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

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Precise pharmacological control of the corpus luteum is important in the manipulation of the oestrous cycle in mares. Angiogenesis plays a key role in the growth and regression of the corpus luteum; therefore, influencing the vasculature of the corpus luteum may offer a novel method for controlling its lifespan. In the present study, changes in angiogenesis and vascular expression of endothelial growth factor (VEGF) were evaluated throughout the luteal phase and after PGF_{2α}-induced luteolysis. Corpora lutea were collected from mares in the early luteal phase (days 3–4), mid-luteal phase (day 10), early regression (day 14), late regression (day 17), and at 12 and 36 h after administration of PGF_{2α} on day 10 of the oestrous cycle. Immunohistochemistry was used to localize Von Willebrand factor and Ki67 in endothelial and proliferating cells, respectively. VEGF mRNA and protein were localized

by *in situ* hybridization and immunohistochemistry. The proliferation index of endothelial cells was intense in the early luteal phase. The early and mid-luteal phases were characterized by a dense network of capillaries. The microvasculature started to regress by day 14. After administration of PGF_{2α}, vasodilation was observed after 12 h, but after 36 h, luteal degeneration was accompanied by a significant decrease in vascularity. VEGF mRNA and protein were expressed mainly in the luteal cells during the early and mid-luteal phases and expression declined at early regression (day 14). However, immunostaining for VEGF protein was high in late luteal regression (day 17) and 36 h after PGF_{2α} administration. These findings indicate a close temporal association between VEGF expression and angiogenesis in the equine corpus luteum during its functional lifespan.

Introduction

The corpus luteum 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). Angiogenesis is essential for normal luteal function, and inhibition of angiogenesis is associated with functional luteal regression (Fraser *et al.*, 2000). Pharmacological manipulation of luteolysis is a key element in oestrous cycle control in clinical equine reproduction. Currently, prostaglandin F_{2α} (PGF_{2α}) can be administered from day 6 of the oestrous cycle onwards to initiate luteolysis (Oxender *et al.*, 1975), but there is no treatment that can induce luteolysis during the entire luteal phase. The process of angiogenesis has not been studied in the equine corpus luteum, but pharmacological control of angiogenesis could offer an alternative approach in shortening the oestrous cycle.

Studies in ruminants have shown that the corpus luteum is one of the most highly vascularized tissues in the body (Bruce and Moor, 1976). Quantitative studies in the ovine corpus luteum have revealed that in the early luteal phase, endothelial cells constitute approximately 85% of proliferating cells and represent > 50% of the cells present in the mature corpus luteum (Reynolds *et al.*, 1994). Luteolysis in ruminants may be associated with changes in the vasculature of the corpus luteum as there is a decrease in luteal vascularity in the late luteal phase (Zheng *et al.*, 1993; Reynolds and Redmer, 1998), and this may be mediated by PGF_{2α} (Nett *et al.*, 1976; Niswender *et al.*, 1976).

Angiogenesis in the corpus luteum 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 corpus luteum (Reynolds and Redmer, 1998; Fraser *et al.*, 2000; Sugino *et al.*, 2000; Kashida *et al.*, 2001), and VEGF mRNA and protein have been found in the corpus luteum of several species (Goede *et al.*, 1998; Hazzard *et al.*, 2000; Wulff *et al.*, 2000). Although VEGF may be induced by hypoxia in most ischaemic tissues, it is

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probably regulated more closely by LH in the corpus luteum (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. VEGF mRNA and protein were localized throughout the luteal phase to establish whether VEGF is temporally associated with angiogenesis in the equine corpus luteum.

Materials and Methods

Animals and tissue collection

Pony mares of mixed breeding, aged 4–12 years and 250–450 kg body weight, were used. The ovaries of the mares were examined daily during oestrus by transrectal ultrasonography to determine the day of ovulation (day 0). The ovary containing the corpus luteum was removed by a colpotomy incision after appropriate sedation and analgesia (Lawler *et al.*, 1999). Corpora lutea were obtained 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 at 12 and 36 h ($n = 3$ each) after i.m. administration of the PGF_{2α} analogue, cloprostenol (Estrumate, 263 µg 500 kg⁻¹, Schering-Plough Animal Health, Middlesex) on day 10 of the oestrous cycle. In an earlier study, it was shown that corpora lutea collected from mares 12 h after PGF_{2α} administration had cellular changes comparable to those seen during early natural regression (day 14), whereas corpora lutea collected at 36 h after PGF_{2α} treatment showed advanced degenerative changes similar to those observed during late natural regression (day 17) (Al-zabi *et al.*, 2002).

The ovaries were transported to the laboratory on ice immediately after surgical removal. The corpus luteum was enucleated from the ovary and dissected free of connective tissue. Tissue samples were fixed in 10% (v/v) neutral phosphate buffered formalin (pH 7.0) for 24 h at room temperature and then embedded in paraffin wax. Serial sections of 4 µm were mounted on to glass microscope slides coated with poly-L-lysine (Sigma, Poole). This study was performed under the approval of the University of Edinburgh Ethical Review Committee and the project licence obtained under the Home Office Animals (Scientific Procedures) Act 1986.

