A histological Study of Angiogenesis and Cell Death in the Equine Corpus Luteum

Ph.D Thesis

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Declaration

This Thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the University of Edinburgh. This Thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiments of this thesis and the work presented in it are entirely my own. Any contribution of others has been fully acknowledged.
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Omar alzi'abi
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<td>acidic fibroblast growth factor</td>
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<td>ang-1</td>
<td>angiopoietin-1</td>
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<tr>
<td>ang-2</td>
<td>angiopoietin-2</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>CL</td>
<td>corpus luteum</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ENOS</td>
<td>endothelial nitric oxide syntheses</td>
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<td>ET-1</td>
<td>endothelin-1</td>
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<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
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<td>FSH</td>
<td>follicular stimulating hormone</td>
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<td>GnRH</td>
<td>gonadotrophin releasing hormone</td>
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<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<td>hCG</td>
<td>human chorionic gonadotrophin</td>
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<td>IFNγ</td>
<td>interferon γ</td>
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<td>IGF-1</td>
<td>insulin like growth factor</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthesis</td>
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<td>LH</td>
<td>luteinising hormone</td>
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<td>PI</td>
<td>proliferation index</td>
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<td>LM</td>
<td>light microscope</td>
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<td>MMP</td>
<td>matrix metalloproteinases</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NOS</td>
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<td>OT</td>
<td>oxytocin</td>
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<td>PAS</td>
<td>periodic acid-schiff</td>
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<td>PCD</td>
<td>programmed cell death</td>
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<td>PGF_2α</td>
<td>prostaglandin F_2α</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>RER</td>
<td>rough endoplasmic reticulum</td>
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<td>smooth endoplasmic reticulum</td>
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<td>TEM</td>
<td>transmission electron microscopy</td>
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<td>TIMP</td>
<td>tissue inhibitor matrix metalloproteinases</td>
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<td>tumour necrosis factor α</td>
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<td>TNFγ</td>
<td>tumour necrosis factor γ</td>
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<td>TUNEL</td>
<td>TdT-mediated X-dUtp nick end labelling</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VPF</td>
<td>vascular permeability factor</td>
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Publications and communications

Papers


AL-zi’abi MO, Fraser H M & Watson E D. Angiogenesis and VEGF expression in the equine corpus luteum. In press.

Abstracts


Abstract

The corpus luteum (CL) is formed from the preovulatory follicle after ovulation and its main function is the secretion of progesterone, which is essential for the establishment of pregnancy. The CL is associated with massive vascularisation followed by rapid regression during luteolysis. In the non-fertile cycle, progesterone production falls and luteal structural regression takes place. If conception occurs, the CL continues to produce progesterone. The CL provides a unique example of tightly controlled angiogenesis and cell death. Little is known about these processes in the equine CL. Elucidation of angiogenesis and luteolysis during natural and PGF2α-induced regression will provide new insights into the control of the corpus luteum, increase our understanding of the physiology of the corpus luteum and may help in advancing and developing other methods for induction of luteolysis.

Corpora lutea were collected from mares in the early luteal phase: Day 3-4 (n=4), mid-luteal phase: Day 10 (n=5), early regression: Day 14 (n=4), late regression: Day 17 (n=4), and 12 and 36 h (n=3 per group) following PGF2α administration on day 10. Luteal angiogenesis was assessed by measuring cell proliferation, endothelial cell content and vascular endothelial growth factor (VEGF) mRNA and protein expression in luteal sections by quantitative immunocytochemistry. For cell death, sections were examined at both histological and ultrastructural levels and TdT-mediated X-dUtp nick end labelling (TUNEL) was used to detect DNA fragmentation. The combination of TUNEL immunostaining and ultrastructural examination provided definitive identification of the types of cell death involved in the CL. It was demonstrated that apoptotic and non-apoptotic mechanisms were involved in the demise of the CL. The presence of fragmented chromatin, pyknotic cells, round dense bodies and phagocytosis were considered as apoptotic features. Other changes (crenation of the nuclear membrane with shrinkage of the nucleus) seen in some luteal cells indicated there is an additional non-apoptotic form of cell death at luteolysis. Angiogenesis is a dynamic process of endothelial cell proliferation and microvessel formation regulated by the production of angiogenic factors, especially VEGF. It was established that the early luteal phase was associated with intense endothelial cell proliferation, expression of VEGF and the growth of the microvascular network. Angiogenesis continued during the mid-luteal phase at a reduced level. However, VEGF remained high and the full microvasculature had been established for optimal progesterone secretion. During regression, the marked progesterone decline was associated with a decline in endothelial cell proliferation, VEGF expression and reduction in vascularity. Tumour necrosis factor α (TNFα). Nitric oxide (NO) and basic fibroblast growth factor (bFGF) immunostaining in the luteal cells during the luteal phase may indicate a paracrine and/or autocrine role for these factors in the CL and may play a role in luteal development and regression. This thesis has described for the first time the cellular changes in angiogenesis and regression post PGF2α-induced regression. The findings revealed massive neutrophils and vasodilation as well as a similar pattern of other cellular changes to those undergone during natural luteolysis. This study increases our understanding of equine CL control. It demonstrates that luteal angiogenesis is important for luteal function and it is likely that VEGF is essential for luteal angiogenesis. Luteolysis and cell death play a crucial role in ovarian cyclicity and the demise of luteal tissue represents both apoptotic and non-apoptotic pathways.
Chapter 1

Introduction
1.1 Overview

Horse breeding has become an international industry and has been influenced by the demands of the thoroughbred racing industry. Manipulation and shortening of oestrous cycles is often requested by breeders to reduce wasted time between oestrus periods. The increasing use of assisted reproductive techniques, such as embryo transfer and artificial insemination in both thoroughbred mares and non-thoroughbred mares has also increased the need for tighter control of the mare’s oestrous cycle. Despite that, little attention has been paid to the physiology of the equine corpus luteum (CL) compared with other domestic species. It is important therefore to focus attention on improving our current understanding of the mechanisms and factors involved in controlling the reproductive system during the oestrous cycle and pregnancy in the mare.

The CL is an endocrine gland with a short life span of approximately 15-20 days in the non-fertile cycle (Daels and Hughes, 1993). Following ovulation, the corpus haemorrhagicum is formed and matures then regresses and finally resorbed and replaced by ovarian stroma (corpus albicans). The mature CL receives one of the greatest blood supplies, per gram of tissue, of any organ (Wiltbank et al., 1988) and demonstrates the highest proliferation rate of endothelium (Jablonka-Shariff et al., 1993). When the CL regresses, it has one of the greatest rates of cell degeneration of any tissue in the body (Zheng et al., 1994). Because luteal angiogenesis and regression are thought to be highly regulated mechanisms, and they occur during each oestrous cycle, the CL provides an outstanding and interesting tissue for investigating the regulation of both angiogenesis and cell death processes. The distinctive features of rapid formation and regression seen in this organ have encouraged reproductive scientists to investigate the biological mechanisms underpinning angiogenesis and blood supply as well as luteal regression and cell death.

Previous studies in other species have sought to elucidate the roles of each type of cell within the CL during each stage of the cycle, the nature of cell-cell communication during angiogenesis and cell death, the mechanisms underpinning the programmed turnover of cellular proliferation and death, the factors controlling
angiogenesis and the types of cell death during CL regression. Considerable progress has been made in this field in primates (including human), laboratory animals and some of the domestic animals (Niswinder et al., 2000). However, the mechanisms of angiogenesis and cell death in the equine CL remain poorly understood and have not yet been reported in the same detail as the other domestic species e.g. cattle, pig and sheep. As information obtained in other species cannot be applied to the horse due to the unique features of its reproductive biology, it is necessary to investigate the distinctive mechanisms of angiogenesis and cell death in order to gain a better understanding of the control of the equine CL which might benefit clinicians in practice.

This study will contribute to the understanding of the control of the equine CL during the oestrous cycle, by investigating the most distinct features of the organ, angiogenesis and cell death. It will also define: the changes in the luteal vascular bed and cellular proliferation rate during the oestrous cycle and after prostaglandin F\textsubscript{2a} (PGF\textsubscript{2a})-induced luteolysis, putative angiogenic factors, the periods during which the CL may be susceptible to inhibitors of angiogenesis, the onset of structural luteolysis, the type of cell death which is involved in both induced and natural CL regression and the morphological and biochemical changes occurring during both natural and induced luteolysis as well as the factors which may involved in cell death.

The following will review the biology of the equine oestrous cycle and what has been reported on the equine CL structure and function in comparison to other species. Angiogenesis, apoptosis and the factors which may be involved in these two processes in the CL will be reviewed in detail with reference to other species.

1.2 The equine ovary
The equine ovary has a unique structure among the domestic mammalian species. It is characterised by: 1- the cortical stroma, which contains different sizes of follicles and corpora lutea, lying internal to the ovarian medulla which surrounds the whole ovary with rich, vascularised loose connective tissue. 2- ovulation of the ovarian follicle occurs at a specific location in the ovary called the "ovulation fossa". 3- the CL is not palpable because its entire structure is embedded in the stroma tissue. 4- formation of secondary CL during pregnancy, 5- the luteal cells are originated from
feature of the mare. These peculiarities of the mare's ovary have presented additional opportunities to study the biological mechanisms of the CL (Mossman and Duke, 1973; Van Niekerk et al., 1975; Levine et al., 1979; Niswender and Nett, 1993).

Fig 1.1. The ovarian medulla surrounds the whole ovary with rich, vascularised and loose connective tissue. The cortical stroma lies internally to the ovarian medulla and contains different sizes of follicles (F) and the corpus luteum (CL). Ovulation occurs at a specific location in the ovary the so-called ovulation fossa (OF).

1.3 The oestrous cycle in the mare
The mare is seasonally polyoestrus. In the Northern hemisphere, mares begin to cycle in April and go through to September; only a few mares show reproductive activity during late autumn and winter (Daels and Hughes 1993). Photoperiod, temperature, and nutrition affect the cycle. The increase in day length in spring stimulates ovarian activity via the pineal gland, which decreases melatonin secretion (Sharp, 1988). Melatonin has a direct stimulatory effect on gonadotrophin releasing hormone in the mare, which leads to an increase in the level of LH and FSH (Sharp, 1988). In contrast, decreasing the daylight length increases melatonin and leads to a decline in the secretion of LH and FSH (Daels and Hughes 1993). Extending the photoperiod in winter by artificial light decreases melatonin levels and stimulates ovarian activity (Sharp and Davis 1993).

The first oestrous cycle of the breeding season shows irregularity in follicular development, but subsequent ovulatory cycles are regular (Ginther, 1990) ranging between 19-22 days in length (Hughes et al., 1972a, 1975, 1980) and on average 21
between 19-22 days in length (Hughes et al., 1972a, 1975, 1980) and on average 21 days long (Hughes et al., 1972). The oestrous cycle in ponies is longer than in horses, being on average 25 days long (Ginther, 1979). Oestrus can be identified as the period of sexual receptivity, which lasts between 3-5 days (Hughes et al., 1980). The signs of oestrus in the mare are due to the absence of progesterone, rather than the increase of oestradiol (Hughes et al., 1972a). The follicles increase in size daily by about 3-4.5 mm during oestrus. Preovulatory follicles range between 35-50 mm in diameter with an average of about 45 mm at the time of the ovulation (Watson et al., 1994).

Ovulation is a process in which the oocyte erupts from the dominant preovulatory follicle and passes to the oviduct. This process is controlled by the hypothalamus, pituitary gland (releasing LH) and the ovary itself. Ovulation is accompanied by diminishing signs of oestrus, and normally occurs 24-48 h before the end of oestrus (Hughes et al., 1972). Furthermore, in contrast to other species, the mare does not exhibit an acute preovulatory LH peak. The LH levels increase for 6-7 days prior to ovulation, reaching a maximum level usually on the day after ovulation, then decrease progressively over 4-5 days (Noden et al., 1974; Geschwind, 1975; Evans and Irvine 1975; Oxender et al., 1977, Watson et al., 1994). Ovulation in the mare occurs at a specific site the "ovulation fossa". Following ovulation, the cavity of the ruptured follicle fills with blood, the remaining granulosa cells and connective tissue to form the corpus hemorrhagicum. The granulosa cells become fully luteinised on day 3 and reach maximum size on day 9 of the cycle (Van Niekerk et al., 1975, Levine et al., 1979).

Progesterone concentrations start to rise in the blood within 24 h of ovulation and reach maximal values within 5 days, then decline during regression. The luteal phase lasts 14-15 days (Van Niekerk et al., 1975). The rise in progesterone concentration inhibits sexual signs so that the mare rejects the stallion, and exerts a negative feedback on GnRH, LH and FSH secretion, which inhibits follicular development. CL regression occurs from day 12 onwards (Van Niekerk et al., 1975), and in another study (Harrison, 1946), on day 14. It has been suggested that PGF$_{2\alpha}$ from the endometrium is responsible for CL regression (Douglas and Ginther, 1976) and has been used pharmacologically to synchronise oestrus and shorten dioestrus (Irvine, 1993). In ruminants, PGF$_{2\alpha}$ is produced by the endometrium and is transported to the
ovary by a counter-current mechanism (Ginther et al., 1972). In the mare the situation is different. The close anatomical arrangement between ovarian and uterine blood vessels does not occur in the mare. Any PGF$_{2\alpha}$ released by uterus must enter the systemic circulation to reach the ovary. However, in other species most of PGF$_{2\alpha}$ is cleared from the circulation in the lungs. The lung clearance therefore could be less effective in equine species and/or small quantities of the PGF$_{2\alpha}$ are required at the level of the ovary. Equine species are 10-18 times more sensitive to the luteolytic effects of PGF$_{2\alpha}$ when given by systemic routes than other species (Douglas and Ginther, 1973; Douglas and Ginther 1957; Oxender et al., 1975). It has been reported that the affinity of luteal cells to PGF$_{2\alpha}$ receptors is 10 times greater than that of receptors from bovine CL (Kimball and Wyngarden, 1977). This high affinity may explain the high sensitivity of the mare to the luteolytic effects of PGF$_{2\alpha}$, which could reduce the need for a local utero-ovarian PGF$_{2\alpha}$ pathway.

1.4 The corpus luteum
The CL is an endocrine gland secreting progesterone, which is essential for establishing and maintaining pregnancy. In the absence of conception the CL undergoes regression and the mare returns to pro-oestrus (follicular phase).

1.4.1 CL Structure

1.4.1.1 Angiogenesis in the CL
Angiogenesis is derived from angio meaning "blood vessels" and genesis meaning "production" and may be identified as neoformation of blood vessels from the pre-existing microvasculature. Angiogenesis has in recent years become the focus of intensive interest in diverse fields of basic scientific and clinical research. Blood vessel growth appears to play a vital role in early development, as well as in various physiological and pathological events. Angiogenesis is a fundamental feature of embryonic development and organogenesis, as circulatory branching is essential in sustaining newly developing structures and tissues. Angiogenesis is physiologically significant in its association with placental, follicular and CL formation, inflammation, wound healing, and tumour growth (Bussolino et al., 1997).

Judah Folkman, the pioneer of angiogenesis research, proposed the following chronology of events in the neovascularization process (Folkman, 1990; Folkman
and Klagsbrun, 1991). 1- local basement membrane degradation of the parent vessel by endothelial cells. 2- locomotion of endothelial cells away from the parent vessel in the direction of an angiogenic stimulus. 3- elongation and alignment of migratory endothelial cells to form a capillary sprout. 4- endothelial cell proliferation in the parent venule and in the capillary sprout. 5- lumen formation. 6- anastomosis of two hollow sprouts to form a capillary loop. 7- onset of blood flow. 8- formation of pericytes.

The structure of the equine ovary is unique, the ovary is surrounded by connective tissue rich of network of blood vessels and this blood supply rich it maximum peak during estrous heat and pregnancy. The surface of the ovary and the mesovarium were supplied with an extensive arterial network, which formed anastomoses with trabeculae of corpus luteum. The blood supply for the ovary comes form the ovarian artery; a branch of the aorta and branches of the uterine ovary supply the ovary too (Ginther et al., 1972). Blood vessel growth is rarely observed in normal adult tissue except in tissues, which exhibit periodic growth and regression such as the CL of the ovary, the human endometrium, hair follicles and the placenta (Reynolds et al., 1992). This process in comparison with pathological events is orderly, limited and tightly regulated (Reynolds et al., 1994; Redmer and Reynolds, 1996). During follicular growth, the theca layer becomes vascularised and rich with blood supply especially when the follicles reach the point of ovulation. After ovulation, the thecal vessels invade the ruptured follicle and form a complex network of vessels to supply the developing CL. This growth of blood vessels in the CL is crucial for progesterone secretion and thus the maintenance of pregnancy (Niswender and Nett, 1988). The mature CL is highly vascular and most of the luteal cells are adjacent to one or more capillaries (Zheng et al., 1993; Redmer and Reynolds, 1996). Quantitative studies have revealed that endothelial cells constitute more than 50% of cells present in the mature CL. This is not surprising, because approximately 85% of CL cells, which proliferate during the early luteal phase are endothelial cells (Reynolds et al., 1994; Christenson and Stouffer, 1996, Nicosia et al., 1995). Moreover, the CL receives most of the ovarian blood supply. This blood flow is highly correlated with progesterone concentrations in the circulating blood (Mangess et al., 1983; Reynolds et al., 1994). During the early luteal phase, intense proliferation was found in the endothelial cells in sheep (Jablonka-Shariff et al., 1993), cow (Zheng et al., 1994), human (Rodger et al., 1997) and marmoset (Young et al., 2000) In contrast, the late
luteal stage has been associated with decreased luteal vascularity (Smith et al., 1996, Reynolds et al., 1994).

1.4.1.2.1 Factors involved in angiogenesis

Angiogenesis is regulated by a number of positive and negative regulators (Table 1.1), which determine if the local vasculature is growing or regressing. A loss of control of the regulating mechanisms could cause a number of diseases that would be characterised by either persistent, unabated neovascularization or vascular insufficiency (Bussolino et al., 1997). Angiogenic factors can be classified according to a variety of functions, including effects on endothelial cells, the mechanism of action, and the molecular form. Many of the angiogenic agents that induce endothelial cell motility are believed to be chemotactic factors, while agents that induce proliferation generally belong to the growth factor family (Folkman and Klagsbrun, 1991). The factors which induce endothelial cell motility or proliferation may act directly on endothelial cells, while others are thought to act indirectly. In the past ten years most attention has been paid to the study of the following angiogenic factors in the CL: basic Fibroblast Growth Factor (bFGF), acidic Fibroblast Growth Factor (aFGF) Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Platelet-derived Growth Factor (PGF), MMP's, TIMP's, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2) and angiogenin.

<table>
<thead>
<tr>
<th>Positive regulators</th>
<th>Negative regulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular endothelial growth factor</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>Placental growth factor</td>
<td>Angiostatin</td>
</tr>
<tr>
<td>Basic Fibroblast growth factors</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>Transforming growth factors</td>
<td>Prolactin 16-kd fragment</td>
</tr>
<tr>
<td>Matrix metalloproteinases</td>
<td>Tissue inhibitor matrix metalloproteinases</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>Platelet factor 4</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>Genistein</td>
</tr>
<tr>
<td>NO</td>
<td>Placental proliferin-related protein</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>Transforming growth factor beta</td>
</tr>
</tbody>
</table>

Table 1.1: A list of some positive and negative regulators of angiogenesis
FGFs are highly potent angiogenic factors shown to stimulate blood vessel development in *in vitro* models. FGF-1 and -2 are bound to low-affinity heparin sulphate sites in the extracellular matrix, and are released by heparitinase or matrix-degrading protease such as plasminogen activator, which then bind to high-affinity cellular receptors to exert their biological effects (Folkman and Klagsbrun, 1991). bFGF plays a role in luteal angiogenesis, an increase of bFGF protein or mRNA in the early stages of the luteal phase. However, bFGF protein or mRNA levels are similar or increased throughout the oestrous cycle in the bovine CL (Zheng *et al.*, 1993). High expression of FGF receptors (FGFR-1) in the luteal cells was found at mid-luteal phase then reduced during late regression in the ovine CL (Reynolds *et al.*, 1996). In addition, strong staining for FGFR-1 was found in endothelial cells during all phases and there was abundant staining in the large microvessels in late regression. The same findings were found for FGFR-2 in the ovine CL (Doraiswamy *et al.*, 1995). The maintenance of the large microvessels in the late stage and the strong expression of the FGF could explain however how these large microvessels in the CL remain while the luteal cells regress. Another study has shown that bFGF stimulates the cell proliferation of rabbit CL endothelial cells *in vitro* (Bagavandoss and Wilks, 1991). It has also been reported that FGF inhibits luteal cell death (Tilly *et al.*, 1992; Yasuda *et al.*, 1995). Thus, it is apparent from the above studies in the CL that FGF stimulates cell proliferation and prevents cell death (Redmer and Reynolds 1996).

Vascular endothelial growth factor (VEGF, also referred to as vascular permeability factor, or VPF) is another heparin-binding endothelial cell growth factor, which has angiogenic activity *in vivo* (Kim *et al.*, 1993). VEGF acts directly on endothelial cells to induce cell proliferation (Kim *et al.*, 1993) and migration (Koch *et al.*, 1994). The action of VEGF is mediated by two tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) found on endothelial cells. Studies have shown that VEGF may be induced by hypoxia to promote angiogenesis in ischaemic tissues (Shweiki *et al.*, 1992). It is possible that the low oxygen tension in embryonic tissues and developing organs likewise induces VEGF to promote neovascularization of these regions. In the CL, it is believed that angiogenesis in the CL is dependent on gonadotrophin stimulation (Yan *et al.*, 1998; Neulen *et al.*, 1998; Dickson and Fraser, 2000). It has been suggested that microvascular hyperpermeability is a crucial
feature of angiogenesis, and that VEGF, as a unique inducer of vessel permeability, may be a ubiquitous player in the neovascularisation process (Shweiki et al., 1992).

Recent investigations have indicated that VEGF plays a pivotal role in the process of angiogenesis in the CL. VEGF is a specific trigger for vascular permeability and vascular endothelial cell protease production, proliferation, and migration which represent the essential elements of angiogenesis in the CL (Ferrara, 1996). The expression of VEGF mRNA by perivascular cells has been documented in female mice during the neovascularization of both ovarian follicles and the CL (Shweiki et al., 1993). VEGF mRNA is highly expressed in the early luteal phase compared with the late luteal phase in monkey CL and can also be reduced by GnRH antagonist treatment (Ravindranath et al., 1992). Human luteal cells have also been shown to express VEGF (Yan et al., 1993, Kammat et al., 1995, Otani et al., 1999). VEGF receptors are highly expressed in endothelial cells in the early stages of the CL formation. The expression of these receptors is not affected by the down-regulation of VEGF expression during regression (Goede et al., 1998). Recently, progress has been made in testing the hypothesis that VEGF is a crucial element for CL angiogenesis. It has been reported that the in vivo treatment with truncated soluble FIT1-3 IgG receptors suppress the growth of blood vessels and endothelial cell proliferation, resulting in a significant and complete inhibition of rat CL angiogenesis. This is the first report to demonstrate the significance of angiogenic inhibitors in CL angiogenesis (Farrara et al., 1998). A more recent finding has supported the suggestion that VEGF is essential for CL angiogenesis. Marmoset monkeys treated with anti-VEGF showed a decrease in endothelial proliferating cells and a reduction in the microvessel bed during early development of the CL, with subsequent luteal function being reduced by 60% compared with controls (Fraser et al., 2000). It has been shown that VEGFR2 (Flk-1/KDR) is the most important receptor mediator of VEGF in the bovine CL (Berisha et al., 2000).

Angiopoietin-1 (Ang-1), an angiogenic factor and a ligand of endothelial cell receptor tyrosine kinase Tie-2, plays a role in blood vessel maturation and stimulating blood vessel growth by cultured endothelial cells in vitro (Koblizek et al., 1998). Angiopoietin-2 (Ang-2) appears to inhibit Ang-1 by binding to Tie-2, preventing any active signals of Ang-1. The ratio of these two factors appears to play a crucial role in regulating blood vessel maturation. High levels of Ang-2 have been
found during CL regression, Ang-1 is expressed only in the early stages of bovine CL formation (Maisonpierre et al., 1997; Goede et al., 1998).

Endothelin-1 (ET-1) is a peptide synthesised and secreted by endothelial cells in response to the changes in blood flow, blood pressure and oxygen tension. Recent studies indicated that the involvement of ET-1 in both angiogenesis and luteolysis. ET-1 has been implicated as a possible mediator of the effects of PGF$_2\alpha$ on blood flow (Girsch et al., 1996). PGF$_2\alpha$ stimulates the local endothelial cells in the CL to secrete ET-1 \textit{in vitro} (Girsch et al., 1996) and \textit{in vivo} (Ohtani et al., 1998). This peptide plays a role in regulating luteal steroidogenesis and it has been reported that ET-1 can inhibit progesterone secretion \textit{in vitro} (Girsch et al., 1996a). Pre-incubation with PGF$_2\alpha$ potentiated the inhibitory action of the ET-1 on progesterone production (Miyamoto et al., 1997). Only large luteal cells responded to ET-1 with, an inhibition of basal and cAMP-stimulated progesterone production, even though receptor subtypes were found in both large and small cells (Girsch et al., 1996a). The involvement of ET-1 in luteal regression suggested that PGF$_2\alpha$ may upregulate ET-1 expression (Girsch et al., 1996).

Several other angiogenic modulators are currently undergoing investigation. For example, TIMP-1 and -2 have been shown to inhibit tumour invasion and angiogenesis in vitro, by inhibiting metalloproteinase activity and thereby blocking tumour and endothelial cell motility (Ray et al., 1994). Quite interestingly, it has been shown recently that TIMP-2 can inhibit bFGF-induced endothelial cell proliferation by an undefined mechanism that is independent of metalloproteinase inhibition (Murphy et al., 1993). Bagavandoss and Wilks (1991) showed that FGF stimulates the proliferation of endothelial cells \textit{in vitro} while PDGF, EGF, ILGF-1, histamine, prostaglandins, sex steroids and interleukin-6 had no effect on endothelial cell proliferation.

Angiogenesis however has yet to be documented in the equine CL. Investigation of endothelial cell proliferation, death and communication with other types of cells within the CL during the oestrous cycle, will lead to a better understanding of endothelial cell function and angiogenesis.
1.4.1.2 Cellular composition of the CL

The equine CL is irregular with a mushroom or gourd shape (Ginther, 1992). The CL is localised inside the ovary. When cut in cross section it exhibits trabeculated appearance, which is a unique feature of the mare. The trabeculae form from the collapsed large follicle at the time of ovulation; the inner walls of the postovulatory follicle are thrown into small and large folds, which invade the cavity of the corpus hemorrhagicum. These trabeculae have a central core of connective tissue and large blood vessels. As the CL develops and the cells start to luteinise, the trabeculae diminish in size and become merely a support for the extensive vascular network passing into the CL (Harrison, 1946; Van Niekerk et al., 1975). The CL in the horse, as in other mammals, contains hormone-producing cells, which are called luteal or steroidogenic cells, as well as non hormone-producing cells e.g. endothelial cells, pericytes, fibroblasts, macrophages and leukocytes.

The origin of the equine luteal cells from the granulosa and theca internal cells of preovulatory follicle remains unclear. The theca internal cells proliferate and develop morphologically and functionally prior to ovulation. After ovulation, the theca internal cells are scattered sparsely at the outer edge of the luteinising granulosa cells and are carried out to the ends of the trabeculae folds of the mural granulosa. These findings indicate that both granulosa cells and theca internal cells in the preovulatory follicle are involved in forming the equine CL (Harrison, 1946). Despite that and quite surprisingly, the same study concluded that the granulosa cells are the only cells that form the CL.

In another study, the theca internal cells were shown to undergo regression, and show condensation, pyknosis, fragmentation and phagocytosis around ovulation (Van Niekerk et al., 1975). These cells are replaced by hypertrophied fibroblasts, which can easily be confused with theca cells, giving the impression that theca cells contribute to luteal tissue. These authors also concluded that the equine CL is derived from granulosa cells only. Van Niekerk et al., (1975) data were based on morphological changes at light microscope level with (x640) magnification. Pierson et al., (1982) reported in their abstract that equine luteal cells originated from both theca internal and granulosa cells; this data were based on a histochemistry technique. Harrison (1946) suggested that the trabeculae of the CL contained cells originated from theca internal. Levine et al (1979) and Roser and Evans, (1983) did
not consider the contribution of the small cells. A recent study showed degenerative features in the theca interna cells and concluded that the luteal cells are derived from the granulosa cells (Kerban et al., 1999). In most domestic mammalian species the luteal cells are derived from both theca internal and granulosa cells, e.g. sheep (McClellan et al., 1975, O'Shea et al., 1980); cattle (Alila and Hansel, 1984); pig (Cornor, 1919); human (Cornor, 1956, Fujiwara et al., 1992). However, the controversy surrounding the origin of luteal cells in the mare remains with some scientists supporting Van Niekerk et al., (1975) findings and others not. Our primary investigation to identify theca interna cells using CD 13 antibody and human CL as a positive control (Fujiwara et al., 1992) failed because CD 13 failed to recognise luteal cells with theca interna origin and did not show any cross reactively with the equine tissue.

Luteal cells
The equine CL has two types of luteal cells: small cells known as dark cells and large cells known as light cells. These two types of cells are thought to originate from the granulosa cells only (Van Niekerk et al., 1975).

Large cells: By day 4 after ovulation, all the granulosa cells are fully luteinised and reach a maximum size on day 9 when the cells measure 37.5µm and the nuclei 10µm in diameter (Van Niekerk et al., 1975). These cells are spherical to polyhedral in shape, lightly stained, have lipid droplets in their cytoplasm, with large spherical to vesicular shaped nuclei with one or more nucleoli. The nuclei have jagged nuclear membranes and sparse heterochromatin, which is restricted to a thin layer on the inner surface of the nuclear membrane. Perichromatin granules were also found in the nucleus (Van Niekerk et al., 1975; Levine et al., 1979) based on examination at EM and LM level. The ultrastructural morphology indicates that these cells are hormone-producing cells (Levine et al 1979): On day 9 these cells contain 1- mitochondria: abundant with round to elongated shape, the cristae of these mitochondria are tubular or lamellar in form, the matrix of the mitochondria in some of the cells are rarefied. 2- golgi apparatus: confined to the perinuclear areas, smooth coated vesicles about 50 nm in diameter. 3- rough endoplasmic reticulum (RER): vesicular to tubular in shape, and distributed between the SER and the mitochondria, the juxtanuclear area has long cisternae of RER 4- smooth endoplasmic reticulum (SER): the predominant organelle in the cytoplasm which has various shapes and is
organised into parallel cisternal arrays or small cisternal whorls. 5- in mid-luteal phase a few lipid droplets are seen as solitary figures. 6- groups of dumbell, padlock-shaped, multivesicular bodies and round dense bodies are abundant, often seen in the peripheral areas of the cytoplasm and surrounded by SER.

**Small cells:** These cells have homogenous condensed eosinophilic cytoplasm with a darkly stained nucleus of about 5-6 μm in diameter. These cells increase in number during the development of the CL. They constitute about 15% of the luteal cell population on day 7, and remain at this percentage until day 10. Thereafter they increase to 25% on day 12 and 60% on day 14, and all the remaining luteal cells after day 16 are dark-stained cells. It is considered that these small cells stop converting to large cells at the start of the regression of the CL (Van Niekerk et al 1975).

It has been suggested that the large luteal cells are derived from the granulosa cells, whereas the small cells originated from the theca interna cells in sheep (McClellan et al., 1975, O'Shea et al., 1980); cattle (Alila and Hansel, 1984); pig (Corner, 1919) and human (Corner 1956; Fujiwara et al., 1992). The morphological and the ultrastructural features of luteal cells are not the same in large and small cells, because of their different function and origin. The most distinctive features between the two cells are: the small cells do not have RER and secretory granules, and there is no evidence of exocytosis in small cells as well as the presence of small numbers of dense membrane-bound granules. In large cells there are several whorls of SER and stacks of RER in their cytoplasm and there is evidence of exocytosis. The features of both cells are described in sheep (O'Shea et al 1979; Farin et al 1986; Rodgers et al 1984), cattle (Parry et al., 1980; O'Shea et al., 1989; O'Shea et al., 1990), pig (Corner, 1919) and human (Corner, 1956). It has been suggested that the large luteal cells exhibit mitotic activity in the early stages of luteal development (0-4 days) but do not proliferate during the mid-luteal phase (O'Shea et al., 1986), while the theca interna cells and non-luteal cells may continue to proliferate until the onset of luteal regression (McClellan et al., 1975). It has been shown that luteal cells and non-luteal cells continue proliferation until day 12 then decrease between day 12-15 (Jablonka-Shariff et al., 1993).
Endothelial cells: The nucleus is irregular with prominent aggregates of heterochromatin, and a large nuclear to cytoplasmic ratio. They have distinct basal lamina and few organelles in the cytoplasm (Levine et al., 1979).

Hypertrophied fibroblasts: These cells have been found in the equine CL in the vicinity of the capillaries, and originate from the stroma cells to replace the degenerated theca interna cells. The nuclei of these cells are vesicular, 10 μm in diameter and have no prominent nucleoli. These cells are spindle shaped with little cytoplasm. These cells decrease gradually in number and on day 6 few of them remain and by day 9 none are present (Van Niekerk et al., 1975).

Macrophages and leukocytes: Macrophages have an irregular shape due to many surface protrusions and distinctive large inclusions as well as heterophagic vacuoles. The nuclei of these cells are also irregular with a dense border of heterochromatin (Paavola, 1979). The number and distribution of both macrophages and white cells in the CL differ among species as well as during the luteal phase (see section 1.4.1.4.9).

1.4.1.4 Luteolysis

CL regression comprises two processes: functional and structural regression. Functional regression refers to the suppression and sharp decline in plasma progesterone levels, while structural regression refers to the loss and elimination of luteal tissue from the ovary. The regression of the CL in domestic mammals is monitored by the decline of progesterone concentrations in the plasma. Luteal regression can be examined morphologically in order to explore the degenerative changes and estimate the degree of luteolysis. Furthermore, ultrasonographic evaluation of the equine CL is an efficient tool to allow visual assessment to determine development, maintenance and regression (Pierson et al., 1985; Townson et al., 1989).

1.4.1.4.1 Progesterone concentrations

The concentrations of progesterone in plasma have been found to increase rapidly after ovulation, to reach a maximal level within 5 days (3-5 ng/ml) remaining until day 11, then decline with CL regression (Van Niekerk et al., 1975). In other studies, the concentration of progesterone has been found to rise to a peak on day 6, and
remain until day 14 or 15 afterwards declining in parallel with luteal regression (Allen et al., 1974; Noden et al., 1975). The variation in these studies might reflect biological variability among different types of breed or technical precision in detecting progesterone level in the plasma.

1.4.1.4.2 Ultrasonographic evaluation
The CL exhibits functional and morphological changes during the oestrous cycle. Frequent Ultrasonographic scanning can be useful to examine and follow the status of the CL from ovulation to regression. The day of ovulation (day 0) can be considered the reference point of CL formation when the large follicle disappears and becomes the corpus haemorrhagicum (Ginther and Pierson, 1984). The ultrasonographic evaluation of luteal change can be accomplished by daily scanning allows us to measure the height and the width of the CL (Pierson and Ginther, 1985); examining the gray-scale values (Pierson and Ginther, 1985); evaluating the percentage of the echoic and non-echoic tissue (Pierson and Ginther, 1985), and by applying computer-assisted image analysis (Townson and Ginther, 1989).

1.4.1.4.3 Histological changes during luteolysis
The decline of progesterone indicates that the steroidogenic cells producing this hormone are subject to functional and structural changes. On day 16 of the equine oestrous cycle, the luteal cells decrease in size, and show two different forms of regression on day 20. Pyknosis characterised by condensation and shrinkage in the cytoplasm and karyolysis characterised by a complete dissolution of the nucleus with loss of chromatin, some nuclei undergoing karyolysis without becoming pyknotic; the early phase of these processes begins from day 12 onwards (Van Niekerk et al., 1975). The ultrastructural features of the luteal cells on day 15 of the cycle show abundant heterochromatin and a dilated nuclear membrane associated with shrinkage in the peripheral mitochondria; some of the mitochondria exhibit rarefied matrices. The SER undergoes vesiculation associated with condensation and dilation. The absence of multivesicular bodies, the swelling of endothelial cells and the partial occlusion of the vessels are signs of degeneration in the equine CL. These changes are described as signs of degeneration in the equine CL (Levine et al., 1979). In another study reduction in the size of the CL and luteal cells was found on day 14 of
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the cycle; but description of the histological changes during regression was not reported (Harrison, 1946).

1.4.1.4.4 Cell death: apoptosis and necrosis

For every cell, there is a time to live and a time to die. As far as cell biology is concerned there are two recognised forms of cell death: physiological (apoptosis) and pathological (necrosis) (Kerr et al., 1972). Necrosis (pathological or accidental cell death) is a process in which the cells are killed or injured by physical or chemical agents, and afterwards undergo a series of changes. The cell membrane loses its selective permeability and ion-pumping capacity as a direct result of membrane damage, which leads to swelling of the cell and its organelles, including the mitochondria. The cell components are released into the extracellular space leading to inflammation of the surrounding tissues. Necrosis lasts a long time and occurs in whole fields of damaged cells. The activation of enzymes such as hydrolases, phospholipases, proteases, RNases, and DNases leads to further degradation of membranes, proteins, RNA and DNA (Vermes and Haanen, 1994).

However, apoptosis (physiological cell death or committing suicide) is a genetically programmed process that controls the balance between cell proliferation and cell death. This programmed cell death (PCD) is an active mechanism, needed to eliminate cells, which represent a threat to the integrity of the organism (e.g. aged, unwanted or sublethally damaged cells) by ingestion by macrophages or nearby cells. Apoptosis is also needed for proper development of the organism (Vermes and Haanen, 1994). The term apoptosis is derived from the Greek word apo "apart" and ptosis "fallen", meaning the shedding of leaves from trees (Stedman's medical dictionary, 1995). PCD occurs during embryogenesis and continues during post neonatal life as "apoptosis" (Vermes and Haanen, 1994).

The cellular morphological changes in apoptosis are loss of cell junctions and specialised membrane structures such as microvilli. The cell membrane and the mitochondria initially remain intact, the plasma membrane shows budding, the cytoplasm condenses and the nucleus coalesces into several large masses, which then break up into membrane bound apoptotic bodies. The endoplasmic reticulum transforms into vesicles that fuse with the cytoplasmic membrane. Apoptosis minimises the release of the cellular contents from dying cells to adjacent cells, and
so does not stimulate an inflammatory response (Wyllie et al., 1980). The difference between apoptosis and necrosis is explained in table 1 and figure 1 below. Biochemically, apoptosis is characterised by degradation of chromatin into large fragments of 50-300 kilobases then into smaller fragments (Steller, 1995). Biochemical studies of apoptosis showed an increase in the levels of the protein clusterin and caspase (Pearse et al., 1992).

1.4.1.4.5 Detection of apoptosis

There is not yet a single specific cytochemical technique available for detection of apoptosis. Techniques which provide data about cell death lack qualitative and quantitative precision due to the difficulties in measuring apoptosis. The only reliable technique available is TEM, by which apoptosis and other types of cell death can be distinguished by examining the ultrastructural changes in the tissue. Common methods, which have been described to detect apoptosis in tissue and in cell suspensions, are:

Techniques Based on Morphological Changes

A-Light microscopy:
Morphological examination is still the standard method for the detection of apoptosis. The proportion of apoptotic cells in a population can be quantified by counting cells exhibiting apoptotic features and immunostained for TdT-mediated X-dUTP nick end labelling technique (TUNEL).

B-Electron microscopy:
The importance use of EM in investigating cell death types has been highlighted by many studies (Wyllie et al., 1980, Fraser et al., 1999). The high power magnification of EM allows us to observe the ultra-cellular changes in degenerated cells which cannot be detected by light microscope. Ultrastructural examination is a reference tool by which apoptosis and other types of cell death can be distinguished. This technique is used to obtain qualitative information on ultrastructural changes during cell death.
Techniques based on DNA fragmentation

A-Detection of endonuclease activity:
This biochemical method is the most common technique used for detection of apoptosis. Endogenous DNA substrate (apoptotic nuclei chromatin) can be used. It is believed that the linker regions between nucleosomes are the DNA targets for apoptotic-endonuclease attack, resulting in fragments of 180-200 bp which may be assessed by the appearance of a ladder of bands on a conventional agarose electrophoresis gel (Wolfe et al., 1996, Young et al., 1998, Pederson et al., 1998). This type of assay is not sensitive enough to detect individual apoptotic cells and is unable to differentiate between cell types undergoing apoptosis.

