has been described in several species, such as porcine (Pitzel *et al.*, 2000; Ribeiro *et al.*, 2006), bovine (Goldberg *et al*., 1996; Zhang *et al.*, 2003), ovine (Ricke *et al*., 2002; Towle *et al.*, 2002), mouse (Waterhouse *et al*., 1993; Hagglund *et* al., 1999; Liu *et al*., 2003), rat (Nothnick *et*

al., 1995; Liu *et al.*, 1999; Li *et al*., 2002), primates (Young e*t al.*, 2002; Chen *et al*., 2006), and human (Duncan *et al*., 1996-1998). MMPs and TIMPs are elevated during the period of extensive tissue and ECM remodelling that occurs as the postovulatory follicle is transformed into CL. After the CL is fully formed, steroidogenesis is maximal during the midluteal period, and MMPs/TIMPs expression and activity are at basal levels. With the onset of structural regression, the MMPs are again called into action for the ECM remodelling and removal of the regressing CL.

The inhibition of MMPs activity in the extracellular environment is principally controlled by TIMPS (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) that are highly abundant in reproductive tissues, locally produced, hormonally regulated, and has been hypothesized to coordinate numerous ovarian and uterine processes including matrix turnover, cell growth, and steroidogenesis. Several evidences indicate that TIMPs, in addition to their classical ability to regulate MMP action, may act as autocrine/paracrine factors in reproductive processes involving cellular proliferation, differentiation, and neovascularization (Fassina *et al.*, 2000).

4. MAINTENANCE OF THE CORPUS LUTEUM

Luteinizing hormone (LH) and growth hormone (GH) are the primary hormones which support the development and function of the CL. However, local angiogenic growth factors (such as IGF-I), PGs, peptide hormones (prolactin and OT) and cytokines should be considered not only as potent regulators of luteal development, but also important factors regulating P4 secretion and lifespan of CL. Moreover, P_4 appears to have an effect on function of the early and mid CL in an autocrine and paracrine fashion (Skarzynski and Okuda, 1999; Rueda *et al.*, 2000; Ferreira-Dias *et al*., 2006a).

Growth hormone (GH) is a pituitary hormone that affects animal growth, metabolism,

lactation, and reproduction. Secretion of GH is stimulated by GH-releasing hormone (GHRH) and ghrelin (acting via the GH secretagogue [GHS] receptor [GHSR]), and inhibited by somatostatin (SRIF). GH secretion is influenced by other peptides/proteins produced in the hypothalamus and in the periphery, including gonadotrophin releasing hormone (GnRH), insulin-like growth factor I (IGF-I), leptin, pituitary adenylate cyclase activating polypeptide (PACAP), and thyrotropin releasing hormone (TRH). Most authors postulated that GH effects on the ovary are a consequence of an increased IGF-I production in the liver (Gong *et al*., 1996). Others authors asserted that GH acts directly via GH-R (receptors) in the cow (Kuehner *et al*., 1993; Lucy *et al*., 1994), sheep (Perrad *et al.*, 1996) rat and woman (Talavera and Menon, 1991) ovaries. In most species, ovarian follicles and corpora lutea are potential sites for GH action because the GH-R is found within granulosa cells as well as in corpora lutea (Spicer and Enright, 1991; Amit *et al.*, 1993). Treatment with GH has been shown to affect follicular dynamics both increasing circulating IGF levels and acting locally to enhance intrafollicular production of IGF by theca or granulosa cells. Besides, GH acts directly into the CL estradiol (E_2), oxitocin (OT) and P_4 secretion (Spicer *et al.*, 1992; Spicer *et al*., 1993; Lucy *et al*., 1994).

Some studies have shown that the administration of GH increases circulating levels of IGF-I in cattle (Rieger *et al.*, 1991; Spicer *et al.*, 1993), pigs (Spicer *et al.*, 1992), horses (Buonomo *et al.*, 1996) and women (Owen *et al.*, 1991). Both IGF-I and IGF-II have stimulatory effects on P_4 secretion of rat (Parmer *et al*., 1991), rabbit (Constantino *et al*., 1991) sheep (Khan-Dawood *et al*., 1994), pig (Yuan *et al*., 1996), cattle (McArdle and Holtorf, 1989), and human (Devoto *et al.*, 1995). IGF-I has potent actions on sterol metabolism that include amplification of steroidogenic acute regulatory protein (StAR) (Balasubramanian *et al.*, 1997) and the delivery and utilization of steroid precursors (Veldhuis, 1989) by both increasing gene expression and enzyme activity of the key steroidogenic enzymes such as cytochrome P450scc, 3β-HSD and aromatase (Adashi *et al*., 1985a; Veldhuis *et al*., 1986; Magoffin *et al.*, 1990; Magoffin and

Weitsman, 1993; deMoura *et al.*, 1997). Moreover, IGF-I increases LH receptor concentrations and the sensitivity to LH improving its luteotrophic effects and stimulates OT secretion (Adashi *et al*., 1985b). IGF-I may acutely stimulate secretion of progesterone through modification of the cytoskeleton, while inhibition of cellular death may help maintain luteal weight (Parmer *et al*., 1991; Lucy *et al.*, 1993). IGF-I and GH receptors were localized in LLC; thus GH and IGF-I may be important to maintain the high basal levels of P_4 secreted from these cells. The IGF-I was localized in the cytoplasm of LLC and SLC and IGF-II in the cytoplasm of pericytes (Amselgruber *et al*., 1994). After angiogenesis was complete the localization of protein changed from EC to the cytoplasm of mainly large luteal cells.

In cow and pig, the highest mRNA expression for IGF-I, IGF-II and IGFR-1 was observed during the early luteal phase in steroidogenic luteal cells. In cow, the highest expression for IGFBP-3 correlates with the highest expression for IGF-I, IGF-II and IGFR-1 during the early luteal phase followed by a clear decrease at the mid-luteal stage (Schams *et al.*, 2002). The IGFBP-3 is supposed to serve as the main carrier and storage reservoir for IGFs within the intravascular compartment.The CL is also a site of IGF-II mRNA expression suggesting a major role for IGF-II as an intra-luteal regulator (Perks *et al*., 1999; Woad *et al.*, 2000). The regulation of luteal function by IGF-II may not be limited to the stimulation of steroid biosynthesis, but may include control of vascular growth and function (IGF-II mRNA expression in luteal vasculature and perivascular cells), and the regulation of vascular and steroidogenic cell interactions (Woad *et al.*, 2000).

The interaction of the IGF receptor with IGF-I or -II has been shown to protect a range of cell types, including ovarian cells, from apoptosis. This impedes transcriptional activation of specific target genes, such as Fas ligand (FasL), an inducer of apoptosis and Bcl-2 family (Yang and Rajamahendran, 2000). In the equine CL, IGFBP-2 expression was increased at luteolysis, and a decrease in the bioavailability of IGFs occurred by inhibiting its binding to the IGF-I

receptor. The increase mRNA IGFBP-2 levels coincided with a decrease in mRNA encoding P450ssc and low circulating P_4 concentration. This would facilitate functional luteolysis in two ways: by preventing IGFs from protecting against apoptosis, and by interfering with their stimulatory effect on steroidogenesis (Watson *at al.*, 2005). In another study, the receptor IGFBP-4 mRNA was expressed by steroidogenic bovine luteal cells and may act to block IGF-stimulation of steroid production, presumably to protect the cell from over-stimulation (Jones and Clemmons, 1995). IGFBP-2 and -3 were predominantly localised to non-steroidogenic luteal cells and showed particular association with the luteal vasculature. IGFBP-2 and -3 produced by the vasculature may act as paracrine regulators of steroidogenesis, since IGFBP-2 and -3 inhibited the stimulatory effects of IGF-I on luteal cell progesterone production (Brown and Braden, 2001).

Luteinizing hormone (LH) released from the pituitary gland is the signal for follicle rupture, for conversion of the mature follicle into the corpus luteum and P_4 production. The pulsatile release of LH by the pituitary is essential for continued development and function of the corpus luteum. In turn, P_4 and estrogen concentrations influence LH pulse amplitude and frequency by negative- or positive-feedback system depending on the species. In the primate many studies evidence that the LH-mediated regulation of gene expression involves both suppression and activation of LH-dependent genes that lead to maintenance of CL function (Yadav *et al.*, 2004; Xu *et al.*, 2005). The LH surge induces Cox-2 expression in the granulosa cells and a subsequent increase in intrafollicular PGs (prostaglandins) production by the cAMP/PKA intracellular effector system establishing a delay between the LH surge and Cox-2 expression (Tsai *et al.*, 1996). This particular molecular mechanism causing this delay in expression allows PGs production to occur only a few hours before the time of ovulation (Sirois and Dore, 1997). Luteal $PGF_2\alpha$ receptors are induced soon after the LH surge, mainly as a result

of LH/cAMP action on steroidogenic cells (Tsai *et al.*, 1996; Anderson *et al*., 2001), while binding of PGE_2 to the EP_2 (PGE_2 Receptor) appears to be essential for normal ovulation (Matsumoto *et al*., 2001).

The LH receptor (LHR) has a pivotal role in CL function through its interaction with LH. The receptor is essential for ovulation and luteinization (Lei *et al.*, 2001). LHR is a member of the G protein-coupled receptor (GPCR) superfamily and consists of an extracellular domain, seven-transmembrane domains connected by alternating intracellular and extracellular loops, and an intracellular carboxyl terminal tail. In the ewe (Hoyer *et al.*, 1984), cow (Alila *et al.*, 1988), woman (Ohara *et al*., 1987), and sow (Tekpetey and Armstrong, 1991), physiological concentrations of LH increase the secretion of P_4 from SLC, but not from LLC. Nevertheless, in the ewe and the cow it has been shown that both cell types contain receptors for LH (Harrison *et al*., 1987; Chegini *et al.*, 1991). Receptors for LH have been identified in equine luteal tissue (Stewart and Allen, 1979), and their numbers have been reported either to increase or to remain constant during diestrus. The binding of LH to its receptor activates adenylate cyclase, leading to increased concentrations of cAMP and ultimately activation of PKA (Protein Kinase A) in SLC that slightly increases release of cholesterol from cholesterol esters (Wiltbank *et al*., 1993) but does not influence concentrations of mRNA or the activity of P-450scc or 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (3β-HSD) (Wiltbank *et al*., 1993; Belfiore *et al*., 1994). The mechanism whereby LH stimulates secretion of P increases steroid production by facilitating transport of cholesterol through the cell and to P-450scc (Wiltbank *et al*., 1993). Besides, activation of PKA increases the amount of steroidogenic acute regulatory protein (StAR) in the phosphorylated form and appears to stimulate the transport of cholesterol (Epstein and Orme-Johnson, 1991; Arakane *et al*., 1997). Thereby, this may be a key step in acute hormonal stimulation of progesterone synthesis. LH is required to maintain normal expression of mRNA, and presumably proteins, encoding StAR, P-450scc, and 3β-HSD. The LLC are

unresponsive to LH but highly responsive to $PGF_{2\alpha}$ and produce high basal levels of progesterone and they produce about 80% of total luteal progesterone secreted during the mid-luteal phase in ruminants (Niswender *et al*., 1985; Diaz *et al.,* 2002).

Both GH and LH secretion appear to be modulated by leptin. Leptin is an adipocyte hormone important in regulating of feed intake, energy homeostasis, and the neuroendocrine axis (among which complex process of puberty) in rodents, humans and large domestic animals. Moreover, the role of hormone/cytokines is crucial for the regulation of a number of diverse physiological processes, such as inflammation (proinflammatory cytokines, such as tumor necrosis factor (TNF-) and interleukin 1 (IL-1), may also directly induce leptin gene expression), angiogenesis, hematopoiesis and immune function (Bennett *et al.*, 1996; Gainsford *et al*., 1996; Loffreda *et al.,* 1998). Leptin receptors (Ob-Rs) have been identified in several tissues and in the hypothalamus, gonadotrope cells of the anterior pituitary (Jin *et al.*, 2000) granulosa, theca, luteal and interstitial cells of the ovary (Karlsson *et al.*, 1997), endometrium (Kitawaki *et al.*, 2000), Leydig cells (Caprio *et al*., 1999), placenta, and mammary gland (Malik *et al.*, 2001; Kawamura *et al.*, 2002) in most species. This multifocal expression of leptin, as well as the dense presence of Ob-Rs at all levels of the hypothalamus-pituitary-gonadal (HPG) axis, implies that the nutritional/leptin regulation of reproduction involves a complex network of interactions at multiple levels to regulate the HPG axis in a paracrine and/or endocrine fashion. It has been found that leptin accelerates gonadotropin-releasing hormone (GnRH) pulsatility, but not pulse amplitude, in arcuate hypothalamic neurons in a dose-dependent manner (Lebrethon *et al*., 2000). Few studies have demonstrated that *in vitro* leptin treatment of a GnRH-secreting neuronal cell line stimulates GnRH release (Magni *et al.*, 1999). In addition, leptin may increase the release of NO from adrenergic interneurons, which then induces GnRH release from GnRH neurons by activating both guanylate cyclase and Cox-1 (Yu *et al.*, 1997). It may directly

stimulate LH and to a lesser extent, follicle-stimulating hormone (FSH), release by the pituitary via NO synthase activation in gonadotropes. Leptin receptor is expressed in human (Löffler *et al.*, 2001), bovine (Nicklin *at al*., 2007) and porcine CL (Ruiz-Cortés *et al.*, 2000) throughout the oestrous cycle. Some authores have showed a increase of leptin-positive luteal cells in early CL, during trasformation from the follicular to postovulatory phase, followed by a high level peak in the lutal phase and a gradual decline during late phase. During follicular growth, leptin seems to act synergistically with GH and IGF-I to influence estadiol production and the luteinization process (Gregoraszczuk *et al.*, 2004). Indeed, in luteinised human granulosa cells, leptin has been shown to stimulate oestrogen-producing activity (Kitawaki *et al*., 1999) by increasing expression and aromatase activity and stimulating FSH and /or IGF-I , which are predominant stimulators of aromatase. Conversely, leptin inhibited oestrogen production stimulated by IGF-I in rat (Zachow and Magoffin, 1997) and by insulin in bovine (Spicer and Francisco, 1997) granulosa cells.

During luteal phase, the synergistic effects synergistic effects of leptin and IGF-I on StAR transcription which leads to a great enough response to increase P_4 synthesis (Ruiz-Cortés *et al.*, 2000). Moreover, it acts as an antiapoptotic factor and at the same time together IGF-I, protects cells from excessive apoptosis by decreasing caspase-3 activity, so supporting the appropiate cell numbers which is necessary for maintenance of homeostasis in the developing CL (Gregoraszczuk and Ptak, 2005; Gregoraszczuk *et al.*, 2006).

Also ghrelin plays an important role in the regulation of GH and LH. Ghrelin, the endogenous ligand of the GH secretagogue receptor type 1a designated as the ghrelin receptor (GRLN-R), is a pleiotrophic regulator involved in a large array of endocrine and non-endocrine functions, among which cells proliferation, energy homeostasis and orexigenic effect (Nakazato *et al.*, 2001) in an antagonistic manner to leptin. The abundant distribution of GRLN-R in the

pituitary gland and hypothalamus may account for the important central effects of ghrelin. In addition to its potent GH-releasing activity (Takaya *et al.*, 2000), this peptide is involved in the modulation of lactotropic, corticotropic, and gonadotropic axes (Fernandez-Fernandez *et al.*, 2005).

Expression of ghrelin has beens demonstrated in an array of tissues and cell types including the stomach, small intestine, pancreas, lymphocytes, kidney, lung, pituitary,brain, placenta and gonads. Indeed, Ghrelin is present in the mammalian and non-mammalian ovary such as human, rat, pig, sheep and chicken ovary (Caminos *et al.*, 2003; Gaytan *et al*., 2004; Sirotkin *et al.*, 2006). This hormone has been shown to inhibit both *in vivo* and *in vitro* LH secretion in rats, and to decrease *in vitro* LH responsiveness to GnRH (Fernandez-Fernandez *et al.*, 2004). In conditions of negative energy balance, such as fasting or anorexia, high plasma levels of ghrelin are associated to decreased LH secretion (Tschop *et al.*, 2000), which is compatible with its suggested inhibitory effect upon reproductive function. Ghrelin is also involved in the control of prolactin secretion in human and rats (Ghigo *et al*., 2001; Tena-Sempere *et al*., 2004).