Fig 1.2 The morphological difference between apoptosis and necrosis. The illustration was performed in paint-shop software using the information in table 1. 1- Normal cell 2- Shrinkage 3- Budding and fragmented DNA 4- Apoptotic bodies and phagocytosis 5- Swelling 6- Vacuolisation, lysis 7- Inflammation and infiltration of leukocytes
B-Labelling of DNA strand breaks:
Apoptosis is associated with endonuclease activation, which leads to extensive DNA cleavage and formation of a large number of DNA strand breaks. The presence of the 3'hydroxyl-termini of the strand breaks can be detected by labelling with tagged nucleotides (e.g. biotin-dUTP, digoxigenin-dUTP, fluorescein-dUTP) in a reaction stimulated by exogenous enzymes such as: terminal desoxynucleotidyl transferase (TdT) or DNA polymerase (Gavrieli et al., 1992). Fluorochrome-conjugated avidin or digoxigenin antibodies are used in a second step of the reaction to make individual cells suitable for detection. The commonly used techniques for the detection of apoptosis are the in situ nick (ISN) labelling technique or TUNEL. Both techniques are applicable for conventional histological sections (Wijsman et al., 1993) and for cell-suspensions using flow cytometry (Gorzyca et al., 1993; Darzynkiewicz and Li, 1996). A simple, single-step procedure has been developed recently; utilising desoxynucleotides directly conjugated to fluorochromes (Li and Darzynkiewicz, 1995; Darzynkiewicz and Li, 1996). This single-step procedure utilises BrdUTP instead of digoxigenin or biotin conjugated triphosphodeoxynucleotides, which increases the sensitivity of the assay by giving a four-fold higher signal. This technique is still widely used in combinations with other methods e.g. EM and DNA extraction in detecting apoptosis in the tissue sections and cell suspension.
<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Origin</strong></td>
<td>genetic, hormonal and growth factors impact</td>
</tr>
<tr>
<td>cells affected</td>
<td>individual scattered cells</td>
</tr>
<tr>
<td>DNA degradation</td>
<td>internucleosome cleavage</td>
</tr>
<tr>
<td>visible features</td>
<td>shrinking</td>
</tr>
<tr>
<td>membrane integrity</td>
<td>initially intact</td>
</tr>
<tr>
<td>the morphology of the surface</td>
<td>protrusions and budding</td>
</tr>
<tr>
<td>changes in the nucleolar</td>
<td>chromatin condensation and margination associated with pyknosis</td>
</tr>
<tr>
<td>Changes in the cytoskeletal organelles</td>
<td>formation of apoptotic bodies</td>
</tr>
<tr>
<td>cytoplasmatic changes</td>
<td>protein denaturation</td>
</tr>
<tr>
<td>protein synthesis</td>
<td>affected by inhibitors</td>
</tr>
<tr>
<td>the period of the process</td>
<td>short, ranges between minutes to hours</td>
</tr>
<tr>
<td>cell abolishing</td>
<td>ingesting by macrophages and nearby cells</td>
</tr>
</tbody>
</table>

Table 1.2 Morphological and biochemical comparison between necrosis and apoptosis
1.4.1.4.6 Apoptosis and luteal regression in mammals

Azmi and O’Shea (1984) described for the first time apoptosis and the degenerative changes in luteal endothelial cells during luteolysis in guinea pigs. Since then, more interest has been focused on apoptosis in the ovary and the CL and a flurry of articles (more than 100) have been published so far on the subject of apoptosis in granulosa cells and luteal tissue in both domestic and laboratory animals. Apoptosis has not yet been reported in the equine CL, but Pederson et al., (1998) showed that the granulosa cells undergo apoptosis in the ovarian follicles.

The majority of the studies describing apoptosis in the CL have utilised morphological examination at light microscope level, DNA extraction and/or TUNEL. Apoptosis has been found in the bovine CL in the late luteal phase and after PGF$_{2a}$ treatment using DNA extraction (Jungel et al., 1993). Luteal cells, endothelial cells and fibroblasts showed positive staining for TUNEL and morphological features of shrinkage and condensation on days 18-21 in the normal bovine cycle (Zheng et al., 1994). In the ovine CL, apoptotic cells were found in regressed CL but not in healthy CL (Kenny et al 1994). Non-luteal cells showed apoptosis earlier than luteal cells in pregnant pigs and after PGF$_{2a}$-induced regression using DNA extraction and TUNEL (Bacci et al., 1996). In human CL, apoptosis was found in large and small luteal cells during regression using morphological examinations and TUNEL (Shikone et al., 1997; Yuan et al., 1997). Apoptosis was present in natural and induced CL regression in monkeys and was increased significantly in the late luteal phase compared to the mid-luteal phase luteal phase with numerous apoptotic bodies appeared in clusters or small irregular bodies in late regression. TUNEL showed positive staining in both luteal cells and non-luteal cells in both natural and induced regression (Young et al., 1997). In another study, two types of cell death, apoptosis and necrosis were found in the luteal cells after treatment with PGF$_{2a}$ and GnRH antagonist (Fraser et al., 1995). The enzymes involved in DNA cleavage (endonuclease activity) during cell death in both granulosa and luteal cells in the rat were also demonstrated (Zeleznik et al., 1989).

The hamster CL regresses rapidly and a high incidence of apoptosis were found on day 3 of the cycle. Luteal, endothelial and neutrophil cells showed positive staining for apoptosis. In addition, hamster CL showed a unique regression feature of a huge
influx of neutrophils on day 3, which was considered to be a marker of luteolysis in this species (McCormack et al., 1998).

However, few studies used ultrastructural examination to detect apoptosis in the CL. During luteal regression in the guinea pig, endothelial cells showed morphological changes that fit the ultrastructural features of apoptosis (Azmi and O’Shea, 1984). Classic features of apoptosis were found in the nuclei of the luteal, endothelial and fibroblast cells of the ovine CL after PGF2α treatment (Sawyer et al., 1990). In dogs, the CL regresses by day 70-90 of the cycle, apoptosis was considered to be a major element causing shrinkage in the luteal cells associated with pyknotic nuclei, disruption of organelles, myelin figures and whorl formation in the SER. Signs of advanced apoptosis seen in the CL included invading macrophages, fibroblasts and collagen by late regression. As the membranes were damaged, the nuclear remnants and the cellular debris were ingested by macrophages or nearby cells (Dore, 1989). Fraser et al. (1999) described morphological changes during natural regression and after GnRH and PGF2α treatment under LM and EM. The findings showed that regression in the marmoset CL appears to involve predominantly non-apoptotic mechanisms.

It is apparent that there are major species differences in the control of CL during luteolysis (Berhman, 1993). This had led to a difficulty in understanding the mechanisms involved in luteolysis in between species and makes the investigation of luteolysis more complicated. The study of CL function and regression not only differs among the species, but there are also some marked variations between studies within species, which most likely reflects differences in the techniques, tissue collection, preservation and processing. For example, apoptosis is significantly increased in mid-luteal phase in the human (Shikone et al., 1997), whereas in the monkey it is observed in the mid-luteal phase but is significantly increased during regression (Young et al., 1997). In the cow, apoptosis is found only on days 18 to 21 (Zheng et al., 1994). Another example is that a recent study showed non-apoptotic changes in the marmoset CL (Fraser et al., 1999) using ultrastructural examination. The same CL samples however showed apoptosis using TUNEL immunostaining (Young et al 1997). Definitive identification of apoptosis relies upon examination of the cells at the ultrastructural level (Wyllie et al., 1980). Recent studies on cell death
during luteolysis questioned the involvement of apoptosis in those studies which utilising TUNEL and DNA extraction (Fraser et al., 1999, Morles et al., 2000).

1.4.1.4.7 Monitors and regulators of apoptosis

Apoptosis is regulated by a number of positive and negative regulators. The balance between the two groups of regulators will determine the state of the tissue. Some of the molecular components of the process of apoptosis have been investigated intensively, particularly the genes which regulate and monitor this process.

It has been suggested that the Fas (APO-1/CD95) receptor, a transmembrane protein, induces apoptosis in the cell when bound to Fas ligand (FasL) (Suda et al. 1994). Fas antigen is thought to be a trigger of apoptosis in luteal cells and ovarian surface epithelial cells. Fas antigen was expressed in both granulosa and luteal cells in the human ovary; these cells were susceptible to Fas mAb-induced apoptosis (Quirk et al., 1995). The expression of Fas and Fas ligand was present in the granulosa cells of atretic follicles and in luteal cells during regression in the rat. This suggested that the Fas receptor and FasL might play an important role in apoptosis during luteolysis (Kim et al., 1998; Roughton et al., 1999).

Bcl2 family of oncoproteins interact to regulate programmed cell death. Various homodimers and heterodimers formed by proteins in this family can either inhibit or promote cell death. Pro-apoptotic members of this family include Bax, Bcl2-ss, and Bad. Apoptotic inhibitors in this family include Bcl2, Bcl2-sl, Bcl2-xb. The proto-gene Bcl2 is localised on the intracellular membranes of mitochondria, endoplasmic reticulum, and nuclei of healthy cells (Reed, 1994). Although the mechanism of Bcl2 action is unknown, biochemical studies have implicated this protein in the regulation of the cellular redox potential. The proto-gene Bcl2 is an inhibitor of apoptosis. Studies on the bovine CL showed that during regression in cattle, mRNA encoding Bax was elevated while mRNA encoding Bcl2 remained unchanged (Rueda et al., 1997). However, Bax and Bcl2 in the human CL were not linked to cell death but had an alternative function (Rodger et al., 1995, 1998). Bcl2 and other members of the Bcl2 family are expected to play an important role in inhibiting apoptosis and prolonging the life span of the CL. The relative levels of Bax and Bcl2 determine the fate of the cell rather than the absolute concentration of either (Oltavi et al., 1993; Yin et al., 1994).
The widely studied p53 tumour suppressor has been found to induce either cell cycle arrest or apoptosis through transcription factor regulation of several genes including the cell cycle inhibitor p21 and the apoptotic inducer Bax. The p53 protein is an anti-proliferation transcription factor that enhances the rate of gene transcription important for p53-dependent function (Tilly et al., 1995). P53 is believed to be involved in DNA repair, apoptosis cell cycle arrest during G1 to S phase, and is present in apoptotic granulosa cell nuclei of atretic follicles in the rat (Tilly et al., 1995). Moreover, p53 was also found in apoptotic atretic follicular cells expressing both Fas and Fas L (Kim et al., 1999). During regression in cattle, mRNA encoding Bax is elevated while mRNA encoding p53 remains unchanged (Rueda et al., 1997). It is unclear whether increases in Bax mRNA are p53 mediated. Another study suggested that p53 and Bcl2 expression in rat CL regression did not act in a diametric manner to regulate programmed cell death (Trott et al., 1997).

Other factors which could be involved in triggering apoptosis are receptors at the cell membrane for growth factors and interleukins, and receptors for steroids and peptide hormones in the cytoplasm. The effects of cytokines such as TNFα and IFNγ on cultured murine luteal cells have been studied. TNFα at high concentrations induced apoptosis and death of the luteal cell. In addition, TNFγ modulates the ability of TNFα to induce apoptosis (Jo et al., 1995). The interleukin-1β converting enzyme family and the Bax family play an important role in the regression of the bovine CL. The increase of interleukin-1β mRNA levels in the regressed bovine CL indicates that this family plays a role in luteolysis (Rueda et al., 1997).

Nitric oxide (NO) is a crucial mediator of macrophage cytotoxicity and apoptosis in addition to being a potent vasodilator factor and an agonist of apoptosis (Lowenstein and Snyder 1992). One study showed that local NO production is involved in the signal transduction mechanisms leading to apoptosis in the human endometrium during menstruation (Tschugguel et al., 1999). There is evidence in the CL that NO induces apoptosis in vitro in human luteal cells (Vega et al., 2000) indicating that NO plays a role as a luteolytic factor in the human CL (Friden et al., 2000). Collectively, apoptosis is induced or suppressed by external signals, by cell-cell interactions and by intrinsic cell biological mechanisms (Amsterdam et al., 1998). Therefore, the major goal of apoptosis research is to identify its molecular components and mechanisms of regulation.
1.4.1.4.4.8 The role of PGF$_{2a}$ in luteolysis

It is known that PGF$_{2a}$ has a significant impact on CL function in both domestic and laboratory animals. In 1969, Pharriss and Wyngarden discovered that the intramuscular injection of PGF$_{2a}$ rapidly diminished the function of the CL in the rat. PGF$_{2a}$ has the same effect on equine CL function (Douglas and Ginther, 1972; Allen et al., 1973). Furthermore, in normal animals, PGF$_{2a}$ is released from the mare's endometrium in pulses (Neely et al., 1979). PGF$_{2a}$ is produced by the endometrium and transported to the ovary via the systemic circulation.

The newly developed CL in the mare is resistant to the luteolytic effects of PGF$_{2a}$ (Douglas and Ginther, 1973; Douglas and Ginther1975b; Oxender et al., 1975). In fact the mare is responsive to PGF$_{2a}$ for only day 6-14 of her cycle (Allan, 1993), which is just half the life span of the CL. Equine species are 10-18 times more sensitive than sheep and cows to the luteolytic effects of PGF$_{2a}$ when given by the systemic routes (Kimball and Wyngarden, 1977). The high affinity of PGF$_{2a}$ receptors in equine luteal cells might explain the high sensitivity of the mare to the luteolytic effects of PGF$_{2a}$, which could compensate for the absence of a local utero-ovarian PGF$_{2a}$ pathway (Douglas and Ginther, 1973; Douglas and Ginther1975; Oxender et al., 1975).

The exact mechanism by which PGF$_{2a}$ induces luteolysis is not well understood. Many theories have been advanced to explain the mechanisms of PGF$_{2a}$-induced luteolysis including a decrease in the number of LH receptors (Behrman et al., 1993), and changes in luteal blood flow (Niswender et al., 1976; Nett et al., 1976). It is not clear however whether this is a cause or consequence of the luteolytic process. The reduction in blood flow is unlikely to be significant enough to cause hypoxia of the luteal cells. Direct cytotoxic action (Fitz et al., 1984; Branden et al., 1988), and a decrease in the diameter of small and large luteal cells (Branden et al., 1988) have also been suggested. PGF$_{2a}$ affects the function of the large luteal cells in the sheep by: 1) activation of protein kinase C (PKC), which inhibits the secretion of progesterone (Wiltbank et al., 1989a, b). 2) causing an influx of extracellular calcium (Wiltbank et al., 1989b; Hoyer and Marison, 1989; Alila et al., 1990; Wegner and Hoyer 1991; Duncan and Davis 1991). There is evidence that PGF$_{2a}$ induces oligonucleosome formation during bovine CL regression (Juengel et al., 1993).
Moreover, large luteal cells appear to be the primary target for PGF$_{2\alpha}$ while small cells appeared to be the physiological target for LH (Fitiz et al., 1982). Collectively, the data on prostaglandin function supports the theory that PGF$_{2\alpha}$ mediates luteolysis by inhibition of progesterone secretion by the large cells via a calcium-mediated intracellular pathway (Hoyer et al., 1992, 1993). Although there is good evidence for all actions, the effects of prostaglandin differ among species and the actions demonstrable in vivo are often different from those observed in vitro (Hoyer, 1991). It is likely that normal luteolysis involves several or all of these actions.

1.4.1.4.9 Immune system involvement in luteolysis

Recently, the role of the immune system in CL regression has received much attention. The CL is highly vascular and thus large numbers of leukocytes circulate through it at all times. Moreover, immune cell populations have been found in the CL of different species, with variation in the type of these immune cells amongst species and throughout the oestrous cycle. Macrophages are thought to be involved in clearing up cellular debris and damaged cells during structural regression and are known to play an essential role in CL regression in the guinea pig (Paavola, 1979). Lymphocytic infiltration of bovine CL was found on day 14 (Lobel and Levy, 1968). It has been found that invasion by macrophages on the day 15 postpartum might play a role in regression of the ovine CL (O'Shea and Wright, 1985). Human CL showed an increase in the number of both T lymphocytes and macrophages in the late luteal phase with the highest number in the degenerated CL (Suzuki et al., 1998). The numbers of proliferating macrophages in the late luteal phase decreased, whereas at mid-luteal phase the number of cells reached its peak (Gaytan et al., 1998). An increase in T lymphocytes prior to luteolysis has been found in the bovine CL (Penny et al., 1999). These workers suggested that the cytokines TNF$\alpha$ and IFN$\alpha$, which are produced by T lymphocytes, are primary luteolytic factors in reducing progesterone production by increasing the production of PGF$_{2\alpha}$. The distribution of immune cells in the equine CL during the early stages of pregnancy and oestrus has been investigated. The numbers of T lymphocytes and macrophages increased in the late luteal stage and the number of CD8$^+$ cells increased before functional luteolysis occurred. In early pregnancy, CD4$^+$ and CD8$^+$ cells and macrophages decreased in numbers (Lawler et al., 1999). CL infiltration by eosinophils was found following PGF$_{2\alpha}$ treatment in sheep (Murdoch, 1987), and also in the equine CL (Lawler et al.,
An influx of neutrophils into the CL was found in golden hamsters to be an indicator for the onset of luteolysis on day 3 (MacCormack et al., 1998).

1.4.2 CL Function
The essential function of the CL is the production of progesterone to establish and maintain and pregnancy. LH and prostaglandin appear to regulate progesterone production. Nevertheless, there are other potential hormones produced by CL which may be involved in regulation of both this gland and the reproductive system e.g. oxytocin, relaxin, prostaglandins and growth factors (Niswender et al., 2000). The role of these factors seems to differ between species

1.4.2.1 Progesterone
Progesterone is a steroid hormone derived from cholesterol, and is converted to pregnenolone by side-chain cleavage enzyme in the mitochondria. Pregnenolone is converted to progesterone by 3β-HSD in SER (Niswender et al., 2000). Progesterone is a small hydrophobic molecule and therefore diffuses freely through the plasma membrane of all cells. The most important factor which stimulates the synthesis and the secretion of progesterone in the CL, irrespective of species, appeared to be LH (Grazul et al., 1985, Mattioli et al., 1985; Rodger et al., 1985). The source of the progesterone in the mare is luteal cells (Van Niekerk et al., 1975; Watson and Sertich, 1990). The endometrium and placenta secrete progesterone during pregnancy (Ganjam et al., 1975). The increase in progesterone production inhibits LH / FSH release from the anterior pituitary and changes the endometrium from proliferative to the secretory and causes structural changes in the vagina with decreased cornification. Progesterone secretion by the CL occurs after ovulation and continues to prepare the endometrium for a possible pregnancy by inhibiting contraction of the uterus and inhibiting the development of new follicles by deceasing LH/FSH release via negative feedback at the hypothalamus. If pregnancy does not occur, secretion decreases towards the end of the cycle, and another oestrus will begin (Niswender et al., 1985, Reynolds 1994).
1.4.2.2 Relaxin

Relaxin is a peptide containing two amino acid chains A and B; the sequences of these amino acids vary among species (Stewart et al., 1982). Relaxin was isolated in 1986 from equine placenta by Stewart and Papkoff (1986). Relaxin has been found in the follicular fluid, granulosa and theca interna cells of the preovulatory follicle and in large luteal cells in mid-luteal phase (Ryan et al., 1997). In other species, relaxin was found in the CL in pigs (Evans et al., 1983), human (Stoelk et al., 1991), cow (Field et al., 1980) and sheep (Renegar and Larkin, 1985). In addition, Ohleth and Bagnell (1995) showed that relaxin could act as a growth factor in pig CL by increasing the proliferation of both granulosa and theca interna cells in vitro. Ryan et al. (1997) suggested that relaxin has an essential role in regulating cyclical ovarian activity in the mare. Relaxin has an inhibitory effect on myometrial contractility in the uterus and the mammary gland in both the pregnant and non-pregnant female by softening the tissue collagen and inhibiting muscle contractility (Sherwood, 1994). The essential role for relaxin in the CL still under investigation and the majority of the reports suggest a role for relaxin in regulating the cyclical ovarian activity.

1.4.2.3 Oxytocin

Oxytocin (OT) is a neuropeptide synthesised mainly by hypothalamic neurones which are located in the supraoptic, paraventricular and arcuate nuclei of the horse (Melrose and Knigge, 1989). OT has been identified in the corpora lutea of some mammals e.g. the monkey (Khan-Dawood et al., 1984), cow particularly in large luteal cells (Wathes et al., 1983, Guldenaar et al., 1984, Kruip et al., 1985), and sheep, were it is restricted also to the large luteal cells (Wathes and Swann et al., 1982; Watkins, 1983). The CL of the mare exceptionally does not secrete OT (Stevenson et al., 1991; Watson et al., 1999).

OT may be involved in luteal regression by stimulating uterine prostaglandin secretion in sheep (Sheldrik et al., 1983), horse (King and Evans, 1987) cow (Newcomb et al., 1977). It has been suggested that OT plays an important role in regulating the onset of luteolysis and the secretion of prostaglandin in ruminants (Flint et al., 1990; Siliva et al., 1991). Furthermore, the relationship between the secretion of the luteal oxytocin and uterine PGF2α, suggests that OT may control luteolysis in ruminants (McCracken et al., 1995). In the mare OT is secreted in pulses during the oestrous cycle and reaches a high level on day 15; thus oxytocin
may be involved in regulating the oestrous cycle by contributing to CL regression via stimulation of uterine prostaglandin synthesis (Tetzke et al., 1987). On the other hand, the presence of hypophyseal OT pulses during luteolysis did not show any correlation with the presence of uterine prostaglandin pulses (Vanderwall et al., 1998). Endometrial concentrations of oxytocin receptor determine uterine PGF$_{2\alpha}$ secretion in cyclic mares and endometrial oxytocin receptor concentrations are reduced in early pregnancy by the conceptus (Starbuck et al., 1998). The main function of oxytocin is stimulating the release of milk after parturition, stimulating myometrial contractions to facilitate parturition and postpartum processes, and gamete transport (Brinsko et al., 1993).

1.4.2.4 Prostaglandin

The luteal cells in the bovine CL secrete prostaglandin and it has been suggested that local prostaglandin may be involved in CL regression (Milvae and Hansel 1983; Rodgers et al., 1988). The human CL is also known to secrete prostaglandin (Patwadhan and Lantheir, 1980). The equine CL secretes prostaglandin F, PGE-2 and 6-keto-PGF-1α in different ratios during the oestrous cycle, after luteal cell incubation in vitro for 24 hrs (Watson and Sertich, 1990). These authors suggested that the local secretion of prostaglandin may play a role in controlling the life span of the CL and that changes in prostaglandin ratios may play an essential role in controlling CL regression.

PGF$_{2\alpha}$ is the most widely used hormone in veterinary practice in the control of oestrous cycle. PGF$_{2\alpha}$ is commonly used because of its luteolytic effect on the CL i.e. PGF$_{2\alpha}$ causes the CL to regress and cease production of progesterone (Allan, 1993). Therefore prostaglandins are only effective during such time as the mare has a functional CL and is cycling normally. PGF$_{2\alpha}$ is used clinically to treat prolonged or maintained CL in mares that exhibit extended dioestrus of 25 to 30 days, and have not been mated or mares that have not conceived. A second use of PGF$_{2\alpha}$ may be to shorten dioestrus. For example instead of breeding during foal heat, a mare would be injected with PGF$_{2\alpha}$ on day five or seven of the following dioestrus. The luteal phase will then be shortened by seven to ten days. A third use of PGF$_{2\alpha}$ can be to maximise heat detection and synchronise oestrus. Oestrus detection in mares can be time
consuming, tedious and often difficult. PGF$_{2\alpha}$ can also be useful in detecting mares with a suboestrus (Irvine, 1993).

1.4.2.5 Oestrogen

Doody et al., (1990) reported that hCG could stimulate luteal secretion of progesterone and oestrogen in the primate CL. It has been demonstrated that neither the bovine or ovine CL secrete oestrogen (Rodgers et al., 1986). Oestrogen secretion in pregnant mares in the first month of conception was at the same level as during dioestrus in non-pregnant mares (Zavy et al., 1984) but increased 2-3 fold between days 35 and 40 of conception. The source of the oestrogen was suggested to be ovarian or feto-placenta but was found by Daels et al., (1991) to be from the CL rather than feto-placental. It has been suggested that oestrogen plays a role in triggering prostaglandin synthesis (Vernon et al., 1981) and there is a temporal relationship between oestrogen and PGF$_{2\alpha}$. The function of oestrogen in the female includes: development of mammary glands, development of the reproductive system and external genitalia, fat deposition on hips and stomach (as a source of energy), triggering of oestrous cycle at puberty and triggering heat and depressing FSH production when the follicle is mature (Austin and Short 1982, Glasser, 1983). Furthermore, estrogens are considered to be the hormones associated with signs of oestrus. Oestrogen compounds are used to induce oestrus in "jump mares", mares used for collection semen collection from stallions in artificial insemination programmes. They have also been used to induce uterine tone in older barren mares, and to cause relaxation of the cervix during induction of parturition (Brinsko et al., 1993).

1.4.2.6 The role of luteinizing hormone (LH)

Luteinizing hormone (LH) is synthesised and released from the anterior lobe of the hypothalamus. LH is a heterodimeric glycoprotein consisting of an 89-amino acid alpha chain and a 115 amino acid beta subunit, which is responsible for its properties. LH is the main luteotrophic hormone in ruminants and primates (Auletta and Flint, 1988). It has been suggested that LH is crucial for controlling the function of the CL in the mid-luteal phase to late luteal phase in cattle (Baird, 1992). It has been found that LH gives trophic support to the mare's CL, and administration of antisera raised against the gonadotrophin fraction of the equine pituitary induces
luteal regression (Pineda et al., 1972). The administration of hCG or equine pituitary extract prolongs the life span of the CL in mares (Ginther, 1972b) and can increase serum progesterone concentrations (Kelly et al., 1988; Watson et al., 1988). Administration of a GnRH antagonist to mares during the luteal stage causes a reduction in progesterone, CL size and early luteal regression (Watson et al., 2000). Moreover, the high concentration of progesterone in the early stages of CL development has a negative feed back effect on LH secretion in the mare (Evans and Irvine, 1975).

The presence and number of LH receptors in the equine CL have been recorded, there is a relationship between progesterone concentration and the number of LH receptors, which increase to day 13 after ovulation, and decline afterwards (Roser and Evens, 1983). Both the small and large luteal cells express LH receptors during the luteal phase, and there are no significant changes in LH receptor affinity between day 3 and 14 of the oestrous cycle (Broadley et al., 1994). In other species, the large cells have few or no LH receptors (Hoyer and Niswender 1985), and there is no change in LH receptor affinity in the corpora lutea in humans (Bramley et al., 1987), cattle and sheep (Diekman et al., 1978; Rao et al., 1979).

1.5 Objectives
There has been no detailed published information on angiogenesis and cell death and their stimuli factors in the equine CL. As shown in this chapter, angiogenesis and cell death have recently come under investigation in a number of different species and there appear to be inter-species differences in the mechanisms controlling angiogenesis and luteal regression. The mare is unique among domestic species with regard to many aspects of reproductive physiology, and because of the commercial importance of a precise control of luteolysis in horse breeding and the increased demand of the horse industry to provide efficient means and techniques for a tighter control of oestrous cycle in the mare, it is important to study changes in the CL that may be specific to the mare. Such a study will be valuable in improving our understanding of the control of the equine CL.

Therefore we have investigated the cellular mechanisms of angiogenesis and cell death in the mare throughout the cycle and following PGF₂α-induced regression. This study was designed 1) to investigate the changes in vascularity and cell proliferation
rate, with particular emphasis on the effect of PGF$_{2\alpha}$-induced luteolysis, hypothesising that PGF$_{2\alpha}$ induces regression by a reduction in blood flow and a decrease of blood vessels resulting in a hypoxia in the luteal cells. 2) to investigate whether the angiogenic mechanism in the mare is regulated by angiogenic factors such as VEGF, FGF and ET-1 and exploring their expression in CL formation and regression. 3) to investigate the point at which structural regression occurred and the types of cell death involved in both natural and induced regression. 4) to confirm light microscope findings of cell death by a combination of ultrastuctural and biochemical techniques. 5) to investigate whether the cell death of luteal and endothelial cells is regulated by Bcl2, Bax, TNF, NOS, cytochrome C and to explore whether these oncoproteins and/or cytokines play a role in luteolysis.

The methods used to achieve the objectives were:

to study the changes in vascularisation and cell proliferation rate at each phase throughout the cycle and following PGF$_{2\alpha}$-induced luteolysis using factor VIII and Ki 67 to detect the microvessels and proliferating cells. (Chapter 3)
to investigate the factors underpinning the angiogenesis mechanism in the mare by immunostaining for VEGF, bFGF, ET-1 and detecting VEGF mRNA by *in situ* hybridisation. (Chapter 4).
to identify the morphological changes and the types of cell death involved in natural and induced regression at light microscope level by using H&E, Oil Red O and PAS stains and TUNEL immunostaining. (Chapter 5).
to re-examine the morphological changes and the cell death types involved in natural regression and induced regression, ultrastructurally. (Chapter 6).
to investigate the oncoproteins and cytokine factors underpinning cell death mechanisms in the mare by immunostaining for Bcl2, Bax, TNF$_{\alpha}$ and NOS. (Chapter 7).
Chapter 2
Materials and Methods
2.1-Animals
Healthy ponies of mixed breeding, genitally normal, aged between 4 and 12 years and weighing 250 to 500 kg were used in this study. The ponies were housed during the summer of 1999 and were under Home Office supervision. The experiments were carried out in accordance with the Animals Scientific Procedures Act 1986. The ponies had normal oestrous cycles and ovulation. The growth of the follicles was monitored throughout oestrus by transrectal ultrasonography using a real-time linear-array scanner equipped with a 5MHz intra-rectal transducer (Aloka 500V, BCF Technologies Ltd, Scotland). During oestrus the ponies were scanned daily to detect the day of ovulation. The ponies were also teased by a stallion to ensure that they were in oestrus. Sometimes PGF$_{2a}$ was used for synchronizing oestrus and to shorten the dioestrus. The day of ovulation (day 0) was considered to be the reference for CL formation, when the large follicle disappeared and became the corpus hemorrhagicum. The ponies were ovariectomised via colpotomy by a chain ecraseur under neuro-analgesia using acepromazine (0.05mg/kg i.m; C-vet Ltd, Edmunds, UK), romifidine (0.05mg/kg i.v; Sedivet, Boehringer Ingelheim Ltd, UK), butorphanol, (0.05mg/kg i.v; Torbugesic, Crawley, UK) and flunixin meglumine (1.1mg/kg i.v; Schering-Plough Animal Health, UK) and lignocaine as a local anesthetic for the ovarian ligament (Watson and Sertich 1990; Lawler et al., 1999). Tetanus prophylaxis was administered subcutaneously prior to the surgery and the ponies received antibiotic cover after the surgery. The ponies were used for two consecutive unilateral ovariectomies, allowing an intervening recovery period of at least two weeks. All the ponies exhibited normal cycle patterns of oestrus and ovulation after they had been ovariectomised on one side.

2.2 Tissue processing and blood collection
The ponies were ovariectomised at a specific time during dioestrus in order to obtain the CL in early luteal phase, Day 3-4 (n = 4), mid-luteal phase, Day 10 (n = 5), early regression phase, Day 14 (n = 4), late regression phase, Day 17 (n = 4), and 12 and 36 h (n = 3 each) following the administration of a PGF$_{2a}$ analogue (Estrumate: 1.2 ml/500kg; equivalent to 263 µg /500kg cloprostenol; Schering-Plough Animal Health, Middlesex, UK) on day 10.
The ovaries were transferred on ice immediately from the surgical theatre to the laboratory. The ovarian cortex layer was removed, and the CL enucleated from the ovary and dissected free of connective tissue. The CL was weighed and cut to give pieces representative of both central and peripheral areas (Fig 2.1 and Fig 2.2). The
samples were fixed in the following fixatives: 1) 5mmx5mm samples were fixed in 4% (v/w) paraformaldehyde freshly prepared in 0.1M phosphate buffered saline (PBS) for 24 hours at 4°C. 2) approximately 1cmx1cm samples were fixed for 24 hours at room temperature in 10% (v/v) neutral phosphate buffered formalin pH (7.0) and Bouin’s fixatives, then processed the following day and embedded in paraffin wax. 3) 1mmx1mm samples were fixed for 3-4 hours in 3% (v/v) glutaraldehyde in 0.1M cacodylate buffer at 4 °C and post fixed with 2% (v/v) osmium tetroxide for 1 hour at room temperature, then washed three times in 0.1M cacodylate buffer and embedded in resin for transmission electron microscopy (TEM). 4) small specimens were snap frozen with dry ice and iso-pentane (BDH Laboratory, UK) and kept at -70 °C. Remaining representative samples were frozen and kept at -70 °C in the freezer prior to being used. This process lasted a maximum of 15 to 20 min from removal the ovary from the animal until CL tissue samples were placed into fixative.

Blood samples were collected from the jugular vein, daily for five days before the ovariectomy, into evacuated heparinised tubes (Becton Dickinson, UK Ltd, Oxford, UK) in order to check progesterone levels and to confirm the phase of the cycle. Samples were centrifuged at 2,000 g for 15 min at 4 °C. The plasma was stored at -20°C prior to being assayed.

2.3 Conventional stains

A- Haematoxylin & Eosin (H&E) staining:

Paraffin sections were stained with conventional H&E staining (Surgipath Europath Ltd, Cambridge, UK). Three sections in each fixative for each CL were subjected to morphological examinations.

Dewax sections, rinse in alcohol, rinse in water.

Stain with Harris' haematoxylin for 2 minutes.

Wash and blue in running tap water for 1 minute.

Differentiate in acid alcohol (1% hydrochloric acid in 70% alcohol) for 10 seconds.
Wash and blue in running tap water - 5 minutes.

Stain with eosin - 1 minutes. *(Eosin (0.5 g, Ethanol 96% - 100 ml Glacial Acetic Acid - 2 drops)*

Wash in tap water, Dehydrate, clear and mount.

*B-Toluidine blue staining:*

Two semi-thin sections 1µm of thickness were cut from two blocks from each CL (peripheral and central), and were stained with 1% toluidine blue for light microscope analysis. Dissolve 1 g Toluidine Blue and 1g Sodium Tetraborate in 100 ml distilled water

Stain semi-thin for 2 – 5 min under heated plate.

Differentiate in 96% alcohol

Wash in tap water

*C- Uranyl acetate staining:*

For electron microscopy examination, ultra-thin sections (silver-grey interference colour 40-70 nm) were obtained, stained with uranyl acetate and counterstained with lead citrate and examined with a Philips CM12 transmission electron microscope.

1) Fix in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2-3 h.

2) Wash: 0.1 M sodium cacodylate buffer 3 x 20 min.

3) Post-fixation: 2% osmium teteroxide in 0.1 M sodium cacodylate buffer for 1 h.

4) Wash: distilled water 3 x 20 min.

5) Dehydration (50 % Acetone 1 x 10 min, 70 % Acetone 1 x 10 min, 90 % Acetone 1 x 10 min and 100% Acetone 3 x 10 min)
5) Dehydration (50 % Acetone 1 x 10 min, 70 % Acetone 1 x 10 min, 90 % Acetone 1 x 10 min and 100% Acetone 3 x 10 min)

6) Embedding: Unicril (overnight) then gelatine capsule.

7) Ultrathin sectioning and grid mounting

8) Staining in Uranyl acetate

Fig 2. 1 Illustrates representative samples in equine CL of both central and peripheral areas for each fixation method. BO: Bouin’s Fixative, BF: Phosphate buffered formalin, PFA: paraformaldehyde, GA: Glutaraldehyde, OTC: Snap frozen, FR: Frozen at −70 °C.
Fig 2.2. CL dissection and the representative samples. a-b: CL dissection, c: mushroom shape of the CL, d: weight of the CL, e: cutting procedure, f: the materials which were collected from the CL and the animal.
D- Oil red O stain
Frozen sections (8\textmu m) were mounted on slides to detect lipid droplets. Sections were left to dry at room temperature for 1h then fixed in formalin buffered saline for 1min. After washing in distilled water, the sections were very quickly rinsed in 70\% alcohol. The slides were stained with Oil red O (Sigma, Poole, UK) solution for 15 min at room temperature then rinsed briefly in 70\% alcohol. The sections were finally washed in distilled water, counterstained with haematoxylin and mounted in glycerol (Filipe and Lake, 1990).

E-Periodic Acid-Schiff reaction (PAS)
Neutrophils in histological sections were detected using PAS reaction. Formalin-fixed paraffin sections (4\textmu m) were mounted on slides, deparaffinised and rehydrated. After washing in water, sections were oxidised for 10 min in 1\% aqueous periodic acid (BDH). The control section was left without oxidisation. The sections were washed in running water for 5 min then immersed in Schiff reagent (Sigma) for 20 min. Finally the sections were washed for 10 min, counterstained in haematoxylin, dehydrated in graded alcohol, cleared in xylene then mounted in DPX (Filipe and Lake, 1990).

2.4 Immunohistochemistry
The following are the general procedures that were consistently used in all immunostaining procedures in this study.

1- Paraffin sections of 4 \textmu m thickness were mounted on slides (Surgipath) coated with BioBond (British Biocell Int), deparaffinised in xylene and rehydrated in 100\%, 90\% and 70\% alcohol, then washed in tap water for 10-15min.

2- Endogenous peroxide activity was blocked by immersing the slides in 3\% (v/v) H\textsubscript{2}O\textsubscript{2} (Sigma) in methanol (BDH) for 30 min.

3- Intensive washing for 10 min in tap water then followed by two washes of 5 min each in 0.01M PBS pH 7.4 (8.5g NaCl, 1.07g Na\textsubscript{2}HPO\textsubscript{4} 2H\textsubscript{2}O, 0.39g NaH\textsubscript{2}PO\textsubscript{4} 2H\textsubscript{2}O and made up in distilled water to 1litre).
4- Unmasking antigens. Often paraffin sections fixed in formalin or paraformaldehyde show masking or even destruction some antigenic epitopes. In order to reveal these antigen sites in paraffin sections, the following treatment was used.

**A-Proteolytic enzyme digestion:**

*Trypsin:* The sections were incubated in 0.1% (v/w) Trypsin (Sigma) in 0.1% (v/w) CaCl₂ pH 7.8 (BDH) at 37 °C for 45 min.

*Proteinase K:* The sections were incubated with 20μg/ml Proteinase K (Sigma) in 20 mM Tris, 2mM CaCl₂ pH 7.8 for 25 min.

**B- Microwave antigen recovery:**

Retrieval solution (0.01M sodium citrate buffer pH 6.0) brought to boiling point in the microwave. Sections were then exposed to two or three 10 min cycles of microwave irradiation at 700 W. Sections were allowed to cool at room temperature for 20 min followed by three washes of 5 min each in distilled water.

**C- Pressure cooker antigen retrieval**

Two litres of retrieval solution (0.01M sodium citrate buffer pH 6.0) were placed in a plastic pressure cooker with an opening pressure of 103 KPa/PSi and brought to the boil in the microwave without sealing the lid. The slides were placed in the pressure cooker, then sealed to full pressure. The heating time began only when full pressure was reached. In this study, heating times of 5-10 min were employed. The cooker was depressurized and cooled under running tap water, the lid was then removed and the hot buffer was flushed out with cold water. Care was taken to ensure that the sections did not dry. Sections were washed three times for 5 min each in distilled water.

5- Two washes with PBS, the slides were blocked for 30 min at room temperature with normal serum (Diagnostics Scotland, Lanarkshire, Scotland), goat serum (NGS) rabbit serum (NRS) or sheep normal serum (SNS) in PBS (1 part NS: 3 parts PBS) depending on which animal the secondary antibody was raised in.
6- Sections were then incubated in a humidified chamber with primary antibody in PBS containing 25% (v/v) normal serum. Negative controls were incubated with PBS containing 2% (v/v) normal serum or IgG instead of the primary antibody.

7- Following a further two washes in PBS of 5 min each; the slides were incubated for 30 min with biotinylated immunoglobulins (Vector Laboratories, Peterborough, UK or Dako, High Wycombe, UK) diluted to 1:100 in PBS containing 2% (v/v) normal horse serum at room temperature in a humidity chamber.

8- Two washes in PBS (5 min each) were followed by incubation for 30 min at room temperature with horseradish peroxidase-avidin biotin complex (Dako or Vector Laboratories) diluted in 0.01M PBS (pH 7.4) according to the manufacturer’s instructions.

9- Sections were again washed twice in PBS followed by visualization with 0.05%(w/v) 3,3’-diaminobenzidine (Sigma) in 0.01M PBS, pH 7.4, and 0.01% (v/v) H2O2 or DAB visualization solution (Dako or Vector Laboratories).

10- The sections were finally washed in distilled water and counterstained with haematoxylin (Vector Laboratories or Surgipath) and dehydrated in graded alcohol, cleared in xylene and mounted in DPX mountant (PDX mixture of Distrene, Plasticiser, Xylene).

2.5 Detection of apoptosis by labelling 3’ends of DNA fragments with digoxigenin-11-UTP (TUNEL).

TUNEL was performed on paraformaldehyde-fixed paraffin-embedded tissue using the technique described by Negoescu et al (1998) with modifications. Sections (4μm) were mounted on slides coated with BioBond. Endogenous peroxide activity was blocked with H2O2 in methanol for 30 min. After two washes of 5 min each in PBS, slides were incubated with Proteinase K (20μg/ml in 20mM Tris, 2mM CaCl2; pH 7.8) for 25 min. Sections were then washed with PBS containing 5 mM EDTA for 10 min at room temperature to stop the proteinase K reaction. After two 5 min washes with PBS, the slides were equilibrated with terminal desoxynucleotidyl transferase (TDT) (Boehringer Mannheim, Germany) buffer containing 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate and 1.5 mM CoCl2, placed on an ice-cold plate and loaded with 2 nM
digoxigenin-11-dUTP (Boehringer Mannheim) and 25 U ml\(^{-1}\) TdT in enzyme buffer (Boehringer Mannheim). Negative control slides were treated with enzyme buffer lacking TdT. The slides were immediately covered with parafilm sheets and incubated at 37 °C for 75 min. The parafilm was then removed and the slides washed twice (5 min each) in standard saline citrate (SSC) buffer to stop the reaction. Following a further 5 min wash with PBS, sections were incubated for 10 min at room temperature with 25% (v/v) normal rabbit serum in PBS. They were then incubated for 90 min at room temperature in a humidified chamber with sheep anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:100 in blocking solution. Following a further two washes in PBS, the slides were incubated for 30 min at room temperature with biotinylated rabbit anti-sheep immunoglobulin (Vector Laboratories) diluted 1:500 in PBS. Two washes in PBS (5 min each) were followed by 30 min incubation at room temperature with horseradish peroxidase-avidin biotin complex (Dako) diluted in PBS. Sections were visualised with 0.05% (w/v) 3,3’-diaminobenzidine (Sigma) in 0.05M Tris-HCl, pH 7.4, and 0.01% (v/v) H\(_2\)O\(_2\).

2.6 In situ hybridization

In situ hybridization was performed using complementary RNA probes for human VEGF. Sense and antisense probes were prepared using a RNA transcription kit (Ambion, Inc, Austen, TX) and labeled with \[^{35}\text{S}]\text{UTP} (NEN Life Science products, Boston, MA). The synthesized probes were purified from free bases using Chroma Spin-100 columns (CLONTECH Laboratories, Inc, Palo Alto, CA). Formalin-fixed sections were mounted on SuperFrost Plus glass slides. Sections were dewaxed in xylene and hydrated through descending concentrations of ethanol. Sections were treated with 0.1N HCl and then digested in Proteinase K (5mg/ml:Sigma, St Louis, MO) for 30 min at 37 °C. The digestion was stopped by treating the slides with 0.2% glycine for 10 min at 4 °C, acetylated with 0.2% (v/v) acetic anhydride in triethanolamine buffer (Sigma), and then washed with in 4x SSC. A prehybridization step was carried out by incubation in prehybridization buffer (50ml/slide) containing 50% (v/v) formamide, 4x SSC, 1x Denhardt’s, 125mg/ml salmon testes DNA, 125mg/ml yeast transfer RNA, and 10mmol/L dithiothreitol at 55 °C in a moist chamber for 2 h. Hybridization was performed in a moist chamber overnight at 55 °C. The hybridization buffer was similar to the prehybridization buffer, additionally but contained 10% (v/w) dextran sulfate. Two sections per slide were exposed to the antisense and sense sequences. After hybridization, slides were rinsed in 4x SSC and then treated with ribonuclease A (20
mg/ml, Sigma) for 30 min at 37 °C to remove all excess probe, desalted in descending concentration of SSC (2 x SSC for 30 min at room temperature, 2 x SSC at 45 °C, and 0.5 x SSC at room temperature) and dehydrated for 2 min each in 50%, 70%, and 90% ethanol containing 0.3 mol/L ammonium acetate. Dry slides were dipped in IlfordG5 liquid emulsion (Ilford imaging, Cheshire, UK), and exposed for 4 weeks and 4 days at 4 °C, and subsequently developed (Kodak D19 Developer, Eastman Kodak Co, Rochester, NY) and fixed (Kodak GBS). All slides were counterstained with haematoxylin.

2.7 Quantification method

Most of the data in this study were quantified in order to explore the differences between the stages of the cycle and to compare the animals receiving PGF2α and the controls. The CL showed considerable size variability at the same stage of the luteal phase. Therefore, relative estimation of the variables per unit area or per field was more applicable and more reproducible than absolute variables in whole CL (Lei et al., 1991; Gaytan et al., 1998, 1999). It was confirmed from preliminary investigations in this study that increasing the number of sections or fields above two sections per CL and four per section did not increase the accuracy of the estimations. There was no difference between the mean of the variables in central and peripheral regions, thus data were combined to represent one variable. The identity of the sections was obscured to ensure blind evaluation.

A-Manual:

Manual quantification was used to count the numbers of cells exhibiting degenerative changes, immunostained or labelled with a maker to estimate the density of the staining, or to estimate the number of positive cells. The sections were selected randomly and counted at 400 magnification. The total number for each phase was expressed as mean±SEM.

B-Image analysis system

Quantimnet image processing and analysis system 500 (Leica, Cambridge, UK) was used to estimate the total area of the staining per unit area or the area fraction or the density of staining. Areas were randomly selected and analysed at 200 magnification. The system was optimised for each individual section based on the density of the stain. The data were expressed as percentage mean ± SEM per unit area.
2.8 Progesterone assay

Progesterone concentrations were determined in plasma by radioimmunoassay as described previously (Watson et al., 2000). Progesterone standards were prepared in ovariectomised mare plasma. The main cross-reactivities of the progesterone antiserum were with 5-pregnan-3,20 dione (9.5%), 11-deoxycorticosterone (6.2%) and 17-hydroxyprogesterone (3.4%). The limit of detection of the assay was 0.5ng/ml and the intra- and inter-assay coefficients of variation were 9.0% and 12.6%, respectively. Displacement curves produced by serial dilutions of plasma and spiking of samples containing low concentrations of progesterone were parallel to the standard curves. The average recovery of a known amount of hormone added to equine plasma was 100% (Watson et al., 2000). Concentrations of progesterone were measured in plasma directly, all samples were assayed in duplicate. All regents diluted with assay buffer phosphate buffered saline 0.1% gelatine 0.05M pH 7.2 (PBSG). Anti-progesterone antiserum was provided by the Scottish antibody production Unit.

Protocol:

Day 1

Tubes were labelled and PBSG added as required:

450μl for standards and unknowns

650μl for nono-specific binding (NSB).

50μl of samples and standards were added.

100μl of I 125 labelled progesterone made up in PBSG containing 8-anilino-1-naphthalene sulfonic acid.

200μl of primary antibody diluted at 1/15, 500

Tubes were vortexed and incubated overnight at 4 °C.
Day 2

To separate free and bound antibody, 100μl donkey anti-rabbit serum at 1:35 incorporating 1/10 EDTA, and 100μl normal rabbit serum at 1/300. Tubes were vortexed and incubated overnight at 4 °C.

Day 3

1 ml cold PBSG was added to each tube and centrifuged for 30 minutes at 3,800 rpm. Supernatant was carefully decanted, and activity in the precipitate counted using a Clinigamma 1271, LKB-Walla (Wallac, Milton Keynes, UK).

Main cross-reactivities of the antiserum were with 5-pregnan-3,20 dione, 11-deoxy-5-corticosterone and 17-hydroxyprogesterone (9.5%, 6.2% and 3.4% respectively). The sensitivity of the assay was 0.5 ng/ml, and the within- and between-assay coefficient variation were 9.0 and 12.16%, respectively.

2.9 Statistical analysis

The differences in early, mid, and early and late regression were analysed by one-way analysis of variance using stage of the luteal phase as the between-subject variable. Data from 12 and 36 h post PGF2α-induced regression was compared with untreated mid-luteal phase (day 10) using a one-way analysis of variance with treatment as the between-subject variable. The data were also subjected to Tukey’s test of multiple comparison among means. Results were considered to be significantly different when p<0.05. The data were expressed as percentage or numbers (mean ± SEM) per unit area or per field. There was no difference between the mean of the variables in central and peripheral regions, thus data were combined to represent one variable. The data from different fixatives were analyzed for each luteal phase separately by one-way analysis of variance using the type of fixative as the between-subject variable.
Chapter Three

3.1 Introduction

Formation and maintenance of the CL are essential for reproductive cyclicity and in the establishment of pregnancy. The mechanisms controlling the formation and maintenance of the equine CL are not yet fully understood. The involvement of local vasculature in the regulation of luteal function in the mare is unclear, but in other species there is strong evidence that the contribution of endothelial cells may be essential (Reynolds et al., 1994; Redmer and Reynolds, 1996; Rodger et al., 1997; Young et al., 2000). Angiogenesis is a process of new blood vessel growth that depends on sprouting and proliferation of capillaries from pre-existing vessels. In the adult, capillary growth occurs rarely and is largely associated with repair of damaged tissues and pathological conditions such as tumour growth. Physiological angiogenesis is almost unique to the female reproductive system, the ovary, uterus and placenta. In the ovary, cyclic angiogenesis occurs in relation to follicular development and CL formation.

The CL is formed from the dominant follicle after ovulation. After ovulation, newly formed blood vessels invade the ruptured follicle cavity and by day 6 the CL becomes highly vascularised (Harrison, 1946). Blood vessels constitute an important element of the CL structure and the CL becomes one of the most highly vascularised tissues in the body (Bruce and Moor, 1976; Wiltbank et al., 1988). Most luteal cells are adjacent to one or more capillaries (Zheng et al., 1993; Redmer and Reynolds, 1996). Endothelial cells constitute large part of the cells present in the mature CL (Reynolds et al., 1994; Christenson and Stouffer, 1996). The CL receives most of the ovarian blood supply which highly important for progesterone secretion and maintenance of the pregnancy (Mangess et al., 1983; Reynolds et al., 1994). Luteal regression is associated with decreased luteal vascularity resulting in the formation of a small relatively avascular, fibrous mass in the sheep (Niswender et al., 1979), cow (Zheng et al., 1993), marmoset (Young et al., 2000) and woman (Lei et al., 1991; Gaytan et al., 1999).

Cell proliferation and tissue growth during early CL formation is equal to or greater than that of a malignant tumor (Reynolds et al., 1994; Zheng et al., 1994; Nicosia et al., 1995; Christenson and Stouffer, 1996; Redmer and Reynolds, 1996). Infusion of ewes with 5-bromo-2-deoxyuridine (BrdU) indicated that luteal cells and non-luteal cells continue to proliferate until day 12 of the cycle (Jablonka-Shariff et al., 1993). High rates of
proliferation have been found in the endothelial cells in many species including the sheep (Jablonka-Shariff et al., 1993), cow (Zheng et al., 1994), human (Rodger et al., 1997) and marmoset (Young et al., 1999). It has been reported that in vivo incorporation of BrdU into cellular DNA during the S-phase of the cell cycle is the most reliable method of detecting cell proliferation (Jablonka-Shariff et al., 1993). Ki67 antibody recognizes an epitope of a nuclear non-histone protein in proliferated cells (Gerdes et al., 1991), and is a reliable in vitro indicator of cell proliferation. Cell proliferation in the equine CL has yet to be documented and there is no information on which type of cell proliferates throughout the cycle, and whether cell proliferation is associated with luteal function.

The temporal patterns of blood vessel growth and regression and cell proliferation have been observed in the CL of a number of species throughout the ovarian cycle. To date, there have been no reports on angiogenesis in the equine CL. The aim of this study is to investigate whether changes in function at different stages of the equine CL are associated with angiogenic activity (neovascularisation and/or endothelial cell proliferation) with particular emphasis on the effects of PGF2α treatment on vascularity and cell proliferation. By assessing the parameters associated with angiogenesis in tissue sections collected at specific time points during the luteal phase, a quantification of endothelial cell proliferation rate using the index of Ki 67 positive cells and endothelial cell content by Von Willebrand factor VIII antigen (vW) will provide detailed information about the rate of angiogenic activity in the equine CL during the luteal phase.

3.2 Materials and Methods

3.2.1 Detection of endothelial cells and blood vessels

Endothelial cells and blood vessels in histological sections were detected using Von Willebrand Factor VIII. Formalin-paraffin sections were treated with 0.1% trypsin (Sigma) in 0.1% CaCl₂ for 45 min at 37°C to unmask the antigen sites, rabbit anti-human Von Willebrand Factor VIII (Dako) was added after dilution to 1:250 for 90 min at room temperature in a humidified chamber.
2.2.2 Detection of cell proliferation by Ki 67 antigen:
Ki67-positive cells were identified as described previously (Rodger et al., 1997) with slight modifications. Formalin-paraffin sections were used and were exposed to three 10 min cycles of microwave irradiation at 700 W in citrate buffer 0.01 M pH 6.0. Monoclonal antibody to the nuclear non-histone antigen, Ki67 (Novocastra, UK) was used and diluted to 1:40 for 3 h at 37°C in a humidified chamber.

2.2.3 Analysis of data

2.2.3.1 Microvessels
Microvessels were quantified in two ways: 1- the density of blood vessels was quantified by estimating the fraction of the blood vessels and their lumens occupied by the luteal tissue per area unit. The density of the microvessels (%) was calculated by examining sections at 400 magnification. At this magnification an eyepiece grid was used, the grid covered an area of 0.063 mm² (25μm x 25μm). Regions from the inner, middle and outer areas of the sections were randomly selected. Each point of the grid superimposed on positive staining or capillary lumens was counted and of the total number of points (ΣPv) was divided by the total numbers superimposed on luteal cells and the rest of luteal tissue (ΣPl) X 100 (Gaytan et al., 1999; Ferrara et al., 1998). 2- the area of factor VIII immunostaining was also calculated by Quantimet image processing and analysis system 500 (Leica, Cambridge, UK). The system was optimized for each individual section based on the density of the stain. The area used in this system was calibrated at x200 magnification. Quantification was performed using unbiased counting rules (Gaytan et al., 1999; Gundersen et al., 1988). Single endothelial cells or clusters of endothelial cells were considered to be individual vessels. The density of the microvessels was expressed as percentage mean ± SEM, whereas the area of factor VIII were expressed as mm² mean ± SEM. However, luteal cell size (hypertrophy and atrophy) during the luteal phase may influence the outcome of factor VIII immunostaining quantification. This has been considered and the same principles (considering the volume variability of the luteal cells during the luteal phase) proposed by Gaytan et al. (1999) in the human CL were applied here to avoid misinterpretation, thus, the density of luteal cells during the luteal phase has been evaluated in a similar method to factor VIII.
3.2.4.3 Proliferation index (PI)

Fields were examined at x400 magnification. The number of Ki 67-labelled nuclei and the total number of nuclei were counted. Two sections per animal and 4 fields per section were counted. The PI was determined by the number of Ki 67-labelled nuclei divided by total number of nuclei x100. The PI was expressed as a percentage mean ± SEM. In order to identify the cell types which exhibited immunostaining for Ki67, the following rules were implemented: endothelial cells were elongated with a long axis of about 4-8 μm, they had little cytoplasm; luteal cell were large round to vesicular shaped, of about 20-40μm in diameter and contained big spherical to vesicular nuclei about 10-15μm in diameter. Cells showing positive staining for Ki67 which did not fit the criteria of endothelial cells or luteal cells were considered to be other types of cells, usually leukocytes and fibroblasts. The number of different cells was counted at x400 and expressed as mean ± SEM per field.

3.3 Results

3.3.1 CL weight and progesterone concentrations

Morphological examination and progesterone concentrations for each CL were used to confirm the cycle stage. In 3 of the 23 CL, the designated stage of the cycle did not conform with the expected morphological appearance and progesterone concentration and were therefore re-classified. In two CL that were collected on day 14 (early regression group), one of the animals had high progesterone levels (9.4 ng/ml) together with a healthy appearance of the luteal cells. This animal was therefore reclassified to the day 10 (mid-luteal phase) group. The other animal had undetectable concentrations of progesterone with histological evidence of marked structural degeneration consistent with the day 17 (late regression) group. It was therefore reclassified as being in the late regression phase. The third CL was collected on day 17, but progesterone concentration was 1.2 ng/ml on the day of the surgery with morphological changes coinciding with those of the early luteal regression group (day 14). This animal was therefore reclassified as being in as early regression. Theses animals have shorter or longer cycle than the others, which reflect interbiological variation between individual animals.

The mid-luteal phase was associated with maximum production of progesterone and concentrations were significantly higher (p< 0.01) than other phases in the cycle. The time of luteolysis was day 14, progesterone levels on day 13 for the four CLs collected in early
Fig 3.1. CL weight (□) plasma and progesterone concentrations (■) throughout the cycle and after PGF₂α administration. Data are expressed as mean ± SEM.

a: significantly (p< 0.01) higher than all other groups.
b: significantly (p< 0.05) lower than all other groups.
c: significantly (p< 0.05) lower than mid-luteal phase (day 10).
regression being 2.1, 5.8, 2.4 and 1 ng/ml. By day 14, progesterone levels had declined sharply to 0.5, 1.2, 0.5 and 0.5 ng/ml respectively. All the CLs collected on day 17 had low concentrations of progesterone (less than 0.5 ng/ml) for two days before ovariectomy. Twelve hours following PGF₂α administration on day 10, progesterone levels had declined significantly (p<0.01) from 6.0 ± 0.01 ng/ml before PGF₂α injection to 1.0 ± 0.01 ng/ml by 12 h after the injection. By 36 h, functional regression was complete and progesterone levels were less than 0.5 ng/ml (Fig 1).

All the collected CLs were weighed without taking the blood clot out. CLs showed changes in weight, shape and colour throughout the cycle. During the early luteal phase, the CLs were irregular in shape, were blood-coloured and contained a large blood clot centrally. By day 10, the CL became mushroom- or gourd-shaped, pink to purple in colour, four of the five CL still contained a clot of blood, although this was smaller than in the early CL, and one CL was solid at this time. By day 14 two of four CL contained a small clot of blood, while the other two CL appeared to be homogeneous. By day 17, the CL became round, cream or yellow in colour, and comprised a solid mass of tissue. The mean weight of luteal tissue was significantly lower (p<0.05) in the late luteal phase (day 17) and 36 h following PGF₂α, than in the early and mid-luteal phase (Fig 3.1).

3.3.2- Von Willebrand factor VIII immunostaining

The immunostaining was found to be limited to endothelial cells of microvessels and capillaries. There was no immunoreactivity in the negative control or in the luteal cells or other type of cells (Fig 3.2b). After ovulation, sprouting endothelial cells invaded the cavity of the corpus hemorrhagicum and began to form a vascular bed between the luteal cells. Individual microvessels could be identified in the early luteal phase (Fig 3.2a). The mid-luteal phase was characterized by a very dense network of fully differentiated capillaries (Fig 3.2b). The density of the microvessels and the area of factor VIII immunostaining increased significantly (P<0.05) compared with early luteal phase (Fig 3.3). During the early regression phase there was a slight decrease in the area and density of the microvessels (Fig 3.2c). By late regression (day 17), the extensive vascular network of the mid-luteal phase had regressed (Fig 3.2d). This resulted in a marked decline (P<0.01) in
Fig 3.2: Factor VIII immunostaining in the equine CL throughout the cycle and following PGF$_{2\alpha}$-induced luteolysis. a) early luteal phase showing individual microvessels, b) mid-luteal phase showing intense network of microvessels, negative control (inset) c) early regression phases, d) late regression, showing reduction in vascularity e) 12 h following PGF$_{2\alpha}$ injection showing vasodilation and marked influx of neutrophils, f) 36 h following PGF$_{2\alpha}$ injection showing vasodilation absent and reduction in microvessels. Scale bar represents 20 μm and 10μm to the inset.
Figure 3.3: a) the density of factor VIII immunostaining and the luteal cells b) the area of factor VIII immunostaining throughout the cycle and after PGF2α administration. The density for each luteal phase was expressed as percentage mean ± SEM, while the area of Factor VIII immunostaining was expressed as mm² mean ± SEM per area.

a: significantly (p<0.01) lower than mid- and early regression luteal phase.
b: significantly (p<0.05) higher than early luteal phase.
c: significantly (p<0.01) higher than early and mid phase.
d: significantly (p<0.01) lower than mid luteal and 12h following PGF2α.
e: significantly (p<0.01) higher than early luteal phase and late regression and 12-36 h post PGF2α.
f: significantly (p<0.01) lower than mid- and early regression luteal phase.
g: significantly (p<0.05) higher than 12 h following PGF2α.

Interestingly, twelve hours following PGF2α administration, microvessels underwent vasodilation (Fig 3.2e), resulting...
microvessels density and area of factor VIII immunostaining. Interestingly, twelve hours following PGF$_{2\alpha}$ administration, microvessels underwent vasodilation (Fig 3.2e), resulting in a significant increase in vessel density (P<0.01). This change was short lived; by 36 h the vasodilation disappeared and there was a sharp decline in the density of the microvessels and the area of factor VIII immunostaining (P<0.01) compared with 12 h post PGF$_{2\alpha}$ (Fig 3.2f).

3.3.2.4. Ki67 immunostaining

Ki 67 immunostaining was observed in all sections examined. The proliferation index of Ki 67 changed throughout the oestrous cycle and following PGF$_{2\alpha}$-induced luteolysis (Fig 3.5). The proliferation index was higher in the early luteal phase than at any other phase of the cycle and declined by more than 70% in mid-luteal phase and early regression (P<0.01). During late regression and 36 h following PGF$_{2\alpha}$ injection, the proliferation index increased significantly (P<0.05). Endothelial cells showed the highest proliferation rate during the early luteal phase (Fig 3.4a); by mid-luteal phase (Fig 3.4b) and early regression the number of endothelial cells that stained positively for Ki 67 (Fig 3.6) dropped significantly (P<0.01). During natural and induced regression, very few endothelial cells were proliferating. Luteal cells stained positively for Ki 67 in the early luteal phase (Fig 3.4a) and numbers of positive staining cells had reduced significantly (P<0.01) by the mid-luteal phase and early regression (Fig 3.6). During late regression and 36 h following PGF$_{2\alpha}$, some luteal cells showed immunostaining (Fig 3.4c,d). Other types of cells (possibly leukocytes and fibroblasts based on morphological appearance), particularly in the regression phase, showed Ki 67 positive staining (Fig 3.4c,d), these cells increased significantly (P<0.01) on day 17 and 36 h following PGF$_{2\alpha}$. 
Fig 3.4: Ki 67 immunopositive cells in the equine CL throughout the cycle and following PGF₂α-induced luteolysis. a) early luteal phase showing intense proliferation in the luteal (thin arrows) and endothelial cells (head arrows), b) mid-luteal phase intense proliferation in endothelial cells (head arrows), negative control (inset), c) late regression, showing unexpected positive Ki 67 in the luteal cells (thin arrows) and other type of cells probably leukocytes (thick arrows). d) 36 h following PGF₂α injection showing positive Ki 67 in the luteal cells (thin arrows) and leukocytes (thick arrows). Scale bar represents 20 μm and 10 μm for the inset.
Fig 3.5: Label index of Ki 67 immunopositive cells in the equine CL throughout the cycle and after PGF$_{2\alpha}$ injection.

a: significantly (p < 0.01) higher than all other groups
b: significantly (p < 0.05) lower than mid-luteal.
c: significantly (p < 0.05) lower than mid-luteal phase.
d: significantly (p < 0.05) higher than mid-luteal phase and early regression
e: significantly (p < 0.05) higher than 12 post PGF$_{2\alpha}$ injection.

Fig 3.6: The number of proliferating cell types per field in the equine CL throughout the cycle and after PGF$_{2\alpha}$ administration.

a: significantly (p < 0.01) higher than mid-luteal and early regression phases.
b: significantly (p < 0.05) lower than mid-luteal phase.
c: significantly (p < 0.01) higher than mid-luteal phase.
d-e: significantly (p < 0.01) higher than early luteal regression and 12h following PGF$_{2\alpha}$, respectively.
f-g: significantly (p < 0.01) higher than early luteal regression and 12h following PGF$_{2\alpha}$, respectively.
3.4 Discussion

To my knowledge, this is the first report demonstrating the temporal changes in vascularity and cell proliferation in the equine CL at different phases of oestrous cycle and following PGF$_{2\alpha}$-induced luteolysis. These changes were suggestive of an important role for endothelial cells in the equine CL. The high vascularity and proliferation of endothelial cells during early and mid-luteal phase suggested intense angiogenesis.

The dramatic reduction in the luteal tissue weight during induced and natural regression supports observations in other species that luteal tissue weight declines during natural regression (Bruce and Moor, 1976; Dharmarajan et al., 1988; Wiltbank et al., 1988; Zheng et al., 1994; Reynolds et al., 1994) and after induced regression (Juengel et al., 1993). However, progesterone level in plasma is an indicator of luteal function. Despite the sharp decline in progesterone levels in early regression and 12hrs following PGF$_{2\alpha}$ treatment; there was no significant reduction in the density of the blood vessels nor in the area of factor VIII immunostaining in these stages. This suggests that functional regression precedes structural regression in the blood vessels in the equine CL. These results support the findings in other species (Deane et al., 1966; Umo, 1975; Braden et al., 1988; Zheng et al., 1994).

Factor VIII and Ki 67 antibodies were used to demonstrate endothelial cells and proliferating cells respectively. Factor VIII is a cascade protein and is produced in cells of endothelial lineage. Studies have shown this antibody to be sensitive and specific in detecting endothelial cells in tissue sections (McComb et al., 1982; Jeanneau et al., 1982). However, Ki 67 antibody is widely used as a marker for cell proliferation and mainly expressed in M-phased of the cell cycle (Gerdes et al., 1991), and has been used to detect cell proliferation in the equine endometrium (Gerstenberg et al., 1999). Studies in a number of tissues have shown Ki 67 immunostaining to be compatible and favorable with the in vivo gold standard technique of bromodeoxyuridine and radiolabelled thymidine incorporation (Sasaki et al., 1988; Kamel et al., 1989).

Endothelial cells of small capillaries were lying between the luteal cells while large arterioles and venules lay in the fibrous capsule and within the trabeculae. The intense
immunostaining of factor VIII during mid-luteal phase demonstrated high vascularisation. The peak in vascularisation and the presence of a fully differentiated network of capillary beds at mid-luteal phase are required for optimal delivery of progesterone precursors to, and progesterone from, the luteal cell. The decline in vascularity did not occur until late regression (day 17). This time course of events was confirmed by observation following PGF$_{2\alpha}$-induced luteolysis. At 36 h there was a significant reduction in factor VIII immunostaining. This may suggest that local blood flow began to decrease from day 14 and 12 h post PGF$_{2\alpha}$ onwards during natural and induced regression, respectively. These findings support the hypothesis that the local blood flow and capillaries bed decline during luteal natural regression in sheep (O'Shea et al., 1977; Niswender et al., 1979), cow (Zheng et al., 1993), marmoset (Young et al., 1999) and human (Lei et al., 1991; Gaytan et al., 1999) guinea pig (Hossian et al., 1983) and following PGF$_{2\alpha}$-induced regression in sheep (Nett et al., 1979) and cow (Juengel et al., 1993). The remaining microvessels during late regression and 36 hrs after PGF$_{2\alpha}$ administration may play a role in resorption of the luteal tissue (Zheng et al., 1993; Reynolds and Redmer 1998). In the trabeculae the blood vessels showed the same pattern of changes as those observed between the luteal cells. Increased blood vessel density during early and mid-luteal phase and decline during regression was observed.

Interestingly, in response to PGF$_{2\alpha}$, unexpected findings were seen at 12 h as the density of the blood vessels increased sharply due to the vasodilation in the blood vessel. This was short lived, as by 36 h the density of the blood vessels declined rapidly. The vasodilation in the vasculature bed caused the high density of the blood vessels at 12 h only. This phenomenon has not been seen in other domestic animals e.g sheep and cow. PGF$_{2\alpha}$ is generally regarded as being a vasoconstrictor agent. However, the mare is particularly sensitive to the effect of administered PGF$_{2\alpha}$ (Douglas and Ginther, 1973; Oxender et al., 1975). The pharmacological dose administered in the present study, may have resulted in this unexpected observation at the ovarian level. Similar findings were found in the CL and ovary in rat, where PGF$_{2\alpha}$ induced vasodilation and functional regression at small doses and structural regression at large doses (Indrei et al., 1999). It is most likely that these findings are compatible with the findings that mares are 10-18 times mare sensitive to PGF$_{2\alpha}$ than other domestic species such as sheep and cows which have been reported as
less sensitive to PGF$_{2\alpha}$ in comparison to the mare due to the lack of utero-ovarian blood vessel connection (Douglas and Ginther, 1973; Douglas and Ginther 1975; Oxender et al. 1975).

We observed high PI during early luteal phase (18.1%), followed by a sharp decrease in mid-luteal phase (4.2%) and during early regression phase (1.3%). Most normal adult tissues proliferate slowly and show PI of less than 0.5% whereas adult tissues with rapid turnover rates, such as epithelial cells and bone marrow can show PI of 1-10% (Cameron et al. 1971). Moreover, malignant tumors can show PI of 11-35% (Bresciani et al., 1974; Baserga, 1985). From these comparisons, it is clear that the PI in the equine CL in early luteal phase is comparable to rapidly growing tumor, while in mid luteal phase, the equine CL was exhibiting the same pattern of constantly renewing tissue such as epithelial cells in kidney and intestine. High proliferation index in early luteal phase was reported in the cow and sheep (Zheng et al., 1994; Jablonka-Shariff et al., 1993) respectively. During late natural regression and 36 h following PGF$_{2\alpha}$, PI started to rise suggesting a second period of proliferation in order to enhance luteal regression. Most of the cells proliferating during regression fitted the morphology of leukocytes and fibroblasts.

We observed endothelial proliferating cells exhibiting the highest level of proliferation during early luteal phase then decreased dramatically in the later phases. Over 70% of proliferating cells were endothelial cells during early luteal phase. The highest proliferation rate of endothelial cells during early luteal phase has been demonstrated in marmoset (Young et al., 1999), in sheep (Jablonka-Shariff et al., 1993) in human (Rodger et al., 1997) and in pig (Reynolds et al., 1994). However, the observed pattern in endothelial cell proliferative rate was compatible with the temporal changes of luteal vascularisation in this study. The high proliferation in the early luteal phase was associated with growing of new microvessels, which leads to increased vascularisation during luteal development, while the presence of dense capillary network at mid-luteal phase was associated with lower rate of proliferation.

Other types of cells probably leukocytes and fibroblasts were immunostained for Ki 67 during late regression and 36hrs following the administration of PGF$_{2\alpha}$. These cells were
identified on morphological basis. Cells showing positive staining for Ki67 which did not fit the criteria of endothelial cells or luteal cells were considered to be other types of cells, usually leukocytes and fibroblasts. Increased numbers of fibroblasts has been reported in the CL in many species including the mare (Harrison, 1946; Van Niekerk et al. 1975), sheep (Farin et al., 1986) cow (Lei et al., 1991), guinea pig (Hossaian and O’shea 1983) and human (Lei et al., 1991). Leukocytes are believed to proliferate during regression, increased numbers of proliferating T-lymphocytes and macrophages were found during regression in the bovine CL (Petroff et al., 1997; Bauer et al., 2001), and in the mare increased numbers of lymphocytes have been associated with both induced and natural regression (Lawler et al., 1999).

Proliferating luteal cells were high during early luteal phase then decreased during mid-luteal and early regression phase. A proportion of cells with the morphological appearance of luteal cells were positive for Ki 67 during late natural regression and 36 h following PGF2α administration. This was unexpected, since a number of studies in other species have demonstrated that luteal cell numbers do not rise in cow, sheep and human (O’Shea et al., 1989; O’Shea et al., 1986; Lei et al., 1991). It is possible that these luteal cells were arrested in one part of the cell cycle and did continue DNA synthesis or mitosis, and /or these cells were in the first stages of DNA degeneration and the futile DNA repairing process resulted in apparent (though false) staining of luteal cells. These positive luteal cells may represent staining of degenerated proteins around the time of cell death.

The following conclusions can be drawn from this study: 1- intense angiogenesis is a feature of the early and mid-luteal phase. 2- reduction in the local luteal microvessels occurs when functional regression is completed and progesterone levels had fallen. 3- luteal regression is associated with a dramatic decrease in proliferating endothelial cells and an increase in leukocyte and fibroblast proliferating cells 4- Vasodilation in microvessels is a temporal feature seen 12 h following PGF2α. In the next chapter further investigation was conducted to study the involvement of VEGF, bFGF and ET-1 in luteal angiogenesis in the mare.
Chapter 4

Angiogenesis in the equine CL (2): Expression of VEGF mRNA and protein and immunostaining of bFGF and ET-1.
4.1 Introduction

The corpus luteum undergoes extensive cellular proliferation and differentiation during luteinisation and involutes rapidly at luteolysis. These changes principally involve intense growth and regression of microvessels (Reynolds et al., 1992; Zheng et al., 1993). Intense luteal angiogenesis was found during CL development and continues at a low rate until luteal regression when endothelial proliferating cells decrease further (Jablonska-shariff et al., 1993, Zheng et al., 1994; Rodger et al., 1997; Dickson and Fraser, 2000; Young et al., 2000). During pregnancy, the endothelial cells must survive for a relatively long period, so it is obvious that the mechanism underpinning luteal angiogenesis is a highly regulated process. However, it is now emerging that angiogenesis is essential for normal luteal function, and inhibition of angiogenesis can trigger functional luteal regression (Fraser et al., 2000). Pharmacological manipulation of luteolysis is a key element in oestrous cycle control in clinical equine reproduction. Currently PGF$_{2\alpha}$ can be administered from day 6 of the cycle to initiate luteolysis (Oxender et al., 1975), but there are no drugs available that can induce luteolysis over the entire luteal phase. The process of angiogenesis has not been studied in the equine CL, but pharmacological control of angiogenesis could offer an alternative way of shortening the oestrous cycle.

Luteal angiogenesis is regulated by a number of growth factors, degradation of the extracellular matrix and cell-cell interactions (Fraser and Lunn, 2001). There is growing evidence that VEGF and bFGF are the main angiogenic factors in the CL (Reynolds and Redmer, 1998). VEGF is an endothelial cell specific cytokine, which is thought to encourage proliferation and migration of the endothelial cells resulting in angiogenesis (Flokman et al., 1991). VEGF is mediated by two tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) (Otani et al., 1999). While VEGF may be induced by hypoxia in most ischaemic tissues (Shweiki et al., 1992), it is probably regulated more closely by LH in the CL of the human (Neulen et al., 1998) and monkey marmoset (Hazzard et al., 1999). The importance of VEGF in luteal angiogenesis has been demonstrated by specific VEGF neutralisation in the rat (Ferrara et al., 1998) and marmoset (Fraser et al., 2000). These studies have shown a suppression of endothelial cell proliferation, restricted development of the microvessels and a decrease in progesterone production during the early luteal phase. Previous studies have demonstrated the presence of VEGF mRNA and protein in the CL of several species including sheep (Redmer et al., 1996; Doraiswamy et al., 1997), cattle (Goede et al.,
Basic FGF is another potential angiogenic factor shown to stimulate blood vessel development *in vitro*. It is bound to low-affinity heparin sulphate sites in the extracellular matrix and mediated by two tyrosine kinase receptors bFGFR-1 and bFGFR-2 (Folkman and Klagsbrun, 1991). The importance of bFGF in luteal angiogenesis has been demonstrated by specific bFGF neutralisation, showing the majority of (82%) of the endothelial mitogenic activity produced by the bovine and ovine and porcine CL was neutralized by bFGF (Grazul-Bilska et al., 1997, Ricke et al., 1995; Redmer and Reynolds 1996). Previous studies have demonstrated the presence of bFGF mRNA and protein in the bovine and ovine CL (Grazul-Bilska et al., 1997, Zheng et al., 1993; Schams et al., 1994; Jablonka-Shariff et al., 1997) and the levels of bFGF protein or mRNA are similar or increased throughout the oestrous cycle in the bovine CL (Zheng et al., 1993; Ricke et al., 1995).

ET-1 is a potent vasoconstrictor identified for the first time in porcine aortic endothelial cells (Yanagisawa et al., 1988). ET-1 is able to induce contractions of vascular and nonvascular smooth muscle and possibly plays a role in both local and systemic regulation of blood flow (Simonson and Dunn, 1990). ET-1 can bind to subtypes of receptors A and B (Arai et al., 1990). ET-1 and its role in the CL have been investigated recently, showing that endothelial cells and their product ET-1 are required for the manifestation of the luteolytic effects of PGF$_{2\alpha}$ (Ohtani et al., 1998). There is strong evidence that luteal ET-1 inhibits luteal steroidogenesis and mediates the effects of PGF$_{2\alpha}$ and causes a reduction in LH-stimulated biosynthesis of progesterone and an increase in PGF$_{2\alpha}$ in bovine and ovine cultured luteal cells (Girsch et al., 1996). The amount of mRNA encoding ET-1 was markedly higher in regression than at any phase of the cycle (Girsch et al., 1996). In cattle, PGF$_{2\alpha}$ administration caused an increase in luteal output of ET-1 with a reduction in progesterone concentrations, while in sheep the combination of ET-1 and PGF$_{2\alpha}$ administration resulted in complete luteolysis with reduction in progesterone and shortening of the luteal phase (Meidan et al., 1999; Milvae et al., 2000).
There is no report on the role of VEGF and bFGF and ET-1 in the equine CL. It is important to investigate whether the changes in endothelial proliferating cells and vascular bed are associated with expressional changes in VEGF and bFGF during the luteal phase and explore the relationship between VEGF and bFGF and angiogenesis in the equine CL. In the present study, we investigated the possible role of VEGF and in angiogenesis by localising VEGF mRNA and protein, as well as bFGF and ET-1 protein by immunostaining throughout the luteal phase.

4.2 Methods and materials

4.2.1 Immunocytochemistry of VEGF, bFGF, ET-1 and MAC 387
Sections of formalin fixed CL were incubated with rabbit anti-VEGF (Cat no: SC-152-G, Santa Cruz Biotechnology Inc., California, USA), diluted to 1:200 in PBS, for 120 min at room temperature or overnight at 4°C in a humidified chamber. Rabbit anti-Bovine bFGF (cat no: F3393) (Sigma) diluted to 1:800 in PBS containing 0.001M Triton x100 for 120 min at room temperature. For ET-1, sections were treated with 0.1% trypsin for 20 min at 37°C then incubated with rabbit anti-bovine ET-1 (Pennsula Laboratories, San Carlos, CA) diluted to 1:500 in PBS for 90 min at room temperature. MAC 378 was used as a marker to detect activated macrophages. Sections were treated with 0.1% trypsin for 20 min at 37°C then incubated with mouse anti-human ET-1 (Dako) diluted to 1:100 in PBS for 60 min at room temperature. Activated macrophages were detected using MAC 387 as the primary antibody (Dako). Sections were treated with 0.1% w/v trypsin for 20 min, and were then incubated with primary antibody diluted to 1:200 for 60 min. Negative controls were incubated with PBS containing 2% normal rabbit IgG instead of the primary antibody or mouse serum for MAC 387. No pretreatment was required for any of the antibodies. For bFGF, the blocking solution and the PBS contained 0.001M of Triton x100.

4.2.2 Quantification: Quanitimet image system was used to estimate the percentage area of the immunostaining (area of the immunostaining stain divided by the total area measured x100) for VEGF, bFGF and ET-1 immunostaining. Areas were analysed at 200 magnification using two sections from each animal and four fields per section. The system was optimised for each individual section based on the density of the stain. The number of MAC 387 positive cells was counted in the cavity of the CL at x400 magnification. VEGF mRNA grains were evaluated by scoring (+++) for intense
expression, (++/++++) for less intense expression (+) weak expression (+/++) very weak expression. The results were expressed as numbers or percentage (mean ± SEM) per unit area or per field.