Ghrelin may be involved in the autocrine/paracrine regulation of luteal function. Its expression mRNA was observed in young and mature corpora lutea of the rat, human, and sheep (Caminos *et al.*, 2003; Gaytan *et al.*, 2003; Miller *et al.*, 2005) ovary. In humans, profile of ghrelin expression in the CL (cytoplasm of granulosa-lutein cells) is roughly coincident with its peak in functional activity within the ovarian cycle. Moreover, the presence of GHS-R1 peptide in both granulosa-lutein cells and theca-lutein cells during the same time frame suggests a potential role of locally produced ghrelin in the control of CL function. In humans, ghrelin has been shown to decrease both basal and hCG-stimulated release of P_4 in mid luteal phase cells. Also, in rat testis, the *in vitro* steroidogenesis has been reported to be negatively influenced by ghrelin (Tena-Sempere *et al.*, 2002) because this hormones was associated with a decrease in

hCG-stimulated levels of mRNAs encoding several key factors in the steroidogenic cascade. Besides inhibiting P_4 secretion, in human luteal cells, ghrelin was also able to decrease luteotrophic PGE_2 release, to increase the luteolytic $PGF_{2\alpha}$ release (Tropea *et al*., 2007) and to decrease both basal and hypoxia-stimulated VEGF release (Tropea *et al.*, 2006). Furthermore, ghrelin was able to modulate the expression of several markers of apoptosis, such as bax and caspase-3 (markers of cytoplasmic apoptosis), bcl-2 (binding protein for bax) and p53 (transcription factors promoting nuclear apoptosis and nuclear DNArepair)(Sirotkin *et al.*, 2006). This negative influence of ghrelin on luteal function could further strengthen its role as a regulatory signal in the integrated control of energy balance and reproduction (Barreiro and Tena-Sempere, 2004).

Local angiogenic growth factors, cytokines, PGs and peptide hormones (OT) should be considered not only as potent regulators of luteal development, but also important factors regulating P_4 secretion and lifespan of CL. P_4 has been shown to act as luteotrophin (Rothchild., 1981), influencing steroid synthesis (Chappell *et al*., 1997; Kotwica *et al*., 2004) in several species. Moreover, P_4 acting as an autocrine factor by a PR-dependent mechanism, P_4 suppresses the onset of apoptosis in the CL in several species (Skarzynski and Okuda, 1999; Kotwica *et al*., 2004). It has been shown that P_4 may suppress apoptosis in bovine luteal cells through the inhibition of Fas and caspase-3 mRNA expression and inhibition of caspase-3 activation (Rueda *et al*., 2000; Okuda *et al*., 2004). Cell-to-cell contact was shown to be necessary for regulating the secretory function of luteal cells. In the mare, P_4 secretion starts early in the luteal structure, with a simultaneous increase in P_4 receptors (PR) in LLC, luteal microvascularization, proliferating cell nuclear antigen (PCNA) expression, and LLC count, until the mid-luteal phase (Roberto da Costa *et al.*, 2005; Ferreira-Dias *et al.*, 2006b) where there is the highest plasma P_4 concentration. In this mare's mid-luteal CL, *in vitro*, long-lasting effect of

P_4 and its precursors may inhibit angiogenic factors production, preparing for CL funcional and structural regression (Costa *et al*., 2006).

It has been shown that the luteotrophic effect of P_4 on CL is mediated by OT and/or prostaglandins (Skarzynski and Okuda, 1999; Kotwica *et al*., 2004). The presence and the release of OT by the CL of a number of species including ruminants, primate, and the pig. In ruminants, large steroidogenic cells seem to be the site of OT synthesis (Fields and Fields, 1986; Theodosis *et al.*, 1987; Fehr *et al.*, 1987). Luteinization stimulated by the preovulatory LH surge appears to be the main trigger for the production of ovarian OT secreted concomitantly with neurophysin and P_4. Cell-to-cell contact plays an important role for the stimulation of P_4 by OT (Okuda and Uenoyama, 1998). P_4 stimulates P_4, OT and PGs secretion by early CL, but at mid-cycle CL, P_4 inhibits $PGF_{2\alpha}$ secretion in bovine CL (Pate, 1996; Skarzynski and Okuda, 1999). The hormones OT and P_4 are components of an autocrine/paracrine positive feedback and they play important roles in regulating the functionality of $PGF_{2\alpha}$ receptors and the intracellular calcium/protein kinase C cascade. In the CL, stimulatory or inhibitory effects may be regulated by autocrine/paracrine mechanisms dependent upon locally produced hormones. In fact, at the end of the luteal phase, $PGF_{2\alpha}$ and OT may interact and activate luteal and non-luteal cells to initiate functional and morphological luteolysis.

In the mare, the endometrium and the neurohypophysis are sources of oxytocin (Stout *et al*., 2000). Also, preovulatory follicle and the CL have been shown to produce small amounts of oxytocin, but the levels are well below that produced by the corpora lutea of ruminants (Watson *et al.*, 1999). In addition, in horses, in contrast with the ruminants, ovarian OT is not thought to be involved in regulation of reproductive tract function (Stevenson *et al.*, 1991). The peptide OT is packaged and stored into secretory granules in the neurohypophysis, luteal cells in several species and in the endometrial epithelial cells of the mare (Bae and Watson, 2003). Those granules fuse with the plasma membrane and release their contents into the intercellular space in

response to an increase in free intracellular calcium concentrations. In mares, uterine OT receptor concentrations were lowest during estrus. At 14-17 days after ovulation, near the time of luteolysis, OT receptors in the endometrium reach a peak, allowing OT to bind to the endometrium and so drive the production of $PGF_{2\alpha}$, produced by the endometrium (Starbuck *et al*., 1998; Stout *et al*., 2000). Moreover, high amplitude pulses of $PGF_{2\alpha}$ were associated with OT pulses within the intercavernous sinus (Vanderwall *et al.,* 1998). So, in this species as ruminants, OT appears to have an important role in initiating $PGF_{2\alpha}$ release and may participate in a positive feedback loop with $PGF_{2\alpha}$, to further amplify PGF release wherein OT stimulates uterine $PGF_{2\alpha}$, and *vice versa* (McCracken *et al*., 1999).

As above-mentioned, PGs may also be involved in the control of CL life by autocrine and paracrine mechanisms. The presence of PGs metabolic enzymes in the CL suggest that intraluteal PGs biosynthesis is selectively directed toward PGE_2 during growth, toward $PGF_{2\alpha}$ during regression, and is non selective during the mature phase of the CL life span (Arosh *et al*., 2004).

PGE_2 has been considered as luteoprotective or luteotrophic in different species. Moreover, it takes a part in many processes such as: mitogenesis, angiogenesis, anti-apoptosis, vasodilation and as an important mediator in endometrial receptivity and immune function at the fetal-maternal interface at the time of establishment of pregnancy (Reynolds *et al.,* 1981; Emond *et al.,* 1998). Prior to ovulation, LH surge induces Cox-2 expression through protein kinase A (PKA) and synthesis of PGE_2 to promote follicle expansion, angiogenensis and ovulation. Luteal PGE_2 may directly promote angiogenesis by several mechanisms. At time of ovulation and during early luteal phase, both VEGF and PGE_2 are produced at high levels. In turn, VEGF may increase Cox-2 and mPGES mRNA expression, which together stimulate PGE_2 production. The increase in PGE_2 levels might affect P_4 production, possibly by enhancing angiogenesis in the

corpus luteum, and further enhancing VEGF mRNA expression (Sakurai *et al.*, 2004).

Furthermore, PGE_2 and PGI_2 increase P_4 secretion by activating the EP_2-cAMP-protein kinase A pathway in human, rabbit, and ruminant LLC cells (Marsh and LeMaire, 1974; Boiti *et al*., 2001; Weems *et al*., 2002). The same luteal $PGF_{2\alpha}$ seems to be luteotrophic during early and mid-luteal phases, but no longer at the late-luteal phase despite the relatively high local production (Miyamoto *et al*., 1993). Prostacyclin may be luteotrophic early in the development of the CL specifically by controlling proliferation of blood vessels (Milvae and Hansel, 1983). In this way, PGE_2 activity guarantees P_4 production, both throught the promotion of angiogenensis and directly by acting on luteal cells. In the mare, luteal tissue from early and mid phases showed the capacity to release PGE_2 *in vitro* (Watson and Sertich, 1990). In this species, PGE_2 production increased from CL collected on days 8–9 to days 12–13, but production of the other prostaglandins did not change over this interval. The ratio of $PGF_{2\alpha}$:PGE_2 released by luteal cells during culture increased as the cycle advanced, reaching high levels between days 14 and 17 post ovulation, immediatly before P_4 levels start to decline.

Noradrenaline (NA), E_2 and nitric oxide (NO) have also been found to stimulate intraluteal PG production. NA stimulates both synthesis and release of $PGF_{2\alpha}$ and PGE_2 in bovine luteal cells. Thus, NA acts directly through its action on P_4 secretion, but also indirectly by stimulating luteal PGs especially in the early CL. So, Estradiol-17β might act in the same indirect way of NA on P_4, via estradiol-induced $PGF_{2\alpha}$ in a paracrine and/or autocrine fashion (Okuda *et al.*, 2001).

Two NOS isoforms were identified in the CL: the endothelial NO synthase, synthesizes intermittent low levels of NO and the inducible form is synthesized after activation by proinflammatory cytokines such as interferon gamma (INFγ), IL-1, or TNF-β, in many cell types including endothelial cells (Albrecht *et al.*, 2003). eNOS and iNOS contributed equally to total NOS activity in young CL despite the fact that eNOS mRNA in this gland was significantly

higher than iNOS, because iNOS is much more robust enzyme than eNOS (Rosiansky-Sultan *et al.*, 2006). Ovarian steroids, such as P_4 and $E_2\beta$, are important modulators of NO synthesis by acting on NOS activity (Figueroa and Massman, 1995; Batra and Al-Hijji, 1998) and its synthesis can depend both on hormones, such as gonadotrophins, and angiogenic factors (Rupnow *et al.*, 2001).

NO has been reported to both enhance and inhibit Cox-2 activity and PG biosynthesis. NO stimulates the activity of both Cox-1 and Cox-2, resulting in the overproduction of PGs (Corbett *et al*., 1993). In different kind of cells a decrease in cellular prostanoid production was observed when endogenously generated NO positively regulated Cox-2 activity. Important *in vivo* interactions between NO and PG are highly dependent on the inherent redox environment (Vidwans *et al.*, 2001). In cultured luteal endothelial cells elevated NO levels up-regulate $PGF_{2\alpha}$ receptors (Rosiansky-Sultan *et al.*, 2006), which in turn are induced by LH surge (Anderson *et al*., 2001). The induction of these receptors in luteal endothelial cells by NO allows to the increase of $PGF_{2\alpha}$ receptors in the developing CL and their modulation during luteolysis.

Also cytokines, such as tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and Interleukins (IL-1α and IL-1β) mainly produced by non-steroidogenic immune cells in the CL (Taniguchi *et al.*, 2002), together with steroid and non steroid hormones, growth factors and eicosanoids, play specific roles in luteal establishment (Reynolds and Redmer, 1999; Berisha and Schams, 2005; Skarzynski *et al.*, 2008; Ferreira-Dias *et al*., 2010). Cytokines effect depends on cell differentiation, as well as the presence of growth factors and other regulatory factors (Orsi *et al*., 2007). In bovine CL interleukins (IL) participate in the $PGF_{2\alpha}$ and PGE_2 luteal stimulation (Nothnick and Pate, 1990). They may have luteotrophic or luteolytic roles depending on the luteal phase. In fact, in the mid luteal stage, both IL-1α and IL-1β, seem to play a role in luteal maintenance because they stimulate PGE_2 and increase the PGE_2: $PGF_{2\alpha}$ ratio production.

This ratio is inverted when IL stimulate $PGF_{2\alpha}$ secretion more than PGE_2 secretion in the late-luteal phase (Nishimura *et al.*, 2004).

TNFα and IFNγ have been shown to increase *in vitro* PGF_2 as well PGE_2 production by bovine luteal cells in the mid luteal phase, in a dose dependent manner (Sakumoto *et al*., 2000; Neuvians *et al*., 2004). Besides, TNFα, IFNγ and their receptors protein was shown in LLC and endothelial cells in several species such as cow (Petroff *et al*., 1999; Sakumoto *et al.*, 2000; Neuvians *et al*., 2004), pig (Wuttke *et al*., 1997), human (Vaskivuo *et al*., 2002) or horses (Galvão, *et al*. 2009) to suggest a luteotrophic effect. In the mare, TNF might control intraluteal pathways both stimulating P_4 and PGE_2 secretion, and inhibiting $PGF_{2\alpha}$ synthesis, during luteal growth and development. In mid luteal phase, TNFα stimulates $PGF_{2\alpha}$ secretion and in late luteal phase TNFα and IFNγ acting alone or sinergically reduced P_4 and PGE_2 secretion and increased $PGF_{2\alpha}$ secretion, supporting the participation of these cytokines on functional luteolysis (Galvão *et al*., 2009).

At the end of the luteal phase, in the absence of pregnancy, the pulsatile release of $PGF_{2\alpha}$ from uterine endometrium and ovary causes a decrease in plasma P_4, which is defined as functional luteolysis and precedes structural regression of the CL (Douglas and Ginther, 1972-1976; Henderson and McNatty, 1975). This process is crucial for CL demise and the resumption of a new ovarian cycle.

5. LUTEOLYSIS

Luteolysis, that is CL regression, is defined as the process in which CL decline in function, reduces in volume and subsequently disappears from the ovary for to allow for a new ovulation. At the and of luteal phase, the release of $PGF_{2\alpha}$ associated to the mediation of local factors such as Endothelin-1, NO and cytokines (TNF_α and INFγ) lead to **functional luteolysis**

characterised by decrease in P_4 production and structural CL involution defined as **structural luteolysis**. Structural CL collapse is associated to changes in blood supply (Myamoto *et al.*, 2005), leucocytes infiltration (Bukovsky *et al.*, 1995; 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; Morales *et al.*, 2000; Gaytan *et al.*, 2002).

The control mechanisms for luteolysis process are complex and different among animal species. In mares, **l**uteolysis normally begins around days 14–16 post-ovulation and is controlled by an oxytocin-dependent release of $PGF_{2\alpha}$ from endometrium (Sharp, 2000), even though the CL starts regressing funcionally from day 10 to 12 onwards. In regard to this, just in the mature CL, *in vitro* long-lasting effect of P_4 and its precursors may inhibit angiogenic factors production, preparing CL for funcional and structural regression (Costa *et al.*, 2006).

The prostaglandin $F_{2\alpha}$ acts at several levels, inducing a drastic decrease in P_4 release from the CL and reducing volume and blood flow to the CL, even though contradictory findings on cow, have reported that $PGF_{2\alpha}$,-induced in blood flow at the beginning of luteolysis may be mediated by the release of NO and PGE_2 (Shirasuna *et al.*, 2004; Miyamoto *et al.*, 2005). In primates, during early luteolysis the decrease in plasma P_4 concentration corresponds to the fall in blood vessels density and endothelial cell proliferation (Stouffer *et al.*, 2001).

In the CL, luteal specific receptor binding sites for $PGF_{2\alpha}$ induced by the LH surge, have been identified in most species. Activation of $PGF_{2\alpha}$ receptors increases intracellular protein kinase C (PKC) and Ca^{2+} to affect luteal regression (McCracken *et al.*, 1999; Diaz *et al.*, 2002). On the one hand the increase in PKC results in decreased secretion of P_4 which leads funtional regression, on the other hand the raise of the Ca^{2+} is involved in apoptosis and cell death, therefore in structural luteolysis (Anderson *et al.*, 2001; Davis and Rueda, 2002; Diaz *et al.*, 2002). In large luteal cells, this mechanism is present throughout the estrous cycle, even though in the early luteal cells and in the CL without luteolytic capacity, $PGF_{2\alpha}$ does not stimulate

increased intraluteal PG production and does not induce other activators of PG production, such as decreased P_4 secretion, increased endothelin-1 production, or increased cytokine production (Wiltbank and Ottobre, 2003). After acquisition of luteolytic capacity, intraluteal $PGF_{2\alpha}$ production activates an autoamplification loop in the mature CL due to $PGF_{2\alpha}$-induced Cox-2 expression and $PGF_{2\alpha}$ induction of other activators of Cox-2 expression.