4.3 Results

4.3.1 Expression of VEGF mRNA
VEGF mRNA was expressed in the luteal cells, being absent from endothelial cells and neutrophils. Intense expression of VEGF mRNA was found in the early luteal phase (Fig 4.1a) and mid-luteal phase (Fig 4.1b, 4.2a). By early and late regression and in the PGF2α-treated animals, the grain density of VEGF mRNA in the luteal cells had decreased markedly (Fig 4.1c-d; Table 1). However, as the density decreased in the luteal cells during early regression, the density increased in non-luteal cells in the cavity of the CL (Fig 4.2b). These cells were identified as macrophages using MAC 387 immunostaining (Fig 4.2c,d). These cells in CL cavity increased significantly during regression (Table 1). The number of non-luteal cells expressing VEGF increased significantly (P<0.05) during regression compared with the early luteal phase. Specific hybridization was not seen when the labeled sense RNA strand was used (Fig 4.1b, inset).

4.3.2 VEGF immunostaining
Immunostaining was located mainly in the cytoplasm of luteal cells in all sections examined throughout the cycle. No positive staining was observed in negative control sections (Fig 4.3b, inset) and no apparent specific staining was detected in endothelial or other cell populations although some immunostaining were found around large blood vessels in the stromal tissue. Strong immunostaining was found in most of the luteal cells the early (Fig 4.3a) and mid-luteal phase (Fig 4.3b). By early luteal regression, a few luteal cells exhibited pale immunostaining (Fig 4.3c). During late regression, strong immunostaining was observed in some luteal cells (Fig 4.3d). Twelve and 36 hours following PGF2α, some luteal cells and neutrophils were immunostained (Fig 4.2e). Other types of cells were found to show immunostaining for VEGF in the central cavity of the CL, these cells are believed to be macrophages (Fig 7f). The percentage area of immunostaining was high during the early and mid-luteal phase (Fig 4.4a) then decreased significantly (P<0.01) during early regression. By late regression, immunostaining increased significantly (P<0.05) compared with early regression.
Twelve hours post PGF$_{2\alpha}$-induced luteolysis, the immunostaining had fallen significantly ($P<0.05$) compared with day 10. The area of immunostaining then increased by 36 h after PGF$_{2\alpha}$ administration.

Fig 4.1 *In situ* hybridisation for VEGF mRNA under dark field. Portions of CL are shown during (a) early, (b) mid-luteal phase, (c) early regression and (d) 36 h after PGF$_{2\alpha}$ injection during mid-luteal phase (day 10). Cells expressing VEGF mRNA are associated with grain clusters (white grains) while amorphous refraction from tissue is gray. Tissues are shown with the cellular luteal cavity on the left hand side. Note the high number of grain clusters in early and mid-luteal phase with a dramatic reduction early regression and 36 h following PGF$_{2\alpha}$ injection. In (d) the cells producing VEGF mRNA were also restricted to the peripheral or central areas of the tissue. Specific grain clusters of VEGF mRNA was not seen when the labeled sense RNA (inset). Scale bar represents 50\(\mu\)m and 20\(\mu\)m for the inset.
Fig 4.2: In situ hybridisation for VEGF mRNA under light field for luteal and non-luteal cells. MAC 387 positive cells in the CL cavity and VEGF immunostaining for non-luteal cells. a) VEGF mRNA section counterstained with H&E shows grains of VEGF expression in the luteal cells in mid-luteal phase. b) non-luteal cells (macrophages) in the cavity of the CL expressing specific VEGF mRNA in early regression. c) CL cavity in the early luteal phase showing macrophages exhibiting immunopositive staining for MAC 387 (activated macrophage marker). d) high number of immunopositive macrophages in the cavity of CL at early regression. e) immunopositive neutrophils for VEGF 36 h following PGF$_{2\alpha}$ injection. f) macrophages showing VEGF immunostaining in the CL cavity at early regression. Scale bar represents 20μm for a, b, c, d and f, and 15 μm for e and 7μm for the inset.
Table 4.1: The density of VEGF mRNA grains throughout the cycle. High expression in early and mid-luteal phase and a decline during regression. The number of non-luteal were expressed VEGF mRNA in the CL cavity increased during regression. The number of MAC 387 positive cells in the CL cavity increased during regression. a: significantly (p<0.05) higher than early and mid-luteal phase.

4.3.3 bFGF immunostaining

bFGF immunostaining revealed a broadly similar pattern of staining to VEGF. Positive immunostaining was seen in the cytoplasm of luteal cells. No positive staining was observed in negative control sections (Fig 4.5b, inset) and no apparent specific staining was detected in endothelial or other cell populations but some immunostaining was seen around large blood vessels throughout the luteal phase (Fig 4.5a,d). Intense staining was found in the growing luteal cells during early luteal phase (Fig 4.5a), while some cells were stained positively in mid-luteal phase (Fig 4.5b). By early luteal regression, a few luteal cells exhibited pale immunostaining (Fig 4.5c). During late regression, strong immunostaining was observed in luteal cells and around the blood vessel in the stromal tissue (Fig 4.5d-e). Twelve and 36 h following PGF$_{2x}$, some luteal cells and neutrophils were immunostained (Fig 4.5f). The percentage area of immunostaining was high during the early luteal phase (Fig 4.4a) then decreased significantly (P<0.01) during mid-luteal phase and early regression. By late regression, immunostaining increased significantly (P<0.01) compared with early regression. Twelve hours post PGF$_{2x}$-induced luteolysis, the immunostaining had fallen significantly (P<0.05) compared with day 10. The area of immunostaining then increased by 36 h after PGF$_{2x}$ administration.

4.3.4 ET-1 immunostaining

ET-1 immunostaining was seen in the endothelial cells and some luteal cells. No positive staining was observed in negative control sections (Fig 4.6b, inset) and no apparent specific staining was detected in other cells. During the early luteal phase (Fig 4.6a) and mid-luteal phase (Fig 4.6b) some microvessels and a few luteal cells showed
Figure 4.3: VEGF protein immunostaining during early luteal phase (a), mid-luteal phase (b), early regression (c) and late regression (d). Note high number of luteal cells showed intense immunopositive staining during early and mid-luteal phase with a dramatic reduction in early regression (c). In (d) some luteal cells in late regression showing strong immunostaining. No apparent staining was found in the negative control (inset). Scale bar represents 50µm and 20 µm for the inset.
Fig 4.4. The percentage area of VEGF and bFGF (a) and ET-1 (b) immunostaining throughout the cycle and after PGF$_{2\alpha}$ administration. Data are expressed as mean ± SEM.

a: significantly (p< 0.01) higher early regression
b: significantly (p< 0.05) lower than mid-luteal phase
c: significantly (p< 0.05) lower than mid-luteal phase and 36 h following PGF$_{2\alpha}$
d: significantly (p< 0.05) lower than early regression
e: significantly (p< 0.05) lower than mid-luteal phase and 36 h following PGF$_{2\alpha}$
f: significantly (p< 0.01) lower than mid-luteal phase and early regression.
g: significantly (p< 0.05) lower than mid-luteal phase and 36 h following PGF$_{2\alpha}$
h: significantly (p< 0.05) higher than early and late regression.
i: significantly (p< 0.05) higher than 36 h following PGF$_{2\alpha}$. 
Fig 4.5. bFGF immunostaining throughout the cycle and following PGF$_{2\alpha}$-induced luteolysis. a) intense immunostaining in the luteal cells and large microvessels (arrowhead) during early luteal phase. b) few luteal cells showing faint immunostaining during mid-luteal phase, negative control, no apparent positive staining (inset). c) pale and dispersed immunostaining in the luteal tissue during early regression. d) intense staining in the luteal cells during late regression. e) intense immunostaining around the blood vessels during regression. f) some luteal and neutrophils immunostained 36 h following PGF$_{2\alpha}$ injection. Scale bar represents 40 μm, e-f scale bar represents 15 μm.
Fig 4.6 ET-1 immunostaining throughout the cycle and following PGF$_{2\alpha}$-induced luteolysis. a) some luteal and endothelial cells immunostained during early luteal phase. b) some luteal and endothelial cells showed immunostaining during mid-luteal phase, no apparent positive staining in the negative control (inset). c) pale immunostaining during early regression. d) 12 h following PGF$_{2\alpha}$ induced regression, some luteal cells and endothelial cells showed immunopositive staining. Scale bar represents 20µm and 10µm for the inset.

intense immunostaining. During early and late regression most of the cells were free of any positive immunostaining, few cells showed very pale immunostaining (Fig 4.5c). Interestingly, at 12 h some immunostaining were seen some microvessels and luteal cells (Fig 4.6d), by 36 h most of cells were free of immunostaining. The percentage area of the immunostaining was high during early and mid-luteal phase (Fig 4.4b) then
decreased significantly \( (p < 0.01) \) during early and late regression. The percentage area of immunostaining decreased significantly 36 h \( (p < 0.01) \) following PGF\(_{2\alpha}\).

**Discussion**

The results have described for the first time the localization and the pattern changes of VEGF and bFGF and ET-1 throughout the cycle and following PGF\(_{2\alpha}\) administration in the mare. The high expression of VEGF mRNA and protein in the luteal cells during early and mid-luteal phase was strongly suggestive of an important role for VEGF in regulation of blood vessel development and CL growth in the mare.

VEGF stimulates vascular permeability, angiogenesis and endothelial cell mitosis (Folkman and Klagsburn, 1991). The peak expression of VEGF mRNA and protein during the early and mid-luteal phase showed a direct temporal association with high endothelial cell proliferation. Our findings strongly indicate a role for VEGF in regulating angiogenesis in the equine CL. The high expression of VEGF is compatible with its established role in stimulating and maintaining the newly formed vasculature in the CL in sheep (Redmer et al., 1996), cattle (Goede et al., 1998; Berisha et al., 2000) humans (Otani et al., 1999; Wulff et al., 2000) and marmoset (Dickson et al., 2001). However during early regression (day 14), VEGF mRNA expression and immunostaining declined in the equine CL, and was associated with a marked decline in endothelial cell proliferation. The time course of these events was also confirmed by our observations after PGF\(_{2\alpha}\)-induced luteolysis. At 12 h there was a sharp decline in VEGF mRNA and protein which reflected the low proliferation rate at this time. These findings suggest PGF\(_{2\alpha}\) had an inhibitory effect on proliferating endothelial cells may by mediated via the decrease in VEGF production.

The role of bFGF in the CL is believed to stimulate proliferation of endothelial and luteal cells (Gospodarowicz et al., 1978; Grazula-Bilska et al., 1995) and prevent cell death during regression (Reynolds et al., 1998). The intense immunostaining of bFGF in luteal cells and large blood vessels during the early luteal phase was followed by a significant decline during mid-luteal phase may indicate a direct and temporal association with high endothelial and luteal cell proliferation during early luteal phase, and the marked decline in luteal cell proliferation during mid-luteal phase. bFGF appeared to plays a major role in cellular turnover in the CL. These findings strongly
indicate a role for bFGF in regulating the equine CL development and are consistent with the main role of bFGF in other species (Gospodarowicz et al., 1978; Grazula-Bilska et al., 1997; Reynolds et al., 1998). Data from previous studies in bovine CL showed intense staining for bFGF during mid-luteal phase (Zheng et al., 1993), while bFGF mRNA in the ovine and porcine CL was stayed constant or even increased throughout oestrous cycle (Riche et al., 1995; Doraiswamy, 1998). This may highlight inter-species difference in the role FGF in the CL. During early regression, bFGF immunostaining declined more than during mid-luteal phase, this was associated with a marked decline in endothelial cell proliferation. This timing of events was also confirmed by our observations after PGF2α-induced luteolysis. At 12 h there was a sharp decline in bFGF immunostaining reflecting the low proliferation rate at this time.

Twelve and 36 h following PGF2α-induced regression there was massive infiltration of the luteal tissue by neutrophils. Although the neutrophils immunostained for VEGF protein, VEGF mRNA expression was not detected. To our knowledge this is the first report of VEGF protein in neutrophils in the CL. Recent studies in the human endometrium also showed strong immunostaining for VEGF protein in neutrophils (Mueller et al., 2000; Gargett et al., 2001). Moreover, bFGF showed the same pattern of immunostaining, some neutrophils showed immunostaining. Neutrophils showed immunostaining for bFGF in rat lung (Arcot et al., 1995) and in nodular sclerosis in Hodgkin's disease (Ohshima et al., 1999). The presence of VEGF immunostaining in neutrophils might suggest that after PGF2α administration, VEGF protein may play a role in non-angiogenic functions in the CL such as the regulation of vascular permeability or mediation of endothelial cell survival (Goede et al., 1998; Berisha et al., 2000).

Non-luteal cells, which we identified as macrophages, showed expression of VEGF mRNA in the cavity of CL throughout the luteal phase. Interestingly the number of these cells increased during regression while luteal cells showed a decrease in VEGF expression. In other species, the luteal cells appear to be the major sites of VEGF production (Christenson and Stouffer, 1997) while VEGF receptors are found in the endothelial cells (Berisha et al., 2000). There is evidence that the human macrophage can produce and express VEGF mRNA (Cho et al., 20001; Cejudo-Martin et al., 2001). It is possible that macrophages trapped in the central clot experience hypoxia which is responsible for activating VEGF mRNA. The increased number of macrophages
expressing VEGF in the CL cavity could raise the possibility of its role in compensating the shortage of oxygen in the luteal tissue during luteolysis.

Non-luteal tissue (stromal tissue and large arterioles) showed positive staining for bFGF particularly during regression. bFGF receptors were found in the luteal cells and in the large microvessels (Reynolds and Redmer, 1998). The present bFGF staining apparent around the blood vessels in the stroma may reflect either receptor uptake or possibly production of bFGF in endothelial cells and stromal tissue (Zheng et al., 1993; Reynolds et al., 1998).

In late regression and 36 h following PGF2α, intense immunostaining for VEGF in degenerated luteal cells was observed. However, these cells were free of VEGF mRNA expression, indicating that VEGF was not being synthesized at this time. It is possible that either VEGF is required for support of the remaining microvessels in clearing up the unwanted luteal tissue during regression, or that this staining represents the presence of residual, non-active/metabolized VEGF (Dickson et al., 2000). A similar scenario was found with bFGF immunostaining during late regression and 36 h following PGF2α. Luteal cells, large microvessels and stromal tissue showed intense immunostaining. These findings support a previous study in the bovine CL (Zheng et al., 1993). It has been suggested that bFGF may be involved in preventing cell death in the CL (Tilly et al., 1992; Yasuda et al., 1995). The high bFGF concentrations during luteal regression may enhance fibroblasts proliferation and subsequently fibrosis (Ohshima et al., 1999).

ET-1 immunostaining was found in the luteal and endothelial cells during early and mid-luteal phase. These findings are in agreement with previous studies that both luteal cells endothelial cells expressed ET-1 in the rat (Usuki et al., 1991), cow (Grish et al., 1996) and human (Apa et al., 1998). We did not expect a decreased ET-1 staining during natural regression or following PGF2α-induced luteolysis. Data from studies in the sheep (Hinckley et al., 2000) cow (Grish et al., 1996a; Grish et al., 1996a; Miyamoto et al., 1997) and human (Apa et al., 1998) suggested a peak in ET-1 level in response to the release of PGF2α from the endometrium or exogenous PGF2α in vivo and in vitro. Our findings did not show the peak of ET-1 during luteolysis. Twelve hours following PGF2α, the density of the staining remained broadly similar to mid-luteal phase. In contrast, early regression (day 14) showed a sharp decrease. This might
indicate a role for ET-1 as a potent vasoconstrictor factor at 12 h post PGF$_{2\alpha}$. Our results did not demonstrate that ET-1 was involved in luteolysis in the equine CL as shown in other species. We expect our findings to show an increase in ET-1 levels during natural and induced luteal regression as in cows (Grish et al., 1996a) and in the human (Apa et al., 1998). However, ET-1 antibody used in this study might not react positively with ET-1A sub-type as been the major mediator to inhibit progesterone secretion from the luteal cells (Miyamoto et al., 1997). Further investigations are needed to use specific techniques to identify levels of ET-1 mRNA.

In conclusion, hormone producing cells are the main source of VEGF and bFGF. This strongly suggests that luteal cells play an important paracrine role in regulation of blood vessel development, growth and regression in the CL. VEGF and bFGF may also play a role in the regulation, vascular permeability or mediation of endothelial cell survival in the equine CL.
Chapter 5

5.1 Introduction

The equine corpus luteum (CL) is functional for about 14-15 days in non-fertile cycles (Daels and Hughes, 1993). The CL must regress functionally to decrease circulating secretion of progesterone and allow the next oestrous cycle to commence; it must also regress structurally in a way that maintains normal ovary size relative to the rest of the reproductive system to allow the preovulatory follicle to grow and ovulate. Luteal regression in the mare is characterised by a decrease in progesterone production (functional regression) and cellular demise of luteal tissue (structural regression) (Van Niekerk et al., 1975). Luteal regression is thought to be brought about by the secretion of uterine PGF2α (Hughes and Ginther, 1975). However, the exact cellular mechanisms involved in luteolysis are not fully understood.

Cells generally die by two recognised mechanisms: necrosis or apoptosis (Kerr et al., 1972). During necrosis, cells develop increased permeability, which leads to cellular swelling, non-selective DNA degradation and inflammation in the surrounding tissues. Apoptosis is a process whereby cells die in a controlled manner. Morphologically, apoptosis is characterised by shrinkage and condensation of chromatin (marginated chromatin) or fragmentation into multiple, small dense bodies. Cells may then break up into discrete membrane-bound structures containing variable amounts of condensed chromatin and/or cytoplasm which are ingested by macrophages or neighbouring cells, or are extruded into the lumen of the blood vessels (Kerr et al., 1972; Wyllie et al., 1980). Alternatively, cells may shrink into a single dense round mass with a densely basophilic pyknotic nucleus (Wyllie et al., 1980). Detailed information on the type of cell death involved in luteal regression can be provided by examining the tissue for morphological appearance of apoptosis/necrosis under light and electron microscopes (Wyllie, 1980). Generation of 180-200bp DNA fragments by specific endonucleases is a characteristic feature of apoptosis and these fragments can be visualised by TUNEL immunostaining (Gavrielli et al., 1992).

Several studies have described apoptosis in luteal regression using DNA electrophoresis or labelling the 3' ends of the DNA fragments in cattle (Juengel et al, 1993; Zheng et al., 1994), sheep (Kenny et al, 1994), pigs (Bacci et al., 1996) women (Shikone et al, 1996; Yuan et al, 1997), monkeys (Young et al., 1997), golden hamster (McCormack et
al, 1998) and rabbits (Dharmarajan et al, 1994). Necrosis has been demonstrated alongside apoptosis in the CL of women and monkeys (Fraser et al. 1995; Shikone et al., 1996).

In mares the CL is reported to start regressing functionally from day 12 onwards, and on day 16 the luteal cells decrease in size. By day 20 two types of cell degeneration were reported a) pyknosis associated with shrunken nuclei and very condensed cytoplasm, and b) karyolysis characterised by lysis of chromatin (Van Niekerk et al 1975). Data from these studies were limited to cellular changes during natural luteolysis. There have been no reports on the types of cell death that take place during PGF\(_{2\alpha}\)-induced luteolysis in the mare. With the observation that forms of cell death other than apoptosis may occur during luteolysis (Fraser et al., 1999; Shikone et al., 1996), it is important to study changes that may be specific to the mare. The goal of the present study was to describe the cellular mechanisms of luteolysis in the mare and to investigate the point at which structural luteolysis occurred and the types of cell death involved in both natural and PGF\(_{2\alpha}\)-induced luteal regression.

5.2 Materials and methods

5.2.1 Histological examination

Three types of fixed tissue with Bouin’s, formalin and paraformaldehyde were stained with conventional H&E stain. Morphological criteria were used (Kerr et al., 1972; Wyllie et al., 1980), to differentiate and quantify cells that were actively undergoing apoptosis at the time of fixation and by identification of dense, small round bodies so-called apoptotic bodies. Apoptotic cells were defined as cells with nuclei containing condensed chromatin that was either a) margined into delineated, densely staining masses aligned with the nuclear membrane (marginated chromatin), b) shrunken into a single, round regularly shaped, dense, homogeneously staining mass (pyknotic appearance), or c) fragmented into multiple homogeneously, dense masses (multiple fragments) situated inside the cells. Apoptotic bodies were considered to be remnants of apoptotic cells defined as discrete membrane-bound structure containing various amounts of condensed chromatin and situated singly or in clusters between apparently viable cells, in the capillaries or extracellular space. Only discrete structures that clearly fitted the above description were counted. Sections obtained from toluidine blue
staining were not be included due to the difficulties in distinguishing between stained red blood cells and round dense bodies.

5.2.2 Quantification method

Individual clusters of dense bodies and pyknotic nuclei, which showed positive immunostaining for TUNEL, or were identified in H & E stained sections, were counted separately at 400 magnification. The results were expressed as mean ± SEM. To quantify the number of neutrophils and macrophages in the luteal tissue, sections were counted at 400 magnification. The total number of neutrophils and macrophages for each phase of the oestrous cycle expressed as mean ± SEM. For quantitative analysis of Oil Red O, Quantimet image processing and analysis system 500 (Leica, Cambridge, UK) was used to measure the proportion of the section stained by Oil Red O (area of the Oil Red O stain divided by the total area measured x100%). Areas were analysed at 200 magnification. The data were expressed as percentage mean ± SEM per unit area.

5.3. Results

5.3.1 Luteal tissue histology

Sections of H&E and toluidine blue staining were examined morphologically. Luteal cells in mid-luteal phase showed a polyhedral to oval shape with abundant cytoplasm, containing irregular or vesicular to spherical nuclei, with one or more nucleoli. These nuclei showed heterochromatin in the nuclear zone, aligned to the nuclear membrane. Some luteal cells showed different sizes of vacuoles in the cytoplasm. Blood vessels and red blood cells were often seen between the luteal cells (Fig 5.1a, 5.2a).

By day 14, luteal cells showed nuclear degenerative changes. Pyknotic cells with dense nuclei were observed between apparently healthy luteal cells (Fig 5.1b, 5.2b). These were classified as apoptotic cells. Discrete membrane-bound structures containing various amounts of condensed chromatin were observed singly or in clusters between apparently viable cells, in capillaries and the extracellular space (Fig 5.1b), were classified as apoptotic bodies. All the luteal cells by day 17 were shrunken in size compared with day 14 and 10. Most of the cells had vacuoles and a foamy appearance. Fibroblasts and connective tissue infiltrating the CL, together
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Fig 5.1. Haematoxylin and eosin-stained sections obtained from formalin fixed luteal tissue (a, b, c and d), paraformaldehyde fixed tissue (e) and bouin’s fixed tissue (f). a) mid-luteal phase showing healthy luteal cells. b) early regression showing pyknotic cells and round dense bodies between healthy cells. c) late regression showing pyknotic cells and round dense bodies and fibroblasts. d) 36 h after PGF$_2$ administration showing pyknotic cells and round dense bodies and neutrophils. e) paraformaldehyde fixed luteal tissue 36 h following PGF$_2$ administration, showing pyknotic cells and round dense bodies, neutrophils and healthy luteal phase. f) bouin’s fixed luteal tissue during early regression showing vacuoles and less detected pyknotic cells and round dense bodies, compared with the morphology differences between (b) early regression formalin fixed section. Thin black arrows indicate healthy luteal cells, thick black arrows indicate neutrophils, white arrows and black arrowheads indicate pyknotic cells and round dense bodies, respectively. White arrowheads indicate fibroblasts. Scale bar represents 20μm for a, b, c, d and f, and 10μm for e.
Fig 5.2. Toluidine blue sections obtained from: a) mid-luteal phase showing healthy luteal cells and blood vessels contained red blood cells. b) early regression showing pyknotic cells between healthy luteal cells. c) late regression showing pyknotic cells, connective tissue and fibroblasts d) 36 h after PGF$_{2\alpha}$ administration regression showing pyknotic and neutrophils. Thin black arrows indicate healthy luteal cells, thick black arrows indicate neutrophils, thick and thin white arrows and arrowheads indicate pyknotic cells, red blood cells and round fibroblast, respectively. Scale bar represents 5µm.
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Fig 5.3. Number of pyknotic cells and round dense bodies in equine luteal tissue during induced and natural regression per field x40 and expressed as mean ± SEM. A) Bouin’s fixed tissue, B) Paraformaldehyde fixed tissue stained with H&E, C) Formalin fixed tissue stained with H&E, D) TUNEL immunostaining stained with stained H&E. Data are shown for CL collected on day 10 and day 14 and 17 of the luteal phase, in addition to those collected at mid-luteal phase 12 hrs and 36 hrs following PGF$_{2\alpha}$.

- a: significantly (p<0.05) higher than mid-luteal phase (day 10).
- b: significantly (p<0.01) higher than late regression phase (day 17).
- c: significantly (p<0.05) higher than mid-luteal (day10) phase and 12 h following PGF$_{2\alpha}$.
- d: significantly (p<0.01) higher than mid-luteal phase (day 10) and 12 h following PGF$_{2\alpha}$.
with intercellular debris and leukocytes were commonly seen. The nuclear changes were similar to day 14 (Fig 5.1c, 5.2c). Twelve hours following PGF$_{2\alpha}$ injection, a huge influx of neutrophils was seen, in addition to a number of cells showing pyknotic nuclei and round dense bodies were observed in the extracellular space. At 36 h the majority of the cells were surrounded by neutrophils and pyknotic cells and clusters of round dense bodies were abundant (Fig 5.1d,e, 5.2d). The morphology of the luteal cells was well preserved in formaldehyde (formalin and paraformaldehyde) fixatives whereas sections fixed in Bouin's showed more vacuolisation (Fig 5.1f) in the cytoplasm and pyknotic nuclei and round dense bodies hard to detect.

During natural or induced luteolysis, the number of pyknotic cells and round dense bodies were similar in all the fixatives. (Fig 5.3). In early regression, pyknotic cells increased (P< 0.05) and remained without a significant change in numbers until late regression. During induced regression, there was an increase in the numbers of pyknotic cells 36 h following PGF$_{2\alpha}$ (P< 0.05). Clusters of round dense bodies increased in early regression (P< 0.01), and declined in late regression (P< 0.01). Following induced regression there was an increase in clusters of round dense bodies 36 h post PGF$_{2\alpha}$ (P< 0.001). However, the number of the pyknotic cells and round dense bodies in Bouin's fixative sections was less than those in formaldehyde fixatives sections (Fig 5.3A).

5.3.4 TUNEL immunostaining
Immunostaining was found to be limited to pyknotic cells and round dense bodies. There was no immunoreactivity endothelial cells, apparently healthy luteal cells. No staining was apparent in negative control sections (Fig 5.4a). There was no apparent difference between number of labelled cells and round dense bodies in the central and peripheral regions, thus, data from these regions were combined. Approximately 95% of pyknotic cells and dense bodies exhibited positive immunostaining with TUNEL. Occasionally, small numbers of positive pyknotic cells were seen on day 10 in some CL (Fig 5.4c). TUNEL immunostaining of cells and dense bodies increased during early regression (Fig 5.4d), late regression (Fig 5.4e), and 36 h following PGF$_{2\alpha}$ injection (Fig 5.4f). The number of labelled pyknotic cells and round dense bodies are shown in Fig (5.3D). There was an increase in labelled pyknotic cells in early and late regression.
Figure 5.4. TUNEL immunostaining in paraformaldehyde-fixed tissue a) negative control of equine CL on day 14, b) hamster CL and atretic follicle (inset), positive staining in both apoptotic cells and bodies. c) mid-luteal phase showing apoptotic body and healthy normal luteal cells. d) early regression showing positive staining in both pyknotic cells and round dense bodies. e) late regression showing immunopositive staining for both pyknotic cells and round dense bodies. f) 36 hrs post PGF<sub>2α</sub> administration, neutrophils, pyknotic cells and round dense bodies show positive immunostaining. Thin black arrows indicate healthy luteal cells, thick white and black arrows indicate pyknotic cells and neutrophils respectively, black and white arrowheads indicate round dense bodies and unstained round dense bodies, respectively. Scale bar represents 20μm and 5μm for the inset.
Pyknotic cell numbers increased significantly 36 h following PGF$_{2\alpha}$ (p<0.05). The number of labelled dense bodies increased significantly between mid-luteal phase and early regression (p<0.01). This increase was short lived and by late regression the number of labelled round clusters had decreased significantly (p<0.01). Labelled round dense bodies increased significantly 36 h following PGF$_{2\alpha}$ injection (p<0.01). Moreover, neutrophils also stained positively at 36 h after PGF$_{2\alpha}$ injection.

### 3.3.4 Oil red O
In the early luteal phase, most of the luteal cells showed intense staining for Oil red O, with the size of positively stained droplets ranging approximately between 0.1-0.6µm in diameter. Positive staining was also observed in some blood vessels and the intercellular space. By mid-luteal phase, few luteal cells showed positive staining (Fig 5.5a). By late regression, very intense staining was observed intracellularly and extracellularly (Fig 5.5b), showing high accumulation of lipid in the regressed CL with lipid droplets ranged in size between 0.1 and 5-7 µm in diameter. Twelve hours after PGF$_{2\alpha}$ administration, few cells showed Oil red O staining, and by 36 h there was moderate accumulation of lipid in most of the luteal cells. The percentage area of lipid (Fig 5.6) decreased dramatically (p<0.01) compared with the early luteal phase and increased sharply (p<0.001) in mid-luteal phase and early regression by late regression.

### 5.3.5 Neutrophils and macrophages:
PAS stain is designed to demonstrate all types of polysaccharides. Neutrophils in the equine CL stained positively with PAS (Fig, 5.5c). In early and mid-luteal phase (Fig, 5.5d), as well as early regression, occasional neutrophils were observed, particularly in the blood vessels. On day 17 the number of neutrophils had increased slightly. Following PGF$_{2\alpha}$ injection at both 12 h and 36 h, the numbers increased sharply (p<0.001) compared with mid-luteal phase (Fig 5.7). Few macrophages were found immunostained positively with MAC 387 during early luteal phase (Fig, 5.5e) and mid-luteal phase. During early regression (Fig, 5.5f) and late regression, there was influx of macrophages (p<0.01). Following PGF$_{2\alpha}$ injection (Fig, 5.5f, inset), the number of macrophages increased significantly (p<0.01) compared with mid-luteal phase (Fig 5.7).
Figure 5.5. Oil red O and PAS histochemical staining and macrophages immunostaining. a) mid-luteal phase, few luteal cells show lipid droplets in the cytoplasm. b) late regression, luteal cells show accumulation of lipid. c) 12 h post PGF$_{2a}$ showing neutrophils stained positively with periodic acid schiff stain post PGF$_{2a}$ administration on day 10. d) mid-luteal phase showing healthy luteal cells with the absence of neutrophils. e) early luteal phase, showing few positive macrophages among luteal cells. f) early regression (day 14), showing massive infiltration of macrophages among luteal cells and trabeculae tissue (inset: showing influx of macrophage after 36 post PGF$_{2a}$ injection). Scale bar represents 20µm and 10 µm for the inset.
Figure 5.7. The number of neutrophils and macrophages during natural and induced regression per x40 field and expressed as mean ± SEM.

- **a:** significantly \( p < 0.001 \) higher than mid-luteal phase.
- **b:** significantly \( p < 0.001 \) higher than early and mid-luteal phase.
- **c:** significantly \( p < 0.001 \) higher than mid-luteal phase.

Figure 5.6. The percentage of area of Oil red O staining throughout the cycle and following PGF\(_{2\alpha}\)-induced regression, expressed as mean ± SEM.

- **a:** significantly \( p < 0.01 \) higher than mid-luteal phase.
- **b:** significantly \( p < 0.001 \) higher than any group during the luteal phase.
- **c:** significantly \( p < 0.01 \) higher than mid-luteal phase and 12hrs following PGF\(_{2\alpha}\).
3.4 Discussion

This study describes cell death during natural and induced luteal regression in the mare, combining the morphological changes observed at the light microscopic level with immunostaining for the 3’end labelling of DNA fragments (TUNEL technique). This is the first description of cellular changes during PGF$_{2\alpha}$-induced luteolysis in the mare. The detection of pyknotic luteal cells and round dense bodies that stained positively with TUNEL during natural and PGF$_{2\alpha}$-induced regression was suggestive of apoptosis. Late regression and 36 h post-PGF$_{2\alpha}$ administration were associated with lipid accumulation and PGF$_{2\alpha}$ administration at 263μg/500kg caused a huge influx of neutrophils.

Nuclear morphological changes in the luteal cells during regression represent cell degeneration. Pyknotic cells and round dense clusters of chromatin in the extracellular space seen in this study have been accepted to be apoptotic changes (Wyllie et al., 1980; Bursch et al., 1985; Clarke 1990; Majno and Joris 1995; Jolly et al., 1997). The pathways of cell degeneration in natural and induced regression are broadly similar. It appears that luteal regression in the mare may utilise apoptosis to eliminate degenerated and unwanted cells from the CL, in agreement with previous studies in other species (Juengel et al., 1993; Kenny et al., 1994; Bacci et al., 1996; Shikone et al., 1996; Young et al., 1997; McCormack et al., 1998).

It has been proposed that the type of fixatives and pre-treatment could influence the number of apoptotic cells and bodies in thyroid tissue sections (Negoescu et al., 1998). Precipitation and cross-linking of different fixatives have been suggested to reflect the detection of apoptosis (Negoescu et al., 1998). In our study, the use of different fixatives demonstrated variability in the numbers of pyknotic cells and round dense bodies. Each fixative has distinct chemical and physical properties which inevitably influence the morphology of the cells. Bouin’s fixed tissue was less satisfactory than formaldehyde fixed tissue, suggesting that prolonged fixation time (24 h) and the strong precipitation caused by picric acid (Drury et al., 1967) resulted in cytoplasmic vacuolisation which created difficulties in recognising the dense round bodies and pyknotic cells. Formaldehyde fixes proteins by forming additive compounds without precipitation or any effect on carbohydrates and glycogen, allowing a wide variety of stains to be applied (Drury et al., 1967). Bouin’s fixative is used for the demonstration
of chromosomes but causes partial or complete lysis of red blood cells and swelling in collagen fibres, but does not cause excessive hardening as formaldehydes. Picric acid in Bouin's precipitates nucleoproteins and causes tissue shrinkage (Drury et al., 1967).

Using H&E and TUNEL staining to quantify changes in apoptotic cells occurring in the CL during regression, the peak increase in round dense bodies in the early luteal phase (day 14), followed by the sharp decrease during late natural regression (day 17) indicates an active removal mechanism. It is not clear whether these round dense bodies are ultimately engulfed by macrophages or flushed out of the CL via the blood supply, but data from previous studies has indicated that elimination by macrophages is likely to occur (Wyllie et al., 1980; Clarke 1990). Macrophage numbers increase during luteal regression in human and cows (Lei et al., 1991) and guinea pig (Paavola, 19790). MAC 387 immunostaining revealed massive infiltration of activated macrophages during natural and induced regression. Presumably, the macrophages contribute to the disappearance of large numbers of these round dense bodies.

MAC 387 antibody was used to detect activated macrophages. A previous study in the mare using conventional method for detecting macrophages by non-esterase staining revealed an increase in macrophages numbers during regression; but the monoclonal DH59B failed to show any increase during regression. The failure of DH59B antigen to recognise and express activated macrophages was suggested (Lawler et al., 1999). Our findings revealed an influx of activated macrophages during induced and natural regression. The presence and the increase of numbers of macrophages during luteal regression may be involved in cleaning up unwanted cells during regression. In addition to their role in phagocytosis in the CL, macrophages secrete a wide variety of products involved in connective tissue break-down and vascular changes, which have also been shown to be ovulatory mediators (Brannstrom et al., 1994). Macrophages are the main source of cytokines like TNFα and interleukin-1, both of which are believed to play a role in luteal regression (Zolti et al., 1990; Roby et al., 1990; Bagavandous et al., 1990).

PGF2α is widely used to induce luteolysis in mares (Allen, 1973; Douglas and Ginther, 1995). Findings from this study showed that individual mares synchronised with PGF2α showed a similar degree of degenerative changes (apoptotic cells and bodies) compared to those going natural regression, which showed some individual-variation. This may be
PGF$_{2\alpha}$ release in individual mares during natural regression. However, two distinctive features in the form of vasodilatation and neutrophil influx were observed after PGF$_{2\alpha}$. The supraphysiological dose of PGF$_{2\alpha}$ used in this study may be responsible for this, hence the immune response was greater post-PGF$_{2\alpha}$ injection (Lawler et al., 1999). It has been shown that ovine luteal cells produce a specific chemoattractant for eosinophils in response to PGF$_{2\alpha}$ injection (Muroch, 1987). One could assume that PGF$_{2\alpha}$ injections may promote the production of specific types of chemoattractant resulting in neutrophil attraction. These suggestions need further investigation.

To our knowledge, this is the first report to show infiltration of neutrophils into the CL following PGF$_{2\alpha}$-induced luteolysis in domestic animals. Massive infiltration of neutrophils was observed 12 and 36 h following PGF$_{2\alpha}$ administration. Influx of neutrophils during luteal regression has been reported during spontaneous regression in the hamster, suggesting that neutrophils may assist in initiating luteal regression in this species (McCormack et al., 1998). Although the essential function of neutrophils is phagocytosis, they also initiate and modify the magnitude and duration of the acute inflammatory processes (Jain, 1986). It has been proposed by studies in the hamster CL that these cells play a role in luteal regression via cytokine secretion (McCormack et al., 1998). Studies on rat luteal cells demonstrated that activated neutrophils inhibit LH stimulation of cAMP and progesterone production in vitro. This inhibition is reversed by addition of catalase and superoxide dismutase. Thus superoxide radicals produced by neutrophils could be involved in functional regression (Carlson et al., 1993, Brannstrom et al., 1994).

In the present study, endothelial cells did not show positive staining or any sign of apoptosis at light microscope level, except when over-treated with Proteinase K (not shown). Previous studies in other species showed positive staining for apoptosis in endothelial cells in marmoset (Young et al., 1997), pig (Bacci et al., 1996) and cow (Zheng et al., 1994). Further investigation is needed to explore the changes in endothelial cells at ultrastructural level.

There was dramatic increase in lipid droplets during luteal regression. This accumulation of lipid was seen in late regression (day 17), but was not yet present in early regression (day 14). This shows that accumulation of lipid is a feature of late
regression, occurring after the onset of structural regression. The time course of these events was confirmed by observations after PGF2α-induced luteolysis. At 12 h there was no lipid accumulation, despite the onset of structural regression, but by 36 h there was a marked lipid accumulation. Accumulation of lipid is associated with CL regression in sheep (Deane et al., 1966, Umo, 1975 Waterman 1988), cow (Priedkalns and Weber 1968), dog (Dore, 1989), sow (Mirecka, 1969 Waterman, 1980), rat (Guraya, 1975), marmoset (Young et al., 1997) and rabbit (Koering 1974). The exact mechanism by which lipid accumulation occurs during regression is not well understood. It has been suggested that degeneration in mitochondria and SER results in a decline in progesterone production and may play a role in the accumulation of lipid (Umo, 1975; Levine et al., 1979). The present study has shown however that accumulation of lipid did not occur directly in response to a decline in progesterone production and the luteolytic effect of PGF2α, but required a period of time to develop.

TUNEL immunostaining was limited to pyknotic cells and round dense bodies. The same pattern of TUNEL immunostaining has been observed in many studies (English et al., 1989; Jolly et al., 1997; Van Wezel et al., 1999 Kim et al., 1999). In this study, both H&E staining and TUNEL showed the same patterns of apoptotic changes. However, there are indications that H&E staining and TUNEL may be over-representing apoptosis. Recent studies in the marmoset CL indicated the involvement of apoptosis using DNA extraction, H&E and TUNEL staining (Young et al., 1997). Resin sections from the same tissues did not show the ultrastructural features associated with classic apoptosis (Fraser et al., 2000). Using TUNEL may generate false staining by picking up cells dying via non-apoptotic mechanisms (Mizoguchi et al., 1998) or can originate from excessive proteinsation with Proteinase K (Negescu et al., 1998; Labat-Moleur et al., 1998). These studies highlight the importance of ultrastructural examination; therefore, the next chapter will investigate and confirm the findings in this chapter by the use of electron microscopy.

In summary, it is possible to draw the following conclusions:
I) structural regression occurs on day 14 of the oestrous cycle and 12 h post PGF2α-induced regression, II) apoptosis may be involved in early and late regression stages, III) accumulation of lipid is a feature of late regression, IV) PGF2α administration causes a huge influx of neutrophils. V) endothelial cells show no sign of apoptosis.
Chapter 6

6.1 Introduction

Cell death is widely recognized to be the major phenomenon responsible for the demise of the CL. The mechanisms by which the luteal cells die in natural and induced luteolysis have been investigated in many species, and the majority of the reports have used TUNEL or DNA extraction and suggest that apoptosis may play a role in luteolysis in cattle (Juengel et al., 1993; Zheng et al., 1994), sheep (Kenney et al., 1994), pigs (Bacci et al., 1996) women (Shikone et al., 1996; Yuan et al., 1997; Shikone et al., 1997), monkeys (Fraser et al 1995; Young et al., 1997).

The evidence of apoptosis in luteal regression is not definitive and has been established using transmission electron microscopy (TEM) in a few species. Many studies utilizing TUNEL to detect apoptosis have reported difficulties with the specificity of this technique (Grasl-Kraupp et al., 1995; Negoescu et al., 1998). A recent study has suggested that the use of paraffin sections could be misleading in detecting the type of cell death present in the marmoset corpus luteum (Fraser et al. 1999). When the same tissue was examined under TEM, non-apoptotic form of cell death was revealed. Very few studies have investigated the demise of the luteal cells ultrastructurally. Sawyer et al. (1990) has shown classical features of apoptosis (crescent accumulation of chromatin in the inner layer of the nuclear membrane in luteal, endothelial and fibroblast cells in the ovine CL. It has been suggested that species differences may exist in the form of cell death and extent of apoptosis during luteolysis (Fraser et al., 1999).

There has been only one report describing the ultrastructural changes in the equine CL (Levine et al., 1979). As early as the mid-luteal phase, some luteal cells showed rarified mitochondria; by day 15 the luteal cells showed dilated nuclear, degeneration in the mitochondria and SER and there was swelling in the endothelial cells. There is growing evidence that the mitochondria play a vital role in steroidogenesis (Niswender and Nett, 1988), as well as in determining the fate of the cell (Brenner and Kroemer, 2000). Mitochondria play a central role in cell death pathways and are involved in the decisive role of the execution phase in the apoptotic pathway. During the execution phase of apoptosis, cytochrome C is released from mitochondria to the cytoplasm activating caspase 3 and 9.
which are subsequent involved in the degenerative events in the cytoplasm and nuclei (Green and Reed, 1998).

Cells which die by apoptosis may pass through three phases in which the cell exhibits different changes: phase 1, cells subject to genetic reprogramming, increased stress proteins, decreased polyamines and increase in Ca2+; phase 2, activation of endonuclease, release of cytochrome C and activation of caspases; phase 3, morphologic changes, formation of apoptotic bodies and phagocytosis of apoptotic bodies. Recent studies in other tissues indicate that disruption of mitochondria could reflect early signs of apoptosis (Kroemer et al. 1997; Xiang et al. 1996; Zamzami et al. 1995; Wilson 1998). Since apoptotic, cell death is regulated by oncoproteins in the mitochondria (Brenner et al., 1998), the release of cytochrome C from the intra-mitochondrial membranes to the cytoplasm was investigated. The study of apoptosis in the CL has become a popular topic in recent years. Surprisingly, very few reports have considered ultrastructural examination, given that the definitive identification of the type of cell death relies upon examination of the cells at the ultrastructural level (Wyllie et al., 1980, Kerr et al., 1995). The importance of the use of robust methods to investigate the cell death in the CL such as TEM came to light after the findings revealed by Fraser et al (1999). Ultrastructural observations of condensed nuclei of crescent or circular shape or fragmented nuclear chromatin will provide convincing evidence for apoptosis rather than TUNEL immunostaining, which often misled many studies.

The types of cell death and the ultrastructural changes occurring during natural, and induced luteal regression in the mare are not fully understood. Previous studies have shown marked variation i) in the control of CL luteolysis among species (Berhaman, 1993), ii) in the types of cell death involved in different cell population in the CL (Augutin et al., 1995), iii) in the outcome of using different techniques to determine the cell death types in the CL during regression (Fraser et al., 1999). The aim of the present study was to confirm our findings in the last chapter, and to explore whether the observed changes seen at light microscope level and TUNEL immunostaining correlate with ultrastructural findings. In addition, the question of whether the degenerative changes in the mitochondria at mid-luteal phase were an early step in structural degeneration was investigated using cytochrome C and tubulin immunostaining as indicators.
6.2 Materials and Methods

6.2.2 Immunocytochemistry of cytochrome C and tubulin
Sections of formalin-fixed CL were exposed to 2 x 10 min cycles of heating in the microwave then incubated with rabbit anti-horse cytochrome C (Cat no SC-7159, Santa Cruz Biotechnology Inc, California, USA), diluted to 1:250 in PBS, for 120 min at room temperature or overnight at 4 °C in a humidified chamber; for tubulin, sections were incubated with rabbit anti-tubulin (cat no T3526) (Sigma) diluted to 1:100 in PBS containing 0.001M Triton X100 for 120 min at room temperature. Negative controls were incubated with PBS containing 2% (v/v) normal rabbit IgG instead of the primary antibody.

6.2.2 Quantification of cytochrome C and tubulin immunostaining.
Sections of cytochrome C immunostaining were examined at x400 magnification. The number of cells exhibiting punctate staining and cytosol stained cells per 100 luteal cells were counted separately and expressed as mean ± SEM. The percentage area of tubulin immunostaining was quantified as described for VEGF and bFGF immunostaining (see chapter 4).

6.3 Results

6.3.1 Ultrastructural observations

Early luteal phase
Luteal cells (Fig 6.1a, b) contained a central, circular nucleus with one or more nucleoli. The heterochromatin was finely granular and uniformly dispersed in the nucleus in both the intra-nuclear zone and aligned to the nuclear envelope. Euchromatin was homogenous and uniformly dispersed. The cytoplasm of these cells showed features consistent with the appearance of hormone-producing cells including vesicles or/and anastomosis of smooth endoplasmic reticulum (SER), electron dense bodies, ribosomes, lipid droplets and mitochondria. SER was found as a series of short anastomosing tubules or vesicles, sometimes showing extensive sheets and arrays of fenestrated cisternae. Mitochondria tended to be in the peripheral regions, but groups of mitochondria were also found throughout the
cytoplasm with a round to elongated shape, and were normal and healthy looking with tubular and lamellar cristae. Groups of lipid droplets were also found in the cytoplasm. Different shape of dense bodies and golgi apparatus were also found in the cytoplasm.

**Mid-luteal phase**

Similar to the early luteal phase, the luteal cells (Fig 6.2a, b) were characterized by an oval to round shape or were irregular in shape. The dominant components in the cytoplasm were large amounts of vesicles or anastomosis of SER. Unexpectedly, mitochondrial matrix was severely rarefied in some cells forming different sizes of vacuoles, these vacuoles were surrounded by SER and sometimes showed close association or coalescence with each other. Electron dense bodies, ribosomes, few lipid droplets, golgi apparatus and rough endoplasmic reticulum were also found in the cytoplasm. Differentiated capillaries were prominent in the luteal tissue, consisting of endothelial cells (Fig 6.2c) with oval to vesicular shaped nuclei in cross sections, and elongated to spindle shaped nuclei in longitudinal sections. The heterochromatin was dispersed and aggregated in the inner layer of nuclear membrane. Complex infoldings of the nuclear envelope were frequently present. Red blood cells and pericytes were seen in some capillaries.

**Early regression phase**

Luteal cells showed clear degenerative changes. Apparent degenerative changes in the SER were observed including variable degrees of vascularisation and dilation or sometimes condensation. Mitochondria in most of the cells were rarefied and distorted and were less dense and most of them were devoid of any cristae. Golgi bodies were seen in stacks between the SER and were predominant in the peripheral area of the cytoplasm. Marked nuclear degenerative changes were observed. Luteal cells showed early stages of margination of chromatin (Fig 6.3a), or had fragmented chromatin, (Fig 6.3b), or appeared to be pyknotic (Fig 6.3c). Single or clusters of dense round bodies, were observed in the extracellular space (Fig 6.3d), or in the capillaries (Fig 6.3g). Other luteal cells showed non-apoptotic changes with shrinkage of nuclei and crenation of the nuclear membrane (Fig 6.3e). Macrophages were also observed with ingested degenerated luteal cells or round dense bodies and debris (Fig 6.3f). Endothelial cells appeared to swell and protrude into the lumen of the blood vessels (Fig 6.3d, h).
Fig 6.1 Early luteal phase. a) showing typical structure of hormone producing cells. Spherical nuclei (N) showed dispersion of heterochromatin (H). The cytoplasm contained healthy mitochondria (MA), lipid droplets (LI) and SER, x3000. b) higher magnification of a luteal cell, showing spherical nucleus with one nucleolus (NI) containing dense heterochromatin (H) dispersed in the nuclear zone and aligned to the nuclear envelope and less dense euchromatin (EU). lipid droplets (LI), healthy mitochondria (MA) and SER in the cytoplasm, x7600.
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(a) [Image 1]

(b) [Image 2]
Fig 6.2. Mid-luteal phase: a) luteal cells with large amount of SER, rarefied mitochondria (RM), vacuoles (V) and dense bodies (DB). Nucleus showed irregular shape compared with early luteal phase and contained more dense heterochromatin (H) and euchromatin (EU), X 2600. b) higher magnification of a luteal cell with rarefied mitochondria and dense bodies (DB), large amount of SER vesicles, rarefied mitochondria (RM) and nucleus showed irregular shape compared with early luteal phase and contained more dense heterochromatin (H) and euchromatin (EU). X 5000. c) capillary lumen surrounded by healthy endothelial cells with elongated flattened shape and jagged nuclear membrane. Dense masses of chromatin are present in the nucleus, X 4000.
Fig 6.3. Early regression (day14). a) luteal cell with early nuclear degenerative changes. Nucleus showed irregular shape with marginated chromatin (CH), sheets of golgi apparatus around the nucleus (GA) X 8600. b) more advanced stage of nuclear degeneration showing fragmented chromatin (CH), rarefied mitochondria (RM) dense bodies (DB) in the cytoplasm. X 7200. c) more advanced stage of nuclear degeneration than (b), showing a pyknotic nucleus (PK) with a mass of condensed chromatin, in the cytoplasm rarefied mitochondria (RM) autophagocytic bodies (AF), rarefied mitochondria (RM) and large vacuoles (V). X 5000 d) cluster of round dense bodies so-called apoptotic bodies (AP), capillary (CAP) with swelling endothelial cells (EN). X 6500.
Fig 6.3. Early regression (day 14). e) luteal cells with non-apoptotic nuclear degenerative changes showing crenation and involution in the nuclear membrane (N), rarefied mitochondria (RM) autophagic bodies (AF). X 2400. f) macrophage cell (MA) engulfing degenerated luteal cell (DLC) containing apoptotic nucleus (AN). X 6800.
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Fig 6.3. Early regression (day 14). g) cross section of a small capillary (CAP) blocked and obstructed with round dense bodies (AB) and pericyte cell (P) with flat, thin, long nucleus X 5200. h) capillary with lumen showing swollen endothelial cells (EN) protruding into the lumen of the blood vessel, debris and degenerated apoptotic bodies (AP), in the corner there is leukocyte cell (LK) X 2400.

Late regression phase
Luteal cells showed shrinkage, massive accumulation of lipid with foamy appearance, disruption in both SER and mitochondria (Fig 6.4a). Nuclear degenerative changes were similar to those found in early regression. Some luteal cells showed fragmented chromatin or pyknotic nuclei (Fig 6.4b), while others showed crenation of the nuclear membrane and the shrinkage of the nuclei (Fig, 6.4c). Capillaries were obstructed by swollen endothelial cells (Fig 6.4d) and apoptotic bodies. Macrophages were seen very often, engulffing whole degenerated luteal cells, or apoptotic bodies containing either primary or secondary lysosomes (Fig, 6.4e). Lymphocytes and a few neutrophils were also seen (Fig, 6.4f).
Fig 6.4. Late regression (day 17). a) Luteal cells showed different degenerative changes. Macrophage (MA) ingesting degenerated luteal cell with fragmented nucleus. Massive lipid (LI) accumulation. Nucleus shows irregular shape of nuclear membrane (N). Degenerated luteal cell with secondary lysosomes (LY). Capillary with swollen endothelial cells (CAP). Apoptotic bodies (AP) in the extracellular space. X 1400. b) Pyknotic nucleus (PK) with very condensed mass of chromatin. Accumulation of lipid droplets (LI). Rarefied mitochondria (RM) and SER. X 4200
Fig 6.4. Late regression (day 17). c) luteal cell showed non-apoptotic degenerative changes with massive lipid accumulation (LI) and rarefied mitochondria (RM) and condensed SER. X 4200. d) blood capillary (CAP) showing swollen endothelial cell (EN). X 6700. e) two macrophage appear to be in active phagocytosis due to the presence of primary lysosomes (LY), engulfing large amounts of debris (D) and apoptotic bodies (AP) X 7000. f) marked influx of leukocytes (LK) probably showing the morphology of lymphocytes. Accumulation of lipid (LI), secondary lysosomes (LY) and neutrophils (NE). X 3600.
**Induced regression**

After PGF$_{2\alpha}$ administration, similar changes to those in natural regression were seen. Some luteal cells showed margination of chromatin or had clumps of fragmented chromatin, or appeared to be pyknotic (Fig 6.5a). Other luteal cells showed non-apoptotic changes with shrinkage in the nuclei and involution of the nuclear membrane (Fig 6.5b). Clusters of dense round bodies were observed in the extracellular space (Fig 6.5c). The endothelial cells appeared to protrude into the blood vessel lumen and were swollen or detached from the plasma membrane (Fig 6.5e). Other cell types such as macrophages were also observed ingesting degenerated luteal cells (Fig 6.5d). Marked influx of neutrophils was seen (Fig 6.5f). These cells had a lobular shaped nucleus with 2-3 segments, darkly dense chromatin, and dark condensed granules in the cytoplasm. Some eosinophils were also seen (Fig 6.5g) containing unique large dark secretory granules in their cytoplasm.

6.3.2 **Cytochrome C immunostaining**

Cytochrome C is normally present in the inter-membrane space of healthy mitochondria. The mitochondrial immunostaining was seen in the cytoplasm of luteal cells and endothelial cells and other cells in all sections examined throughout the cycle. The luteal cells showed intense punctated staining in the peripheral regions of the cytoplasm during the early luteal phase (Fig 6.6a). By mid-luteal phase (Fig 6.6b) and early regression (Fig 6.6c) most of the luteal cells showed strong cytosolic immunostaining presumably due to the release of cytochrome C from the mitochondria to the cytoplasm. In late regression, few cells showed cytosol immunostaining but there was some staining between the degenerated cells and debris (Fig 6.6d). Twelve hours and 36 h following PGF$_{2\alpha}$, most of the luteal cells showed cytosol immunostaining. The highest numbers of luteal cells showing punctated staining were seen during early luteal phase (Fig 6.7), followed by a significant decrease (p< 0.01) in the following phases. Mid-luteal phase and early regression phase showed the highest number (p< 0.01) of luteal cells with cytosolic staining.
Fig 6.5. Induced regression a) luteal cell with large amount of SER, rarefied mitochondria (RM). Nucleus containing fragmented dense chromatin, swollen endothelial cell (EN), neutrophils (NE) and pericytes (P) X 4000. b) other luteal cell contained dense bodies (DB), large amount of condensed SER. Nucleus has crenation and involution in the nuclear membrane X 5200.
Fig 6.5. Induced regression c) cluster of apoptotic bodies (AP) surrounded by a membrane in the extracellular space, autophagocytic vacuoles (AU). X 8000. d) macrophage ingesting large amounts of debris and apoptotic bodies (AP) X 7000.
Fig 6.5. Induced regression e) endothelial cells (EN) appeared to protrude into the blood vessel lumens swollen and detached from the plasma membrane. X 3600. f) massive influx of neutrophils (NE), lipid droplets (LI) and rarified mitochondria (RM). X 4000
Fig 6.5. Induced regression at 36 h following PGF$_{2\alpha}$. g) eosinophils (ES) with nucleus (N) clumps and dark condensed secretory granules x4000

6.3.3 Tubulin immunostaining

Tubulin is a major component of the cellular skeleton and part of the microtubule structure in the cytoplasm. Tubulin positive immunostaining was seen in the cytoplasm of luteal cells in all the sections examined throughout the cycle. Luteal cells showed strong and intense staining during early luteal phase (Fig 6.8a). By mid-luteal phase, a number of luteal cells were immunostained (Fig 6.8b). During early and late regression, few cells were immunostained (Fig 4.1c,d). Following PGF$_{2\alpha}$-induced regression the immunostaining was similar to those at mid-luteal phase. The percentage area of the immunostaining was high during early luteal phase then decreased significantly (p< 0.01) during mid-luteal and luteal regression (Fig 6.9).
Fig 6.6 Cytochrome C immunostaining in the luteal cells throughout the cycle. Cytochrome C is normally present in the inter-membrane space of healthy mitochondria. a) Luteal cells contained healthy mitochondria with intense punctuate immunostaining during the early luteal phase, inset shows higher magnification of mitochondrial punctuated immunostaining. b) Luteal cells contained rarefied mitochondria exhibiting cytosol immunostaining during the mid-luteal phase, inset shows higher magnification of mitochondrial cytosolic immunostaining, these cells are believed to show cytochrome C release from the mitochondria to the cytoplasm resulted in cytosolic immunostaining. c) Cytosol immunostaining during early regression, one apoptotic cell showing strong cytosol immunostaining (arrow), inset shows no immunostaining (negative control). d) Late regression, few cells showed cytosol immunostaining in the intercellular space (arrowhead). Scale bar represents 10μm and 2μm for the insets.
Fig 6.7. The number of luteal cells with punctate and cytosol immunostaining with anti-horse cytochrome C throughout the cycle and after PGF$_{2\alpha}$ administration. Data are expressed as mean ± SEM.

a: significantly (p< 0.01) higher than all other groups.
b: significantly (p< 0.01) higher than early luteal phase.
c: significantly (p< 0.01) lower than early regression.
Fig 6.8. Tubulin immunostaining with cytoplasmic staining throughout the luteal phase.

a) early luteal phase with intense immunostaining of microtubules in the luteal cells, negative control free of immunostaining (inset). b) mid-luteal phase, few luteal cells showing intense immunostaining of microtubules in the cytoplasm c) scattered and faint immunostaining in the luteal cells and extracellular space during early regression. d) late regression, dispersed immunostaining in the intercellular space and stromal tissue. Scale bar represents 50 µm and 20 µm for the inset.
Figure 6.9. The percentage of area of tubulin immunostaining throughout the cycle and following PGF$_{2\alpha}$-induced regression, expressed as mean ±SEM.

a: significantly (p< 0.01) higher than all groups.

b: significantly (p< 0.01) higher than early and late regression.
6.4 Discussion

We have described cell death at the ultrastructural level during natural and induced luteal regression in the mare. The detection of different stages of nuclear degenerative changes (marginated chromatin, fragmented chromatin and pyknotic nuclei) in the luteal cells and the present of round dense bodies during natural and PGF2α-induced regression, were strongly suggestive of apoptosis. However, the shrinkage of the nuclei and the crenation and involution of the nuclear membrane in the luteal cells was suggestive of another form of cell death.

Rarefied mitochondria indicative of minor degenerative changes were observed in some luteal cells in the mid-luteal phase (day 10). This is around the time of the onset of declining progesterone concentrations in the oestrous cycle in the mare (Van Niekerk et al., 1975). By day 14, and 12 h following PGF2α administration on day 10, marked structural degenerative changes (apoptosis) were observed after circulating progesterone concentrations had fallen to less than 1 ng/ml. In our previous findings, we showed that the sharp decline in progesterone levels in early regression phase and 12 h following PGF2α preceded the decline in vascularity. Minor structural changes (rarefied mitochondria) were evident in the mid-luteal phase, but overall findings demonstrated that marked structural changes (nuclear degenerative) did not precede the fall in progesterone concentrations. This is in agreement with a previous report in the equine CL (Van Niekerk et al., 1975) and in other species (Umo, 1975; Juengel et al., 1993).

It has been proposed that the degeneration of the mitochondria in the mid-luteal phase in the mare is an early step in the decline in progesterone levels, since the mitochondria are involved in steroidogenesis (Levine et al., 1979). Recent studies in other tissues indicate that disruption of mitochondria could reflect early signs of apoptosis (Kroemer et al. 1997; Xiang et al. 1996; Zamzami et al. 1995; Wilson 1998). Since apoptotic cell death is regulated by oncoproteins in the mitochondria (Brenner et al., 1998), this period of regression may be the key time for further study of changes in the expression of the oncoproteins that initiate the cell death process.
To explore whether the rarefied mitochondria in mid-luteal phase are degenerative changes reflecting cell death pathway and/or a cause of functional luteolysis, the release of cytochrome C from the intra-mitochondrial membranes to the cytoplasm was studied. During the early luteal phase, luteal cells showed peripheral punctate immunostaining of cytochrome C and a normal and intact appearance of mitochondria at TEM level. By the mid-luteal phase, luteal cells showed cytosol immunostaining and rarefied mitochondria ultrastructurally. The changed pattern of cytochrome C immunostaining from punctated during early luteal phase to cytosolic during mid-luteal phase, clearly suggests a relationship between the cytosol immunostaining and the observed rarefied mitochondria, which reflect structural damage in the mitochondria in the mid-luteal phase. Previous studies have shown that apoptosis was associated with the release of cytochrome C in the granulosa cells in the ovary (Robles et al., 1999; Hsu et al., 2000) and in many other tissues such as neurons (Sugawara et al., 1999), heart (Cesselli et al., 2001), liver (Higuchi et al., 2001) and smooth muscle (Granville et al., 2001). The release of cytochrome C from mitochondria to cytosol is responsible for the activation of effector caspases and consequently the demise of the cell (Reed et al., 1998; Robles et al., 1999; Li et al., 2000; Shah et al., 2000; Dauffenbach et al., 2000 Abu-Qare et al., 2001). These studies suggested that mitochondria act not only as a cellular powerhouse, but also play a decisive role in apoptotic and necrotic death pathways.

Tubulin is part of the cytoskeletal matrix of the SER and mitochondrial structure in the luteal cells (Murdoch, 1996). Tubulin serves as a carrier-mediator for many inter/intra-cellular nutrients, e.g. tubulin is involved in the transport of cholesterol to the inner mitochondrial membrane for the side chain cleavage enzymes which been considered to be a crucial step in steroidogenesis (Gemmell et al., 1977; Carnegie et al., 1988). Tubulin immunostaining was carried out in order to assess the integrity of the microtubules. During the early luteal phase, intense immunostaining of tubulin was found in most of the luteal cells, when SER and mitochondria had a healthy appearance ultrastructurally in these cells. By mid-luteal phase, there was reduction in the immunostaining. This reduction in the immunostaining may indicate a reduction in microtubular density in the luteal cells. However, these findings most likely reflect structural damage in the mitochondrial microtubules. Previous reports have used tubulin alongside ultrastructural observation to assess the integrity of the mitochondria and SER in the neurons of grass sickness horses (Griffiths et al., 1993) and in the CL post PGF2α injection (Murdoch, 1996). These studies suggested a direct link between the
structural damage of SER and mitochondrial microtubules and the reduction in the tubulin immunostaining density.

Other changes (shrinkage of the nuclei with crenation and involution of the nuclear membrane) seen in some luteal cells during luteal regression in the mare may indicate that there is an additional non-apoptotic form of cell death at luteolysis. It has been proposed that autophagocytosis and nonlysosomal disintegration occur in the regressing CL of the marmoset (Fraser et al., 1999). In autophagocytosis, the nucleus disintegrates and is digested by autolysosomes, while in nonlysosomal disintegration, organelles swell and formation of vacuoles and cytoplasmic destruction is followed by nuclear disintegration. However, these changes were not observed in the present study. Terminal differentiation has been reported to be another type of cell death, whereby certain cells types such as the outer squamous layers of skin and the granulosa cells next to the antrum of atretic ovarian follicles undergo nuclear destruction or expulsion before the cessation of cellular function leads to cell death, with condensation in the mitochondria and other organelles (Stenn, 1983; van Wezel et al., 1999).

Ultrastructural observation confirmed our findings at light microscope level that endothelial cells did not show any signs of apoptosis, but showed signs of swelling and detachment from the walls of the blood vessels in agreement with Levine et al. (1979). Reports from previous studies showed apoptosis in endothelial cells ultrastructurally during regression in guinea pigs (Azmi and O’Shea, 1984) and in the sheep following PGF_{2α}-induced regression (Sawyer et al., 1991), but not in bovine CL (Augustin et al., 1995; Modlich et al., 1996). These studies strongly suggest that there are species differences in the fate of endothelial cells during luteolysis, and that the vasculature in bovine and equine CL regresses in a similar manner.

In the previous chapter, we suggested that phagocytosis might to be responsible for the disappearance of round dense bodies (apoptotic bodies) or that they might be flushed out of the CL via the blood supply; but data from the present study and previous studies has indicated that elimination by macrophages is likely to occur (Wyllie et al., 1980; Bursch et al., 1985; Clarke 1990; Majno and Joris 1995; Columbano, 1995 Jolly et al., 1997; Van Wezel et al., 1999). The increased numbers of macrophages during luteal regression seen
ultrastructurally and immunocytochemically in the present study, and in agreement with a previous study in the mare (Lawler et al., 1999), suggest a role for macrophages in the removal of apoptotic bodies.

The presence of a marked influx of neutrophils following PGF$_{2\alpha}$-induced luteolysis has been confirmed ultrastructurally. Neutrophils appeared to be active and mature showing lobular shaped nuclei and with cytoplasm containing dense secretory granules responsible for the secretion of proteases and toxic cytokines and many other biochemical products (Jain et al., 1997). In addition to neutrophils, there was an influx of eosinophils 36 h following PGF$_{2\alpha}$ injection in agreement with Lawler et al., (1999) and others including pig (Standaert et al., 1991) and sheep (Murdoch, 1987). Eosinophils believed to play a role luteal regression throughout cytokine secretion and may make the process of regression more faster (Murdoch, 1987). These studies suggested a role for eosinophils in the initiation of luteal regression (Murdoch, 1987). Invasion of leukocytes (probably lymphocytes) was found during late regression, in agreement with recent finding in the equine CL (Lawler et al., 1999).

Lipid accumulation during late regression and 36 h following PGF$_{2\alpha}$ was evident ultrastructurally. Lipid droplets throughout the luteal phase appeared as pale or sometimes as grey colour in different phases of cycle. These findings have previously been reported in the equine CL (Levine et al., 1979). Different types of lipid have been demonstrated in the CL throughout the cycle of many species including rat (Guraya, 1975, 1977), pig (Waterman, 1984) and sheep (Waterman, 1988). Biochemical experiments showed the level of cholesterol and/or its esters, triglycerides and phospholipid in the luteal cells might differ throughout the luteal phase (Guraya, 1975, Waterman, 1988).

In conclusion, we have shown that ultrastructural degenerative changes in the equine CL may be initiated by day 10 (mitochondrial rarefaction) and apoptotic bodies and cells were present by day 14 of the cycle and 12 h post PGF$_{2\alpha}$ injection. Non-apoptotic changes were also observed in some luteal cells during regression. Phagocytosis is a feature of late regression. Endothelial cells showed no sign of apoptosis, but displayed swelling and protrusion into the lumen of the blood vessels. A marked influx of neutrophils was observed after PGF$_{2\alpha}$-
induced regression. In the next chapter, further investigation was conducted to examine whether oncoproteins and cytokines such as Bcl2, Bax, TNFα and NOS are involved in luteal regression in the mare.
Chapter 7

Cell Death in the Equine CL (3). The expression of TNF$_{\alpha}$, iNOS, Bcl2 and Bax: Immunocytochemical study.
Introduction

Luteolysis is regulated by a series of factors tightly controlling the fate of cells. Previous studies suggested that luteolysis may represent apoptosis as a form of programmed cell death (Sawyer et al., 1990). Apoptosis is regulated by molecules, which decide the status of the cell. Several molecules such as cytokines, growth factors, peptide hormones, reactive oxygen species (ROS) (Feuerstein et al., 1999) and steroids (Billing et al., 1993; Evansstorm and Cidlowski, 1995) have been reported to regulate apoptosis in different tissues. Over the past few years, cytokines, tumour suppressor genes, death genes and survival genes have been found to play a role in the fate of human luteal cells (Matsubara et al., 2000). Little information is known on the mechanisms responsible for activating cell death in the equine CL. Cytokines and oncoproteins involved in luteal regression in the mare remain unclear. It is important to investigate the role of some of these factors in order to improve our understanding of luteal regression in the mare.

Cytokines are involved in triggering apoptosis in the CL. The most recent data showed that TNFα and nitric oxide (NO) have a role in luteal regression. TNFα is a non-glycoslated protein and macrophages are believed to be the dominant source of TNFα in the CL in late stages (Roby and Terranova, 1989; Bagavandoss et al., 1990). There is growing evidence that TNFα and inflammatory cells in the CL may promote luteal regression (Bagavandoss et al., 1990; Adashi et al., 1990). Luteal cells have been reported to contain immunoreactivity for TNFα (Bagavandoss et al., 1990) and play a role as an autocrine luteotropin. It has been demonstrated that TNFα inhibits gonadotrophin-supported progesterone production in murine (Adashi et al., 1990), porcine (Pitzel et al., 1993), and bovine (Benyo and Pate, 1992) luteal cells and stimulates PGF2α synthesis by bovine luteal cells (Bagavandoss et al., 1990). High concentrations of TNFα induced apoptosis and death in the luteal cells in vitro (Jo et al., 1995).

Nitric oxide (NO) is synthesised enzymatically by nitric oxide synthase (NOS) converting L-arginine to L-citrulline. NO is a powerful vasodilator and a crucial mediator of macrophage cytotoxicity and apoptosis (Lowenstein and Snyder 1992; Mebmer et al.,
1994). Two isoforms, endothelial NOS (eNOS) and inducible NOS (iNOS) have been detected in the ovarian tissue of rat and mouse (Van Voorhis et al., 1995; Oslon and Jones-Burton, 1996; Jablonka-Shariff and Olson, 1997). Human luteinised granulosa cells showed eNOS mRNA expression (Van Voorhis et al., 1994). NO production is involved in the signal transduction mechanisms leading to apoptosis in the human CL. Studies have shown that NO induces apoptosis in vitro in human luteal cells (Vega et al., 2000) suggesting that NO may play a role as a luteolytic factor in the human CL (Friden et al., 2000). The more recent finding in rat CL showed that NO has a double function in CL development and regression (Motta et al., 2001).

The role of mitochondria in cell death is well documented. Oncoproteins of the Bcl2 family interact to regulate programmed cell death, and are found in the mitochondria and endoplasmic reticulum (Monaghan et al., 1992). Bcl2 is believed to inhibit the occurrence of apoptosis in human follicular lymphoma (Croce, 1986). Bax is an oncoprotein with a degree of homology to Bcl2 (Oltvai et al., 1993), and is believed to induce apoptosis. Bax itself forms homodimers to induce apoptosis, but it is the relative levels of Bcl2 and Bax that determine the fate of a cell, rather than the absolute concentration of either (Oltvai et al., 1993; Yin et al., 1994). Bcl2 expression during luteolysis in women (Rodger et al., 1995) and in the rat (Trott et al., 1997) did not act in a diametric manner to regulate cell death in the CL. However, during luteolysis in cattle, mRNA encoding Bax was elevated while Bcl2 remains unchanged (Rueda et al., 1997).

The presence of high numbers of leukocytes in the luteal tissue during regression may contribute to luteolysis via cytokine secretion (Lawler et al., 1999). TNFa (Friedman et al., 2001) and NO (Friden et al., 2000) are thought to be involved in luteolysis in many species by inducing apoptosis in vitro. It is important to investigate and explore the role of TNFa and NO in the equine CL. In the present study, we investigated the possible role of TNFa and NO by investigating the immunolocalisation of TNFa, eNOS, iNOS, Bcl2 and Bax throughout the cycle and following PGF2α injection, to explore the role of these factors in luteal regression in the CL.
Materials and Methods

7.2.2 immunocytochemistry of Bcl2, Bax, TNFα and iNOS

TNFα
Sections of formalin fixed tissue were incubated for 120 min at room temperature in a humidified chamber or overnight at 4 °C with goat anti-TNFα (Santa Crus Biotechnology) diluted to 1:250 in PBS. Negative controls were incubated with PBS containing normal goat IgG instead of the primary antibody.

NOS
Sections were immersed in citrate buffer and exposed to three cycles of 5 min each in the microwave, then incubated for 120 min at room temperature with rabbit anti-human eNOS and rabbit anti-mouse iNOS (Transduction Laboratories, Lexington, USA) diluted to 1:500 in PBS. Negative controls were incubated with PBS containing normal rabbit IgG instead of the primary antibody.

Bcl2 and Bax
Formalin-fixed sections were exposed to 5 min in a pressure cooker, then incubated for 150 min at 37 °C with rabbit anti-Bcl2 (Bcl2: PC 68T) and Bax (Bax: PC66T, Oncogene, Boston, USA), diluted to 1:1000 in PBS. Negative controls were incubated with PBS containing normal rabbit normal IgG instead of the primary antibody.

7.2.2 The percentage area of Bcl2, Bax, TNFα, iNOS and eNOS immunostaining: Quantimet image system was used to estimate the percentage area of the immunostaining (area of the immunostaining stain divided by the total area measured x100). The system was optimised for each individual section based on the density of the stain. The results were expressed as percentage mean ± SEM per unit area.
7.3 Results

7.3.1 TNFα immunostaining

Positive immunostaining was found in the cytoplasm of luteal cells in all sections examined throughout the cycle. Non-luteal cells, including neutrophils and probably macrophages and/or lymphocytes were also immunostained during regression. No positive staining was observed in negative control sections (Fig 7.1b, inset) and no apparent staining was detected in endothelial cells. Some growing luteal cells exhibited immunoreactivity during the early luteal phase (Fig 7.1a) and by the mid-luteal phase (Fig 7.1b) a few cells were immunostained. Pale immunostaining was found in a few luteal cells during early regression; by late regression most of the luteal cells showed immunostaining (Fig 7.1c) as well as some non-luteal cells (Fig 7.1c, inset). Twelve and 36 h following PGF2α-induced regression some luteal cells showed strong immunostaining including those that underwent apoptosis (Fig 7.1d) as well as some neutrophils (Fig 7.1d, inset). The percentage area of TNFα immunostaining was high during the early luteal phase then decreased significantly (p< 0.01) at mid-luteal phase and early regression (Fig 7.2). By late regression and 36 h following PGF2α there was significant increase in TNFα immunostaining (p< 0.01) compared with early regression and 12 h following PGF2α, respectively.

7.3.2 NOS immunostaining

iNOS immunostaining was seen in the cytoplasm of luteal cells in all sections examined throughout the cycle. No positive staining was observed in negative control sections (Fig 7.3b, inset). Positive staining was found in some luteal cells during the early luteal phase (Fig 7.3a) and a few mature luteal cells during the mid-luteal phase (Fig 7.3b). During natural and induced regression, luteal cells were densely immunostained (Fig 7.3c). The percentage area of iNOS immunostaining (Fig 7.4) was significantly (p< 0.01) higher during regression in comparison with the early and mid-luteal phase. eNOS immunostaining was limited to a few luteal cells and endothelial cells during early (Fig 7.3d) and mid-luteal phase (Fig 7.3e). Little immunostaining was found during natural and induced regression (Fig 7.3f). The percentage area of eNOS immunostaining (Fig 7.4) was significantly (p< 0.05) higher during early and mid-luteal phase in comparison with regression.
Fig 7.1. TNFα immunostaining during luteal phase and following PGF$_{2\alpha}$-induced regression. a) some luteal cells showed cytoplasmic immunostaining during early luteal phase. b) by mid-luteal phase few luteal cells showed immunopositive, negative control (inset) free of any immunostaining. c-d) late luteal regression and 36 h post PGF$_{2\alpha}$ injections showing immunopositive luteal cells, luteal cells with apoptotic appearance showed strong positive staining (thin black arrow). Immunostaining for TNFα were found in non-luteal cells probably lymphocytes and macrophages (white arrowhead, c: inset) and neutrophils (black arrowhead, d: inset) after PGF$_{2\alpha}$-induced regression. Scale bar represents 40 µm and 5 µm for the insets.
Fig 7.2. The percentage area of TNFα immunostaining throughout the cycle and after PGF₂α administration. Data are expressed as percentage (mean ± SEM).

a: significantly (p< 0.01) higher mid-luteal phase.
b: significantly (p< 0.01) higher than mid-luteal phase and early regression.
c: significantly (p< 0.05) higher than mid-luteal phase and 12 h following PGF₂α.

7.3.3 Bcl2 and Bax immunostaining

Bcl2 and Bax immunostaining was found in the cytoplasm of luteal cells in all sections examined throughout the cycle. Bcl2 and Bax immunostaining were broadly similar. No positive staining was observed in negative control sections (inset). During early luteal phase some luteal cells were immunostained while the others were pale or were free of immunostaining (Fig 7.5-6a). By mid-luteal phase, most of the luteal cells showed strong and more intense immunostaining than those in early luteal phase (Fig 7.5-6b), this remained without change during early regression. In late regression, the immunostaining was found in most of the luteal cells and in the extracellular space (Fig 7.5-6c). During PGF₂α-induced regression most of luteal cells showed strong immunostaining similar to mid-luteal phase and early regression (Fig 7.5-6d). The percentage area of Bcl2 and Bax immunostaining was broadly similar throughout the luteal phase. Intense immunostaining was found during mid-luteal phase (Fig 7.7) then decreased significantly during late regression (p< 0.05).
Fig 7.3. iNOS immunostaining during early luteal phase (a), mid-luteal phase (b), 36h following PGF2α injection (c). Luteal cells showed immunostaining during early luteal phase while very few cells showed positive staining during mid-luteal phase. During natural and induced regression most of luteal cells showed strong immunostaining. No positive immunostaining was found in the negative control (b: inset). eNOS immunostaining was restricted to some endothelial cells and very few luteal cell during early (d) and mid-luteal phase (e). During early regression, pale immunostaining was found in few luteal cells (f). Scale bar represents 50 μm and 10 μm for the inset.
Fig 7.4. The percentage area of iNOS and eNOS immunostaining throughout the cycle and after PGF$_{2\alpha}$ administration. Data are expressed as percentage (mean ± SEM).

a: significantly (p< 0.05) higher than mid-luteal phase and early regression.
b: significantly (p< 0.05) higher than early and late regression.
c: significantly (p< 0.01) higher than early, mid-luteal phase and early regression.
d: significantly (p< 0.05) higher than mid-luteal phase and 12 h post PGF$_{2\alpha}$. 
Fig 7.5. Bcl2 immunostaining during early luteal phase (a), mid-luteal phase (b), late regression (c) and 36 h following PGF$_{2a}$induced regression (d). a) A number of luteal cells showed cytoplasmic immunostaining during early luteal phase. Positive control (inset) obtained from horse tonsil, lymphocytes stained positively for Bcl2. b) By mid-luteal phase most of the luteal cells showed strong immunopositive, negative control (inset) free of any immunostaining. c-d) late luteal regression and 36 h post PGF$_{2a}$ injection showing immunopositive luteal cells, some immunostaining was found in the extracellular space. Scale bar represents 50 µm and 10 µm for the insets.
Fig 7.6. Bax immunostaining during early luteal phase (a), mid-luteal phase (b), late regression (c) and 36 h following PGF$_2$$_a$-induced regression (d). Note cytoplasmic immunostaining for Bax, with dense staining in each phase of the cycle and reduction during late regression. Scale bar represents 50 μm.
Fig 7.7. The percentage area of Bcl2 and Bax immunostaining throughout the cycle and after PGF$_{2\alpha}$ administration. Data are expressed as percentage (mean ± SEM).

a-b: significantly (p< 0.05) higher than all other groups.

c-d: significantly (p< 0.05) lower than early regression.

e-f: significantly (p< 0.05) lower than mid-luteal phase.
7.4 Discussion
The present study has described the immunolocalisation of Bcl2, Bax, TNFα, iNOS and eNOS in the equine CL. The association of TNFα and iNOS intense immunostaining and luteolysis, suggests an important role for TNFα and NO in cell death. The pattern of eNOS immunostaining during early and mid-luteal phase may indicate a role for NO in CL development and angiogenesis. Bcl2 and Bax immunostaining did not appear to act in diametric manner to regulate cell death.