Intraluteal autocrine loop is present in most species. Studies in most species (Tsai and Wiltbank, 1997), and also in the mare (Beg *et al.*, 2005), indicate that the intraluteal $PGF_{2\alpha}$ is involved in an autocrine loop to amplify and intensify the luteolytic effect of same $PGF_{2\alpha}$.

5.1. ANTISTEROIDOGENIC ACTION

Prostaglandin $F_{2\alpha}$ could decrease P_4 synthesis by a number of intracellular mechanisms. $PGF_{2\alpha}$ causes a rapid decrease in mRNA encoding the receptor for LH (Guy *et al.*, 1995), while it seems not to affect concentrations of mRNA encoding GH-R mRNA (Juengel *et al*., 1997) and binding of IGF-I to luteal tissue (Perks *et al*., 1995). A decrease in LH receptors mRNA in response to $PGF_{2\alpha}$ has been reported in cattle (Tsai *et al.*, 2001), sheep (Guy *et al.*, 1995), and mare (Roser *et al*., 1982), was simultaneous with a decrease in serum and luteal P_4. In any case, downregulation of receptors for luteotropic hormones does not appear to be a mechanism by which $PGF_{2\alpha}$ decreases secretion of P_4 from the CL, rather it may interfere with the ability of LH to activate PKA (Garverick *et al.*, 1985). Thus, the ability of $PGF_{2\alpha}$ to suppress PKA activity may lead to a fall in P_4 secretion from LLC. Morevoer, $PGF_{2\alpha}$ antisteroidogenic action is performed on several steps of the process of steroidogenesis, acting on, before and after cholesterol transport to mitochondria, as sterol carrier protein (SCP-2), which may reduce the transport of cholesterol to the mitochondria and both *in vivo* and *in vitro*, by reducing the levels of StAR mRNA (Pescador *et al*., 1996). Also in mare, as in other species, treatment with $PGF_{2\alpha}$ leads to a decrease in StAR mRNA in the luteal tissue (Beg *et al.*, 2005).

5.2. ENDOTHELIN-1 AND VASOACTIVE PEPTIDES

Prostaglandin $F_{2\alpha}$ caused luteolysis by a reduction in ovarian or CL blood flow, so depriving the gland of nutrients, substrates for steroidogenesis, and luteotrophic support (Niswender *et al.*, 2000). Because endothelial cells express receptors for $PGF_{2\alpha}$ (Mamluk *et al*., 1998), this hormone is likely to act directly on this cell population. $PGF_{2\alpha}$ causes degeneration of luteal endothelial cells (Sawyer *et al.*, 1990), resulting in a marked reduction in capillary density, thereby reducing blood flow to the luteal parenchyma. In mare's late luteal phase the decrease in endothelial cell mitogenesis, in the presence of $PGF_{2\alpha}$ (Ferreira-Dias *et al.*, 2006b) could be due both to a lack of synthesis of angiogenic factors and a rise in anti-angiogenic factors, such as angiostatin, endostatin (Maisonpierre *et al*., 1997; Espinosa Cervantes and Rosado Garcia, 2002), thrombospondins (Hazzard *et al.*, 2002) and platelet factor 4 (Perollet *et al.*, 1998), which may be modulating luteal regression. Some anti-angiogenic factors might be involved in luteal formation to avoid excessive vascular development and to mediate endothelial cells apoptosis during regression (Tamanini and De Ambrogi, 2004).

Endothelins (ETs) and Angiotensin II (Ang II), potent vasoconstrictive peptides, play a crucial role in functional luteolysis, both decreasing luteal blood flow sinergically with $PGF_{2\alpha}$ and inhibiting luteal P_4 synthesis.

In the cow, ET-1 peptide concentrations in early CL tissue are considerably lower than in CL at the mid luteal phase (Girsh *et al*., 1996b) and increases (Ohtani *et al.*, 1998; Klipper *et al.*, 2004; Rosiansky-Sultan *et al.*, 2006) during late luteal phase. Instead, pre-propeptide, ppET-1 mRNA was similar during the early and mid luteal phases (Girsh *et al*., 1996b), suggesting that endothelin-converting enzyme (ECE-1) expression, present in steroidogenic and endothelial cells, might vary with CL age. There may be a positive feedback system in which $PGF_{2\alpha}$, oxitocin and vasopressin augment ET-1 production and release by endothelial cells, and it also induces ET-1 and ET-1 receptors (ET-A) up-regulates expression in the same cells (Mamluk *et*

al., 1999) and vice versa. ET-1 further enhances $PGF_{2\alpha}$ synthesis. Synergy $PGF_{2\alpha}$ and ET-1 activity promotes the decrease in luteal blood flow, in concert with numerous other luteal modulators, among Ang II (Kobayashi *et al.*, 2001). Moreover, several evidences relate that ET-1, via binding ET-A receptors, located on large and small luteal cells, alters the normal pattern of prostanoid synthesis (Miyamoto *et al.*, 1996; Hinckley *et al.*, 1997; Ohtani *et al.*, 1998) and mediates the inhibitory activity of $PGF_{2\alpha}$ on LH-stimulated P_4 synthesis (Iwai *et al.*, 1991; Kamada *et al.*, 1995). Thus, ET-1 acts on P4 inhibition both direclty and indirectly throught drop in the blood supply in the CL.

5.3. NITRIC OXIDE

Nitric oxide (NO) acting as potent vasodilator and inhibiting P_4 production by luteal endothelial cells, has been suggested to be an important mediator of luteolytic action of $PGF_{2\alpha}$ in the cow (Skarzynski *et al.*, 2000b; Korzekwa *et al.*, 2004), rabbit (Boiti *et al.*, 2003), rat (Motta *et al.*, 1999), and human (Vega *et al.*, 1998). Steroidogenic, endothelial and immuno cells are source to the NO production (Skarzynski *et al.*, 2003; Weems *et al.*, 2004). Many experiments showed that $PGF_{2\alpha}$ stimulates eNOS mRNA expressions and/or NOS acivity in the luteal tissue of sheep (Vonnahme *et al.*, 2006b), rabbit (Boiti *et al.*, 2003), rat (Motta *et al.*, 2001), and cow (Shirasuna *et al.*, 2008). In the cow, even though $PGF_{2\alpha}$ increases eNO expression and consequently enhances blood flow in the mature CL periphery, this does not occur in the center (Shirasuna *et al.*, 2008). This is probably due to the different blood vessels typology present in CL during luteal regression, richer in arteriole-venous than in capillary vessels, in which abundant pericytes and smooth muscle are more sensitive to vasodilation (Hojo *et al.*, 2009).

The prostaglandin $F_{2\alpha}$ causes an increase in eNOS activity by increasing intracellular-free Ca^{2+} and at the same time increasing luteal blood flow/shear stress, which may contribute to

the upregulation of ET-1 in endothelial cells (Morawietz *et al.*, 2000; Shirasuna *et al.*, 2008). In this way, NO may act in concert with $PGF_{2\alpha}$ and ET-1 in the structural luteolyis.

The action of NO on the CL may be either luteotrophic or luteolytic and are strictly dependent on the stage of CL, species, and cell-to-cell contact and composition. Several studies have showed that increased NO production during early stages of the cycle and pregnancy is likely to play a role in CL development and angiogenesis (Skarzynski *et al*., 2000b; Weems *et al.*, 2004; Vonnahme *et al.*, 2005a; Rosiansky-Sultan *et al.*, 2006; Ferreira-Dias *et al.*, 2011). On the contrary, during late luteal phase, a NO donor decreased P_4 production by luteal cells in human (Friden *et al.*, 2000), rabbit (Gobbetti *et al.*, 1999), rat (Motta *et al.*, 2001), and cow (Skarzynski and Okuda, 2000; Jaroszewski *et al.*, 2003; Korzekwa *et al.*, 2004). Both 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. P_4 decline during regression could be essential for triggering off cellular apoptosis (Okuda *et al.*, 2004). NO not only acts directly inhibiting P_4 production, but also inducing apoptosis in luteal cells by increasing DNA fragmentation and Fas, Bax and Caspase-3 mRNA expression (Korzekwa *et al.*, 2006), pro-apoptotic proteins.

In the cow, NO stimulated bax mRNA expression, but did not influence on Fas expression or bcl-2 mRNA, decreasing in this way the ratio of bcl-2 to bax (Korzekwa *et al.*, 2006). Moreover, NO stimulates expression and activity of caspase-3, proteins that are one of the main executors of the apoptotic process (Vaux and Korsmeyer, 1999; Lakhani *et al*., 2006). Once activated, caspase-3 follows inactivation of enzymes involved in DNA repair, DNA fragmentation, cleavage of the key cellular proteins, such as cytoskeletal proteins, that leads to the typical morphological changes of apoptosis untill cell death (Jänicke *et al.*, 1998)

5.4. IMMUNE-MEDIATED EVENTS AND CYTOKINES

Immune cells, infiltrating the CL, have been implicated in the process of luteal regression. The presence of several inflammatory cell types such as mast cells (Jones and Hsueh, 1981; Mori, 1990), macrophages (Paavola, 1979; Brannstrom and Norman, 1993) and lymphocytes (Brannstrom and Norman, 1993) has been demonstrated in the ovary throughout the estrous cycle in most species and their number increases at the time of luteolysis. Neutrophils, macrophages and T-lymphocytes predominate in the CL around the time of luteolysis and may be directly involved in the destruction of luteal cells and subsequent loss of P_4 secretion (Brannstrom *et al.*, 1994; Pate *et al.* 2001). In the mare, $CD4^+$ and $CD8^+$ cytotoxic T cells are present in the CL during luteolysis (Lawler *et al.*, 1999). Also cytokines, produced by these immune cells, such as TNFα, IFNγ, IL-1 (Nothnick and Pate, 1990; Pate, 1995; Davis and Rueda,, 2002; Skarzynski *et al.*, 2008; Korzekwa *et al.*, 2008) and FasL (Fas Ligand) are involved in luteal regression (Suda *et al.*, 1993; Galvão *et al.*, 2010). Even though immune cells have been accepted as the source of cytokines, a recent work from our group has shown the presence of FasL and its receptor FAS in large steroidogenic and endothelial CL cells throughout the luteal phase, in the mare (Galvão *et al*., 2010).

Mechanisms regulating the immune cell trafficking include the production and release of chemokines, a group of chemoattractant proteins (Zlotnic *et al.*, 1999). A variety of cells including endothelial cells, fibroblast and T-lymphocytes, activated by subluteolytic release of $PGF_{2\alpha}$, produce monocyte chemotactic protein-1 (MCP-19, one member of the chemokine family of cytokines, that act as potent chemotactic agent for monocytes/macrophages (Bowen *et al.*, 1996; Tsai *et al.*, 1997; Penny, 2000). So, macrophages infiltrate the parenchyma and blood vessels during luteal regression, phagocyte degenerative luteal cells (Paavola, 1979; Pepperell *et al.*, 1992), get involved in the degradation of the extracellular matrix (Parker, 1991), and produce cytokines in particular TNFα (Pate, 1995; Petroff *et al.*, 1999; Pate *et al.* 2001). TNFα

itself can induce both cell proliferation and cell death, depending on which receptor it binds to (TNFRI or TNFRII) (Boldin *et al*., 1995; Hsu *et al*., 1995). The cytokine TNFα, acting on its receptor 1 (TNFR1), is an important apoptosis inductor of luteal (steroidogenic and endothelial cells) tissue regression (Korzekwa *et al*., 2008). TNFRI belongs to TNFα superfamily receptors known as death receptors, containing an intracellular death domain required for signaling pathways associated with apoptosis via death domain (Bodmer *et al*., 2002). Also IFNγ, deriveted from T- lymphocytes, acting on its receptor IFNR1, reduced bovine luteal cells viability (Taniguchi *et al.*, 2002) and, together with TNFα, stimulates presentation of major histocompatibility complex (MHC) antigens on the surface of luteal cells (Fairchild and Pate, 1989; Cannon *et al*., 2006).

Moreover, TNF can inhibit gonadotrophin-stimulated P_4 and androgen production by theca (Andreani *et al.*, 1991; Zachow *et al*., 1993) and also inhibits P_4 and aromatase (and E_2 secretion) in granulosa cells (Adashi *et al.*, 1989). Besides, evidence based on *in* vitro studies on luteal cells, has shown that TNFα and IFNγ cooperate together on cell death, by increasing $PGF_{2\alpha}$ levels and reducing P_4 production in many species as rat (Gorospe *et al.*, 1988; Kuruso *et al.*, 2007), mouse (Adashi *et al.*, 1990), pig (Pitzel *et al.*, 1993), cow (Benyo and Pate, 1992; Sakumoto *et al.*, 2000; Petroff *et al.*, 2001) and mare (Galvão *et al.*, 2010). TNF was also able to stimulate ET-1 production by endothelial cells, as well as $PGF_{2\alpha}$ release, thereby establishing a local positive feedback together with other factors, such as NO, which could accelerate the luteolytic cascade in the cow CL (Okuda *et al*., 1999).

The Fas antigen (Fas), like TNFα, belongs to tumor necrosis factor family of cell surface receptors (Nagata and Golstein, 1995) and engagement of the receptor Fas with its ligand (Fas ligand; FasL) induces apoptosis (Sakamaki *et al*., 1997; Taniguchi *et al.*, 2002). FasL is expressed at high levels on activated T lymphocytes (Suda *et al.*, 1993) and mediates apoptosis of target cells (Nagata, and Golstein, 1995). The cytokine FasL and its receptor FAS were

expressed in CL of mice (Sakamaki *et al*., 1997), rats (Slot *et al.*, 2006), and humans (Kondo *et al*., 1996), cows (Taniguchi *et al.*, 2002) and mare (Galvão *et al.*, 2010) throughout the luteal phase. 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 (Galvão *et al.*, 2010).

The cytokines act in a synergic way on luteal cell viability reduction. Indeed, TNF and IFNγ or IFNγ alone increased Fas expression in cow (Taniguchi *et al.*, 2002), murine luteal cells (Komatsu *et al*., 2003), human granulosa lutein cells (Quirk *et al.*, 1995) and mare CL (Galvão *et al.*, 2010) enhancing FasL cytotoxic effect on many ovary cells.

In equine mid luteal phase CL, cell stimulation with FasL alone significantly reduced P_4 concentration (Galvão *et al.*, 2010) as in pseudopregnant mice (Sakamaki *et al*., 1997), in contrast with the cow (Pru *et al.*, 2002) where FasL stimulation of bovine steroidogenic luteal cells had no effect on P_4 production. Moreover, *in vitro* luteal cell stimulation with FasL alone, was responsible for a decrease in both luteotrophic factors P_4 (Roberto da Costa *et al*., 2005) and PGE_2 (Schams *et al.*, 1995), while the luteolytic hormone $PGF_{2\alpha}$ production increased. So, the decline of P_4 during the regression phase increases luteal cell apoptosis via Fas and Fas-ligand system.

5.5. APOPTOSIS

Programmed cell death or apoptosis, is a normal component of the development and health of multicellular organisms and essential for cell number and tissue size control and homeostasis maintenance (Danial and Korsmeyer, 2004). Morphologically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a “horse-shoe” like appearance. Cells continue to shrink, packaging

themselves into discrete membrane-bound structures containing condensed chromatin or cytoplasm, allowing for their removal by macrophages or neighboring cells, or are extruded into the lumen of blood vessels (Kerr *et al.,* 1972; Wyllie *et al*., 1980). Cellular shrinkage may leads to formation of single dense round mass with a densely basophilic pyknotic nucleus (Wyllie *et al.,* 1980). Terminal differentiation, instead, is considered a specialized type of apoptosis in which cells lose their nuclei (denucleation) but remain functional (Stenn, 1983). On the contrary, necrosis is another form of cell death in which uncontrolled cell death leads to cellular swelling, non selective DNA degradation, lysis of cells, inflammatory responses in the surrounding tissue (Kerr *et al.,* 1972).

Two major intracellular apoptosis signaling cascades have been characterized, namely, the death receptor pathway and the mitochondrial pathway. Apoptosis can be initiated via several cytokine receptors including TNF super family receptors (TNFRs). The apoptotic process is characterized by two distinct signaling cascades: extrinsic or death receptor pathway and intrinsic or mitochondrial pathway (Nagata, 1997; Adam and Cory, 1998). In the extrinsic pathway, apoptosis is triggered when cytokine death receptors, on the cellular surface, are activated by specific ligands, such as TNFα and Fas ligand (Fas L), which belong to tumor necrosis factor super family. TNFα and Fas L binding TNFR1 and FAS, respectively, trigger apoptotic events. 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 signaling complex (DISC), which propagates the signaling 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 (Slot *et al*., 2006). Caspase family regulates the selective destruction of structural and functional key proteins in the cell (Chinnaiyan and Dixit, 1996; Cryns and Yan, 1998; Thornberry and Lazabnik, 1998). Caspase-3 is not only the central effector, but is also

responsible for the apoptotic phenotype (Rueda *et al.*, 1999).

In apoptosis intrinsic pathway, internal stimuli originated within the cell promote changes in mitochondrial permeability (Adams and Cory, 1998). Mitochondria play an important role both in the regulation of cell death and in amplifying the apoptotic signaling from the death receptors (Kroemer *et al.*, 1998). The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- (such as Bax, Bad and Bid) and anti-apoptotic (bcl-2 and bcl-XL), Bcl-2 proteins family, which in turn are activated by apoptotic signals such as cell stress, free radical damage or growth factor deprivation. Following cellular signal, the balance between pro- and antiapoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins, leading to the formation of pores in the mitochondria called Permeability Transition pore (PT) and the release of pro-apoptotic proteins, as cytochrome C, into the cytoplasm of the cell. The result is the formation of the apoptosome and the activation of the caspase cascade (Cain *et al.*, 1999).

Apoptosis is determinant for normal reproductive function and ovarian cyclicity (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 (Guo *et al.*, 1988; Gaytan *et al.*, 2001), rabbit (Goodman *et al.*, 1998; Dharmarajan *et al.*, 1999;), sheep (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.*, 2007). In the mare, in the early luteal phase (14 days) the abundant presence of pyknotic cells and round dense clusters of chromatin in the extracellular space, show the evolution of an apoptotic process in action (Al-zi'abi *et al.*, 2002).

The enzyme caspase is involved in luteal regression in cow (Rueda *et al.*, 1997; Okuda *et al.*, 2004; Liszewska *et al.*, 2005), sheep (Reuda *et al*., 1999), rabbit (Abdo *et al.*, 2005), woman (Khan *et al.*, 2000), mice (Carambula *et al*., 2002), and mare (Ferreira-Dias *et al*., 2007). In bovine luteal cells, inhibition of intraluteal P_4 action appears to be the first step in the initiation of structural luteolysis and induction of apoptosis, increasing Fas and caspase-3 expression (Okuda

et al., 2004; Liszewska *et al.*, 2005). In the mare, caspase-3 expression increases in the mid luteal phase CL, when also P_4 production is at its highest level (Ferreira-Dias *et al*., 2007). In fact, in this species, as in mice, caspase-3, might not be the direct mediator of P_4 impairment associated with structural luteolysis (Ferreira-Dias *et al*., 2006), but P_4 decrease may be explained either by a reduction in the total number of large luteal cells in late luteal phase (Roberto da Costa *et al.*, 2005) or by an autocrine-paracrine negative feedback on P_4 receptors during CL regression or by a desensitization mechanism exercised by the same P_4 (Diaz and Wilkbank, 2004). This work suggests that the effector caspase-3 of apoptosis, might play an important role during luteal tissue involution in the mare, even though its relationship with P_4 remains to be clarified (Ferreira-Dias *et al.*, 2007). In addition, in the mid luteal phase, the degeneration of the mitochondria, that reflects an early step in the decline in P_4 concentrations (Levine *et al*., 1979) and apoptosis signs (Kroemer *et al*., 1988). So, in the mare, as in ruminants (Juengel *et al.*, 1993; Zheng *et al.*, 1994) and monkeys (Young *et al.*, 1997), structural changes in the CL did not precede P_4 decrease concentration.

In the mare during luteal regression the decrease of apoptotic structures indicates an active removal by macrophages (Manjong and Joris, 1995). Besides, in some luteal cells in the equine CL, the crenation of the nuclear membrane with shrinkage of the nucleus may indicate the presence of an additional non-apoptotic form of cell death at luteolysis knowed as terminal differentiation (Al-zi'abi *et al.*, 2002). On the contrary, endothelial cells, in bovine, do not appear to show any morphological signs of apoptosis, but they depict signs of swelling and detachment from the walls of blood vessels (Augustin *et al*., 1995; Modlich *et al*., 1996), while during CL regression in guinea-pig (Azmi and O'Shea, 1984) and in sheep after $PGF_{2\alpha}$-induced regression (Sawyer *et al*., 1990), endothelial cells are subjected to apoptosis. In fact, in the mare, *in vitro* studies have suggested that $PGF_{2\alpha}$ may play a role on vascular regression of the CL during the late luteal phase (Ferreira-Dias *et al.*, 2006b).

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EXPERIMENTAL SECTION

AIMS

The overall goal of this thesis is to understand metabolic and endocrine control of luteal function in the mare. This might bring about new approaches to increase fertility that involve the use of growth and angiogenic factors that might be considered as luteotrophic in the mare.

Our main hypothesis was that endogenous pleiotropic metabolic factors, such as ghrelin and leptin, mainly involved in food intake and appetite, might also play an important role on the regulation of gonadal functions in the mare. The first experiment was an attempt to examine a possible physiologic regulation of mare corpus luteum (CL) by leptin and ghrelin on (i) angiogenic activity that could modulate *in vitro* endothelial cell proliferation (BAEC) and nitric oxide (NO) production; and (ii) luteal secretory capacity concerning progesterone and prostaglandins. Moreover, we evaluated the presence of leptin, ghrelin and their receptors in luteal tissue by gene identification.

In the second experiment we evaluated growth hormone (GH) and insulin growth factor-I (IGF), which may participate in the regulation of luteal function in some species. The interest was created due to the scarce information on the subject in the mare, and because of the close relationship between the hormones (leptin and ghrelin) analyzed in the first experiment and GH and IGF-I. The purpose of this study was therefore to investigate the role of GH and IGF-I on NO and angiogenic activity that can modulate *in vitro* BAEC proliferation, by equine CL, throughout the luteal phase. Furthermore, gene identification of GH and IGF-I in luteal tissue was also performed.

FIRST EXPERIMENT: LEPTIN & GHRELIN

INTRODUCTION

The CL is a transient endocrine gland that represents a major endocrine component of the ovary and whose primary function is to produce progesterone (P_4), a hormone that allows for the maintenance of pregnancy. In a short time after ovulation, an intricate interaction between luteotrophic and luteolytic mediators enables a fast angiogenesis, cell proliferation and differentiation, growth of CL followed by a rapid regression process characterized by changes and degeneration of vascular and steroidogenic cells (Reynolds *et al.,* 1992; Ginther, 1992a; Stouffer *et al*., 2001; Davis *et al.,* 2003). Thus, in the mare, a number of growth/angiogenic factors, P_4, cytokines such as Tumor necrosis factor (TNF_α), and NO (nitric oxide) mediated prostaglandin E_2 (PGE_2) production might also be involved in this complex mechanism of CL formation (Roberto da Costa *et al*., 2005; Ferreira-Dias *et al*., 2006a; Ferreira-Dias *et al.,* 2011). In the absence of gestation, $PGF_{2\alpha}$ and other factors, such as P_4, $TNF_{\alpha,}$ FasL and NO might contribute for luteal regression (Ferreira-Dias *et al.,* 2006a; Galvão *et al.,* 2010).

Lately, growing evidence indicates that the hormones ghrelin and leptin may also participate in the regulation of reproductive function, participating in this complex process (Karlsson *et al.*, 1997; Smith *et al*., 2002; Barreiro and Tena-Sempere, 2004). In the mare, increased blood concentrations of leptin might be associated with the maintenance of ovarian activity during seasonal anestrus (Gentry *et al.,* 2002; Fitzgerald *et al.*, 2002; Ferreira-Dias *et al.,* 2005), spring seasonal recrudescence of ovarian activity and seasonal cyclicity (Ferreira-Dias *et al*., 2005) and during foaling and lactation (Heidler *et al*., 2010). Leptin and ghrelin are two of the primary afferent signals in integration of body-weight homeostasis (Zhang *et al.*, 1994;

Houseknecht *et al.*, 1998; Gale *et al.*, 2004; Horvath *et al.*, 2004).

Both leptin and ghrelin may operate as endocrine–paracrine mediators connecting energy balance and reproduction axis (Cunningham *at al*., 1999; Smith *et al*., 2002; Barreiro and Tena-Sempere, 2004). These mediators of appetite act on the central nervous system (CNS), especially the hypothalamus and pituitary, influencing GnRH pulsatility as well as FSH and LH production and secretion. Besides, expression of leptin, ghrelin, and their receptors in various reproductive organs, such as the ovary (Karlsson *et al.*, 1997), testis (Caprio *et al*., 1999; Tena-Sempere *et al.*, 2002), endometrium (Kitawaki *et al.*, 2000), embryo and placenta (Malik *et al.*, 2001; Kawamura *et al.*, 2002-2003) evidence the important roles in male and female reproduction.

Leptin is an adipocyte-derived hormone under the control of the obesity (ob) gene (Zhang *et al.*, 1994). Leptin signalling is accomplished via membrane receptors that belong to the Class I cytokine family (Tartaglia *et al*., 1995). Six isoforms of leptin receptor have been identified in mammals (Lollman *et al.*, 1997) that derived from an alternative splicing of a single db gene transcript. They differ in the length of cytoplasmatic sequency and can be divided into three classes: long (OB-Rb), short (OB-Ra, -Rc, -Rd, -Rf) and soluble (OB-Re) **(**Tartaglia *et al*., 1995; Caprio *at al*., 2001). The primary biological role can be attributed to the long form (OB-Rb), containing a complete intracellular domain, capable of activating the JAK-STAT signalling pathway and is responsible for most of the biological effects of leptin (Bjorback *et al*., 1997). The short isoforms have a truncated intracellular domain and are generally considered to lack signalling capability (Tartaglia *et al.*, 1995). Ob-Ra has, however, been shown to serve a transport function for leptin (Hileman *et al.*, 2002). Ob-Re, that consists only of the extracellular domain, represents the circulation form of Ob-R (soluble Ob-R). It has been shown to account for the majority of the serum leptin-binding activity playing a key role in the regulation of the bioactivity of leptin (Li *et al.*, 1998). The expression of ovarian leptin receptors and theirs direct

involvement in ovarian function has been demonstrated in human (Karlsson *et al*., 1997; Löffler *et al*., 2001), mouse (Kikuchi *et al.*, 1999), rat (Duggal *et al*., 2000), porcine (Ruiz-Corte's *et al*., 2000), and bovine (Nicklin *et al.*, 2007). OB-Rb is expressed in human (Löffler *et al.*, 2001), bovine (Nicklin *at al*., 2007), and porcine (Ruiz-Corte's *et al*., 2000) CL throughout the oestrous cycle. Leptin levels are correlated with P_4 and vary during the human menstrual cycle, reaching its peak during the luteal phase (Ludwig *et al.*, 2000).

In the cow (Mann and Blache, 2002; Sarkar *et al.*, 2009) and pig CL (Ruiz-Corte's *et al.*, 2000) leptin and its receptor expression increases in association with luteinization and declines coincidental with luteal regression. Several studies in the porcine CL have shown that expression patterns of leptin and its receptor correspond to the pattern of P_4 secretion, suggesting a possible positive effect of leptin on luteal steriodogenesis (Ruiz-Corte's *et al.*, 2000). Leptin is thought to act mainly in a synergistic fashion with insulin growth factor-I (IGF-I) and growth hormone (GH), promoting the luteinization process (Gregoraszczuk *et al., 2004*). In the cow, IGF-I increases during mid- and late-luteal phases (Schams *et al*., 2002) and has direct stimulatory effects on key components of the steriodogenic pathway, increasing P_4 secretion (Sauerwein *et al.*, 1992) and in particular inducing of StAR mRNA expression (Mamluk *et al*., 1999). Increased P_4 synthesis may be due both through the direct activity of leptin on induction of StAR transcription, as the result of sterol regulatory element binding protein 1(SREBP-1) modulation (Ruiz-Corte's *et al*., 2003) and indirectly through synergistic effects of leptin and IGF-I on StAR transcription (Ruiz-Cortés *et al.*, 2000). Furthermore, the combined action between leptin and IGF-I decreases caspase-3 activity and therefore protects CL cells from excessive apoptosis and supports homeostasis of the organ (Gregoraszczuk and Ptak, 2005; Gregoraszczuk *et al.*, 2006).

Leptin promotes angiogenic processes via activation of leptin receptor (Ob-R) in endothelial cells (Bouloumie *et al.*, 1998; Sierra-Honigmann *et al.*, 1998; Park *et al.*, 2001), suggesting a crucial role of Ob-Rb in leptin-mediated control of cell differentiation (Bouloumie

et al., 1998). Besides, both *in vitro* and *in vivo* study model of angiogenesis have shown that leptin enhances the formation of capillary-like tubes and neovascularization, acting on endothelial cells proliferation and survival (Bouloumié *et al.*, 1998; Sierra-Honigmann *et al.*, 1998). Besides, another *in vitro* study, used to elucidate the effect of leptin on VEGF and bFGF expression, showed that leptin increased the secretion of VEGF in HUVECs (Human Umbilical Vein Endothelial Cells) in a dose-dependent manner, but it had little effect on the secretion of bFGF (Sierra-Honigmann *et al.*, 1998). In addition, leptin enhanced the expression of some members of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)(Sierra-Honigmann *et al.*, 1998).

Ghrelin, the endogenous ligand of the GH secretagogue receptor type 1a designated as the ghrelin receptor (GRLN-R), is a pleiotropic regulator secreted mainly by oxyntic glands of the stomach (Kojima *et al.*, 1999; Hayashida *et al.*, 2001). This hormone is involved in a large array of endocrine and non-endocrine functions, among which regulation of GH, cell proliferation, expression of several markers of apoptosis (Sirotkin *et al.*, 2006), energy homeostasis and orexigenic effect, in an antagonistic manner to leptin (Arvat *et al*., 2002; Cummings and Shannon, 2003). Some evidence suggests that ghrelin may play a role in the central regulation of reproduction. Indeed, it has been shown to inhibit both *in vivo* and *in vitro* LH secretion in rats in conditions of negative energy balance, such as fasting or anorexia, and to decrease *in vitro* LH responsiveness to GnRH (Fernandez-Fernandez *et al.*, 2004).

Expression of ghrelin has been demonstrated in an array of tissues and cell types including steroigenic tissues such as placenta, testis and mammalian and non-mammalian ovary in human, rat, pig, sheep and chicken ovary (Caminos *et al.*, 2003; Gaytan *et al*., 2003; Miller *et al.*, 2005; Sirotkin *et al.*, 2006). Ghrelin expression mRNA was observed in young and mature CL in the rat, human, and sheep (Caminos *et al.*, 2003; Gaytan *et al.*, 2003; Miller *et al.*, 2005).

In humans, in the mid luteal phase ghrelin has been shown to decrease both basal and hCG-stimulated release of P_4 (Tropea *et al.*, 2006). In the male gonad, specifically in rat testis, direct inhibitory effect of ghrelin on *in vitro* steroidogenesis has been recently suggested as well (Tena-Sempere *et al.*, 2002). Besides inhibiting P_4 secretion, in human luteal cells, ghrelin was also able to decrease luteotrophic PGE_2 release, to increase the luteolytic $PGF_{2\alpha}$ release (Tropea *et al.*, 2007) and to decrease both basal and hypoxia-stimulated VEGF release (Tropea *et al.*, 2006). On the contrary, other authors have shown the expression of ghrelin and its receptor in human microvascular endothelial cells and that ghrelin stimulates proliferation, migration, and angiogenesis of this cells (Li *et al.*, 2007).

Both leptin and ghrelin have shown an influence on NO activity. The first hormone may increase NO production in the pituitary (Baratta *et al.*, 2002) inducing expression of LHRH and LH secretion via nitricoxidergic mechanisms (Yu *et al.*, 1997) and in serum (Beltowski *et al.*, 2002). Ghrelin actives eNOS in endothelial cells through GHSR-mediated phosphorylation and activation of several protein kinases (Xu *et al.*, 2008).