This is the first report to describe the immunolocalisation of TNFα in the equine CL. Luteal cells, neutrophils and macrophages/lymphocytes were immunostained for TNFα. Previous studies in the CL believed that luteal cells and macrophages are the main source of TNFα in the human CL (Roby et al., 1990, Zolti et al., 1990) and in the bovine (Roby and Terranova, 1989; Zolti et al., 1990), ovine (Ji et al., 1990), pig (Zhao et al., 1998) and rabbit (Bagavandoss et al., 1988, 1990) CL. Neutrophils are the main source of many cytokines including TNFα (Jain, 1993), and neutrophils have shown immunostaining for TNFα in many other tissues including human endometrium (Von Wolff et al., 1999), blood (Djeu et al., 1990), the liver (Ohlinger et al., 1993) and periodontal tissue (Miyauchi et al., 2001).

Changes in TNFα immunostaining were observed throughout the luteal phase. During early and mid-luteal phase, scattered luteal cells were immunostained. This may suggest a role for TNFα in CL development and maintenance, and the presence of TNFα in the luteal cells, may indicate a paracrine and/or autocrine luteotrophic role for TNFα in the CL (Adashi et al., 1989; 1990). Previous studies presumed that TNFα may be one of the factors mediating luteotropic function (Kirsch et al., 1981, 1983), and may play a role in stimulating luteal progesterone secretion (Terranova et al., 1991). A recent study showed an increase in granulosa proliferating cells in the pig when treated with TNFα in vitro (Prange-Kiel et al., 2001). However, in the present study the intense immunostaining in late regression suggested an interesting role for TNFα in luteal regression. The role of TNFα in luteal regression has been investigated and different hypotheses have been suggested.
including the stimulatory effect on PGF$_{2\alpha}$ release \textit{in vitro} (Zolti et al., 1990), inhibition of LH action (Benyo et al., 1992), induction of apoptosis and the initiate of structural regression (Jo et al., 1995; Prange-Kiel et al., 2001, Petroff et al., 2001, Friedman et al., 2001). The peak increase in TNF$\alpha$ immunostaining at late regression (day 17) does not support any of these hypotheses because structural luteal regression was evident in early regression (day 14). TNF$\alpha$ seemed to be involved in the removal and clearance of degenerated luteal cells, as demonstrated in the ovine CL (Ji et al., 1991). During late regression and 36 h following PGF$_{2\alpha}$ injection, luteal cells with pyknotic appearance showed intense immunostaining for TNF$\alpha$. These findings suggest a direct role for TNF$\alpha$ in luteal cell demise.

Other cells such as neutrophils showed immunostaining for TNF$\alpha$ during induced regression at 12 and 36 h. The presence of TNF$\alpha$ in neutrophils may be important to luteolysis (Brannstrom et al., 1994). In addition, TNF$\alpha$ is regarded as a vasodilation agent (Takahashi et al., 1999; Shibata et al., 1996). It is possible that the presence of TNF$\alpha$ in the neutrophils may play a role in increasing blood vessel permeability at 12 h post PGF$_{2\alpha}$ injection, causing the vasodilation observed at this time. However, macrophages have been reported to be the main source of TNF$\alpha$ in many tissues including the CL (Bagavandoss et al., 1988). Many previous studies (Bagavandoss et al., 1988; Pate, 1994; Penny et al., 1999; Lawler et al., 1999) including our observations at EM level and MAC 387 immunostaining showed an increased in the number of macrophages during regression. This may suggest a role for macrophages in luteal regression.

eNOS and iNOS immunostaining appeared to show changes in the density and localization throughout the luteal phase. eNOS was restricted to the early and mid-luteal phase, while some endothelial cells and a few luteal cells were immunostained. Previous studies showed immunostaining of eNOS in endothelial and luteal cells in rodents (Van Voorhis et al., 1995; Jablonka-Shariff and Olson, 1997) and human (Van Voorhis et al., 1994; Vega et al., 1998; Friden et al., 2000). iNOS was found to be limited to the luteal cells during the normal luteal phase and following PGF$_{2\alpha}$-induced regression, in agreement with other
studies in the human CL, where luteal cells showed immunoreactivity for iNOS (Vega et al., 1998; Friden et al., 2000).

Our findings of eNOS and iNOS immunostaining during early and mid-luteal phase may indicate a role for NO in angiogenesis. The restricted immunostaining of eNOS in early and mid-luteal phase may indicate a role for eNOS in CL development and angiogenesis. We may also assume a protective role for NO during the early and mid-luteal phase. These suggestions may support the recent findings of Friden et al. (2000) and Motta et al. (2001) which revealed a role for NO in increasing progesterone production. NO is known to mediate many physiological and pathological functions (Motta et al., 1999), including angiogenesis, vascular permeability and cell death. NO production induced angiogenesis and vascular permeability via VEGF action in wound healing (Lee et al., 1999) and in lung development (Parker et al., 2000). It has been reported that VEGF enhances the expression of eNOS in cultured endothelial cells, which are believed to be important in the process of VEGF-induced angiogenesis (Papapetropoulos et al., 1997; Bouloumie et al., 1999). In contrast, inhibition of in vivo NO production, reduces angiogenesis and vascular permeability (Papapetropoulos et al., 1997). Many studies have demonstrated a role for eNOS in angiogenesis and cell proliferation (Jadeski et al., 2000) and it is considered to be an anti-apoptotic factor (Knowles and Moncada, 1994; Dimmeler and Zeiher, 1997).

During natural regression and induced regression, intense immunostaining of iNOS was evident. This may suggest a role for NO in luteal regression and cell death. iNOS has been considered to be a pro-apoptotic factor (Knowles and Moncada, 1994; Dimmeler and Zeiher, 1997). Recent in vitro studies in CL showed that NO may play a role as a luteolytic factor in rat and human luteal cells (Vega et al., 2000; Friden et al., 2000; Motta et al., 2001). Intra-luteal injections of the NO blocker (Nomega-Nitro-L-Arginine Methyl Ester (L-NAME): an inhibitor of nitric oxide synthase (NOS) in cows, showed increased production of progesterone during regression. This may indicate that NO plays a direct luteolytic role in the bovine CL (Jaroszewski and Hansel, 2000). Moreover, PGF$_{2\alpha}$-induced regression may upregulate NOS activity and, consequently, the production of NO, which acutely inhibits progesterone release (Gobbetti et al., 1999). This may suggest that NO may be involved in participating in functional regression by inhibiting steroidogenesis during
regression in bovine (Jaroszewski and Hansel, 2000) and in rabbit CL (Gobbetti et al., 1999). In another study, enhancement of ovarian NO activity was observed during PGF$_{2\alpha}$-induced regression in rats (Motta et al., 2001).

The best known oncoproteins in regulation of apoptosis are Bcl2 and Bax (Oltvai et al., 1993; Yin et al., 1994). They are involved in the critical execution stage of cell death. The mechanisms by which Bcl2 and Bax regulate apoptosis are still under intensive investigation. Several hypotheses have been proposed including the involvement in regulating the release of the execution caspases from the mitochondria to the cytoplasm, whereby the activation of caspase 3 and 9 takes place (Mancini et al., 1998). Other studies proposed that the relative levels of Bcl2 and Bax, not the absolute concentration of either, would determine the fate of a cell (Oltvai et al., 1993; Yin et al., 1994). Our findings showed that the immunostaining of Bcl2 and Bax were broadly similar throughout the cycle and following PGF$_{2\alpha}$-induced regression. Hormone-producing cells showed intense staining for both Bcl2 and Bax during mid-luteal phase, when apoptosis is rarely found. Unexpectedly, the density of staining remained unchanged during early regression when apoptosis occurrence reached its peak. Our understanding of Bcl2 action is in preventing cell death while Bax induces it. If Bcl2 and Bax were to play a role in the fate of the equine luteal cells, we have to expect increased in Bax immunostaining at luteolysis. However, this was not evident in our study and other studies in the CL have shown unchanged expression of Bcl2 in mature and regressed CL in the human (Rodger et al., 1995) and rat (Trott et al., 1997). Controversially, studies in cows showed a rise in Bax expression, which may be involved in cell death in the bovine CL (Rueda et al., 1997) and rat CL (Tilly et al., 1996). In the present study the rise in Bax immunostaining does not correlates with apoptosis occurrence, so we cannot rule out the involvement of Bax in cell death on the basis of the hypothesis of Oltvai et al., (1993). Further investigations are needed to elucidate the role of Bax in the equine CL.

In summary, our findings showed for the first time the immuno-localization of TNF$\alpha$, NO, Bcl2 and Bax in the equine CL. It is likely that TNF$\alpha$ and iNOS may be involved in luteal regression. eNOS showed association with CL development and angiogenesis. However, changes in Bcl2 and Bax ratio do not show an association with apoptosis or cell death.
Chapter 8

General Discussion
This thesis has described the cellular mechanisms of angiogenesis and cell death in the equine CL throughout the luteal phase and following PGF$_{2\alpha}$-induced luteolysis. A discussion of the findings has been presented in the relevant chapters. Therefore, this chapter provides an opportunity to summarise the main findings of the thesis, to clarify unexplained results and highlight the areas which need further investigation in the future, and discuss the clinical implications of this research.

8.1 The main findings

Although endocrine ovarian events which take place during the equine cycle have been investigated previously, many mechanisms and regulatory pathways such as those controlling corpus luteum formation, angiogenesis and regression are still unclear. Information concerning angiogenesis and cell death in the equine CL was limited to a few reports describing the basic morphological changes in the CL during the luteal phase (Harrison, 1946; Van Niekerk et al., 1975; Levine et al., 1979), and little information is available on the cellular mechanisms involved in PGF$_{2\alpha}$-induced regression. This thesis was designed to investigate the cellular mechanisms of angiogenesis and cell death in order to increase our understanding of CL control in the mare. A number of important findings have been described: 1) Luteal angiogenesis and regression can be evaluated and quantified effectively in tissue sections, using histological and semiquantitative immunocytochemistry methods by measuring specific parameters associated with neovascularisation, such as endothelial cell content, endothelial cell proliferation and growth factor expression. The combination of TUNEL immunostaining and ultrastructural examination can provide more definitive identification of the type of cell death involved in the CL. 2) The early luteal phase displays an intense period of angiogenesis showing the highest rate of proliferating endothelial cells and intense VEGF expression, which results in the formation of an extensive microvascular network by the mid-luteal phase. In contrast, natural and induced luteal regression was associated with marked decrease in angiogenesis showing the lowest level of vascularisation, endothelial proliferating cells and VEGF expression. 3) We have demonstrated that apoptotic and non-apoptotic mechanisms were involved in the demise of the CL. 4) The presence of TNF$\alpha$, iNOS and bFGF immunostaining in the luteal cells during the luteal phase may indicate a paracrine and/or autocrine role for these factors in
luteal development and regression. 5) Finally, this thesis reports for the first time the effect of PGF$_{2\alpha}$ on cellular changes of angiogenesis and luteolysis.

The collection of corpora lutea at specific time points during the luteal phase, showed that there was a considerable variation in the luteal mass at the same luteal phase. Despite the observed variability in the CL mass, there was significant and apparent dramatic reduction in the luteal tissue weight during induced and natural regression. For this reason, our quantification procedure of the immunostaining was carried out per unit area or per field, which is more applicable and reproducible than absolute variables in whole CL mass (Lei et al., 1991; Gaytan et al., 1998, 1999). The use of semiquantitative immunocytochemistry was demonstrated to be reliable in measuring angiogenesis in the CL and evaluating the morphological changes associated with luteal development and maintenance. The Ki 67 antibody is widely used for evaluating cell proliferation and is mainly expressed in the M-phase of the cell cycle and has been shown to compare favourably with the in vivo gold standard technique of bromodeoxyuridine and radiolabelled thymidine incorporation (Sasaki et al., 1988; Kamel et al., 1989). Measuring endothelial cell content (area and density of factor VIII immunostaining) using unbiased sampling and taking into account the volume changes of luteal cells (hypertrophy and atrophy) throughout the luteal phase (Gaytan et al., 1999), was demonstrated to be valid method in quantifying blood vessels in the CL. However, quantifying VEGF and bFGF and other proteins localised in the luteal cells by immunohistochemistry was a relative estimation and not an absolute value. It is worthwhile to mention that at least correlation between staining intensity in the tissue sections and amount of protein present may exist. The area of the immunostaining would consider the number of luteal cells in which the protein is present, and semiquantitative immunocytochemistry based on this parameter is valid.

An attempt to co-localise Ki 67 and factor VIII would provide more accurate findings on endothelial proliferating cells. Unfortunately, we were disappointed by the failure of a co-localisation experiment due to the different treatments in demasking sections resulting in a high background. Despite that, it was found that the early luteal phase in the equine CL was associated with intense endothelial cell proliferation, and high expression of VEGF as the newly developed luteal microvasculature and the formation of blood vessel bed were underway. By the mid-luteal phase, in spite of high VEGF expression, the endothelial cell
proliferation continued at a lower rate with the formation of a dense network of differentiated capillaries along with the presence of mature vessels and small capillaries. It is worth noting that although levels of proliferation in the mid-luteal phase are considerably lower than early luteal phase levels, the rate of angiogenesis remains higher than that of most other normal adult tissues in which turnover time to replace endothelium is measured in years (Hanahan and Folkman, 1996).

Luteal regression was associated with a further decline in endothelial cell proliferation, decrease in VEGF production and regression of the microvessels. It was also found that PGF$_{2\alpha}$-induced regression was associated with a decline in endothelial cell proliferation, VEGF production and vascularity. Although there is a reduction in luteal vascularity in the regressed CL, some small microvessels are maintained and probably play an important role in resorption of the luteal tissue (Zheng et al., 1993; Reynolds and Redmer 1998, Young et al., 2000). On the other hand, unexpected intense VEGF immunostaining was observed during both late regression and 36 h following PGF$_{2\alpha}$-induced regression. It is possible that hypoxia at this particular time could explain this strong staining (Berisha et al., 2000) or there might have been non-specific staining due to the presence of residual, non-active metabolised VEGF or lutein cell debris.

VEGF production in the equine CL was found in luteal cells and leukocytes (Fig 8.1). VEGF mRNA and protein were expressed in all the luteal cells during the early luteal phase, which may serve a paracrine role in regulation of blood vessel growth and regression. However, macrophages in the cavity of the CL were found to express VEGF mRNA and protein and neutrophils were found to show immunostaining for VEGF protein following PGF$_{2\alpha}$-induced regression. The expression of VEGF in these cells does not appear to promote angiogenesis; it could also reflect some of the non-angiogenic functions of VEGF, such as the regulation of vascular permeability or mediation of endothelial cell survival. Other studies have demonstrated the expression of VEGF in endothelial cells and pericytes in culture (Yonekura et al., 1999) and in pericytes in vivo in the ovine CL (Reynolds et al., 2000; Redmer et al., 2001); the type of VEGF was not mentioned in these studies. It is possible that these pericytes expressed predominantly other forms of VEGF (C or D), which might explain why they were not detected with the antibody (specific for
VEGF-A) used in this study. There is no detailed previous report on VEGF expression in neutrophils nor in macrophages in the CL, thus, the exact role for these cells in angiogenesis in the CL requires further investigation. There was no apparent production of VEGF in the endothelial cells in our study; however, there are a few reports which have demonstrated the production of VEGF by endothelial cells (Nicosia et al., 1997; Yamagishi et al., 1997).

It was documented that bFGF is secreted through the conventional pathways in the tissue (Burgess and Maciag, 1989). It is apparent that bFGF does not function as freely diffusible factors but instead is found largely in the cytoplasm of secreting cells and in the basement membrane and in the extracellular matrix perhaps bound to heparin sulphate. It is has been proposed that bFGF may be released from cells and extracellular matrix, in response to tissue injury (Damon et al., 1990), and would then participate in the repair process. We demonstrated the presence of bFGF in luteal cells and large blood vessels during the luteal phase. Endothelial cells and vascular smooth muscle cells are capable of producing bFGF in the CL (Doraiswamy, 1998). Luteal cells and microvessels were found to be immunostained with bFGF intensely in the mid-luteal phase (Zheng et al., 1993). This was not demonstrated in the present study. Most of the immunostained cells were luteal cells throughout the luteal phase, which could suggest an inter-species difference regarding the secretion of bFGF in the CL. However, bFGF appeared to have a dual function in the CL. It is probable that bFGF is involved in continuous remodelling of the CL associated with CL formation and regression (Reynolds et al., 1998); bFGF can induce endothelial cell proliferation in the CL (Bagavandoss and Wilks, 1991) and can suppress CL cell populations from undergoing apoptosis (Tilly et al., 1992).

The growing interest in understanding the angiogenic process in the CL has led to the development of a strategy to inhibit luteal angiogenesis, which might provide a novel approach to post-ovulatory fertility control. So far, there have been few successful studies to demonstrate luteal angiogenesis inhibition (Ferrara et al., 1998; Fraser et al., 2000). Questions have been raised in this regard including: the secondary and the side effects of the these drugs on the body, the effects on other body tissue, the complete suppression of progesterone production, the effect on the prevention of pregnancy, the effect on foetal angiogenesis and the effect on ovarian angiogenesis reversibility. However, there is
increased interest in elucidating the endogenous anti-angiogenic factors in the CL. The inhibition of these factors or prolonged expression of VEGF could result in continued angiogenic activity. This would assess the association between angiogenesis and luteolysis, investigating whether inhibition of vascular regression would help in prolonging the lifespan of the CL.

We have demonstrated the cellular changes and types of cell death during natural and induced regression, combining TUNEL technique and the gold standard method of ultrastructural examination. Our findings revealed the presence of apoptotic and non-apoptotic features in the luteal cells (Fig 8.2). Features of apoptosis were found including: 1) cells with nuclei containing condensed chromatin that was either aligned with the nuclear membrane (marginated chromatin), or shrunken into a single, round regularly shaped (pyknotic appearance) or fragmented into multiple homogeneously dense bodies (multiple fragments). 2) the presence of apoptotic bodies: discrete membrane-bound structure containing various amounts of condensed chromatin and situated singly or in clusters.
Fig 8.2: Cellular and molecular changes during CL development and regression in the equine CL. (↑) increase, (↓) decrease.
between apparently viable cells, in the capillaries or extracellular space. The present of macrophages, and phagocytic activity on the degenerated luteal, debris and apoptotic bodies. The above features are in agreement with the standard features of apoptosis described previously (Kerr et al., 1972; Wyllie et al., 1980).

Other cell death changes were also found in the luteal tissue. Crenation of the nuclear membrane with shrinkage of the nucleus in some luteal cells may indicate there is an additional non-apoptotic form of cell death at luteolysis. Autophagocytosis and nonlysosomal disintegration are believed to be other types of cell death, which have been reported in the CL of the marmoset (Fraser et al 1999) and in neurons (Clarke, 1990). In the case of autophagocytosis, the nucleus disintegrates and is digested by autolysosomes, while in nonlysosomal disintegration, organelles swell, there is formation of vacuoles and cytoplasmic destruction is followed by nuclear disintegration. These changes were not observed in the present study. Terminal differentiation has been reported to be another type of cell death, whereby certain cells types such as the outer squamous layers of skin and the granulosa cells next to the antrum of atretic ovarian follicles undergo nuclear destruction or expulsion before the cessation of cellular function leads to cell death (Stenn, 1983; van Wezel et al., 1999). It has been suggested that these cells were undergoing the type of cell death known as terminal differentiation where progressive condensation of nuclear material eventually results in pyknosis and nuclear destruction or expulsion (van Wezel et al., 1999). Recently, different reports have raised questions about other types of cell death where cytoplasmic changes are the major feature, so called cytoplasmic cell death. In this type of cell death, the nucleus changes were absent, while vast changes occur in the cytoplasm: lysosomes increase in number and redistribute in the cytoplasm; large autophagic vacuoles form; and organelles are frequently degenerated in specific sequence. Very late in the process, most of the cytoplasm is filled with vacuoles or has been pinched off (Bursch et al., 2000, Uchiyama, 2001).

Apoptosis is a highly regulated and genetically programmed process. Cells dying by apoptosis go through three phases of change (Table 8.1). The apoptotic process can be subdivided into at least three different stages: 1) initiation, the pro-apoptotic stimuli trigger activation of the molecular machinery of apoptosis, 2) execution, the molecular executioner factors is fully activated and 3) degradation, cell acquires the hallmarks of apoptotic
morphological changes. It still appears difficult to draw fixed boundaries among cell death factors, which could be involved in apoptosis. Many of the accepted causative factors of apoptosis e.g withdrawal of trophic factor, oxidative stress and protease activation can also induce necrosis. Hypoxia is a classic reversible inducer of necrosis which can cause apoptosis depending on the strength of the inducers (Columbano, 1995). Over the past few years tumour suppressor genes, death genes and survival genes have been found to play a role in the fate of luteal cells and ovarian granulosa cells in some species (Amsterdam et al. 1998).

<table>
<thead>
<tr>
<th>D1 (Reversible)</th>
<th>D2 (Irreversible)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Genetic</td>
<td>- Morphologic changes</td>
</tr>
<tr>
<td>- Reprogramming</td>
<td>- Protein crosslinking by transglutaminase</td>
</tr>
<tr>
<td>- Change in protein profile</td>
<td>- Formation of apoptotic bodies</td>
</tr>
<tr>
<td>- Increased stress proteins</td>
<td>- Phagocytosis of apoptotic bodies</td>
</tr>
<tr>
<td>- Decreased polyamines</td>
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<tr>
<td>- Chromatin changes</td>
<td></td>
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<tr>
<td>- Increased Ca2+</td>
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</table>

Table 1

Phases of apoptosis and its features

We have observed rarefied mitochondria as early as the mid-luteal phase, which might reflect early signs of apoptosis (Kroemer et al. 1998; Wilson 1998). Recent studies have shown that common pathways of cell death take place in the mitochondria and then oncoproteins determine whether the cell dies by apoptosis or necrosis (Brenner et al. 1998). Further investigation was conducted to determine whether the rarefied mitochondria in mid-luteal phase are degenerative changes that reflect cell death pathway or/and an initial cause of functional luteolysis. Cytochrome C immunostaining revealed that cytochrome C indeed was released in most of the luteal cells at mid-luteal phase; our attempt to confirm this at the ultrastructural level by immuno-gold staining failed because the required fixation procedure for immuno-gold was not performed at an early stage of the project. Immuno-gold staining needs tissue free of osmium tetroxide fixative. The reduction in tubulin immunostaining in the mid-luteal phase was another indication of mitochondrial disruption in luteal regression.
Numerous pro-apoptotic stimuli converge on mitochondria and provoke the release of factors (such as cytochrome C) that trigger caspase activation and cell death as a consequence (Fig 8.3). Caspases, a family of cysteine proteases with aspartate substrate specificity, are produced as inactive zymogens. Caspases participate in the molecular control of apoptosis in several guises; caspase activation events occur during the signalling and demolition phases of cell death. Evidence is accumulating to suggest that activation events are initiated by molecules that promote caspase aggregation and facilitate autoactivation (Thornberry et al., 1998). It has been proposed that initiator caspase 9 either directly or indirectly activates executioner caspase-3. Expression of caspase-3 has been found in ovarian leukocytes and in atretic follicles (Van Nassauw et al., 1999). In contrast, healthy granulosa cells possess almost exclusively the inactive forms of the enzymes whereas in apoptosis, granulosa cells process the procaspase-3 to the active enzyme (Robles et al., 1999). Further investigation is required to establish the involvement of these caspases in the equine CL.

Fig 8.3: Apoptosis and the role of mitochondria
Findings in this thesis are the first to describe changes in the vasculature and cell death after PGF$_{2\alpha}$-induced regression. The pathways of cell degeneration in natural and induced regression appeared to be broadly similar (table 8.2). However, there was unique infiltration of neutrophils 12 and 36 h following PGF$_{2\alpha}$ administration. Two distinctive features of acute inflammation, vasodilatation and neutrophil influx were observed. Although the essential function of neutrophils is phagocytosis, they have also been observed to initiate and modify the magnitude and duration of acute inflammatory processes (Jain, 1986). This indication of acute inflammation is not necessarily associated with necrosis and appears to play a role in luteal regression via cytokine secretion by the inflammatory cells as suggested in the hamster (McCormack et al., 1998). Influx of neutrophils during luteal regression has also been reported during spontaneous regression in the hamster, suggesting that neutrophils may assist in initiating luteal regression (McCormack et al., 1998). A recent study by Lawler et al. (1999) on immune cells in the equine CL revealed an increase in numbers of eosinophils, macrophages and lymphocytes 24 h following PGF$_{2\alpha}$ administration, however, no mention was made of a neutrophil influx and no description was found of neutrophil influx in their report. The equine CL appears to show multiple-invasion of high numbers of neutrophils, eosinophils, macrophages and lymphocytes following PGF$_{2\alpha}$-induced regression compared with natural regression where small number of these cells were found (Lawler et al., 1999). In sheep, there is an increase in eosinophils (Murdoch, 1987) while macrophages and eosinophils invade the regressing CL in cattle (Penny et al., 1999). In fact, it appears that the dose, absorption and clearance of PGF$_{2\alpha}$ in different species may determine the type of leukocytes that invade the CL, and the type of chemoattractants secreted by the luteal cells. However, our results from tissues taken post PGF$_{2\alpha}$-induced regression indicate that the processes occurring during induced and natural regression are principally similar with regard to the involvement of apoptosis and the marked reduction in angiogenesis. As the standard dose of PGF$_{2\alpha}$ used to induce luteolysis is of a supraphysiological nature, it would seem that the natural order of events is disrupted resulting in unexpected changes (an influx of neutrophils and vasodilation). We do believe that our findings have increased our understanding of PGF$_{2\alpha}$ effect at the cellular level in equine CL, and this will help in developing additional or alternative therapies for induction of luteolysis in the future. Many chemicals, cytokines, molecules proteins and
genes have been proposed to play a role in cell death and apoptosis (Table 8.3). The immunocytochemical localisation for

<p>| Table 8.2 |
| Summary of the cellular and molecular changes during natural regression and PGF$_{2\alpha}$-induced regression |</p>
<table>
<thead>
<tr>
<th>Induced regression</th>
<th>Natural regression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apoptotic cells</strong></td>
<td>Present</td>
</tr>
<tr>
<td><strong>Apoptotic bodies</strong></td>
<td>Present</td>
</tr>
<tr>
<td><strong>Lipid</strong></td>
<td>Moderate accumulation</td>
</tr>
<tr>
<td><strong>PAS</strong></td>
<td>Massive influx of neutrophils</td>
</tr>
<tr>
<td><strong>Factor VIII</strong></td>
<td>Vasodilation at 12 h post PGF$_{2\alpha}$, reduction at 36 h in the area and density of FVIII immunostaining</td>
</tr>
<tr>
<td><strong>Ki 67</strong></td>
<td>Reduction in endothelial and luteal cell proliferation rate and increase in other cell types (fibroblasts and leukocytes)</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td>Present in high numbers</td>
</tr>
<tr>
<td><strong>VEGF protein</strong></td>
<td>Intense immunostaining in some luteal cells and neutrophils</td>
</tr>
<tr>
<td><strong>VEGF mRNA</strong></td>
<td>Reduction in VEGF grains</td>
</tr>
<tr>
<td><strong>bFGF</strong></td>
<td>Intense immunostaining in some luteal cells and neutrophils</td>
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<tr>
<td><strong>ET-1</strong></td>
<td>Absent</td>
</tr>
<tr>
<td><strong>eNOS</strong></td>
<td>Absent</td>
</tr>
<tr>
<td><strong>iNOS</strong></td>
<td>Intense immunostaining</td>
</tr>
<tr>
<td><strong>TNF</strong></td>
<td>Intense immunostaining in some luteal cells and neutrophils</td>
</tr>
<tr>
<td><strong>Eosinophils</strong></td>
<td>infiltration</td>
</tr>
</tbody>
</table>
ET-1, TNFα, iNOS and eNOS in the CL in the present study revealed a potential role of these factors in CL function. It has been demonstrated in ruminants that ET-1 inhibits luteal steroidogenesis and mediates the effects of PGF2α. ET-1 caused a reduction in LH-stimulated biosynthesis of progesterone and an increase in PGF2α and subsequently induction of regression (Grish et al., 1996; Miyamoto et al., 1997; Hinckley et al., 2001). ET-1 immunostaining was seen in the luteal and endothelial cells during early and mid-luteal phase. We did not observe a peak in ET-1 immunostaining during regression, based on this primary study, suggesting that the ET-1 role in CL function is species-dependent.

Our findings for TNFα immunostaining indicate that TNFα plays a role in both luteal development and regression. Many functions of TNFα in the CL have been proposed in different species and different studies including: TNFα serves as a paracrine and/or autocrine luteotrophin (Adashi et al., 1989; 1990); TNFα may play a role in stimulating luteal progesterone secretion (Terranova et al., 1991); TNFα increases granulosa proliferating cells in the pig when treated with TNFα in vitro (Prange-Kiel et al., 2001) and TNFα induces apoptosis and initiates structural regression (Jo et al., 1995; Petroff et al., 2001; Friedman et al., 2001). The intense immunostaining of TNFα in late but not in early regression may indicate a role for TNFα in the removal and clearance of degenerated luteal cells as seen in the sheep CL (Ji et al., 1991). We showed that eNOS immunostaining was restricted to the early and mid-luteal phase in some endothelial cells and a few luteal cells. This may indicate that NO production may play a role in CL development and angiogenesis. In contrast, iNOS immunostaining showed a peak during regression. This could indicate a role as a luteolytic factor in the equine CL as been has suggested in the human (Vega et al., 1998; Friden et al., 2000) and rat (Motta et al., 2000).

8.2 Research implications

The major function of the CL is the secretion of progesterone, which brings about endometrial and myometrial receptivity for implantation after conception. Vascularisation in the CL is required to provide luteal tissue with oxygen, nutrients, and substrates for
progesterone and growth factor biosynthesis, and it facilitates the removal of end products of metabolism. Our findings showing the intense angiogenesis in the CL suggest that the early and mid-luteal phase may represent the periods during which the equine CL may be susceptible to angiogenic inhibitors.

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Table 3
Apoptosis inducing signals

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Physical Insults</th>
<th>Cells</th>
<th>Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotherapeutic agents</td>
<td>Neutrons, x-rays</td>
<td>Cytolytic</td>
<td>TNFα</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Neutrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free-radicals</td>
<td>Neutrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>Neutrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Neutrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azide</td>
<td>Neutrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Neutrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV-radiation, heat shock</td>
<td>Neutrons</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Understanding the regulation of physiological angiogenesis in the CL is clinically relevant. Examples where our findings might be clinically beneficial include: 1) inadequate luteal function may be associated with decreased luteal vascularisation, therefore luteal inadequacy caused by deficient follicular or luteal angiogenesis may be treated by pro-angiogenic agents to stimulate neovascularisation. 2- Tumour growth and metastasis is highly dependent on angiogenic activity, and this dependence on angiogenesis has long been proposed as an attractive therapeutic target. Therefore, angiogenic inhibitors are currently being developed primarily for treatment of vascular solid tumours. Ovarian tumour growth and metastases may be suppressed by angiogenic inhibitors which do not kill tumour cells directly but operate indirectly by inhibiting vascularisation of the tumour and tumour cells die by hypoxia or from the deprivation of angiogenic factors. 3- the ability to manipulate luteal angiogenesis and consequently luteal function could lead to novel approaches in post-ovulatory control. Pharmacological manipulation of luteolysis is a
key element in oestrous cycle control in clinical equine reproduction. PGF$_{2\alpha}$ can be administered from day 6 of the cycle to initiate luteolysis (Oxender et al, 1975), but there are no drugs available that can induce luteolysis over the entire luteal phase. Pharmacological control of angiogenesis could offer an alternative way of shortening the oestrous cycle.

The official breeding season for thoroughbreds starts before mares begin to cycle naturally therefore, there has been a high degree of veterinary input in mare breeding over the past years. This led to increase the demand for methods to advance the onset of the normal oestrous cycle, using photoperiod, and hormonal manipulation. Manipulation and shortening of the oestrous cycle is often used to reduce the time between oestrous cycles which increase the chance for tighter control of the mare’s oestrous cycle and oestrus periods. PGF$_{2\alpha}$ is accepted to be the hormone responsible for initiation of the luteolytic process in the mare (Allen, 1973; Douglas and Ginther, 1975; Oxender et al, 1975). In practice, exogenous PGF$_{2\alpha}$ is the commonest method of short-cycling in the mare. Understanding the cellular changes following PGF$_{2\alpha}$ administration in the mare would be beneficial for veterinarians. A better understanding of the luteolytic process in the mare can only help in advancing and developing other methods for induction of luteolysis. This study may provide new insights into manipulation of luteolysis as well as increase our understanding of the physiology of the CL in order to develop new pharmacological drugs that induce luteal regression all over the entire luteal phase.

8.3 Future research investigations

There are many questions and areas requiring further investigations. The following are some suggested areas where further information could help us understand the CL.

1- Physiological angiogenesis in the CL is tightly regulated and angiogenic activity decreases with the age of CL. We demonstrated that VEGF and bFGF are likely to be pro-angiogenic factors in the CL. This may highlight the need to investigate endogenous anti-angiogenic factors in the CL in a similar way to pro-angiogenic factors (Table 1.1).
2- Results indicated that the early and mid-luteal phase may represent the periods during which the CL may be susceptible to angiogenic inhibitors. This may raise the question of applying clinical trials to investigate the effect of angiogenic inhibitors on the life span of the CL.

3- Recent reports have shown a role for pericytes (endothelial supporting cells) playing role in the angiogenic activity of the CL. It may be informative to look at these cells and their contribution to regulating the vasculature in the equine CL.

4- We reported the presence of other types of cells positive for Ki 67 during the regression phases and suggested that these cells are probably fibroblasts and leukocytes. Therefore, it is worth investigating the identity of these cells in regression by dual immunocytochemistry using Ki 67 with other leukocyte, macrophage and fibroblast markers.

5- Our primary and basic findings on ET-1 immunostaining do not indicate the involvement of ET-1 in luteal regression as has been well documented in other species. Further study using more robust techniques are required to explore the role of ET-1 in the equine CL. Clinical trials of the effect of ET-1 on the CL would be also beneficial since many reports indicate a direct involvement of ET-1 in luteolysis in ruminants.

6- More investigation is required to confirm our findings of cytochrome C release at the mid-luteal phase using immuno-gold staining under the electron microscope.

7- In situ hybridisation studies are required to investigate the role of TNFα and its receptors and it may be that an in vitro study would help us to understand TNFα function and the luteolytic effects of TNFα on cultured luteal cells.

8- Our primary findings on the role of NO in the CL were indirect by using the NOS isoforms which are responsible for NO syntheses. It would be appropriate and more
direct to use an *in vitro* study and demonstrate the luteolytic effect of NO on cultured luteal cells.

### 8.5 Conclusion

In conclusion, the studies reported in this thesis have investigated the cellular mechanisms of angiogenesis and cell death in the equine CL. The findings reported demonstrate that luteal angiogenesis is important for CL function. Indeed, endothelial cell proliferation, endothelial cell content and VEGF expression showed that the early and mid-luteal phase exhibit intense angiogenesis; and this suggests that angiogenesis is important for the luteal development in the mare. During luteal regression, apoptotic features were observed in the luteal cells, and this suggests that apoptosis may be involved in luteolysis in the mare. Other changes (carnation of the nuclear membrane and shrinkage of the nucleus) are features not characteristic of apoptotic cell death. TNFα, bFGF and NO may be involved in luteal development and regression. The effect of PGF₂α on the CL at a cellular and molecular level has been described. The data showed broad similarities to natural luteolysis, with additional features including, massive influx of neutrophils and vasodilation in microvessels were associated with PGF₂α-induced regression. Progress has been made in understanding the cellular mechanisms involved in luteal angiogenesis and cell death. Studies reported in this thesis provide new insights into the control of the equine CL. This has been a largely neglected area of research in the equine CL, and the number of potential factors necessitating investigation are numerous. Although there is still a considerable amount of work to be done in this area before we can hope to develop better methods of artificial controlling and/or manipulating the equine oestrous cycle, every step forwards, no matter how small, gets us closer to this ultimate goal.
Appendix

Solutions

PBS: Phosphate buffered saline (pH 7.2)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2HPO4</td>
<td>1.48 g</td>
</tr>
<tr>
<td>NaH2PO4</td>
<td>0.43 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>7.2 g</td>
</tr>
<tr>
<td>NaN3</td>
<td>1.3 g</td>
</tr>
</tbody>
</table>

Made up to 1 litre with distilled water then adjusted to pH 7.2.

TBS: Tris buffered saline (pH 8.2)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ml distilled water</td>
<td></td>
</tr>
<tr>
<td>0.242 g (20 mM) Tris (tris-hydroxymethyl-aminomethane)</td>
<td></td>
</tr>
<tr>
<td>0.13 g (20 mM) NaN3 (preservative)</td>
<td></td>
</tr>
<tr>
<td>0.9 g (225 mM) NaCl</td>
<td></td>
</tr>
</tbody>
</table>

Sodium cacodylate buffer solution (0.1M, pH 7.2)

10.7 g of sodium cacodylate in 1 litre of distilled water.

Adjust to pH 7.2 with HCl.

SSC buffer (pH 7.0)

175.3 g NaC

88.23 g tri-sodium-citrate in

900 ml of distilled water
Adjust to pH 7.0 with HCl.

**Fixatives**

**4% Paraformaldehyde**

Heat 100 ml PBS to 60°C.

Add 8 g of paraformaldehyde.

Add NaOH until paraformaldehyde dissolves.

Make up 200 ml and cool under tap.

Adjust pH to 7.0.

**Bouins's fixative**

70 ml Saturated aqueous solution of picric acid
25 ml Formalin
5 ml Glacial acetic acid

**10% formalin fixative**

100 ml Formalin (40% aqueous solution of formaldehyde)
100 ml Sodium dihydrogen orthophosphate (monohydrate) 4g
6.5 g Disodium hydrogen orthophosphate (anhydrous)
900 ml Distilled water
Tissue processing for EM

1) Fix in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2-3 h.

2) Wash: 0.1 M sodium cacodylate buffer 3 x 20 min.

3) Post-fixation: 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h.

4) Wash: distilled water 3 x 20 min.

5) Dehydration (50 % Acetone 1 x 10 min, 70 % Acetone 1 x 10 min, 90 % Acetone 1 x 10 min and 100% Acetone 3 x 10 min)

6) Embedding: Unicril (overnight) then gelatine capsule.

7) Ultrathin sectioning and grid mounting

8) Staining in Uranyl acetate

Staining

Haematoxylin & eosin (H&E) Staining

Dewax sections, rinse in alcohol, rinse in water.
Stain with Harris' haematoxylin for 2 minutes.
Wash and blue in running tap water for 1 minute.
Differentiate in acid alcohol (1% hydrochloric acid in 70% alcohol) for 10 seconds.
Wash and blue in running tap water - 5 minutes.
Stain with eosin - 1 minutes. (Eosin (0.5 g, Ethanol 96% - 100 ml Glacial Acetic Acid - 2 drops)
Wash in tap water, Dehydrate, clear and mount.
P.A.S.

Dewax sections, rinse in alcohol, rinse in distilled water.

Oxidise in 1% periodic acid for 5 min

Rinse in distilled water, wash in tap water for 1 minute and rinse in distilled water again.

Treat with Schiff’s reagent 20 min

Rinse in distilled water then wash in tap water for 5-10 minutes.

counterstaining- 1 minute.

Blue in tap water - 5 minutes.

Dehydrate, clear and mount.

Toluidine Blue

Dissolve 1 g Toluidine Blue and 1g Sodium Tetraborate in 100 ml distilled water

Stain semi-thin for 2 – 5 min under heated plate.

Differentiate in 96% alcohol

Wash in tap water

Oil Red O

Oil Red O solution (60% aqueous triethyl phosphate and 0.5% Oil Red O solution)

Stain 5-20 minutes in Oil Red O solution

Rinse well in distilled water

Counterstain

Rinse well in distilled water, drying and Mount in glycerin
Progesterone assay

Concentrations of progesterone were measured in plasma directly, all samples were assayed in duplicate. All regents diluted with assay buffer phosphate buffered saline 0.1% gelatine 0.05M pH 7.2 (PBSG). Anti-progesterone antisera was provided by the Scottish antibody production Unit.

Protocol:

Day 1

Tubes were labelled and PBSG added as required:

450μl for standards and unknowns

650μl for nono-specific binding (NSB).

50μl of samples and standards were added.

100μl of 1\textsuperscript{125} labelled progesterone made up in PBSG containing 8-anilino-1-naphthalene sulfonic acid.

200μl of primary antibody diluted at 1/15, 500

Tubes were vortexed and incubated overnight at 4 °C.

Day 2

To separate free and bound antibody, 100μl donkey anti-rabbit serum at 1:35 incorporating 1/10 EDTA, and 100μl normal rabbit serum at 1/300. Tubes were vortexed and incubated overnight at 4 °C.