Even though these hormones have been demonstrated to affect ovarian function, the role of leptin and ghrelin on equine CL is still unclear. Thus, the objectives of the present study were to evaluate the role of the hormones leptin and ghrelin on NO, angiogenic factor(s) production that can modulate *in vitro* endothelial cell proliferation (BAEC), secretory function (P_4 and prostaglandins) by the equine corpus luteum (CL). Furthermore, gene identification of ghrelin and leptin in luteal tissue was also accomplished.

MATERIALS AND METHODS

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 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). Mares' reproductive and clinical histories were unknown, but they were in good physical condition as determined by veterinary inspection.

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*. 2007a; Roberto da Costa *et al*. 2008, Galvão *et al*. 2010). Briefly, luteal tissue was considered as: early luteal phase CL (presence of corpus hemorrhagicum, P4> 1ng/ml, Early CL; n=6), or mid luteal phase CL (CL associated with follicles 15 to 20 mm in diameter and P_4> 6 ng/ml, Mid CL; n=6).

Jugular blood vein samples (10ml) were collected into heparinized tubes (monovettes® Sarstedt, Numbrecht, Germany) and transported on ice to the laboratory. Plasma obtained by blood centrifugation was kept frozen (−20 ◦C) until P_4 assays were performed. Mares were considered to be in the luteal phase when their plasma P4 concentration was >1 ng/ml, a CL was present. Plasma obtained by blood centrifugation was kept frozen (−20 ◦C) until progesterone assays were performed. Immediately after collection, luteal samples were included in specific solutions: (i) RNAlater (AM7020, Ambion, Applied Biosystems, CA, USA) for gene expression quantification; and in (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.

Luteal tissue viability assessment

Equine luteal tissue was collected from ovary and sectioned longitudinally from the centre of the CL toward its periphery. Briefly, connective tissue and blood clots were removed,

and small portions of corpora lutea were cut and rinsed thoroughly thrice in sterile PBS containing streptomycin (40μmg/ml) (all reagents from Sigma, St. Louis; MO, USA).

Luteal tissue was minced into small (approximately 1mm3) explants (CH= 6; Mid-CL = 6), weighed, and 50 mg of tissue were placed in 3 ml of culture medium in polystyrene culture tubes (Sarstedt, Numbrecht, Germany). The culture medium consisted of Dulbecco's modified eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 [v/v], D-8900; Sigma, USA) and was supplemented with 0.1% (w/v) bovine serum albumin (BSA) (#735078; Roche Diagnostics GmbH Mannheim, Germany) and 20 μg/mL gentamicin (G1397, Sigma, USA). The assay was run in triplicate. Explants were pre-incubated for 1h in a tissue incubator at 37ºC in humidified atmosphere (5% CO_2 and 95% air) (Biosafe Eco-Integra Biosciences, Chur, Switzerland), on a shaker (Titertek; Huntsville, AL, USA; 150 rpm).

Following pre-incubated period, cells from different luteal stages (Early CL, n=6; Mid CL, n=6) were incubated for 24 h under the same experimental conditions with: (i) media without hormones – control; or with (ii) Leptin (20 or 200 ng/mL); (iii) Ghrelin (100 ng/mL); or (iv) Leptin+Ghrelin (20 ng/mL + 100ng/mL; 200ng/mL+100ng/mL) (all reagents from Sigma, St. Louis, MO, USA). Conditioned media by luteal tissue explants and from negative controls were stored at -70°C to be later analyzed for their ability to stimulate bovine aortic endothelial cell (BAEC) *in vitro* proliferation; and for and nitric oxide (NO) and prostaglandins (PG) production.

Endothelial cell proliferation assay

The effects of different stimulations *(i.e.* control, Leptin, Ghrelin and Leptin+Ghrelin) on angiogenic activity of early and mid luteal phase CL explants conditioned media were assessed by the evaluation of BAEC proliferation using alamarblue reagent (Serotec, Oxford, UK). This reagent is designed to measure quantitatively the proliferation of various animal cell lines based

on their metabolic activity in response to a chemical reduction of a growth substrate and detection of cellular incorporation of a fluorometric/colorimetric growth indicator (Lancaster and Fields 1996). Normal cellular metabolic activity causes chemical reduction of the alamarblue reagent. Thus, continued cell proliferation maintains the reduced environment, while cell growth inhibition results in an oxidized environment. Protocol optimization specifically for BAEC was performed before (Ferreira-Dias, *et al* 2006a). Thus, the optimal reading time for the BAEC is after 5h incubation with the conditioned media from equine luteal tissue 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* 2006). Briefly, BAEC (2×10^4 cells/ml) were incubated in 24- well plates (Nuclon-Nunc®) 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 another 48 h. The conditioned media was 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). The BAEC proliferation response 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.* 2006a). Alamarblue percentage reduction was determined according to alamarblue technical datasheet.

Determination of NO metabolites

Assessment of *in vitro* NO production by cultured luteal tissue explants was determined as previously described (Jaroszewski, *et al.,* 2003). Briefly, after thawing the media obtained

from *Experiment 1*, 50µl of each sample were added to a 96well 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, Hercules, CA, USA). The nitrite content was calculated based on a standard curve made with $NaNO_2$. Nitric oxide production (ng/mg of tissue) was calculated and data subjected to statistical analyses.

Hormones 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, and antiserum was used at a final dilution of 1:100,000 for P_4 concentration assessment (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.

Assessment of PGE_2 and $PGF_{2\alpha}$ concentrations followed the methodology previously described (Skarzynski *et al.*, 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 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.

Polymerase Chain Reaction

The PCR was performed in order to assess leptin and ghrelin and their receptors gene

transcription in corpora lutea throughout the luteal phase. Total RNA from the luteal tissue was extracted (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 the manufacturer's protocols. Later on, RNA electrophoresis through a 1.5% gel agarose and ethidium bromide staining were performed, evaluation of RNA concentration was done spectrophotometrically (260 nm and 280 nm) and RNA quality assessed by visualization of 28S and 18S rRNA bands. Reverse Transcription was accomplished by using Reverse Transcriptase Superscript III enzyme (ref.18080093, Invitrogene, Gibco, Carlsbad, USA), from 1 g total RNA in a 20µl reaction volume, using oligo (dT) primer (27-7858-01, GE Healthcare, Buckinghamshire, UK). Due to the lack of mRNA sequence for *leptin, ghrelin* and their respective *receptors* genes in the horse, primer design was determined in Gene Bank Sequence Data Base (Gene DB). Degenerated primers 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 degenerated primers designed using different internet-based interfaces, such as Primer-3 (Rozen and Skaletsky, 2000) and Primer Premier software (Premier Biosoft Int., Palo Alto, CA, USA) (Feng *et al.*, 1993).

A series of conventional PCR reactions were carried out using a default thermocycler (Applied Biosysthems, California, USA), using FideliTaq DNA polymerase master mix (71180, USB, Cleveland, USA), according to the following thermal cycling protocol: 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 for extension 45 sec at 68ºC; and finally 5 min at 68ºC for finalization. The PCR reaction products were electrophoresed in Agarose gel (1%) (BIO-41025, Bioline, Luckenwalde, Germany) and then were stained with ethidium bromide (17896, Thermo, USA) showing a specific and single product. cDNA samples in duplicate were sent for

sequenciation (Stabvida, Lisbon, Portugal) after purification with GFX PCR DNA and Gel Band Purification Kit (28903470, GE Healthcare, Buckinghamshire, UK). Sequences homology was confirmed and submitted to the GenBank at the National Center for Biotechnology Information (NCBI). Specific primers were then chosen for these target genes and housekeeping gene.

To avoid genomic DNA amplification, primers were designed for two different exons and all primers design followed specific guidelines (Xiaowei and Seed, 2006). Under our experimental conditions, three potential housekeeping genes (HKG) were initially considered (hypoxanthine phosphoribosyltransferase-1, β-actin and β2-microglobulin-B2M) to establish the most stable internal control gene. B2M gene transcription was unaffected by our experimental conditions, and therefore, elected as HKG.

Statistical analysis

Data regarding plasma P_4 concentrations and *in vitro* NO, P_4, PGE_2 and $PGF_{2\alpha}$ concentrations in luteal tissue culture media and BAEC proliferation assays, 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).

RESULTS

Endothelial cell proliferation assay

In the present study, no difference was observed on BAEC mitogenesis when these cells were incubated with conditioned media from Early-CL submitted to any of the treatment tested. However, in the Mid-CL, ghrelin (100ng/ml) and the lowest leptin dose (20ng/ml), when used alone, stimulated BAEC proliferation ($p<0.05$). Nevertheless, the highest leptin concentration (200mg/ml), alone or associated with ghrelin (100mg/ml), resulted in a decrease in endothelial cell proliferation ($p<0.05$)(Fig. 1).

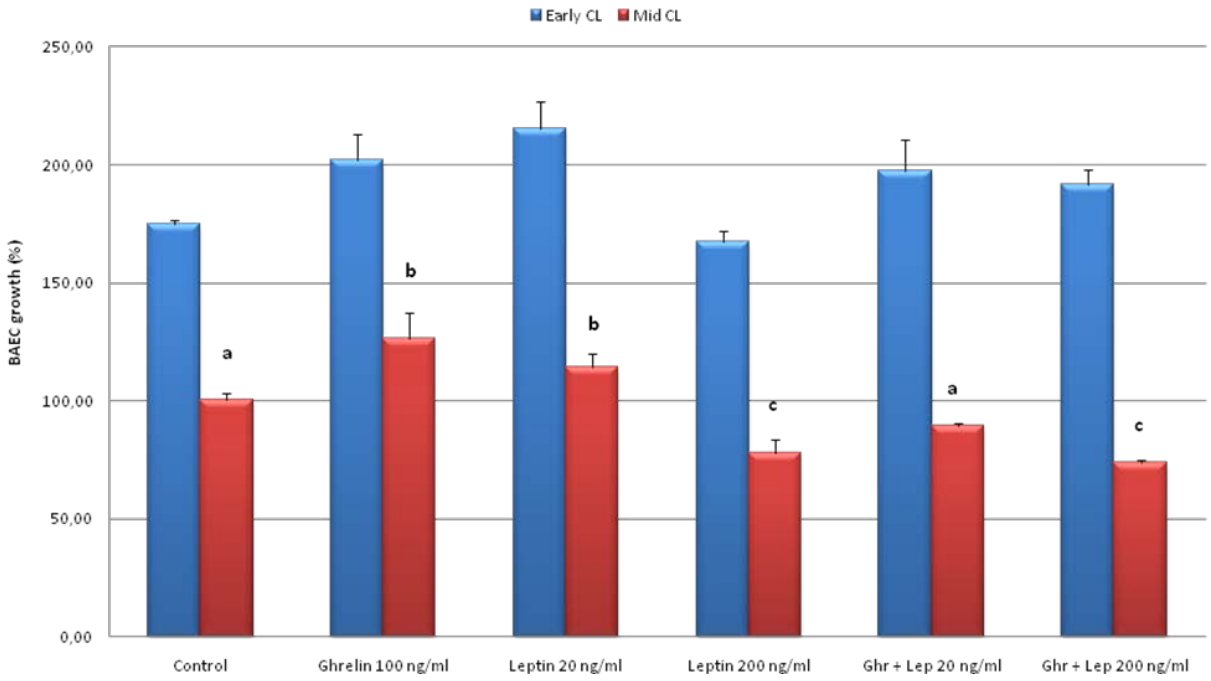


Fig. 1. *In vitro* BAEC proliferation (%) when stimulated by equine Early-CL and Mid-CLconditioned media in absence (control) or presence of leptin and/or ghrelin. Bars represent mean±SEM. Different letters (a, b and c) mean significant differences at $p<0.05$.

Nitric oxide production

Regardless of the treatments, NO production from luteal tissue explants after a 24h culture showed that it was always higher in the Mid-CL than in the Early-CL regardless of the treatment ($p<0.05$). Leptin and ghrelin alone at the lowest dose (20ng/mL) showed no effect on NO production during early and mid luteal phases. Only at the highest dose of Leptin (200 ng/ml) and Ghrelin (100 ng/ml), alone or combined, increased NO, for both Early and Mid-CL explants ($p<0.01$)(Fig. 2).

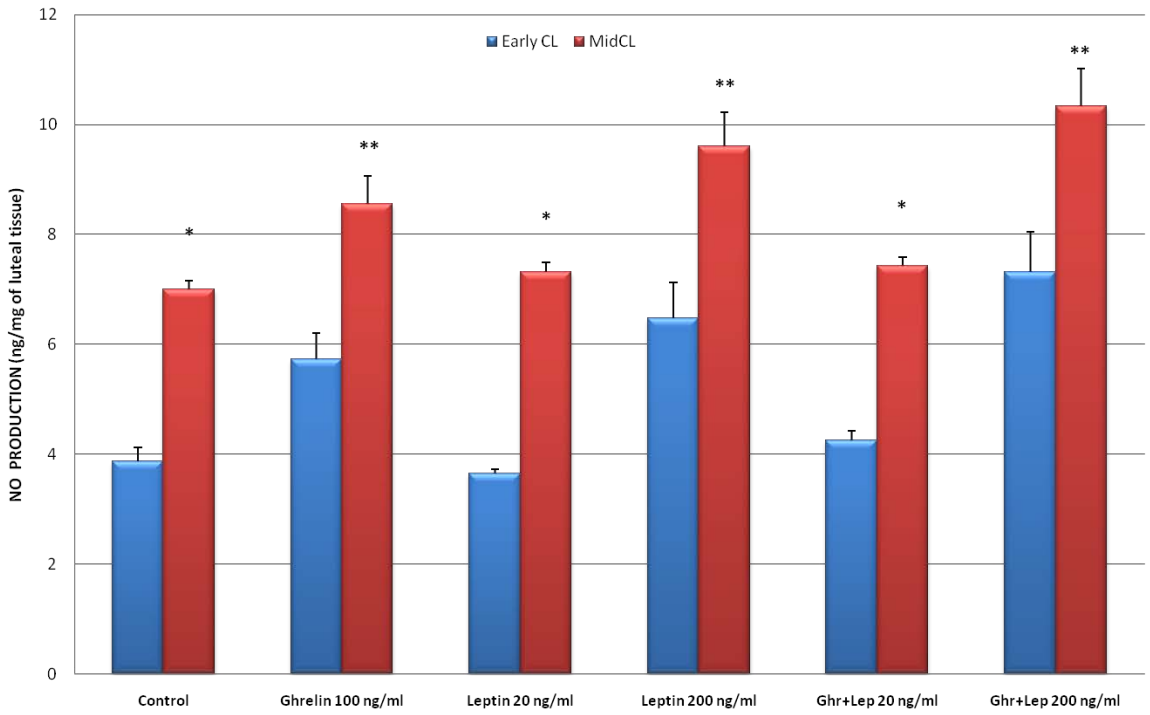


Fig. 2. *In vitro* NO production by equine Early-CL (blue bars) and Mid-CL (red bars) conditioned media in absence (control) or presence of leptin and/or ghrelin. Bars represent mean±SEM. Asterisks mean significant differences at $p<0.01$ (*).

Hormones determinations

The evaluation of leptin and ghrelin on equine luteal cells steroidogenic capacity showed that, in the early and mid luteal phases, no differences were observed for both factors on P_4 production (fig. 3). Regarding PGE_2 output from luteal cells from both early and mid luteal phases, the lowest leptin dose (20ng/ml) stimulated its production when compared to negative control ($p<0.05$). Also, during the early-luteal phase, when leptin was used at the same dose in association with ghrelin at 100 ng/ml, PGE_2 production was increased ($p<0.01$)(fig 4). During the early-luteal phase, $PGF_{2\alpha}$ secretion by luteal cells was not affected by any treatment, while during the mid luteal phase, the highest doses of ghrelin (100ng/ml) and ghrelin associated with the lowest doses of leptin (20ng/ml) increased $PGF_{2\alpha}$ production ($p<0.001$), with respect to control (Fig. 5).

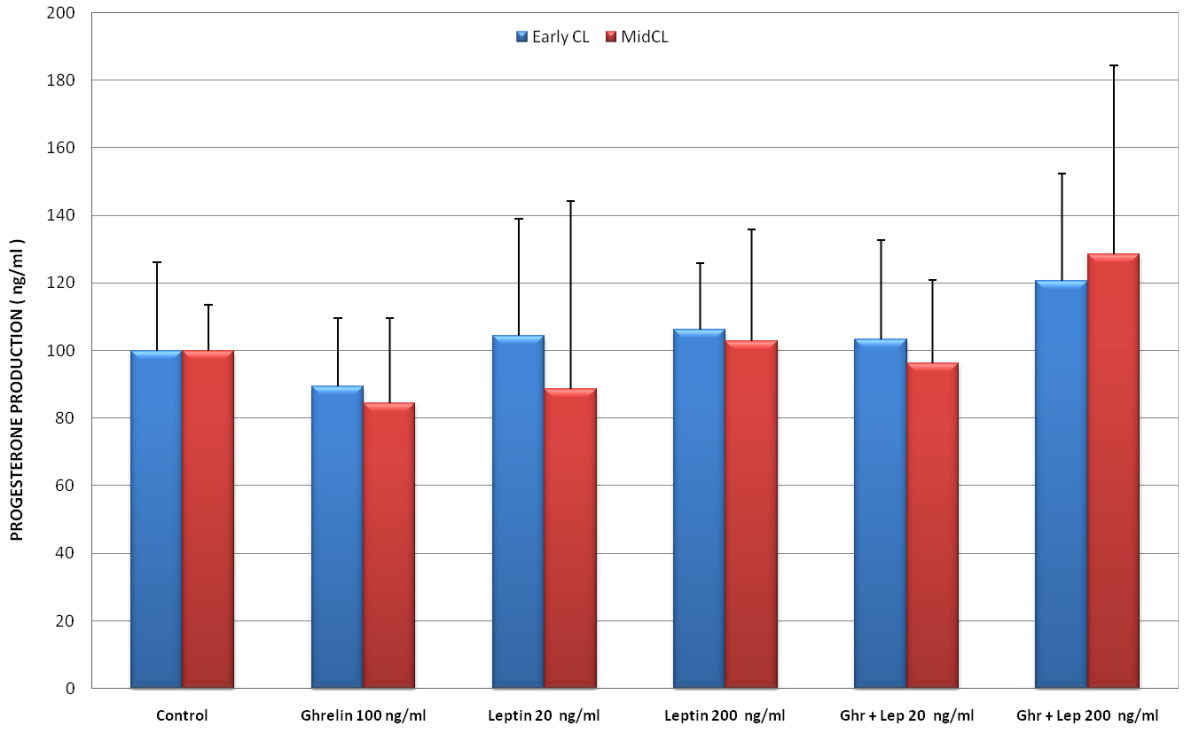


Fig. 3. *In vitro* P_4 production by equine Early-CL (blue bars) and Mid-CL (red bars) conditioned media in absence (control) or presence of leptin and/or ghrelin. Bars represent mean±SEM.

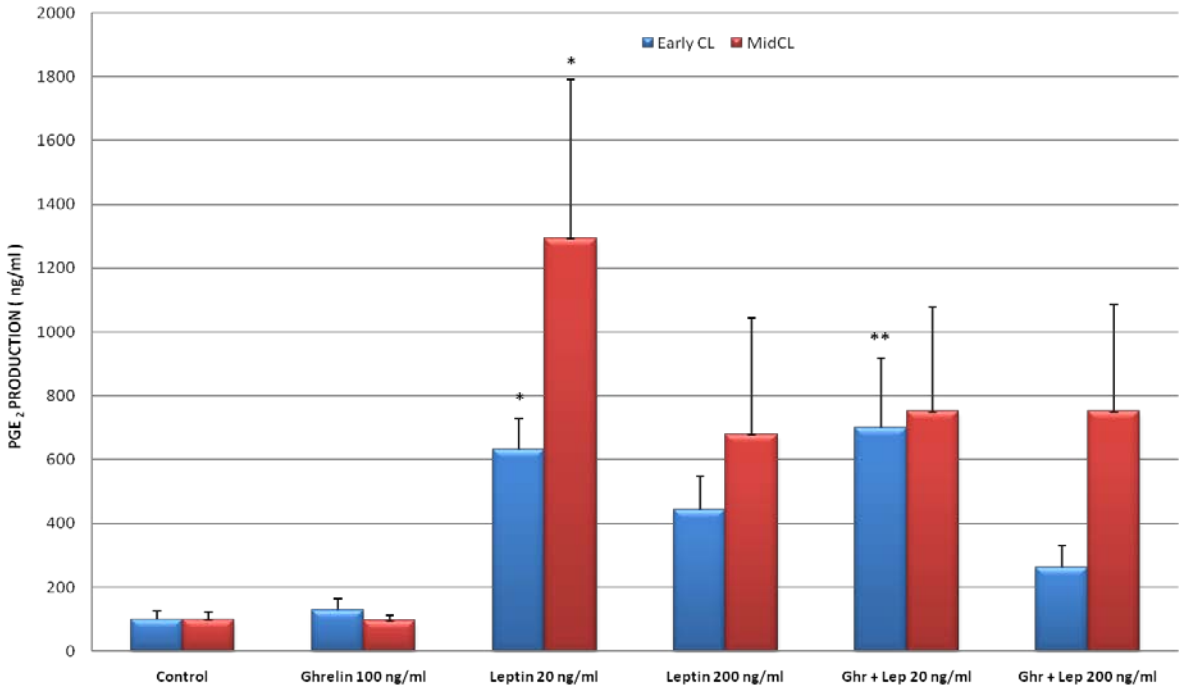


Fig. 4. *In vitro* PGE_2 production by equine Early-CL (blue bars) and Mid-CL (red bars) conditioned media in absence (control) or presence of leptin and/or ghrelin. Bars represent mean±SEM. Asterisks mean significant differences at $p<0.05$ (*) and $p<0.01$(**).

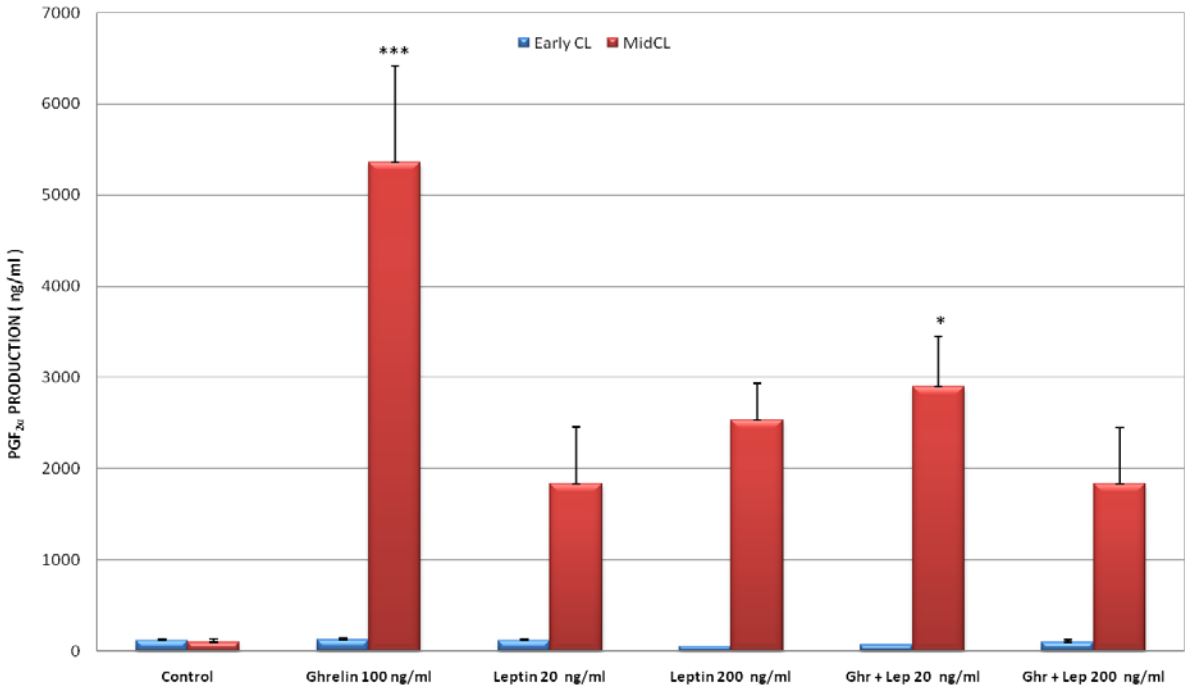
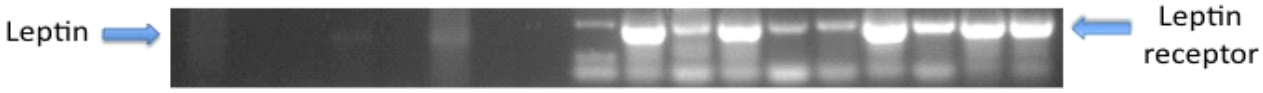


Fig. 5. *In vitro* $PGF_{2\alpha}$ production by equine Early-CL (blue bars) and Mid-CL (red bars) conditioned media in absence (control) or presence of leptin and/or ghrelin. Bars represent mean±SEM. Asterisks mean significant differences at $p<0.05$ (*) and $p<0.001$(***).

Polymerase Chain Reaction

In this study, using primers for leptin, ghrelin and respective receptors we observed the presence of ghrelin, its receptors and leptin receptor in all samples of equine CL obtained throughout the luteal phase (Fig. 6). Nevertheless, leptin expression was completely absent in CL collected at any stage of the luteal phase (Fig. 6).



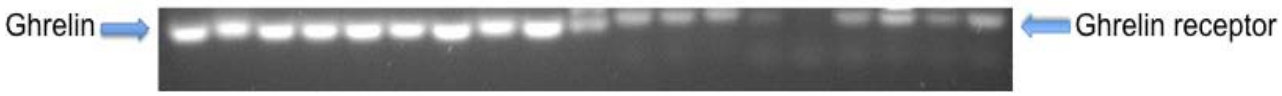


Fig. 6. Representative PCR reaction showing that leptin mRNA expression was inexistent on the equine CL, while leptin receptors, ghrelin and its receptors mRNA were expressed throughout the luteal phase.

DISCUSSION

This study was an attempt to examine a possible physiologic regulation of the equine CL by leptin and ghrelin on angiogenic development, NO production and luteal secretory capacity concerning progesterone and prostaglandins. Morevover, to the best of our knowledge, the presence of ghrelin and its receptor, and leptin receptor was reported for the first time in the equine CL throughout the luteal phase.

Several evidences have been shown the presence of leptin and theirs receptors in the ovary in most species, particularly in human (Löffler *et al.*, 2001), porcine (Ruiz-Corte's *et al.*, 2000), and bovine (Nicklin *et al.*, 2007). Concerning the expression of ghrelin and its receptors in the CL, it has been shown in rat, human, and sheep (Caminos *et al.*, 2003; Gaytan *et al.*, 2003; Miller *et al.*, 2005). In the present study, we demonstrated the expression of leptin receptor, ghrelin and its receptor in mare CL throughout the luteal phase, while leptin mRNA expression was inexistent. In light of these results we can assume that in the mare, leptin might act on the CL, even though it is not produced there. Indeed, as in other species, in the horse, leptin is a hormone produced by adipose tissue and its peripheral concentration reflects fat mass and a partial sequence of the equine leptin and leptin receptors gene from adipose tissue have been demostrated in a previously study (Buff *et al.*, 2002). In fact, in the mare, this metabolic hormone plays an important role on spring seasonal recrudescence of ovarian activity and seasonal cyclicity (Ferreira-Dias *et al.*, 2005). Besides, this might suggest that leptin of exogenous origin (adipose tissue), could promote luteal growth and luteinization process acting

in a synergistic fashion with insulin growth factor-I (IGF-I) and growth hormone (GH), as previously demostrated in porcine CL (Gregoraszczuk *et al., 2004*). Moreover, leptin capacity to induce angiogenesis (Bouloumie *et al.*, 1998), was also observed in the Mid-CL, at the lowest leptin dose tested. This endothelial cell proliferation might contribute for CL growth and luteinization process in the mare ovary, when luteal vascular development reaches its maximum (Ferreira-Dias *et al*., 2006b).

Regarding ghrelin, several evidence indicates the role of ghrelin as an endogenous ligand of the growth hormone secretagogue receptor (GHS-R) and involved in the control of GH secretion (Kojima *et al*., 1999-2001; Casanueva and Dieguez, 1999). Indeed, the existence of a local ghrelin-GH axis in the ovary, where GH increase locally produces ghrelin secretion, seems to be evident in other species. Ghrelin, in association with GH, stimulated estradiol synthesis and secretion by ovarian follicles and prevented apoptosis in porcine ovary (Rak and Gregoraszczuk, 2008). In our study, the presence of ghrelin and its receptor in the mare CL might indicate a potential regulatory role of locally produced ghrelin in the control of luteal function, as already shown in human CL (Gaytan *et al.*, 2003), as well as an influence on CL growth acting together with leptin and GH, also on the control of apoptosis.

In confirmation of this possible impact on angiogenesis, leptin and ghrelin, alone or combined, increased NO, for both early and mid luteal phases, even though their production was always higher in the Mid-CL than Early-CL. Several studies have shown that increased NO production during early stages of the ovarian cycle and pregnancy is likely to play a role in CL development and angiogenesis (Skarzynski *et al*., 2000b; Weems *et al.*, 2004; Vonnahme *et al.*, 2005a; Rosiansky-Sultan *et al.*, 2006; Ferreira-Dias *et al.*, 2011). NO, which is primarily an endothelial product and a potent vasodilator, can stimulate VEGF production and angiogenesis (Chin *et al.*, 1997; Frank *et al*., 1999), and at the same time VEGF, present in luteal perivascular cells, can stimulate endothelial NO synthase (eNOS) expression and thus NO production (Van

der Zee *et al*., 1997; Bouloumie *et al*., 1999), maximizing angiogenesis mechanism during CL development. In the mare, NO stimulates early CL secretory function (P_4 production and PGE_2), as well as angiogenic activity (Ferreira-Dias *et al*., 2011). Indeed, it was recently found in the mare CL that eNOS is expressed in endothelial cells of luteal arterioles and capillaries early in the luteal phase but that its expression is greatly reduced by mid cycle (Ferreira-Dias *et al.*, 2011). On the contrary, in the our study, the largest increase in NO occurred in mature CL, especially when leptin and ghrelin were used in high doses alone or combined. Therefore, the raise of the NO synthesis in equine CL by leptin and ghrelin, in particulary during mid luteal phase, could indicate a match and mutual influence between NO and angiogenic factors such as VEGF and consequently on angiogenesis process. Nevertheless, an important increase in NO production during mid luteal phase might be associated with an increase in the production of the luteolytic $PGF_{2\alpha}$.

In this study, the potential control of luteal secretory function by leptin and ghrelin could result, not as much by a direct effect of these hormones on the production of P_4, which seems to remain unaffected in early and mid luteal phase, but through the production of PGE_2 and $PGF_{2\alpha}$. Even though *in vitro* P_4 production by equine luteal tissues explants was unchanged between luteal phases or treatments, it shows the incubation adequacy for the maintenance of normal luteal function under exogenous hormone influence (Redmer *et al*., 1988). Interestingly, the metabolic hormones studied showed different actions on eicosanoids secretion by equine CL explants at early and mid luteal phases. Soon after ovulation, a low leptin concentration (20ng/ml), either alone or associated with ghrelin, stimulated PGE_2 secretion. This leptin effect was continued in the mid luteal phase CL, when vascular and non-vascular development is known to reach its maximum in mare luteal tissue (Ferreira-Dias *et al*., 2006b), suggesting a luteotrophic action of this hormone during CL growth and differentiation (Miyamoto *et al.* 1993; Okuda *et al.*, 1998; Ferreira-Dias *et al*., 2011). Nevertheless, in the Mid-CL it appears that ghrelin abolished leptin

luteotrophic action, since their association no longer stimulated PGE_2 secretion. It is worth noting that, in contrast to leptin, ghrelin may act as a luteolytic hormone, since in the Mid-CL at its highest concentration (100ng/ml), caused a raise in *in vitro* $PGF_{2\alpha}$ production. These data are in agreement with a previous study on human luteal cells, where ghrelin was able to decrease luteotrophic PGE_2 release and to increase the luteolytic $PGF_{2\alpha}$ release (Tropea *et al*., 2007). However, ghrelin luteolytic effect appeared to be reverted by leptin at its highest concentration (100ng/ml), but not at the low concentration (20ng/ml) tested, which might indicate a dose dependent response/antagonism effect to this hormone.