Day 3

1 ml cold PBSG was added to each tube and centrifuged for 30 minutes at 3,800 rpm. Supernatant was carefully decanted, and
activity in the precipitate counted using a Clinigamma 1271, LKB-Walla (Wallac, Milton Keynes, UK).

Main cross-reactivities of the antiserum were with 5-pregnan-3,20 dione, 11-deoxycorticosterone and 17-hydroxyprogesterone (9.5%, 6.2% and 3.4% respectively). The sensitivity of the assay was 0.5 ng/ml, and the within- and between-assay coefficient variation were 9.0 and 12.16%, respectively.


References


Folkman J (1990). What is the evidence that tumours are angiogenesis dependent. Journal of the National Cancer Institute, 82, 4-6.


Kim JM, Yoon YD and Tsang BK (1999). Involvement of the Fas/Fas ligand system in p53-mediated granulosa cell apoptosis during follicular development and atresia. Endocrinology, 140, 2307-2317.


References


References


References


In mares, little information is available on the type of cell death that occurs during natural and induced luteal regression. Corpora lutea were collected from mares in the early luteal phase, days 3-4 (n = 4); mid-luteal phase, day 10 (n = 5); early regression, day 14 (n = 4); late regression, day 17 (n = 4); and 12 and 36 h (n = 3 per group) after PGF$_{2\alpha}$ administration on day 10. Histological and ultrastructural sections were examined and TUNEL was used to detect DNA fragmentation. In early luteal regression, there were more pyknotic luteal cells and extracellular round dense bodies compared with the mid-luteal phase. By late regression, there was a significant decline (P < 0.01) in the number of round dense body clusters and a marked accumulation of lipid. Twelve and 36 h after PGF$_{2\alpha}$ administration, changes were similar to those seen in natural regression, but there was also a marked infiltration of neutrophils. Accumulation of lipid was not apparent until 36 h after PGF$_{2\alpha}$ administration. Ultrastructural examination revealed rarefaction and distortion of the mitochondrial cristae in most of the luteal cells by the mid-luteal phase. Luteal cells showed shrinkage, accumulation of lipid with foamy appearance, and disruption in both smooth endoplasmic reticulum and mitochondria during natural and induced regression. Some luteal cells showed fragmented or pyknotic chromatin characteristic of apoptosis. Other luteal cells showed creation of the nuclear membrane and shrinkage of the nucleus, features not characteristic of apoptotic cell death. In late regression, capillaries were obstructed by swollen endothelial cells and round dense bodies. These results show that structural regression may be initiated as early as the mid-luteal phase, and is clearly visible by day 14 in natural regression and 12 h after induced regression. Apoptosis did appear to be involved in luteolysis in the equine corpus luteum, but non-apoptotic changes were also observed in some luteal cells during regression. Accumulation of lipid was a late feature of luteal regression.

Introduction

The equine corpus luteum is functional for about 14-15 days during the non-fertile cycle (Daels and Hughes, 1993). Luteal regression, characterized by a decrease in progesterone production (functional regression) and cellular demise of luteal tissue (structural regression), is thought to be brought about by secretion of uterine PGF$_{2\alpha}$ (Douglas and Ginther, 1975). However, the precise cellular mechanisms involved in luteolysis are not fully understood.

In general, cells die by three recognized mechanisms: apoptosis, necrosis (Kerr et al., 1972) or terminal differentiation (Stenn, 1983). During necrosis, cells develop increased permeability, which leads to cellular swelling, non-selective DNA degradation and inflammation in the surrounding tissues. Apoptosis is a process whereby cells die in a controlled manner. Morphologically, apoptosis is characterized by shrinkage and condensation of chromatin (marginated chromatin) or fragmentation into multiple, small dense bodies. Cells may then break up into discrete membrane-bound structures containing variable amounts of condensed chromatin or cytoplasm, which are then ingested by macrophages or neighbouring cells, or are extruded into the lumen of the blood vessels (Kerr et al., 1972; Wylie et al., 1980). Alternatively, cells may shrink into a single dense round mass with a densely basophilic pyknotic nucleus (Wylie et al., 1980). Terminal differentiation is another type of cell death, whereby certain types of cell, such as the outer squamous layers of skin and the granulosa cells next to the antrum of atretic ovarian follicles, undergo nuclear destruction or expulsion before the cessation of cellular function leads to cell death (Stenn, 1983; van Wezel et al., 1999).

Most recent studies on luteal cell death have used 3' end-labelling of the DNA fragments (TUNEL) to determine the type of cell death (Zheng et al., 1994; Bacci et al., 1996; Young et al., 1997; McCormack et al., 1998). In situations in which apoptosis is the major form of cell death in a tissue, this technique can assist in quantifying the degree of apoptosis (Negoeescu et al., 1998). However, when necrosis...
Sections (4 μm) were mounted on slides coated with BioBond (British Biocell Int, Cardiff), deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by immersion in 3% (v/v) H₂O₂ in methanol for 30 min. After two washes of 5 min each in 0.01 mol PBS pH 7.4, slides were incubated with proteinase K (Sigma, Poole) (20 μg ml⁻¹ in 20 mmol Tris HCl pH 7.4, 2 mmol CaCl₂ 1⁻, 1.5 mmol CoCl₂ 1⁻, placed on an ice-cold plate and loaded with 2 mmol digoxigenin-11-dUTP 1⁻ and 25 U ml⁻¹ TDT in enzyme buffer. Negative control slides were treated with enzyme buffer lacking TDT. The slides were immediately covered with paraffilm sheets and incubated at 37°C for 75 min. The paraffilm was then removed and the slides were washed twice (5 min each) in standard saline citrate buffer to stop the reaction. After a further 5 min wash with PBS, sections were incubated for 10 min at room temperature with 25% (v/v) normal rabbit serum in PBS, and then incubated for 90 min at room temperature in a humidified chamber with sheep anti-digoxigenin antibody (Boehringer, Mannheim) diluted 1:100 in blocking solution. After two further washes in PBS, the slides were incubated for 30 min at room temperature with biotinylated rabbit anti-sheep immunoglobulin (Vector Laboratories, Peterborough) diluted 1:500 in PBS. Two washes in PBS (5 min each) were followed by 30 min incubation at room temperature with horseradish peroxidase-avidin biotin complex (Dako, High Wycombe) diluted in PBS according to the manufacturer’s instructions. After two further washes in PBS, sections were visualized with 0.05% (w/v) 3,3'-diaminobenzidine (Sigma) in 0.05 mol Tris-HCl 1⁻, pH 7.4, and 0.01% (v/v) H₂O₂. The sections were then washed in distilled water, lightly counterstained with haematoxylin, dehydrated in graded alcohol, cleared in xylene and mounted in DPX (dixtrine plasticiser xylene).

Detection of lipid droplets using oil red O stain

Frozen sections (8 μm) were stained with oil red O (Sigma) and then counterstained with haematoxylin (Filipe and Lake, 1990).

Detection of neutrophils using periodic acid–Schiff reaction

Formalin-fixed sections were stained with periodic acid–Schiff (PAS) reagent (Sigma) for 10 min and then counterstained with haematoxylin (Filipe and Lake, 1990).

Quantification method

The above stains were used on each tissue block, a minimum of three times. Two representative sections (peripheral and central) from each corpus luteum were selected and four fields per section were examined at ×400 magnification. The identity of the sections was obscured to ensure blind counting. Individual clusters of dense bodies and pyknotic nuclei that showed positive immunostaining for TUNEL, or that were identified in haematoxylin and eosin stained sections, were counted separately. The results were expressed as mean ± SEM. Sections were counted at ×400 magnification to quantify the number of neutrophils. The total number of neutrophils for each stage of the oestrous cycle was expressed as mean ± SEM. Quantimet image processing and analysis system 500 (Leica, Cambridge) was used to measure the proportion of the section stained by oil red O (area of the oil red O stain divided by the total area measured × 100). Areas were analysed at ×200 magnification. The system was optimized for each individual section on the basis of the density of the stain. The data were expressed as percentage mean ± SEM per unit area. Two sections per corpus luteum and four per section were used in the quantification, as increasing the number of sections above two sections per corpus luteum and four fields per section did not alter the result. There was no difference between the mean of the variables in central and peripheral regions, and therefore data were combined to represent one variable.

Progesterone assay

Progesterone concentrations were determined in plasma by radioimmunoassay as described by Watson et al. (2000). The limit of detection of the assay was 0.5 ng ml⁻¹ and the intra- and interassay coefficients of variation were 9.0 and 12.6%, respectively.

Statistical analysis

The differences in early, mid-luteal, early and late regression corpora lutea were analysed by one-way analysis of variance using stage of the luteal phase as the between-subject variable. Data from untreated mid-luteal phase corpora lutea were compared with corpora lutea after induced regression using a one-way analysis of variance with treatment as the between-subject variable. The data were then subjected to Tukey’s test of multicomparison among means. Results were considered to be significantly different when P < 0.05.

Results

Corpus luteum mass and progesterone concentrations

Morphological examination and progesterone concentrations for each corpus luteum were used to confirm the cycle stage. In three of the 23 corpora lutea, the designated stage of the cycle did not conform to the expected morphological appearance and progesterone concentration and these corpora lutea were therefore re-classified. In two corpora lutea that were collected on day 14 (early
pyknotic cells and round dense bodies in TUNEL-stained sections were not included in the quantitative analysis. In early regression, the number of pyknotic cells increased \((P < 0.05)\) and remained without significant change until late regression. During induced regression, there was an increase in the number of pyknotic cells in the corpora lutea collected \(36\ h\) after PGF\(_{2\alpha}\ (P < 0.05)\) compared with those collected after \(12\ h\). Clusters of round dense bodies increased in early regression \((P < 0.01)\), and declined in late regression \((P < 0.01)\). After induced regression there was an increase in clusters of round dense bodies \(36\ h\) after PGF\(_{2\alpha}\) \((P < 0.01)\) compared with \(12\ h\) after PGF\(_{2\alpha}\).

**Oil red O**

In the early luteal phase, most of the luteal cells showed intense staining for oil red O. By the mid-luteal phase, fewer luteal cells showed positive staining (Fig. 2e). In late regression (day 17), very intense staining was observed both intracellularly and extracellularly (Fig. 2f), reflecting high accumulation of lipid in the regressing corpus luteum. Twelve hours after PGF\(_{2\alpha}\) administration, few cells showed oil red O staining and by \(36\ h\) there was moderate accumulation of lipid in most of the luteal cells. The percentage area of lipid (Fig. 4) in mid-luteal phase and early regression was decreased markedly \((P < 0.01)\) compared with in the early luteal phase. By late regression, the percentage area of lipid had increased sharply \((P < 0.001)\).

**Neutrophils**

PAS stains all types of polysaccharides. Neutrophils in the equine corpus luteum stained positively with PAS (Fig. 2g). In early and mid-luteal phases (Fig. 2h), as well as in early regression, occasional neutrophils were observed, particularly in the blood vessels. On day 17, the number of neutrophils had increased slightly (Fig. 4). After PGF\(_{2\alpha}\) injection at both \(12\) and \(36\ h\), the number of neutrophils increased sharply \((P < 0.001)\).

**Ultrastructural changes**

Ultrastructural examination during the early luteal phase (Fig. 5a) showed luteal cells with central or peripheral, oval-to-round shaped nuclei with one or more nucleoli. Heterochromatin was finely granular and uniformly dispersed in the nucleus. The cytoplasm contained vesicles or smooth endoplasmic reticulum (SER), electron dense bodies, ribosomes and lipid droplets. Mitochondria were round-to-elongated, and were normal in appearance with tubular and lamellar cristae. In the mid-luteal phase, luteal cells showed features similar to cells in the early luteal phase. However, the mitochondrial matrix was rarefied in some luteal cells. During regression, some luteal cells showed early stages of margination of chromatin (not shown), had fragmented chromatin (Fig. 5b), or appeared to be pyknotic (Fig. 5c). Single or clusters of dense round bodies (Fig. 5d) were observed in the extracellular space or in the capillaries (Fig. 5f). Other luteal cells showed non-apoptotic changes with shrinkage, crenation and involution of the nuclear membrane (Fig. 5e). The luteal cells showed accumulation of lipid, and disruption in both SER and mitochondria (Fig. 5b,c,e).

Endothelial cells were seen in the capillaries with oval-to-vesicular shaped nuclei, and contained aggregated dispersed heterochromatin with complex infoldings of the nuclear envelope during the mid-luteal phase (Fig. 6a). During regression, the endothelial cells appeared to protrude into the blood vessel lumens and were swollen or detached from the plasma membrane (Figs 5d and 6b). Other cell types, such as macrophages, were abundant, engulfing apoptotic bodies or degenerated luteal cells (Fig. 6c). A marked influx of neutrophils was seen only during induced regression (Fig. 6d).

**Discussion**

This report has described cell death during natural and induced luteal regression in mares, combining, for the first time, morphological changes observed under light and electron microscopy with immunostaining for the 3' end labelling of DNA (TUNEL technique). This is also the first description, to the author's knowledge, of cellular changes during PGF\(_{2\alpha}\)-induced luteolysis in mares. The detection of pyknotic luteal cells and round dense bodies that stained positively with TUNEL during natural and PGF\(_{2\alpha}\)-induced regression was strongly indicative of apoptosis. However, the detection of the nuclear membrane and the shrinkage of the nuclei in the luteal cells were indicative of an additional form of cell death, possibly terminal differentiation.

In the present study, rarefied mitochondria indicative of minor degenerative changes were observed in some luteal cells in the mid-luteal phase (day 10), which is around the time of onset of declining progesterone concentrations in the oestrous cycle in mares (Van Niekerk et al., 1975). By day 14, and by \(12\ h\) after PGF\(_{2\alpha}\) administration on day 10, marked structural degenerative changes were detected and circulating progesterone concentration had fallen to \(< 1\ ng\ ml^{-1}\). Overall, these results indicate that marked structural changes did not precede the decrease in progesterone concentrations. These results are in agreement with studies in ruminants (Umo, 1975; Juengel et al., 1993; Zheng et al., 1994) and monkeys (Young et al., 1997).

The degeneration of the mitochondria in the mid-luteal phase in mares is an early step in the decline in progesterone concentrations, since the mitochondria are involved in steroidogenesis (Levine et al., 1979). Recent studies in other tissues indicate that disruption of mitochondria reflects early signs of apoptosis (Kroemer et al., 1998; Wilson, 1998). Since apoptotic cell death is regulated by oncoproteins in the mitochondria (Brenner et al., 1998), this period of regression may be the key time for further study of changes in the expression of the oncoproteins that initiate the cell death process.
In the present study, both TUNEL technique and morphological examination under light and electron microscopy demonstrated the presence of pyknotic cells and round dense clusters of chromatin in the extracellular space, characteristic of apoptosis. The pathways of cell degeneration in natural and induced regression appeared to be broadly similar. It appears that luteal regression in mares may involve apoptosis in the elimination of degenerated and unwanted cells from the corpus luteum, in agreement with a previous ultrastructural study in sheep that showed apoptosis in luteal cells (Sawyer et al., 1991).

In the present study, TUNEL immunostaining was limited to pyknotic cells and round dense bodies. Paraffin wax-embedded histological sections of marmoset corpus luteum have been shown to exhibit apoptosis both morphologically and immunohistochemically (by TUNEL) (Young et al., 1997). However, resin sections from the same tissue did not show the ultrastructural features associated with classic apoptosis, but rather autophagy and nonlysosomal disintegration, and it may be that TUNEL over-represented apoptotic degeneration by staining non-apoptotic chromatin (Fraser et al., 1999). Although many studies have used TUNEL to detect apoptosis, few have investigated the death of the luteal cells ultrastructurally. In the present study, results from haematoxylin and eosin staining and TUNEL were confirmed by electron microscopy, highlighting the importance of ultrastructural examination of tissues in investigating cell death during regression.

When haematoxylin and eosin and TUNEL staining were used to quantitate changes in the corpus luteum during regression, the peak increase in round dense bodies occurred in the early luteal phase (day 14), and was followed by the sharp decrease during late natural regression (day 17), indicating an active removal mechanism. It is not clear whether ultimately these round dense bodies undergo phagocytosis or are flushed out of the corpus luteum via the blood supply, but data from the present and previous studies indicate that elimination by macrophages is likely (Wyllie et al., 1980; Manjog and Jons, 1995). The increased numbers of macrophages during luteal regression, observed ultrastructurally in the present study and immunocytochemically in that of Lawler et al. (1999), indicate a role for macrophages in the removal of apoptotic bodies.

The crenation of the nuclear membrane with shrinkage of the nucleus seen in some luteal cells during luteal

dense bodies show positive immunostaining. (e) Oil red O staining in mid-luteal phase corpus luteum showing lipid droplets in a few luteal cells. (f) Accumulation of lipid droplets in late regression corpus luteum. (g) Mid-luteal phase corpus luteum stained with PAS showing healthy normal cells and no neutrophils. (h) Corpus luteum 12 h after PGF2α-induced luteolysis, showing a huge influx of neutrophils associated with vasoconstriction. Thin black arrows indicate healthy luteal cells, thick white and black arrows indicate pyknotic cells and neutrophils, respectively, black and white arrowheads indicate round dense bodies and unstained round dense bodies, respectively. Scale bar represents 20 μm and 10 μm (inset).

**Fig. 3.** Number of pyknotic cells (a) and round dense bodies (b) per field at x 400 magnification expressed as mean ± se in equine luteal tissue (n = 3–4 animals for each phase) during induced and natural regression. (a) TUNEL immunostaining; (b) paraformaldehyde-fixed tissue stained with haematoxylin and eosin. *Significantly (P < 0.05) higher than during mid-luteal phase (day 10); **significantly (P < 0.01) higher than during mid-luteal phase (day 10); ***significantly (P < 0.001) higher than during mid-luteal phase (day 10) and 12 h after PGF2α administration; †significantly (P < 0.01) higher than during mid-luteal phase (day 10) and 12 h after PGF2α administration.

**Fig. 4.** The area fraction for oil red O stain (■) and number of neutrophils (■) in equine luteal tissue, expressed as mean ± sem, during natural and induced regression. *Significantly (P < 0.01) higher than during mid-luteal phase (day 10); **significantly (P < 0.001) higher than during mid-luteal (day 10) and early regression phases (day 14); †significantly (P < 0.01) higher than during mid-luteal phase (day 10) and 12 h after PGF2α administration; ‡significantly (P < 0.001) higher than during mid-luteal phase (day 10).
Cell death in the equine corpus luteum

Fig. 6. Ultrastructural changes in the equine corpus luteum throughout late regression and after PGF2α-induced luteolysis. (a) Healthy endothelial cells (en) in mid-luteal phase showing elongated flattened shape with jagged membrane and dense mass of chromatin in the nucleus. (b) Endothelial cells appeared to protrude into the blood vessel lumens swollen (sen) and detached from the plasma membrane (den) during induced regression. (c) Macrophage (ma) engulfing whole degenerated luteal cell (die) during late regression phase. (d) Massive influx of neutrophils (nt), lipid droplets (li) and rarified mitochondria (rm). 36 h after PGF2α administration. Scale bar represents (a,d) 2, (b) 3 and (c) 1 μm.

regression in mares may indicate the presence of an additional non-apoptotic form of cell death at luteolysis. It is possible that these cells were undergoing the type of cell death known as terminal differentiation in which progressive condensation of nuclear material results eventually in pyknosis and nuclear destruction or expulsion (van Wezel et al., 1999). In the regressing corpus luteum of marmosets, autophagocytosis and nonlysosomal disintegration may occur (Fraser et al., 1999). In autophagocytosis, the nucleus disintegrates and is digested by autolysosomes and, in nonlysosomal disintegration, organelles swell and formation of vacuoles and cytoplasmic destruction is followed by nuclear disintegration. However, these changes were not observed in the present study in mares.

TUNEL and ultrastructural examination confirmed that endothelial cells did not show any morphological signs of apoptosis, but that they did show signs of swelling and detachment from the walls of the blood vessels. Ultrastructural studies have shown that apoptosis occurs in endothelial cells during regression of the corpora lutea in guinea-pigs (Azmi and O'Shea, 1984) and in sheep after PGF2α-induced regression (Sawyer et al., 1990), but that it does not occur in cows (Augustin et al., 1995; Modlich et al., 1996). These studies strongly indicate that there are species differences in the fate of endothelial cells during luteolysis, and that the vasculature in bovine and equine corpora lutea regresses in a similar manner.

This is the first report, to the authors' knowledge, showing infiltration of neutrophils into the corpus luteum after PGF2α-induced luteolysis in domestic animals. An intense infiltration of neutrophils was observed 12 and 36 h after PGF2α administration. Influx of neutrophils during
changes in ovine luteal cells in response to PGF2α. Domestic Animal Endocrinology 7: 229–237

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Characterization of morphology and angiogenesis in follicles of mares during spring transition and the breeding season

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The mare is a seasonal breeder and undergoes a period of ovarian transition in spring between winter anoestrous and cyclicity. During spring transition LH concentrations are low and many mares have successive large anovulatory follicular waves which reach the size of preovulatory follicles. Follicular angiogenesis is essential for growth and health of preovulatory follicles. The aim of the present study was to investigate the morphology and vascularity of transitional anovulatory follicles. On gross inspection, the wall of transitional follicles was visibly less well vascularized than that of preovulatory follicles. Histologically, it could be seen that the theca was only poorly developed in transitional follicles. Immunostaining for factor VIII showed that there were significantly ($P < 0.05$) fewer blood vessels in the theca of transitional follicles. There was substantially less ($P < 0.001$) proliferative activity, measured by immunostaining for Ki67, in the endothelial cells and granulosa cells of transitional follicles compared with preovulatory follicles. Preovulatory follicles had a heavy band of immunostaining in the theca for vascular endothelial growth factor (VEGF), whereas staining was sparse in the transitional follicles. It was concluded that the poor vascularity and development of the theca layer in transitional follicles could be related to low circulating LH, and possibly other trophic hormones, and are likely to be the key factors in explaining the steroidogenic incompetence of transitional anovulatory follicles.

Introduction

The mare is a seasonal breeder and most mares enter a period of anoestrous during the short days of winter. During this time only very small follicles are present in the ovaries. In many mares there is a transitional period between deep winter anoestrous and normal ovarian cyclicity, when larger follicles grow and regress. In approximately 50% of mares, sequential anovulatory follicular waves then develop, with the dominant follicle reaching a diameter similar to that of a preovulatory follicle (Ginther, 1990). These follicles fail to ovulate because of suppression of GnRH secretion by inhibitory neuronal mechanisms that result in lack of LH stimulation (Fitzgerald and Mellbye, 1988; Aurich et al., 2000). Furthermore, these large transitional follicles are thought to be steroidogenically incompetent as circulating oestradiol concentrations are very low, and incubated granulosa cells from anovulatory transitional follicles produce significantly lower concentrations of oestradiol than do granulosa cells from preovulatory follicles (Davis and Sharp, 1991).

As daylength increases, an increase in GnRH secretion results in FSH secretion and follicle growth (Freedman et al., 1979; Turner et al., 1979). LH secretion does not increase as pituitary reserves are low in winter (Silvia et al., 1987). However, shortly before first ovulation there is a large increase in circulating LH which coincides temporally with an increase in plasma oestrogen concentrations. It is thought that the increase in circulating oestradiol is the key event in increasing LH synthesis and secretion at the pituitary, or in increasing GnRH release (Sharp et al., 1991). The reason for the apparently low steroidogenic capacity of transitional follicles is unclear. There is significant in vitro conversion of labelled androstenedione to oestrogens by transitional follicles (Seamans and Sharp, 1982) and so it appears that these follicles have sufficient quantities of the aromatizing enzyme, P450arom. There is circumstantial evidence that the rate-limiting step in oestrogen synthesis in transitional follicles may be insufficient amounts of androgen substrate caused by low concentrations of P450C17. Concentrations of progesterone were similar in large follicles throughout transition and into cyclicity, but concentrations of androgen did not increase until the onset of cyclicity (Davis and Sharp, 1991). Although there may be deficiencies in steroidogenic enzymes in transitional follicles, an alternative explanation may be that the development of the transitional follicles is not as advanced as that of preovulatory follicles, and they may be less well endowed with blood vessels, thus limiting availability of substrate for steroidogenesis. Indeed, there is anecdotal evidence that, on gross inspection, transitional follicles appear to be relatively avascular (Sharp and Davis, 1993).

The regulatory role of gonadotrophins in follicle development has been well documented; however, the mechanisms acting locally within the follicle that translate hormonal stimulation into growth and differentiation are...
not well understood. Blood vessel development has a crucial role in follicular maturation (Richards, 1980) and, during follicle growth, a rich capillaryplexusdevelopsin the thecal layer surrounding the avascular granulosa cells. Studies in primates have shown that the density of the vascular network of follicles destined to ovulate is at least double that of follicles destined to become atretic (Zelevznik et al., 1981). This increased vascularity results in increased delivery of gonadotrophins to preovulatory follicles. Ravindranath et al. (1992) showed that there is an apparent association between the capacity of a follicle to produce angiogenic factors and follicle selection. Angiogenesis requires proliferation and migration of vascular endothelial cells. Several intraovarian regulators have been implicated in angiogenesis. Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that stimulates blood vessel formation and enhances microvascular permeability with 50,000 times the potency of histamine (Dvorak et al., 1995). Expression of VEGF mRNA and protein has been reported in follicles and corpora lutea of various species (Ravindranath et al., 1992; Gordon et al., 1996; Yamamoto et al., 1997; Barbont et al., 2000; Touteaud et al., 2000; Kashida et al., 2001). There appears to be a clear difference in patterns of expression among species.

Very little is known about the relative vascularity of equine ovulatory and anovulatory transitional follicles, although it is clear that the degree of vascularity may be a critical factor in determining their subsequent fate. Therefore, in the present study we used immunohistochemistry to detect the presence of VEGF in transitional and preovulatory follicles. The proliferative activity in granulosa cells and endothelial cells was investigated by determining expression of the Ki67 antigen, and the distribution and neoinformation of blood vessels was confirmed by immunolocalization of the endothelial cell marker, Von Willebrand factor VIII.

Materials and Methods

Six Pony mares of mixed breeding, weighing 344–445 kg and aged 3–19 years, were used. The reproductive history of the mares was unknown, but all were in winter anoestrus at the beginning of the period of study. The ovaries of the mares were examined three times a week by transrectal ultrasonography from the beginning of February until the onset of follicular waves. During one of the follicular waves, the ovary containing the dominant follicle was removed by a colpotomy incision after appropriate sedation and analgesia (Watson and Sertich, 1990) on the day after the dominant follicle reached 30 mm in diameter. The mares were then monitored by transrectal ultrasonography until first ovulation, which occurred between 13 April and 10 May. The remaining ovary was removed at either the second or third subsequent oestrus on the day after the dominant follicle reached 30 mm in diameter. Care was taken to remove ovaries only when the diameter of the follicle was increasing each day.

Immediately after ovarioctomy, excised ovaries were transported on ice to the laboratory for dissection. After identification of the largest follicle, the tunica albuginea was removed carefully with fine scissors and forceps, and the follicle diameter was measured and recorded. A syringe and fine gauge needle were used to aspirate follicular fluid, which was stored at −20°C until assayed for oestradiol. The follicle was re-inflated with air and the anterior follicle wall was cut open. The appearance of the follicle wall was recorded. One piece of follicle wall, approximately 0.5 cm × 0.5 cm, was immersed in freshly prepared 4% (w/v) paraformaldehyde. The tissue was kept at 4°C and transferred 24 h later to 70% (v/v) alcohol until it was embedded in paraffin wax.

Immunostaining

Immunostaining was performed using the following antibodies: rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human Von Willebrand factor VIII (Dako Laboratories, High Wycombe) and a monoclonal antibody directed against the nuclear non-histone antigen, Ki67 (Novocastra, Peterborough). Paraffin wax sections (4 μm thickness) were mounted on slides coated with BioBond (British Biocell, Cardiff), deparaffinized and rehydrated. The sections were treated with 0.3% (v/v) hydrogen peroxide and incubated for 30 min with 35% (w/v) goat serum to block non-specific binding. The sections were incubated with primary antibody or 2% normal rabbit serum (VEGF and factor VIII) or 2% normal mouse serum (Ki67). Dilution and incubation times and temperatures for the different antibodies were: 1:200 for 120 min at room temperature for VEGF; 1:250 for 90 min at room temperature for factor VIII; and 1:40 for 3 h at 37°C for Ki67. The slides were subsequently incubated for 30 min with biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Peterborough) or goat anti-mouse immunoglobulins (Vector Laboratories) for Ki67, diluted to 1:100 in phosphate buffered saline (PBS), or to 1:200 for Ki67. The slides were washed in PBS, treated with avidin–peroxidase complex (Vector Laboratories) and stained with 0.05% (w/v) 3,3′-diaminobenzidine containing 0.01% (v/v) hydrogen peroxide. Counterstaining was performed with Mayer’s haematoxylin and the sections were rinsed with Scott’s tap water.

Area of Von Willebrand factor VIII immunostaining

The Quantimet image processing and analysis system 500 (Leica, Cambridge) was used to measure the area of immunostaining for factor VIII. Regions from the inner, middle and outer areas of the sections were randomly selected. Two sections and three fields per section were counted. The system was optimized for each individual section based on the density of the stain. The area used in the analysis was calibrated at ×200 magnification. The area of factor VIII immunostaining for each group was expressed as mean ± SEM per unit area.
Labelling index for cell proliferation

Sections were examined at \( \times 400 \) magnification. The number of nuclei positively stained for Ki67 and the total number of nuclei were counted in two sections per animal and four fields per section, and the percentage of positively stained nuclei (label index) was calculated.

Hormone assays

Concentrations of progesterone and oestradiol were measured directly in follicular fluid using assays described previously (Corrie et al., 1981; Glasi et al., 1989; Law et al., 1992; Watson et al., 2000; Riley et al., 2001). Assay sensitivity was 0.5 ng ml\(^{-1}\) for progesterone and 8 pg ml\(^{-1}\) for oestradiol. Intra- and interassay coefficients of variation were 9.0 and 12.6\%, respectively, for progesterone, and 4.6 and 7.8\%, respectively, for oestradiol. Displacement curves produced by serial dilutions of follicular fluid and curves produced by addition of hormone to follicular fluid samples were parallel to the respective standard curves.

Statistical analyses

Hormone concentrations in follicular fluid from transitional and preovulatory follicles from the same mares, as well as follicle size, were compared by paired \( t \) test. Hormone data were log-transformed before analysis. The area of staining for factor VIII and the proportion of cells staining positively for Ki67 in transitional and preovulatory follicles were compared using a paired \( t \) test.

Results

Size of follicles at removal was not significantly different between the transitional (33.8 ± 2.3 mm) and preovulatory (34.1 ± 1.5 mm) groups. The colour of the walls of the transitional follicles was yellowish to pale pink, and that of the preovulatory follicles was orange to red. The concentration of oestradiol in follicular fluid was significantly lower \((P < 0.01)\) in the transitional follicles (345 ± 112.3 ng ml\(^{-1}\)) than in the preovulatory follicles (1063 ± 169.2 ng ml\(^{-1}\)). Similarly, progesterone concentrations were significantly lower \((P < 0.05)\) in transitional follicles (6.2 ± 1.7 ng ml\(^{-1}\)) than in preovulatory follicles (33.6 ± 12.8 ng ml\(^{-1}\)).

Histological findings

Both anovulatory and preovulatory follicles had a well-organized layer of granulosa cells in contact with the basement membrane (Fig. 1). The granulosa cells appeared to be randomly distributed in the upper layers. There did not appear to be any difference in the thickness of the granulosa layers in anovulatory or preovulatory follicles, although there was some variability between follicles. Occasional mitotic nuclei were visible in both transitional and preovulatory follicles, and a few pyknotic nuclei were observed. The theca interna in preovulatory follicles comprised a thick layer of plump polyhedral cells with a pale nucleus and cytoplasm (Fig. 1a), whereas the theca layer of the anovulatory follicles was thin and poorly developed in most parts with only sparse foci of polyhedral cells (Fig. 1b). Most of the theca cells retained a fibroblast-type appearance with a spindle-shaped darkly staining nucleus.

Immunohistochemical localization of VEGF, factor VIII and Ki67

Immunostaining for VEGF was confined mainly to the theca interna. The granulosa layer remained mainly unstained, with a few scattered positively stained cells. In preovulatory follicles, the entire theca interna layer stained strongly and diffusely for VEGF (Fig. 2a). Immunostaining in the transitional follicles was scant, with patchy staining in the thin theca layer (Fig. 2b).

Immunostaining for factor VIII was confined to endothelial cells of blood vessels in the theca interna. The theca was well supplied with blood vessels in the preovulatory follicles, whereas the transitional follicles had a relatively avascular theca. A significantly larger \((P < 0.05)\) area of
tissue was stained for factor VIII in the preovulatory follicles than in the transitional follicles (Figs 3 and 4). Positive staining for Ki67 was confined to cell nuclei and was frequently present in the granulosa cells and thecal endothelial cells of preovulatory follicles (Fig. 5a). No staining was visible in endothelial cells of transitional follicles and only occasional granulosa cells were stained (Fig. 5b). The difference in labelling index for Ki67 between preovulatory and transitional follicles was highly significant (Fig. 4).

Observations for immunohistochemical staining were consistent across all six animals studied. No positive immunostaining was observed in negative control sections (Fig. 6).

**Discussion**

The results of the present study show for the first time clear differences in morphology and vascularization between large follicles collected during spring transition (anovulatory) and the breeding season (preovulatory) from mares. The major differences reported in this study, together with previously reported differences in circulating gonadotrophin concentrations, could play a key role in determining the subsequent fate of these two types of follicle.

A follicle diameter of approximately 35 mm was chosen because it is known that equine preovulatory follicles are responsive to exogenous gonadotrophin stimulation at this size and ovulate within 48 h (Duchamp et al., 1987). All of the follicles collected in the present study were still growing and were not regressing; this was confirmed by histological evaluation of granulosa and theca cell morphology. The morphological differences in the theca interna layer of the preovulatory and transitional follicles were marked. The preovulatory follicles had the characteristic plump polyhedral cells that have been described previously (Kenney et al., 1979; Kerban et al., 1999). It has been suggested that these cells have undergone luteinization (Kenney et al., 1979) and it is known that follicular progesterone concentrations increase during the preovulatory period in mares (Watson and Sertich, 1990). Furthermore, follicular progesterone concentrations were increased in the preovulatory follicles sampled in the present study. It has also been suggested that a thick theca layer may be essential in the growing pre-
ovulatory follicle for provision of androgen substrate to maintain follicular oestriadiol synthesis. Therefore, it seems likely that, as this layer is only poorly developed in transitional follicles, the lack of androgen substrate will contribute substantially to the low oestradiol concentrations measured in these follicles. Hence, the morphological findings of the present study explain the low steroidogenic capacity of incubated walls of transitional follicles (Davis and Sharpe, 1991) and indicate strongly that the deficiency in theca P450C17 suggested by Davis and Sharpe (1991) is directly attributable to poor thecal development.

Poor vascularity, characterized by visibly pale follicular walls, has been reported to be a sign of atresia in equine follicles (Kenney et al., 1979; Modawska and Okolski, 1997; Pedersen, 2000). In the present study, the lining of transitional follicles was paler than that of preovulatory follicles. This observation was confirmed by immunostaining for factor VIII, which identifies endothelial cells. There was a significantly smaller area of factor VIII immunostaining in the theca of transitional follicles compared with preovulatory follicles; this does not necessarily mean that there was a greater number of individual blood vessels, as the same vessel may intersect the plane of the section any number of times. However, the results of the present study indicate strongly that there is increased vascularity in the preovulatory follicles compared with transitional follicles as a result of increased numbers of vessels, increased size of the vessels or increased tortuosity of the vessels, or a combination of these factors. Furthermore, the transitional follicles contained very little VEGF, which is important in promoting angiogenesis, whereas VEGF appeared to be abundant in the preovulatory follicles. VEGF also has the ability to increase the permeability of the microvasculature (Murohara et al., 1998). Thus, in preovulatory follicles, the richer blood supply in combination with increased permeability of blood vessels will allow increased provision of oxygen, nutrients and substrates, as well as circulating gonadotrophins, which are essential for follicular health, growth and steroidogenesis. In addition, the rich blood supply to preovulatory follicles will allow more follicular steroids and other hormones to leave the follicular fluid and access the circulation.

Fig. 4. Area of staining for factor VIII (■) and percentage of cells positive for Ki67 (□) in transitional and cyclical preovulatory follicles from six mares. *P < 0.05 and **P < 0.001 compared with cyclical value.

Fig. 5. Immunohistochemical staining for Ki67 in (a) a preovulatory follicle and (b) a transitional equine follicle. Note positive nuclear staining in granulosa cells (horizontal arrows) and in endothelial cells in the preovulatory follicles only (vertical arrows). Scale bars represent 40 μm.

Fig. 6. Negative control section of an equine preovulatory follicle stained for Ki67 in which the primary antibody was substituted with 2% normal rabbit serum. Negative control sections for the other antigens similarly lacked positive staining. Scale bar represents 50 μm.
The VEGF protein was clearly present in the thecal cells of equine follicles, which is similar to human follicles (Gordon et al., 1996; Yamamoto et al., 1997). VEGF protein and mRNA encoding VEGF have been identified in both granulosa and theca cells from follicles of cows (Berisha et al., 2000) and pigs (Barboni et al., 2000). Isolated granulosa cells from various species secrete VEGF in culture (Christenson and Stouffer, 1997; Hazzard et al., 1999; Barboni et al., 2000). These differences may in part reflect species differences or disparity in stages of follicle maturity at the time of collection, but may also indicate that VEGF is being produced in the granulosa cells and that the protein then passes to the theca layer where the blood vessels are located. Post-transcriptional regulation of VEGF production by gonadotrophins may take place in periovulatory follicles (Hazzard et al., 1999).

The growth of new blood vessels can also be monitored by measuring proliferation of endothelial cells (Jablonska-Shariff et al., 1993; Rodger et al., 1997). Immunohistochemical detection of the proliferation marker Ki67 has been used previously as a monitor of mitotic activity in equine endometrium (Gerstenberg et al., 1999). Cells with the morphology of endothelial cells that stained positively for Ki67 were present only in the theca of preovulatory follicles, which is indicative of active proliferation of blood vessels in these follicles. Many of the granulosa cells in these follicles were also positively stained, whereas staining was infrequent in the granulosa of the transitional follicles, indicating active cell division in the preovulatory follicles in contrast to the transitional follicles. In the marmoset corpus luteum, it appears that luteal cells that stain positively for Ki67 do not co-label with the steroidogenic cell marker 3β-HSD (Young et al., 2000), indicating that steroidogenic cells may not produce steroid hormones and express cell cycle antigens simultaneously. It is not known whether this is also true in the follicle, but the preovulatory follicles in the present study with high proliferation indices were markedly more steroidogenically active than the transitional follicles. It has been shown that activation of VEGF production is more dependent on the dynamic status of the follicle and its growth rate than on its diameter (Barboni et al., 2000). This finding correlates well with the high degree of proliferative activity in the preovulatory follicles in the present study.

The low vascularization in the transitional follicles in the present study, together with the low proliferative index, low VEGF protein content and follicular steroids are consistent with early follicular atresia, although this was not evident histologically. As seen in the present study in equine follicles, endothelial cell proliferation has been strongly correlated with expression of VEGF in theca cells during follicle growth (Yamamoto et al., 1997). Follicle health depends on the presence of a rich network of capillaries in the innermost part of the thecal wall, such that oxygen and nutrients can be transported into the follicle, particularly at the cumulus-oocyte complex. Early signs of atresia are characterized by disappearance of this inner vascular layer (Hay et al., 1976). Hypoxia is commonly implicated in stimulation of VEGF in most tissues, but in ovarian cells, a relationship has been shown between gonadotrophin stimulation of cultured luteinized granulosa cells and VEGF production (Christenson and Stouffer, 1997; Hazzard et al., 1999). Other studies have shown in vivo that the intense angiogenesis in the early preovulatory corpus luteum is dependent on gonadotrophin stimulation of the luteal cells (Dickson and Fraser, 2000) and VEGF concentrations in follicular fluid from prepuberal gilts increase after eCG and hCG treatment (Barboni et al., 2000). As follicle health and growth are also dependent on gonadotrophin stimulation, it is likely that the low circulating concentrations of LH, characteristic of the transitional breeding season (Freedman et al., 1979), failed to stimulate the synthesis of angiogenic factors, including VEGF. Other trophic hormones such as growth hormone, which is known to be low in mares during seasonal anoestrus (Aurich et al., 1999) and is involved in follicle growth and viability (Kirkwood et al., 1990), may also be involved. The low concentrations of angiogenic factors then led to poor thecal vascularization and vascular permeability, both of which are essential for trophic support of the actively dividing follicular cells. In turn, poor vascularity will contribute to inadequate delivery of gonadotrophins and other trophic factors to the follicle to sustain development. Therefore, in these mares, the low concentrations of thecal VEGF could result in failure of further development and subsequent atresia of transitional follicles. In primates, VEGF production has been implicated in follicle selection during the menstrual cycle (Ravindranath et al., 1992). From the results of the present study it appears likely that VEGF could also be a key factor in regulating gonadotrophin-dependent follicular growth in equine preovulatory follicles.