Vascularization of the reproductive system during cyclic period is essential to guarantee the necessary supply of nutrients and hormones to promote follicular growth and CL formation, as well as its physiologic function until complete regression. In the mare, Al-zi'abi *et al.* (*2003*) have shown how during the early and mid-luteal phases, the peak expression of VEGF mRNA and protein indicate a temporal association with high endothelial cell proliferation and the presence of a dense capillary network. Especially in the mid-luteal phase, the high expression of VEGF mRNA is compatible with its established role in stimulating and maintaining the newly formed vasculature in the CL (Dickson *et al*., 2001). In the present study, there was no effect of treatments in early-luteal phase on BAEC proliferation, showing that these metabolic factors could not influence angiogenic activity in this phase. Instead, when the equine CL was already formed, leptin and ghrelin might affect angiogenesis in a dose-dependent manner. In the mid luteal phase, it appears that ghrelin, when used alone, was able to stimulate endothelial cell mitogenesis. Besides, low leptin concentration also had a stimulatory effect on luteal angiogenic activity (endothelial cell proliferation), that was abolished at its high concentration and when combined with high ghrelin concentrations. However, since no parallel change in NO was present, it seems that this stimulation of endothelial cell proliferation by low dose leptin in the Mid-CL might not be directly mediated by NO, but through PGE_2 stimulation, as referred by

others (Sakurai *et al.*, 2004). Nevertheless, when high leptin and ghrelin concentrations were used, NO production increased by Mid-CL, which might have been responsible for the inhibitory effect on endothelial cell proliferation, through $PGF_{2\alpha}$ action, as described for the cow and mare (Jaroszewski *et al.*, 2003; Skarzynski *et al*., 2003; Ferreira-Dias *et al.*, 2006b). The eicosanoid $PGF_{2\alpha}$ appears to be the main cause of luteal regression together with NO, since this latter is considered an important mediator of luteolysis in several species (Vega *et al*., 1998; Motta *et al*., 1999; Vega *et al*., 2000; Skarzynski *et al*., 2000b; Boiti *et al.*, 2003; Korzekwa *et al*., 2004). As a matter of fact, microvascular endothelial cells are the first ones to undergo apoptosis in the CL in response to the luteolytic signal (Davis *et al*., 2003). Besides, NO actions on steroid and prostaglandin secretion are very variable according to NO concentration and animal species (Grasselli *et al*., 2001; Basini and Tamanini, 2001; Skarzynski *et al*., 2003; Weems *et al*., 2005). In addition, even though NO may be involved in the autocrine/paracrine luteolytic cascade induced by PGF2α in the cow (Skarzynski *et al*., 2003), it may be antiluteolytic and prevent luteolysis in the ewe (Weems *et al*., 2005). In the rat, this vasodilator molecule has a dual role, being protective in the mid luteal phase and pro-oxidant/luteolytic in the late luteal phase (Motta *et al*., 2001).

As mentioned before, leptin seems to have an influence both on angiogenesis, in particular on VEGF activity in endothelial cells (Sierra-Honigmann *et al.*, 1998), and NO production (Huang *et al*., 2005). Specifically, leptin stimulates NOS activity in white adipocytes in humans (Mehebik *et al.*, 2005). Also ghrelin appears to be involved in the same mechanisms (Li *et al.*, 2007; Xu *et al.*, 2008), even though others authors suggest this hormone inhibits VEGF release (Tropea *et al.*, 2006). In the present study, another possibility for explaining ghrelin and leptin stimulatory effect on BAEC proliferation in the Mid-CL could be due to NOS stimulation since NO production was also increased. The fact that the combination of higher doses of leptin

and ghrelin appeared to inhibit BAEC proliferation might be due to the overlap of the already proven inhibitory effect of ghrelin on VEGF release (Tropea *et al.*, 2006).

In conclusion, this study showed the presence of ghrelin, its receptor and leptin receptor and in all CL luteal stages. In light of these results we can suggest that both metabolic hormones studied, leptin and ghrelin, play specific roles on prostaglandin production, NO and angiogenic activity. In disagreement with several other reports, these hormones might not have a direct influence on P_4 production. Nevertheless, in a dose dependent manner, leptin and ghrelin might be potential regulators of CL vascular and non vascular growth by modulating $PGE_{2,}$ $PGF_{2\alpha}$ and NO secretion, during early and mid luteal phases. Thus, leptin showed a predominant luteotrophic action, while ghrelin presented a luteolytic action. However, further studies are needed to understand the physiologic role of these metabolic peptides on luteal function in the mare.

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SECOND EXPERIMENT: GH & IGF-I

INTRODUCTION

Growth hormone (GH) is a pituitary hormone that affects animal growth, metabolism, lactation, and reproduction, whose production is stimulated by GH-releasing hormone (GHRH) and ghrelin - acting via the GH secretagogue (GHS) receptor (GHSR) - and many other factors including leptin (Mizuno *et al.*, 1999). GH and LH are necessary hormones for normal CL development (Schams *et al.*, 1999). GH acts directly on CL influencing estradiol (E_2), oxitocin (OT) and P_4 secretion (Spicer *et al.*, 1992-1993; Lucy *et al.*, 1994) by its own receptor GH-R identified in human, ovine, bovine and rat luteal tissue (Carlsson *et al.*, 1993; Lucy *et al.*, 1993; Sharara and Nieman, 1994; Juengel *et al.*, 1997a,b). Nevertheless, many of the effects of GH are mediated by insulin-like growth factor I (IGF-I) which is synthesized in the liver and ovary in response to GH (Gong *et al.*, 1996). Insulin-like growth factor I synergizes with gonadotrophins (LH and FSH) to stimulate growth and differentiation of ovarian cells. Besides, the IGF system is thought to play a key role in follicle growth, selection for dominance, ovulation and follicle regression (Gong *et al.*, 1993; Ginther *et al.*, 2001; Ginther *et al.*, 2003).

The IGF system may regulate luteal angiogenesis, steroidogenesis and apoptosis. This system may have indirect or direct effects on angiogenesis by stimulating actions for VEGF production in luteal cells as well as by proliferation and differentiation of EC (Reynolds and Redmer, 1998; Berisha *et al.*, 2000). It is known that GH and IGF-I act in synergistic faschion with leptin in the luteinization process (Gregoraszczuk *et al.*, 2004). In the matter of steroidogenesis, growing evidence confirms that IGF-I acts augmenting LH receptors and its sensitivity to the LH resulting in increasing luteotrophic effects (Adashi *et al.*, 1985). Besides, it

also stimulates directly P_4 secretion in rat (Parmer *et al*., 1991), rabbit (Constantino *et al*., 1991) sheep (Khan-Dawood *et al*., 1994; Hastie and Haresign, 2006), pig (Yuan *et al*., 1996), cattle (McArdle and Holtorf, 1989), and human (Devoto *et al.*, 1995). Indeed, IGF-I concentration increases during mid- and late-luteal phases in the cow (Schams *et al*., 2002) and appears to stimulate key components in the steroidogenic pathway, especially throughout the amplification of StAR mRNA expression (Balasubramanian *et al.*, 1997; Mamluk *et al*., 1999) and the delivery and utilization of steroid precursors (Veldhuis, 1989) leading to increased P_4 (Sauerwein *et al.*, 1992). In addition, GH and IGF-I by having anti-apoptotic effects act indirectly by increasing P_4 secretion, and directly, through the inhibition of caspase-3 activity (Ptak *et al*., 2004). Concerning GH, one possible anti-apoptotic mechanisms is based upon the up-regulation of Bcl-2 expression which controls the cytochrome c release from mitochondria, thus prevents the activation of caspase cascade, among which the effector caspase-3 (Haeffner *et al.*, 1999; Mitsunaka *et al*., 2001). In the case of IGF-I stimulates P_4 production, bcl-2 family gene expression and decreased apoptosis impeding transcriptional activation of specific target genes, such as Fas ligand (FasL), an inducer of apoptosis (Yang and Rajamahendran, 2000) and caspase-3 (Ness and Wood, 2002; Saeki *et al.*, 2002).

In some species, GH and IGF system may play a key role in regulation of luteal function (Devoto *et al.*, 1995; Niswender *et al.*, 2000; Schams *et al*., 2002; Hastie and Haresign, 2006). Nevertheless, in the mare this is unclear and there is no information on the role of the GH and IGF system in the equine CL. Thus, the purpose of this study was therefore to investigate the role of GH and IGF-I on nitric oxide (NO) and angiogenic factor(s) production that can modulate *in vitro* endothelial cell proliferation (BAEC), by equine corpus luteum (CL), throughout the luteal phase. Furthermore, gene identification of GH and IGF-I in luteal tissue was also performed.

MATERIALS AND METHODS

Collection of equine corpora lutea

During the breeding season, ovaries and venous blood were collected *post mortem* from randomly designated cyclic mares at the local abattoir. The animals were euthanized after stunning according the European Legislation concerning welfare aspects of animal stunning and killing methods (EFSA, AHAW/04-027). The mares were in good physical condition as determined by veterinary inspection. Since mares' reproductive and clinical histories were not known, estrous cycle stage was determined based on good macroscopic condition, plasma progesterone (P_4) levels, on follicle size and on the morphological appearance of the CL (Roberto da Costa *et al*. 2007a; Roberto da Costa *et al*. 2008, Galvão *et al*. 2010). Luteal tissue was considered as: early luteal phase CL (presence of corpus hemorrhagicum, P4> 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=5) 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=4).

Blood samples (10ml) were collected from the jugular vein into heparinized tubes (monovettes® Sarstedt, Numbrecht, Germany) and transported on ice to the laboratory. Plasma obtained by blood centrifugation was kept frozen (−20 °C) until progesterone assays were performed. Immediately after collection, luteal samples were included in specific solutions: (i) RNAlater (AM7020, Ambion, Applied Biosystems, CA, USA) for gene expression quantification; and in (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.

Luteal tissue viability assessment

Equine luteal tissue (CL) was collected from ovary and sectioned longitudinally from the centre of the CL toward its periphery. Connective tissue and blood clots were then removed, and small portions of corpora lutea were cut and rinsed thoroughly three times in sterile PBS containing streptomycin (40μmg/ml) (all reagents from Sigma, St. Louis; MO, USA). Luteal tissue was minced into small (approximately 1mm3) explants (CH= 6; Mid-CL = 5; Late-CL=4), weighed, and 50 mg of tissue were placed in 3 ml of culture medium in polystyrene culture tubes (Sarstedt, Numbrecht, Germany). The assays were run in triplicate. Luteal tissue were pre-incubated for 1 h in a tissue incubator at 37ºC in humidified atmosphere (5% CO_2 and 95% air) (Biosafe Eco-Integra Biosciences, Chur, Switzerland), on a shaker (Titertek; Huntsville, AL, USA; 150 rpm).

The culture medium consisted of Dulbecco's modified eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 [v/v], D-8900; Sigma, USA) and was supplemented with 0.1% (w/v) bovine serum albumin (BSA) (#735078; Roche Diagnostics GmbH Mannheim, Germany) and 20 µg/mL gentamicin (G1397, Sigma, USA).

Following the pre-incubation period, cells from different luteal stages (Early CL, n=6; Mid CL, n=5; Late-CL, n=4) were exposed under the same experimental conditions for 24 h to: (i) media without hormones – negative control; or with (ii) equine LH (positive control; 100ng/mL); (iii) equine GH (20 or 100 ng/mL); (iv) IGF (30 or 50ng/mL); (v) IGF+LH (30+100ng/mL; 50+100ng/mL); or (vi) IGF+GH (30+20ng/mL; 50+100ng/mL) (all reagents from Sigma, St. Louis, MO, USA). Conditioned media by luteal tissue and from negative controls were stored at -70°C to be later analyzed for their ability to stimulate bovine aortic endothelial cell (BAEC) *in vitro* proliferation and nitric oxide (NO) production.

Endothelial cell proliferation assay

The effects of different cell stimulations (i.e. control, LH, GH, IGF, IGF+LH and IGF+GH) on angiogenic activity from early, mid and late-CL conditioned media were assessed after evaluation of BAEC proliferation using alamarblue reagent (Serotec, Oxford, UK). Normal cellular metabolic activity causes chemical reduction of the alamarblue reagent. In this way, continued cell proliferation maintains the reduced environment, while cell growth inhibition results in an oxidized environment.

Protocol optimization for BAEC was performed before (Ferreira-Dias *et al.,* 2006a), being the optimal reading time at 5h incubation of BAEC with the conditioned media from equine luteal tissue explants cultures. 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 protocol (Ferreira-Dias *et al.,* 2006a). Briefly, BAEC (2×10^4 cells/ml) were incubated in 24- well plates (Nuclon-Nunc®) 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 another 48 h. The conditioned media was 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). The BAEC proliferation response 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*., 2006a). Alamarblue percentage reduction using abs was determined according to alamarblue technical datasheet.

Determination of NO metabolites

Evaluation of *in vitro* NO production by cultured luteal tissue was determined according to the methodology previously described (Jaroszewski *et al*., 2003). Briefly, after thawing the media originating from *Experiment 2*, 50µl 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, Hercules, CA, USA). The nitrite content was calculated based on a standard curve made with $NaNO_2$.. Nitric oxide production (ng/mg of tissue) was calculated and data subjected to statistical analyses.

Polymerase Chain Reaction

The PCR was performed in order to assess GH and IGF-I and their receptors gene transcription in corpora lutea throughout the luteal phase. Total RNA from the luteal tissue was extracted (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 the manufacturer's protocols. Later on, RNA electrophoresis through a 1.5% gel agarose and ethidium bromide staining were performed, evaluation of RNA concentration was done spectrophotometrically (260 nm and 280 nm) and RNA quality assessed by visualization of 28S and 18S rRNA bands. Reverse Transcription was accomplished by using Reverse Transcriptase Superscript III enzyme (ref.18080093, Invitrogene, Gibco, Carlsbad, USA), from 1 g total RNA in a 20µl reaction volume, using oligo (dT) primer (27-7858-01, GE Healthcare, Buckinghamshire, UK). Due to the lack of mRNA sequence for *IGF-I, GH* and their respective *receptors* genes in the horse, primer design was determined in Gene Bank Sequence Data Base (Gene DB). Degenerated primers 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 degenerated primers designed using different internet-based interfaces, such as Primer-3 (Rozen and Skaletsky, 2000) and Primer Premier software (Premier Biosoft Int., Palo Alto, CA, USA) (Feng *et al.*, 1993).

A series of conventional PCR reactions were carried out using a default thermocycler (Applied Biosysthems, California, USA), using FideliTaq DNA polymerase master mix (71180, USB, Cleveland, USA), according to the following thermal cycling protocol: 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 for extension 45 sec at 68ºC; and finally 5 min at 68ºC for finalization. The PCR reaction products were electrophoresed in Agarose gel (1%) (BIO-41025, Bioline, Luckenwalde, Germany) and then were stained with ethidium bromide (17896, Thermo, USA) showing a specific and single product. cDNA samples in duplicate were sent for sequenciation (Stabvida, Lisbon, Portugal) after purification with GFX PCR DNA and Gel Band Purification Kit (28903470, GE Healthcare, Buckinghamshire, UK). Sequences homology was confirmed and submitted to the GenBank at the National Center for Biotechnology Information (NCBI) (Table2). Specific primers were then chosen for these target genes and housekeeping gene. To avoid genomic DNA amplification, primers were designed for two different exons and all primers design followed specific guidelines (Xiaowei and Seed, 2006). Under our experimental conditions, three potential housekeeping genes (HKG) were initially considered (hypoxanthine phosphoribosyltransferase-1, β-actin and β2-microglobulin-B2M) to establish the most stable internal control gene. B2M gene transcription was unaffected by our experimental conditions, and therefore, elected as HKG.

Statistical analysis

Data regarding *in vitro* NO concentrations in luteal tissue culture media and BAEC proliferation assays 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).

RESULTS

Endothelial cell proliferation assay

In the present study, in the Late-CL, IGF+LH (30, 50+100ng/mL) and IGF+GH (50+100ng/mL) decreased BAEC proliferation when compared to LH stimulation ($p<0.05$).