It is possible to draw three main conclusions on the differences between transitional and preovulatory follicles: (i) the histological morphology of these two types of follicle is clearly different; the thecal layer is only poorly developed in transitional follicles and the proliferative index of the granulosa cells is markedly depressed in transitional follicles; (ii) thecal vascularization is sparse in transitional follicles in association with the absence of proliferative activity in the vascular endothelial cells; and (iii) VEGF, one of the main angiogenic factors, is present in abundance in the theca of preovulatory, but not transitional, follicles. These inter-related factors could explain the low steroidogenic capacity of transitional follicles and why these follicles fail to progress to ovulation during the spring anovulatory period in mares.

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References


Christensen LK and Stouffer JE (1997) Follicle-stimulating hormone and luteinising hormone/chorionic gonadotrophin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. Journal of Clinical Endocrinology and Metabolism 82 2135-2142

Corrie JET, Hunter WM and Macpherson JS (1981) A strategy for radioimmunoassay of plasma progesterone with the use of a homologous site-labeled radioglandular Clinical Chemistry 27 594-599


Dickson SE and Fraser H (2000) Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate Journal of Clinical Endocrinology and Metabolism 85 2339-2344


Glasser AE, Irvine DS, Wickings EJ, Hilier SG and Baird DT (1986) A comparison of the effects on follicular development between colon spinatum citrate, its two separate isomers and spontaneous cycles Human Reproduction 252-256


Hazard TM, Maldenckers TA, Chaffin CL and Stouffer RJ (1999) Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophins and steroids in macaque granulosa cells during the periovulatory interval. Molecular Human Reproduction 5 1115-1121

Jablonska-Shariff A, Grazulis-Biska AT, Redmer DA and Reynolds LP (1993) Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle Endocrinology 133 1871-1879


Kirkwood RN, Thacker PA, Guedo BL and Lherfeldt B (1989) Effect of exogenous growth hormone on the follicular luteal phase on hCG binding by porcine luteal tissue Canadian Journal of Animal Science 79 719-721


Richards JS (1985) Maturation of ovarian follicles: action and interaction of pituitary and ovarian hormones on follicular cell differentiation Physiological Reviews 60 51-89


Rodger FE, Young FM, Fraser HM and Illingworth PJ (1997) Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotrophin rescue, in the human corpus luteum Human Reproduction 12 1723-1729


Sharp DC, Grubauh WR, Weithenauer J, Davis SD and Wilcox C (1991) Effect of steroid administration on pituitary luteinizing hormone and follicle-stimulating hormone in ovarioctomized pony mares in the early spring pituitary responsiveness to gonadotropin-releasing hormone and pituitary gonadotropin content. Biology of Reproduction 44 983-990

Silvia PJ, Squires EL and Nett TM (1987) Pituitary responsiveness of mares challenged with GnRH at various stages of the transition into the breeding season Journal of Animal Science 64 790-796


Turner DD, Garcia MC and Gันther OJ (1979) Follicular and gonadotropin changes throughout the year in pony mares American Journal of Veterinary Research 40 1694-1700


Watson ED, Pedersen HG, Thomson SRM and Fraser HM (2000) Control of follicular development and corpus luteum function in the mare: effects of a potent GnRH antagonist Teratology 54 599-609


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Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum

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Short title: angiogenesis in the equine corpus luteum.

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Precise pharmacological control of the corpus luteum is important in manipulation of the oestrous cycle in the mare. Angiogenesis plays a key role in growth and regression of the corpus luteum, therefore influencing the vasculature of the corpus luteum may offer a novel method of controlling its lifespan. In the present study the changes in angiogenesis and VEGF expression were evaluated throughout the luteal phase, and also after PGF$_{2\alpha}$ administration. Corpora lutea were collected from mares in the early luteal phase (Day 3-4), mid-luteal phase (Day 10) early regression (Day 14), late regression (Day 17), and 12 and 36 h following administration of PGF$_{2\alpha}$ on day 10. Immunohistochemistry was used to localize Von Willebrand factor VIII and Ki67 in endothelial and proliferating cells, respectively. VEGF mRNA and protein was localized by *in situ* hybridization and immunohistochemistry. The proliferation index of endothelial cells was intense in the early luteal phase then decreased to low levels by day 14. The early and mid-luteal phases were characterized by a dense network of capillaries. The microvasculature had begun to regress by day 14. Following administration of PGF$_{2\alpha}$ vasodilation was observed at 12 h. However, after 36 h, luteal degeneration was accompanied by a significant decrease in vascularity. VEGF mRNA and protein was expressed mainly in the luteal cells during the early and mid-luteal phase and declined sharply on day 14. However immunostaining for VEGF protein was high in late luteal regression (day 17) and 36 h after PGF$_{2\alpha}$ administration. These findings show a close temporal association between VEGF expression and angiogenesis in the equine CL during its functional lifespan.
Introduction

The corpus luteum (CL) undergoes extensive cellular proliferation and differentiation during luteinization and involutes rapidly at luteolysis. These changes involve intense growth and regression of microvessels (Reynolds et al., 1992; Zheng et al., 1993). It is now emerging that angiogenesis is essential for normal luteal function, and inhibition of angiogenesis can trigger functional luteal regression (Fraser et al., 2000). Pharmacological manipulation of luteolysis is a key element in oestrous cycle control in clinical equine reproduction. Currently PGF$_{2\alpha}$ can be administered from day 6 of the cycle onwards to initiate luteolysis (Oxender et al., 1975), but there are no drugs available that can induce luteolysis over the entire luteal phase. The process of angiogenesis has not been studied in the equine CL, but pharmacological control of angiogenesis could offer an alternative approach to shortening the oestrous cycle.

Studies in ruminants have shown that the CL is one of the most highly vascularised tissues in the body (Bruce and Moor 1976). Quantitative studies in the ovine CL have revealed that in the early luteal phase, endothelial cells constitute approximately 85% of proliferating cells and represent more than 50% of the cells present in the mature CL (Reynolds et al., 1994). Luteolysis in the ruminant may be associated with changes in CL vasculature as there is a decrease in luteal vascularity in the late luteal phase (Zheng et al., 1993; Reynolds et al., 1998), and this may be mediated by PGF$_{2\alpha}$ (Niswender et al., 1976; Nett et al., 1976).

Angiogenesis in the CL is regulated by a number of growth factors (Fraser and Lunn, 2000). There is growing evidence that vascular endothelial growth factor (VEGF) is the main angiogenic factor in the CL (Reynolds and Redmer, 1998, Fraser et al., 2000), and VEGF
mRNA and protein have been demonstrated in the CL of several species (Redmer and Reynolds, 1996b; Goede et al., 1998; Hazzard et al., 2000; Wulff et al., 2000). While VEGF may be induced by hypoxia in most ischaemic tissues, it is probably regulated more closely by LH in the CL (Neulen et al., 1998; Dickson and Fraser 2000).

In the present study, the pattern of endothelial cell proliferation throughout the luteal phase of the oestrous cycle and after induced luteal regression was investigated. In order to establish whether VEGF is temporally associated with angiogenesis in the equine CL, VEGF mRNA and protein were localised throughout the luteal phase.

Materials and Methods

Animals and tissue collection

Pony mares of mixed breeding, aged between 4 and 12 years and weighing 250 to 450 kg were used. The ovaries were examined daily during oestrus by transrectal ultrasonography to determine day of ovulation (day 0). The ovary containing the CL was removed via a colpotomy incision after appropriate sedation and analgesia (Lawler et al., 1999). CLs were obtained in early luteal phase, Day 3-4 (n = 4), mid-luteal phase, Day 10 (n = 5), early regression phase, Day 14 (n = 4), late regression phase, Day 17 (n = 4), and 12 and 36 h (n = 3 each) after intramuscular administration of the PGF2α analogue, cloprostenol (Estrumate, 263μg/500kg, Schering-Plough Animal Health, Middlesex, UK) on day 10 of the cycle. The ovaries were transferred to the laboratory on ice immediately after surgical removal. The CL was enucleated from the ovary and dissected free of connective tissue. Tissue samples were fixed in 10% neutral phosphate buffered formalin (pH 7.0) for 24 h at room temperature and were then embedded in paraffin wax. Sections (4 μm) were then mounted onto glass microscope slides coated with poly-L-lysine (Sigma, Poole, UK).
**Immunohistochemistry**

The endothelial cells were identified by Von Willebrand factor VIII antigen and cell proliferation by Ki67 as described previously (Rodger et al., 1997) with slight modifications. Sections were treated with 0.1% trypsin for 45 min at 37°C then incubated with rabbit anti-human Von Willebrand factor VIII (Dako, High Wycombe, UK) at a dilution of 1:250 for 90 min at room temperature. For Ki 67, sections were exposed to three x10 min cycles of microwave irradiation at 700 W in citrate buffer (0.01M pH 6.0). The sections were incubated with a monoclonal antibody to the nuclear non-histone antigen, Ki67 (Novocastra, Peterborough, UK) diluted to 1:40 for 3 h at 37°C. For VEGF, sections were incubated with rabbit anti-VEGF (Santa Cruz Biotechnology, California, USA), diluted to 1:200 in PBS, for 120 min at room temperature or overnight at 4°C. Activated macrophages were detected using MAC 387 as the primary antibody (Dako). Sections were treated with 0.1% w/v trypsin for 20 min, and were then incubated with primary antibody diluted to 1:200 for 60 min. Negative control sections were incubated with 2% (v/v) normal rabbit serum for factor VIII and VEGF or 2% (v/v) normal mouse serum for Ki 67 and MAC 387. Sections were visualized with 0.05% (w/v) 3,3′-diaminobenzidine containing 0.01% (v/v) H₂O₂ (Sigma, Poole, UK) and counterstained with haematoxylin.

**In situ hybridization for VEGF mRNA**

The method used for in situ hybridization was as described previously (Wulff et al., 2000) with slight modifications. Briefly, complementary RNA probes for human VEGF were used. Sense and antisense probes were prepared using a RNA transcription kit (Ambion, Inc, Austin, TX) and labelled with [³⁵S]UTP (NEN Life Science Products, Boston, MA). Sections were treated with 0.1N HCl and then digested in proteinase K (5mg/ml, Sigma) for 30 min at
37°C. A prehybridization step was carried out by incubation in prehybridization buffer containing 50% formamide, 4 x SSC, 1 x Denhardt's, 125mg/ml salmon testis DNA, 125mg/ml yeast transfer RNA, and 10 mmol/L dithiothreitol at 55°C in a moist chamber for 2 h. Hybridization was performed in a moist chamber overnight at 55°C. The hybridization buffer was similar to the prehybridization buffer, but contained 10% (w/v) dextran sulfate in addition. Two sections per slide were exposed to the antisense and sense sequences. Dry slides were dipped in Ilford G5 liquid emulsion (Ilford imaging, Cheshire, UK), exposed for 4.5 weeks at 4°C, and subsequently developed (Kodak D19 Developer, Eastman Kodak Co, Rochester, NY) and fixed (Kodak GBS). All slides were counterstained with haematoxylin. For light field photography the coverslips were removed and the sections stained with haematoxylin and eosin.

Quantification methods

Microvessels:

Microvessels were quantified in two ways: 1. density of blood vessels, to estimate the proportion of luteal tissue occupied by blood vessels. The density of the microvessels (%) was calculated by examining sections at x 400 magnification. An eyepiece grid was used which covered an area of 0.063 mm² (25μm x 25μm). Regions from the inner, middle and outer areas of the sections were randomly selected. Each point of the grid superimposed on positive immunostaining or on capillary lumens was counted and the total number of points was divided by the total number superimposed on luteal cells and the rest of luteal tissue x 100% (Ferrara et al., 1998; Gaytan et al., 1999), and 2. the area of factor VIII immunostaining was calculated by Quantimet image processing and analysis system 500 (Leica, Cambridge, UK). The system was optimized for each individual section based on the density of the stain. The area used in this system was calibrated at x200 magnification. Quantification was performed
using unbiased counting rules (Gundersen et al., 1988). Single endothelial cells or clusters of endothelial cells were considered to be individual vessels. The density of the microvessels was expressed as a percentage mean ± SEM, whereas the area of factor VIII was expressed as mean ± SEM mm².

Proliferation index:

Fields were examined at x400 magnification. The number of Ki 67-labelled nuclei and the total number of nuclei were counted. Two sections per animal and 4 fields per section were counted. The proliferation index was determined by the number of Ki 67-labelled nuclei divided by total number of nuclei x100 and expressed as a percentage mean ± SEM. In order to identify the cell types immunostained for Ki 67, the following classifications were used: endothelial cells were elongated with a long axis of about 4-8 µm and had little cytoplasm; luteal cells were large, round to vesicular shaped cells, of 20-40µm in diameter, and contained large spherical to vesicular nuclei of 10-15µm in diameter. Cells showing positive staining for Ki67 which did not fit the criteria described for endothelial cells or luteal cells were considered to be other types of cells, usually leukocytes and fibroblasts. The number of different cells were expressed as mean ± SEM per field.

VEGF mRNA:

Grain density for VEGF mRNA was evaluated by scoring (+++ for intense expression, (+) for moderate expression (+) for low expression and (-/+ for very weak expression.

The percentage area of VEGF immunostaining:

The Quantimet image-analysis system was used to calculate the percentage area of the immunostaining (area of the immunostaining divided by the total area measured x 100). Areas
were analysed at x200 magnification using two sections from each animal and four fields per section. The results were expressed as percentage mean ± SEM per unit area.

**MAC 387 positive cells:**

Positive cells in the cavity of the corpora lutea were counted at x400 magnification and expressed as mean ± SEM per field.

**Statistical analysis**

The differences in early, mid-luteal, early and late regression CL were analysed by one-way analysis of variance using stage of the luteal phase as the between-subject variable. Data from untreated mid-luteal phase CL (day 10) were compared with CL after induced regression using a one-way analysis of variance with treatment as the between-subject variable. The data were subjected to Tukey's test of multicomparison among means. Results were considered to be significantly different when P < 0.05.

**Results**

**Factor VIII immunostaining**

Factor VIII immunostaining was limited to endothelial cells of microvessels and capillaries (Fig 1). After ovulation, sprouting endothelial cells invaded the cavity of the corpus hemorrhagicum and began to form a vascular bed between the luteal cells (Fig 1a). The mid-luteal phase was characterized by a dense network of fully differentiated capillaries (Fig 1b), the density of the microvessels and the area of factor VIII immunostaining being significantly greater (P<0.05) than in the early luteal phase (Fig 2). During early regression there was a slight decrease in the area and density of the microvessels. By late regression (day 17), the
extensive vascular network of the mid-luteal phase had regressed leaving a few long vessels (Fig 1c). This resulted in a marked decline (P < 0.01) in microvessel density and area of factor VIII immunostaining. Twelve hours following PGF$_{2\alpha}$ administration, microvessels underwent vasodilation (Fig 1d), resulting in a significant increase in vessel density (P < 0.01). However by 36 h the vasodilation had virtually disappeared. At this time there was a sharp decline in the density of the microvessels and the area of factor VIII immunostaining (P < 0.01) compared with 12 h post PGF$_{2\alpha}$. No immunostaining was present in negative control sections.

**Ki 67 immunostaining**

Ki 67 immunostaining was observed in all sections examined. The proliferation index of Ki 67 changed throughout the oestrous cycle and after PGF$_{2\alpha}$-induced luteolysis (Fig 3). The proliferation index was highest in the early luteal phase and declined by more than 70% in the mid-luteal phase and early regression (P < 0.01). During late regression and 36 h following PGF$_{2\alpha}$ injection, a significant increase in the proliferation index was observed (P < 0.05). Endothelial cells showed the highest proliferation rate during the early luteal phase (Fig 4a, b); by mid-luteal phase (Fig 4c) and early regression (Fig 4d) the number of endothelial cells that stained positively for Ki 67 dropped significantly (P < 0.01; Fig 5). During natural and induced regression, very few endothelial cells were proliferating. Luteal cells stained positively for Ki 67 in the early luteal phase (Fig 4a) and numbers of positively staining cells had reduced significantly (P < 0.01) by the mid-luteal phase and early regression (Fig 5). During late regression and 36 h following PGF$_{2\alpha}$, some luteal cells showed immunostaining (Fig 4e). Other types of cells (probably leukocytes and fibroblasts based on morphological appearance), particularly in the regression phase, showed Ki 67 positive staining. These cells increased significantly (P < 0.01) on day 17 and 36 h following PGF$_{2\alpha}$ (Fig 5). No immunostaining was present in negative control sections (Fig 4f).
Expression of VEGF mRNA

VEGF mRNA was expressed in the luteal cells and was absent from endothelial cells and neutrophils. Intense expression of VEGF mRNA was found in the early luteal phase (Fig 6a) and mid-luteal phase (Fig 6b, 7a). By early and late regression and in the PGF$_{2\alpha}$-treated animals, grain density representing VEGF mRNA in the luteal cells had decreased markedly (Fig 6c,d; Table 1). However, as the grains decreased in the luteal cells during early regression, high VEGF mRNA expression was observed in some non-luteal cells in the cavity of the CL (Fig 7b). These cells were identified as macrophages using MAC 387 immunostaining (Fig 7c,d). The number of non-luteal cells expressing VEGF increased significantly ($P < 0.05$) during regression compared with the early luteal phase. Hybridization was not seen when the labelled sense RNA strand was used (Fig 6b, inset).

VEGF immunostaining

Immunostaining was located mainly in the cytoplasm of luteal cells. Moderate immunostaining was found in most of the luteal cells during the early luteal phase (Fig 8a). At this stage, some endothelial cells also exhibited positive staining. In the mid-luteal phase, strong positive staining for VEGF was observed but tended to be confined to luteal cells located adjacent to the trabeculae (Fig 8b). By early luteal regression, only a few luteal cells exhibited pale immunostaining (Fig 8c). During late regression, strong immunostaining was observed in some luteal cells scattered throughout the CL and in some sections the staining was diffuse, with the trabeculae also stained. Twelve and 36 hours following PGF$_{2\alpha}$, some luteal cells and many neutrophils were immunostained (Fig 8d). The neutrophils were frequently found clustered round blood vessels. Some of these vessels also contained strongly stained neutrophils (Fig 8e). At all stages, macrophages and other non-luteal cells were found
to exhibit immunostaining for VEGF both in the central cavity of the CL and in the adjacent capsule. The percentage area of immunostaining was high during the early and mid-luteal phase (Fig 9) then decreased significantly (P < 0.01) during early regression. By late regression, immunostaining increased significantly (P < 0.05) compared with early regression.

Twelve hours after PGF$_{2\alpha}$ administration, the immunostaining had decreased significantly (P < 0.05) compared with day 10. The area of immunostaining then increased by 36 h after PGF$_{2\alpha}$ administration. No immunostaining was present in the negative control sections (Fig 8f).

**Discussion**

This report describes angiogenesis and cell proliferation throughout the cycle and following PGF$_{2\alpha}$-induced luteolysis in the equine CL. The marked endothelial cell proliferation during the early and mid-luteal phase demonstrated intense angiogenesis. The high expression of VEGF mRNA and protein in the luteal cells during early and mid-luteal phase was strongly suggestive of an important paracrine role for VEGF in regulation of blood vessel growth and development in the CL of the mare. Natural and induced luteolysis was accompanied by a decline in expression of mRNA encoding VEGF and a reduction in endothelial cell proliferation.

The high proliferation of endothelial cells in the early luteal phase led to increasing vascularisation during luteal development. The observed peak in vascularity and the presence of a dense capillary network by the mid-luteal phase will be required for optimal delivery of progesterone precursors to, and progesterone from the luteal cells, at a time when maximum progesterone concentrations are observed (Al-zi’abi et al., 2002). These findings agree with
previous reports in other species (Niswender et al., 1979; Gaytan et al., 1999; Young et al., 2000). In the present study, decreased endothelial cell proliferation during late regression, and 36 h following PGF$_{2\alpha}$, coincided with an observed decline in microvessels and progesterone concentrations (Al-zi’abi et al., 2002).

VEGF stimulates increased vascular permeability, angiogenesis and endothelial cell mitosis (Folkman and Klagsburn, 1991). The peak expression of VEGF mRNA and protein during the early and mid-luteal phase in the present study showed a direct temporal association with high endothelial cell proliferation and presence of a dense capillary network. Furthermore the location of the protein as visualized by immunohistochemistry also suggested a close association with angiogenesis. In the early luteal phase, when angiogenesis was at its peak, there was diffuse immunostaining throughout the CL, including immunostaining in the endothelial cells. In the mature CL, strong immunostaining for VEGF was present in cells bordering the trabeculae where the blood vessels enter the luteal tissue. The high expression of VEGF mRNA during the mid-luteal phase is compatible with its established role in stimulating and maintaining the newly formed vasculature in the CL (Dickson et al., 2001). Therefore our findings strongly indicate a role for VEGF in regulating angiogenesis in the equine CL. During early regression (day 14), VEGF mRNA expression and immunostaining declined in the equine CL, and were associated with a marked decline in endothelial cell proliferation. The time course of these events was also confirmed by our observations after PGF$_{2\alpha}$-induced luteolysis. At 12 h after PGF$_{2\alpha}$ administration, there was a sharp decline in VEGF mRNA and protein accompanied by low endothelial cell proliferation rates. These findings suggest that either PGF$_{2\alpha}$ has a direct inhibitory effect on proliferating endothelial cells and this may coincide with the decrease in VEGF production, and/or that PGF$_{2\alpha}$ inhibits endothelial cell proliferation by decreasing VEGF expression.
Twelve hours and 36 h following PGF$_{2\alpha}$-induced regression there was massive infiltration of the luteal tissue by neutrophils. Although many neutrophils exhibited positive immunostaining for VEGF protein, expression of mRNA encoding VEGF was not detected. To our knowledge this is the first report of VEGF protein in neutrophils in the CL. Recent studies in the human endometrium also showed strong immunostaining for VEGF protein in neutrophils and a role in angiogenesis was proposed (Mueller et al., 2000, Gargett et al., 2001). In the present study, VEGF immunostaining in neutrophils was not associated with a rise in proliferating endothelial cells. This might suggest that after PGF$_{2\alpha}$ administration, VEGF protein may play a role in non-angiogenic functions in the CL such as the regulation of vascular permeability, vasodilation, or mediation of endothelial cell survival (Goede et al., 1998; Berisha et al., 2000).

Non-luteal cells, which we identified as macrophages, showed expression of VEGF mRNA and protein in the cavity of the CL throughout the luteal phase. Interestingly the number of these cells increased during regression while luteal cells showed a decrease in VEGF expression. There is evidence that the human macrophage can produce and express VEGF mRNA (Cho et al., 20001; Cejudo-Martin et al., 2001). It is possible that macrophages trapped in the central clot experience hypoxia which is responsible for activating VEGF mRNA.

In late regression, intense immunostaining for VEGF in degenerated luteal cells was observed. However, VEGF mRNA expression was absent, indicating that VEGF was not being synthesized at this time. It is possible that either VEGF is required for support of the remaining microvessels in clearing up the unwanted luteal tissue during regression, or that
this staining represents the presence of residual, non-active/metabolized VEGF as suggested by Dickson et al. (2000) using the same antibody.

Vasodilation as well as influx of neutrophils was observed 12 h following PGF<sub>2α</sub> injection. PGF<sub>2α</sub> is generally regarded as being a vasoconstrictor. The mare is particularly sensitive to the effect of administered PGF<sub>2α</sub> (Douglas and Ginther, 1973; Oxender et al., 1975). It is possible that the combination of the pharmacological dose of PGF<sub>2α</sub>, the sensitivity of mares to PGF<sub>2α</sub>, and the increased vascular permeability caused by VEGF at 12 h following PGF<sub>2α</sub> injection, may have resulted in this unexpected observation at the ovarian level.

Other types of cells with the morphological appearance of leukocytes and fibroblasts stained positively for Ki 67 during late regression and 36 h following the administration of PGF<sub>2α</sub>. These findings support other recent data, which showed increased numbers of proliferating T-lymphocytes and macrophages during regression in the bovine CL (Petroff et al., 1997), and in the mare increased numbers of lymphocytes have been reported during both induced and natural regression (Lawler et al., 1999). Fibroblasts also have been reported to increase in number in the regressing CL in many species, including the mare (Van Niekerk et al., 1975). A proportion of cells with the morphological appearance of luteal cells was positive for Ki 67 during late regression and 36 h following PGF<sub>2α</sub> administration. This was unexpected, since a number of studies in other species have demonstrated that luteal cells do not proliferate in late regression (O’Shea et al., 1986; Lei et al., 1991). These positive luteal cells may represent staining of degenerated proteins around the time of cell death.

In conclusion, luteal regression is associated with a dramatic decrease in proliferating endothelial cells and an increase in proliferating cells of other types, probably leukocytes and
fibroblasts. The pattern of VEGF expression is strongly suggestive of a paracrine role in regulation of blood vessel development, growth and regression in the CL. Manipulation of angiogenesis in the equine CL may provide a useful alternative method of controlling the oestrous cycle in the mare.

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References


Bruce NW and Moor RM (1976) Capillary blood flow to ovarian follicles, stroma and corpora lutea of anaesthetized sheep Journal of Reproduction and Fertility 46 299-304


Dickson SE and Fraser HM (2000) Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate Journal of Clinical Endocrinology and Metabolism 85 2339-2344
Dickson SE, Bicknell R and Fraser HM (2001) Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor Journal of Endocrinology 168 409-416


Fraser HM and Lunn SF (2000) Angiogenesis and its control in the female reproductive system British Medical Bulletin 56 787-797


Hazzard TM, Molskness TA and Chaffin CL (1999) Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval *Molecular Human Reproduction* **5** 1115–1121


Oxender WD, Noden PA, Bolenbaugh DL and Hafs HD (1975) Oestrus, ovulation and plasma hormones after PGF$_{2\alpha}$ in mares *Journal of Reproduction and Fertility Supplement* 23 251-255


Reynolds LP and Redmer DA (1998) Expression of the angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in the ovary *Journal of Animal Science* 76 1671-1681

Reynolds LP, Grazul-Bilska AT, Killilea SD and Redmer DA (1994). Mitogenic factors of corpora lutea *Progress of Growth Factor Research* 5 159-175


Rodger, FE, Young, FM, Fraser, HM and Illingworth, PJ (1997) Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotrophin rescue, in the human corpus luteum *Human Reproduction* 12 1723-1729
Van Niekerk CH, Morgenthal JC and Gerneke WH (1975) Relationship between the morphology of and progesterone production by the corpus luteum of the mare Journal of Reproduction and Fertility Supplement 23171-175


Legends to Figures

**Figure 1.** Factor VIII immunostaining during (a) early luteal phase, (b) mid-luteal phase, (c) late regression, and (d) 12 h following PGF$_{2\alpha}$. Endothelial cells and microvessels show positive immunostaining. Note intense factor VIII immunostaining during early and mid-luteal phase and sharp reduction in vascularity in late regression. In (d) note vasodilation in the microvessels. Scale bar represents 50μm.

**Figure 2:** a) Density of factor VIII immunostaining and b) the area of factor VIII immunostaining throughout the cycle and after PGF$_{2\alpha}$ administration. The density for each stage of the luteal phase was expressed as percentage mean ± SEM, while the area of Factor VIII immunostaining was expressed as mean ± SEM per unit area.

a: significantly (P < 0.05) higher than early luteal phase.

b: significantly (P < 0.01) lower than mid-luteal phase and early regression.

c: significantly (P < 0.01) higher than early and mid-luteal phase.

d: significantly (P < 0.01) lower than mid-luteal phase and 12 h following PGF$_{2\alpha}$.

**Figure 3:** Percentage of Ki 67 immunopositive cells in the equine CL throughout the cycle and after PGF$_{2\alpha}$ injection.

a: significantly (P < 0.01) higher than all other groups.

b: significantly (P < 0.05) lower than mid-luteal phase.

c: significantly (P < 0.05) lower than mid-luteal phase.
Figure 4. Ki 67 immunostaining during (a and b) early luteal phase, (c) mid-luteal phase, (d) early regression, (e) 36 h following PGF$_{2α}$-induced regression, and (f) negative control. Proliferating cells showed dark brown staining while non-proliferating cells stained with haematoxylin. Note the high number of endothelial cells showing proliferation during the early luteal phase followed by a decline during the mid-luteal phase. Numbers of other cell types showing positive staining increased after PG-induced regression. Scale bar represents 50 µm in a, c, d, e and f, and 25 µm in b.

Figure 5: The number of proliferating cell types per field in the equine CL throughout the cycle and after PGF$_{2α}$ administration.

a: significantly (P < 0.01) higher than mid-luteal and early regression phases.
b: significantly (P < 0.05) lower than mid-luteal phase.
c: significantly (P < 0.01) higher than mid-luteal phase.
d and e: significantly (P < 0.01) higher than early luteal regression and 12 h following PGF$_{2α}$, respectively.

Figure 6: In situ hybridisation for VEGF mRNA under dark field microscopy. Portions of CL with the cellular luteal cavity on the left hand side are shown during (a) early, (b) mid-luteal, (c) early regression and (d) 36 h after PGF$_{2α}$ injection during mid-luteal phase (day 10). Cells expressing VEGF mRNA are associated with grain clusters (white grains) while amorphous refraction from tissue is grey. Note the high number of grain clusters in early and mid-luteal phase with a dramatic reduction during early regression and 36 h following PGF$_{2α}$ injection. In CL (d) the cells producing VEGF mRNA were also restricted to the peripheral or central areas of the tissue. Grain clusters were not seen when the labelled sense RNA probe was used (inset). Scale bar represents 50 µm.
Figure 7: *In situ* hybridisation for VEGF mRNA under light field microscopy for luteal and non luteal cells, and immunostaining for MAC 387 positive cells. a) VEGF mRNA section counterstained with H&E showing black grains representative of VEGF expression in the luteal cells in mid-luteal phase (arrows). b) non-luteal cells (macrophages; white arrows) in the cavity of a CL collected 12 h after PGF$_{2\alpha}$ administration expressing specific VEGF mRNA. Inset shows cavity from CL collected at early regression. c) CL cavity in the early luteal phase showing some macrophages exhibiting immunostaining with MAC 387. d) immunostaining of macrophages in the cavity of CL at early regression using MAC 387. Scale bar represents 20µm.

Figure 8: VEGF protein immunostaining during (a) early luteal phase, (b) mid-luteal phase, (c) early regression, (d) 36 h after PGF$_{2\alpha}$, (e) 36 h after PGF$_{2\alpha}$ showing neutrophils in luteal tissue and blood vessels (arrows), and (f) negative control. Scale bar represents 50µm in a, b, c, d and f, and 25µm in e.

Figure 9: The percentage area of VEGF immunostaining throughout the cycle and after PGF$_{2\alpha}$ administration. Data are expressed as mean ± SEM.

a: significantly (P < 0.01) higher than early regression

b: significantly (P < 0.05) lower than mid-luteal phase

c: significantly (P < 0.05) lower than mid-luteal phase, early regression and 36 h post PGF$_{2\alpha}$
Figure 2

(a) Density of blood vessels (%)

Day 3  Day 10  Day 14  Day 17  12 h post PGF  36 h post PGF

(b) Area (mm$^2$) of factor VII immunostaining

Day 3  Day 10  Day 14  Day 17  12 h post PGF  36 h post PGF
Figure 3

Day 3  Day 10  Day 14  Day 17  12h post PGF  36h post PGF

Kι67 positive cells (%)
Figure 5

The number of proliferating cell types per x40 field

- Endothelial cells
- Luteal cells
- Other cells

- D3
- D10
- D14
- D17
- 12h post P
- 36h post P
Table 1 The density of VEGF mRNA grains in the luteal cells through the luteal phase and after regression, and expression of VEGF mRNA in the central cavity.

<table>
<thead>
<tr>
<th>Stage of the luteal phase</th>
<th>day 3</th>
<th>day 10</th>
<th>Day 14</th>
<th>Day 17</th>
<th>12 h post PGF_{2α}</th>
<th>36 h post PGF_{2α}</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF mRNA grains in luteal cells</td>
<td>+++</td>
<td>++/+++</td>
<td>+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>No. of non-luteal cells in CL cavity expressing VEGF mRNA per field</td>
<td>1.6 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>6.8 ± 1.3^a</td>
<td>No cavity</td>
<td>7.1 ± 6.0^a</td>
<td>5.9 ± 1.5^a</td>
</tr>
<tr>
<td>No of MAC 387 positive cells in the cavity of the CL per field</td>
<td>1.1 ± 0.3</td>
<td>0.7 ± 0.4</td>
<td>5.3 ± 1.4^a</td>
<td>No cavity</td>
<td>6.5 ± 1.2</td>
<td>7.1 ± 1.3^a</td>
</tr>
</tbody>
</table>

a: significantly (p< 0.05) higher than early and mid-luteal phase
before PGF₂α. The number of follicles 5mm in diameter in the penultimate wave before PGF₂α which failed to ovulate in response to PGF₂α was higher in response to the second compared to first injection of PGF₂α (P<0.05). The proportion of luteal structures detected relative to the number of ovolutions was higher in the early compared to late group (86 and 60%, respectively; P<0.05). The time from PGF₂α to the detection of luteal structures (6.4 ± 0.4 d) was not significantly different in the early and late groups nor in response to the first and second injections. We concluded that impaired luteogenesis in response to PGF₂α given late compared to early in the oestrous cycle could partly explain the variability in fertility following PGF₂α treatment of cyclic ewes.

229. Effects of the corpus luteum on antral follicular development in the ewe.
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The purpose of this study was to use daily transrectal ultrasonography to examine the effects of the corpus luteum (CL), from days 3 to 16 after ovulation, on ovarian antral follicle dynamics (follicles ≥3 mm in diameter), in unilaterally ovariectomised ewes. At different times of the transition (days 3 to 16 after ovulation, on ovarian antral follicle dynamics (follicles ≥3 mm in diameter), in unilaterally ovariectomised Western white-faced ewes, at different times of the year (transitions into and out of the breeding season, mid-breeding season, and anoestrous ewes) nucleated with induced ovulation. Mean daily numbers of 3-mm follicles that did not grow any larger were significantly reduced in the CL-containing ovaries of ewes at all periods of study except for the transition to anoestrous. Non-CL-bearing ovaries of ewes in the transition to anoestrous had fewer (P<0.05) follicles growing from 3 to ≥5 mm in size compared to the mid-breeding season and mid-anoestrous, but the numbers of follicles in CL-containing ovaries did not vary among study periods. The duration of different phases of follicle lifespan as well as the maximum size and mean growth rate of all non-ovulatory follicles growing to 5 mm in size, did not differ with the stage of the annual reproductive cycle, but the follicle lifespan was shorter (P<0.05) in CL-containing ovaries of anoestrous ewes. Serum levels of progesterone (P₄) were lower during both transitional periods and mid-anoestrous than during the mid-breeding season (P<0.001), and were lower during anoestrous than during both transitional periods (P<0.05). We concluded that CL locally suppressed the growth of ovarian antral follicles to a 3-mm size-range except during the transition to anoestrous in ewes, but that there was no inhibitory effect of the CL on the growth of ovarian follicles to larger diameters. Neither the presence of the CL nor variations in peripheral concentrations of P₄ across the breeding season altered the length of the lifespan of large antral follicles emerging throughout dioestrus. CL/luteal P₄ appeared to maintain follicle development to ouulatory sizes during the transition to anoestrous and shorten the lifespan of large antral follicles in mid-anoestrous ewes, through a local/intraovarian mechanism.

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Luteolysis has been shown to be characterised by variable degrees of apoptosis in cattle, sheep, pigs and humans. No detailed studies have been carried out on cell death during luteolysis in the mare. The aim of this study was to investigate the forms of cell death in both natural and induced luteal regression. Morphological changes in CL at various stages of the cycle were examined: Day 10 (n=4), Day 14 (n=4), Day 17 (n=4), and 12 and 36h (n=3 each) following PGF₂α administration on day 10. Conventional histological stains were employed in addition to TUNEL technique to detect the fragmented DNA. TEM was also performed to examine the ultrastructural changes. Few cells showed signs of apoptosis on day 10, while apoptotic cells increased dramatically on day 14 and 17 associated with massive accumulation of lipid on day 17 only. Interestingly, 12 h after PGF₂α injection, an influx of neutrophils and vasodilatation was observed with few cells showing apoptotic features. By 36 h after PGF₂α, there was no significant change in neutrophil numbers but an increase in apoptotic cells accompanied by accumulation of lipid. As early as day 10, electron microscopic observations showed clear degenerative changes in the mitochondria of some luteal cells. During luteolysis some luteal cells showed irregular nuclear shape, ruffled membrane, myelin bodies and autophagocytic vacuoles, whilst other cells appeared to show apoptotic features. These results indicate that apoptosis is likely involved in luteolysis in the mare, but other types of cell death might also be involved.

231. Luteal protein secretion during early pregnancy in rat.
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The present study was designed to examine the role of luteal proteins in the functional differentiation of corpora lutea (CL) in the early stages of pregnancy. Colony bred mated adult female Sprague Dawley rats were used in this study. Animals were maintained under standard husbandry conditions. On different days of pregnancy (day 1: day of sperm positive vaginal smear) ovaries were dissected out from the animals, transferred to chilled phosphate buffered saline (pH-7.4). CL were enucleated from the ovary under the magnifying head-gear, their weights/animal recorded and homogenized in distilled water in an all glass homogenizer. The homogenate was then centrifuged at 3000 rpm under refrigeration and the supernatant was processed for protein analysis by SDS-PAGE. A 7.5% SDS-Polyacrylamide concentration gave good resolution with approximately 100μg of protein loaded per well and passing 25 μA AC current. Protein bands were identified employing gel densitometry following commassie blue staining. Preliminary studies on the luteal protein pattern though different stages of pregnancy indicated that a low molecular weight protein (66-70 kDa) appears between day 6-8 post-coitum and which disappears after day 10 post coitum. Above studies suggest that this particular protein may have a
CHANGES IN THE VASCULATURE OF THE EQUINE CORPUS LUTEUM THROUGHOUT THE OESTROUS CYCLE AND AFTER PGF$_{2\alpha}$ INDUCED LUTEOLYSIS

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INTRODUCTION: Changes in microvessels have been suggested to play an essential role in CL growth and regression. Understanding the process of luteal angiogenesis in the mare could provide a more precise control over the oestrous cycle. The current study evaluated the vascular changes in the equine CL throughout the oestrous cycle and after PGF$_{2\alpha}$ administration.

MATERIALS AND METHODS: CLs were obtained during early (Day 4, n=4), mid (Day 10, n=4), early regression (Day 14, n=4), late regression (Day 17, n=4), and 12 and 36h (n=3 each) following PGF$_{2\alpha}$ administration on Day 10 and fixed in Formalin. Blood vessels were identified by immunohistochemical staining of Factor VIII. An estimation of blood vessel volume density in both trabeculae and luteal tissue, as well as the percentage of vessels with lumen, was determined by morphometry.

RESULTS AND DISCUSSION: After ovulation, sprouting endothelial cells invaded the cavity of the CL and continued to grow until the mid luteal phase. The mid luteal CL was characterised by a dense network of fully differentiated capillaries with elongated flattened endothelial cells; vascularization was increased significantly (P< 0.05) compared with the early luteal CL. During late natural luteolysis, there was massive degeneration and a decline of approximately 50% in the volume density of luteal tissue, accompanied by a significant decrease in capillary density (P< 0.05). Interestingly, 12 h after PGF$_{2\alpha}$ injection, influx of neutrophils and vasodilation associated with an increase in vasularity was observed (P< 0.001). These changes were short lived, and by 36 h there was a decline in the volume density of the microvessels without a decline in neutrophil numbers. These findings suggest a temporal pattern of blood vessel growth and regression during the ovarian cycle. To our knowledge, this is the first report showing an increase in blood vessels and vasodilation 12 h after PGF$_{2\alpha}$ administration which may reflect the high sensitivity of the mare to PGF$_{2\alpha}$. 