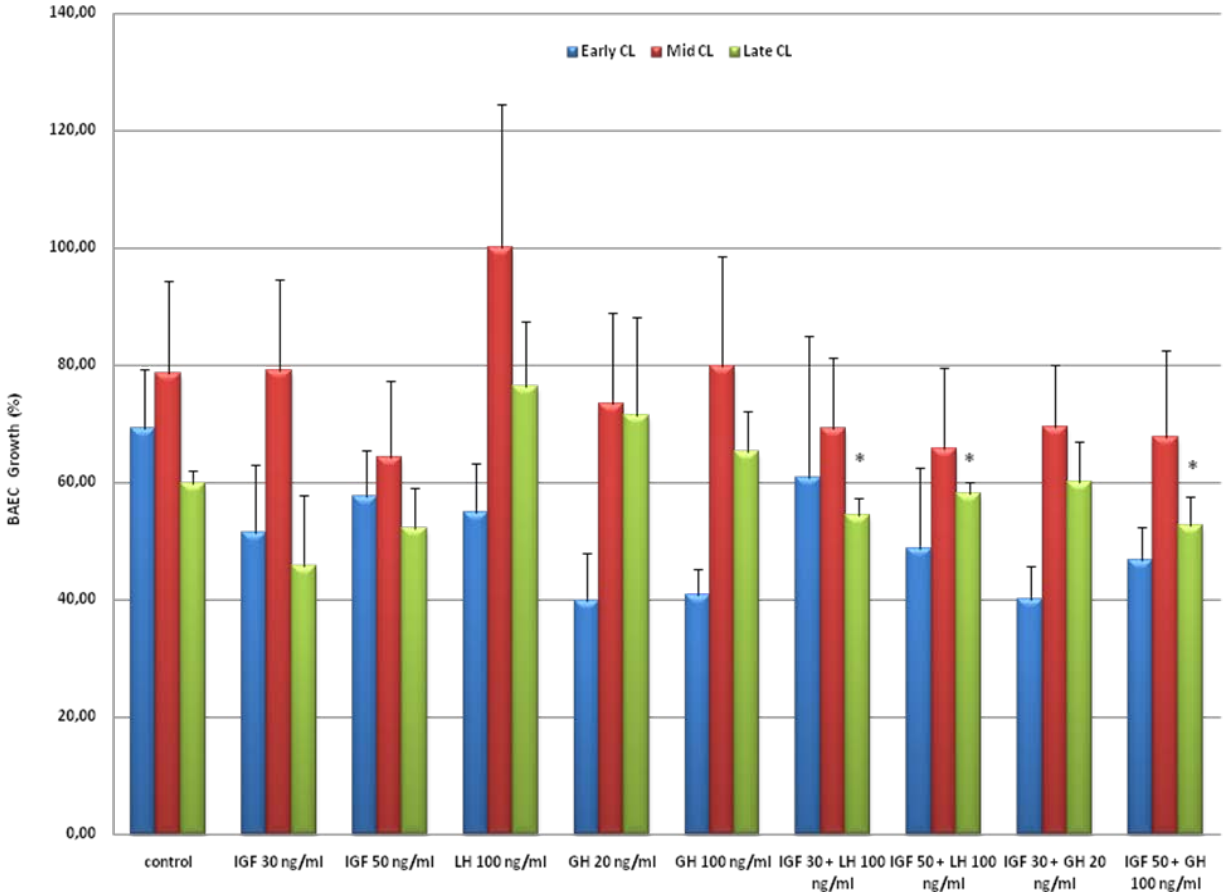


Fig. 1. *In vitro* BAEC proliferation (%) when stimulated by Early, Mid and Late luteal phase, in the presence or absence (negative control) of LH (positive control), GH, IGF-I and their associations. Bars represent mean±SEM. Asterisks mean significant differences at $p<0.05$ (*).

Nitric oxide production

In the Mid-CL, NO production was higher in IGF+LH group (50+100ng/mL) than in control and IGF (50ng/mL)($p<0.05$). In the Late-CL, IGF+LH (50+100ng/mL) increased NO production with respect to IGF (30, 50ng/mL), LH, GH (20ng/mL) and IGF+GH (30+20ng/mL)($p<0.05$). Also, in IGF+GH (50+100ng/mL) treatment, NO was higher than in all groups ($p<0.001$).

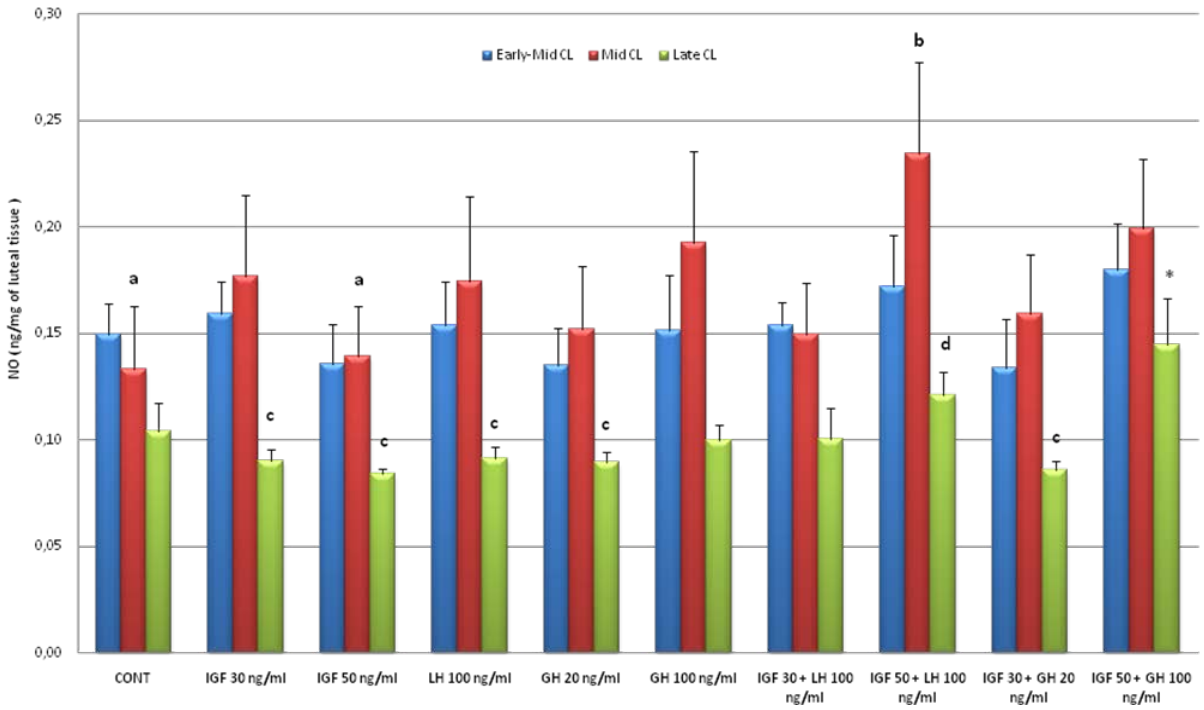
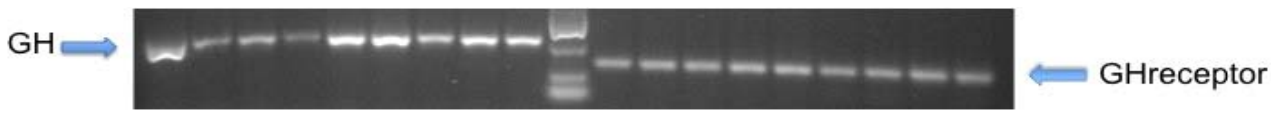


Fig. 2. *In vitro* NO production by Early, Mid and Late luteal phase, in the presence or absence (negative control) of LH (positive control), GH, IGF-I and their associations. Values reported are means±SEM of reagent reduction. Values (a, b, c and d) differ significantly ($p<0.05$; LSD test). Asterisk means significant differences (*), NO was higher than in all groups ($p<0.001$).

Polymerase Chain Reaction

In the present study, PCR analyses for evaluating GH, IGF-I mRNA expression and respective receptors showed that both factors and their receptors were present in the equine CL throughout the luteal phase (Fig. 3).



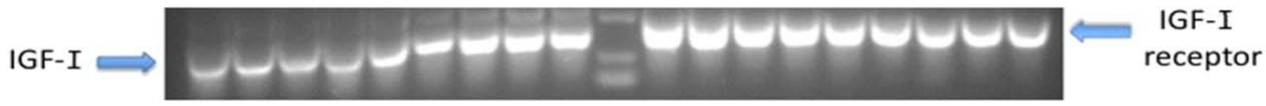


Fig. 3. Representative PCR reaction showing the GH, IGF-I and their receptors mRNA expression in the equine CL, throughout the luteal phase.

DISCUSSION

The results of the present study showed the presence of GH and IGF and their receptor in the equine CL obtained throughout the luteal phase. Like in other species, such as in human (Sharara and Nieman, 1994; Iñiguez *et al.*, 2001), ovine (Juengel *et al*., 1997a,b), bovine (Lucy *et al.*, 1993; Woad *et al*., 2000) and rat (Carlsson *et al*., 1993), both GH and IGF and their receptors were expressed throughout the luteal phase in the mare. In the mare is well-know the crucial role of GH and IGF-I in the follicular growth and selection for dominance (Ginther *et al.*, 2004). Moreover, it was shown that IGF and GH stimulate *in vitro* NO production and influenced angiogenic activity in mare´s CL. Since in the late luteal phase CL the associations between IGF and LH or GH decreased BAEC proliferation and increased NO production, these peptides might mainly contribute to vascular luteal involution possibly mediated by NO.

Several studies evidenced how GH and IGF-I act in a synergistic fashion with LH, affect luteal development and functions, including establishment, maintenance and regression of the CL, especially emphasizing the important role on P_4 production (Spicer *et al.*, 1992-1993; Lucy *et al.*, 1994). Indeed, GH acts on P_4 production both directly on luteal cells, and indirectly by IGF production that in turn exerts a luteotrophic action by an amplification of steroidogenic acute regulatory protein (StAR) (Balasubramanian *et al.*, 1997; Mamluk *et al*., 1999), via increased avaiability of steroids precursors (Veldhuis, 1989) by both increasing gene expression and

enzyme activity of the key steroidogenic enzymes such as cytochrome P450scc, 3β-HSD and aromatase (Adashi *et al*., 1985a; Veldhuis *et al*., 1986; Magoffin *et al.*, 1990; Magoffin and Weitsman, 1993; deMoura *et al.,* 1997), improving LH luteotrophic effects (Adashi *et al*., 1985b), and throughout an anti-apoptotic effects (Ptak *et al*., 2004). Their presence in the mare during the luteal phase might indicate a similar physiologic role, since biological activities of these molecules are dependent on their expression profiles, even though data are still not available to confirm this hypothesis. Apart from the confirmed effect of these factors on P_4 activity, GH and IGF system might modulate the angiogenic process during CL development, while other evidences underline their role in the regression phase (Neuvians *et al.,* 2003; Hastie and Haresign, 2006). Human GH was reported to be angiogenic *in vitro* (Rymaszewky *et al*., 1991), whereas both bovine and chicken GH were shown to be angiogenic *in vivo* (Gould *et al.*, 1995). Also the IGF system may rather have indirect effects on angiogenesis in the bovine early CL by stimulatory actions for VEGF production in luteal cells (Schams *et al.,* 2001) as well as by proliferation and differentiation of endothelial cells. Nevertheless, in our study, when mare CL obtained during the late luteal phase was stimulated *in vitro* with IGF associated with the higher doses of LH or GH, showed a decrease of the capacity to induce BAEC proliferation when compared to LH stimulation. Therefore, these data might suggest the involvement of these peptides on vascular luteal involution, even though in the cow Amselgruber *et al.* (1994) assumed for IGF- II a major role in coordinating angiogenic processes and vessel maintenance and that its loss during luteolysis may affect primarily nourishing of the vascular system. On the contrary, other studies in the early regressing CL have shown that an increase in IGF-I mRNA may be associated with immune cell activity during the cellular destruction and phagocytosis and therefore to be associated with structural luteal regression (Pate and Keyes, 2001). Thus, it appears the this study, might somehow explain the findings of the present study in the mare CL.

The hypothesis of an involvement of GH and IGF-I in vascular involution in mare is in

part reinforced from the results obtained on NO production. Since NO acts as a potent vasorelaxant and since it inhibits P_4 production by luteal cells, it has been suggested to be an important mediator of luteolysis in the cow (Skarzynski *et al*., 2000b; Korzekwa *et al*., 2004), rabbit (Boiti *et al.*, 2003), rat (Motta *et al*., 1999), and human (Vega *et al*., 1998, 2000). NO acts directly inhibiting P_4 production, modulates $PGF_{2\alpha}$ action and induces apoptosis in luteal cells by increasing DNA fragmentation and expression of FAS, BAX and Caspase-3 mRNA, pro-apoptotic proteins (Korzekwa *et al.*, 2006). In the present study, already during mid luteal phase, IGF+LH associated showed the highest NO production than control and IGF at the dose of 50ng/mL. The same IGF association mentioned, as well as its association with GH, was also responsible for a high NO concentration in the late luteal phase, time when there is maximum luteolytic activity of NO and $PGF_{2\alpha}$ on vascular tissue.

Costa *et al.* (2006) have been shown in mare's mid-luteal CL, how long-lasting *in vitro* effect of P_4 and its precursors may inhibit angiogenic factors production, preparing for CL functional and structural regression. It is possible that the high concentration of NO in this phase may pave the way to the beginning of the functional and structural regression of the CL, as it might be also plausible that elevate NO concentration associated to high levels of VEGF mRNA and protein in the mid CL of the mare (Al-zi'abi *et al.*, 2003) may suggest an intense angiogenesis process. In fact, NO may play a role in equine CL growth during early luteal development, when vascular development is more intense (Ferreira-Dias *et al.*, 2011), or be involved in vascular regression as it is shown in the present work. On the other hand, it was recently found in the mare CL that expression of nitric oxide synthase (eNOS) is greatly increased in the late luteal phase (Ferreira-Dias *et al.*, 2011), which might corroborate NO involvement in luteolysis. Nevertheless, in the cow two NOS isoforms were identified in the CL: the eNOS that synthesizes intermittent low levels of NO and the inducible form (iNOS) that is synthesized after activation by proinflammatory cytokines such as interferon gamma (INFγ),

IL-1, or TNFα, in many cell types including endothelial cells (Albrecht *et al.*, 2003). It was shown that eNOS and iNOS contributed equally to total NOS activity in young cow CL, despite the fact that eNOS mRNA in this gland was significantly higher than iNOS (Rosiansky-Sultan *et al.*, 2006). The presence of several inflammatory cell types such as mast cells (Jones and Hsueh, 1981; Mori, 1990), macrophages (Paavola, 1979; Brannstrom and Norman, 1993) and lymphocytes (Brannstrom and Norman, 1993) has been demonstrated in the ovary throughout the estrous cycle in most species and their number increases at the time of luteolysis. Also, in the mare CL $CD4^+$ and $CD8^+$ cytotoxic T cells are present (Lawler *et al.*, 1999), as well as cytokines produced by these immune cells, such as TNFα, IFNγ, IL-1 (Nothnick and Pate, 1990; Pate, 1995; Davis and Rueda, 2002; Skarzynski *et al.*, 2008; Korzekwa *et al.*, 2008) and FasL (Fas Ligand) especially during luteal regression (Suda *et al.*, 1993; Galvão *et al.*, 2010). In the present study, the high NO concentration produced by mid luteal phase CL when stimulated by IGF and LH together, might be also explained by the augment of iNOS activity in response to proinflammatory cytokines produced *in situ* (Albrecht *et al.*, 2003), and already present in mature CL.

In conclusion, the present work showed the presence of GH and IGF-I and their receptors in equine CL throughout the luteal phase. Both GH and IGF-I increased NO production in mid and late luteal phase. Particularly in this last luteal phase, it resulted in a decrease in BAEC proliferation suggesting their involvement in vascular regression via nitric oxide. Nevertheless, further studies should be performed in order to clarify if, IGF-I and GH in spite of their luteotrophic and angiogenetic role, might also participate in vascular regression through NO production.

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