Immunohistochemistry

The endothelial cells were identified by Von Willebrand factor (vWF) antigen and cell proliferation by Ki67 as described by Rodger *et al.* (1997), with slight modifications. The sections were treated with 0.1% (w/v) trypsin for 45 min at 37°C and incubated with rabbit anti-human vWF (Dako, High Wycombe) at a dilution of 1:250 for 90 min at room temperature. For

Ki67, sections were exposed to three ×10 min cycles of microwave irradiation at 700 W in citrate buffer (0.01 mol l⁻¹, pH 6.0). The sections were incubated with a monoclonal antibody to the nuclear non-histone antigen, Ki67 (Novocastra, Peterborough) diluted to 1:40 for 3 h at 37°C. For VEGF, sections were incubated with rabbit anti-VEGF (Santa Cruz Biotechnology, CA), 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). The sections were treated with 0.1% trypsin for 20 min and 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 vWF and VEGF or 2% (v/v) normal mouse serum for Ki67 and MAC 387. Sections were visualized with 0.05% (w/v) 3,3'-diaminobenzidine containing 0.01% (v/v) H₂O₂ (Sigma, Poole) and counterstained with haematoxylin.

In situ hybridization for VEGF mRNA

The method used for *in situ* hybridization was as described by Wulff *et al.* (2000) with slight modifications. In brief, complementary RNA probes for human VEGF were used. Sense and antisense probes were prepared using an RNA transcription kit (Ambion Inc., Austin, TX) and labelled with [³⁵S]UTP (NEN Life Science Products, Boston, MA). The sections were treated with 0.1 mmol HCl l⁻¹ and then digested in proteinase K (5 mg ml⁻¹, Sigma) for 30 min at 37°C. A prehybridization step was carried out by incubation in prehybridization buffer containing 50% (v/v) formamide, 4 × standard saline citrate, 1 × Denhardt's, 125 mg salmon testis DNA ml⁻¹, 125 mg yeast transfer RNA ml⁻¹ and 10 mmol dithiothreitol l⁻¹ 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 sulphate. Two sections per slide were exposed to the antisense and sense sequences. Dry slides were dipped into Ilford G5 liquid emulsion (Ilford Imaging, Mobberly), 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–eosin.

Quantification methods

Microvessels. Microvessels were quantified in two ways: (i) density of blood vessels, to estimate the proportion of luteal tissue occupied by blood vessels and (ii) the area of vWF immunostaining. The density of the microvessels (%) was calculated by examining sections at ×400 magnification. An eyepiece grid was

Fig. 1. Von Willebrand factor (vWF) immunostaining in the equine corpus luteum during (a) the early luteal phase, (b) the mid-luteal phase, (c) late regression and (d) 12 h after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration. Endothelial cells and microvessels show positive immunostaining. Note intense vWF immunostaining during the early and mid-luteal phase, and reduction in vascularity in late regression. In (d) note vasodilation in the microvessels. Scale bar represents 50 μm .

used which covered an area of 0.063 mm² (25 μm $\times$ 25 μm). Regions from the inner, middle and outer areas of the sections were selected randomly. Each point of

the grid superimposed on positive immunostaining or on capillary lumina was counted and the total number of points was divided by the total number superimposed

Fig. 2. (a) Density of Von Willebrand factor (vWF) immunostaining and (b) the area of vWF immunostaining in the equine corpus luteum throughout the early luteal phase, days 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 i.m. administration of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogue. The density for each stage of the luteal phase was expressed as the percentage mean $\pm$ SEM, whereas the area of vWF immunostaining was expressed as mean $\pm$ SEM per unit area. ^aValue significantly ($P < 0.05$) higher than early luteal phase; ^bvalue significantly ($P < 0.01$) lower than mid-luteal phase and early regression; ^cvalue significantly ($P < 0.01$) higher than all other groups; ^dvalue significantly ($P < 0.01$) lower than mid-luteal phase.

on luteal cells and the remainder of the luteal tissue $\times 100\%$ (Ferrara *et al.*, 1998; Gaytan *et al.*, 1999). The area of vWF immunostaining was calculated using the Quantimet Image Processing and Analysis System 500 (Leica, Cambridge). The system was optimized for each individual section on the basis of the density of the stain. The area used in this system was calibrated at $\times 200$ magnification. Quantification was performed using unbiased counting rules (Gundersen *et al.*, 1988). Single or clusters of endothelial cells were considered to be individual vessels. The density of the microvessels was expressed as a percentage mean $\pm$ SEM, whereas the area of vWF was expressed as mean $\pm$ SEM.

Proliferation index. Fields were examined at $\times 400$ magnification. The number of Ki67-labelled nuclei and the total number of nuclei were counted. Two sections per animal and four fields per section were counted. The proliferation index was determined by the number of Ki67-labelled nuclei divided by the total number of nuclei $\times 100$ and expressed as a percentage mean $\pm$ SEM. The types of cell immunostained for Ki67 were identified using the following classifications: 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 20–40 μm in diameter, and contained large spherical to vesicular nuclei of 10–15 μm in diameter. Cells that stained positive for Ki67 that did not fit the criteria described for endothelial cells or luteal cells were considered to be other types of cell, usually leucocytes or fibroblasts. Lymphocytes were identified as approximately 10 μm in diameter with round nuclei and scant cytoplasm. Plasma cells had abundant cytoplasm with an eccentric nucleus containing characteristic cartwheel chromatin. Neutrophils were identified by their segmented nucleus and neutrophilic cytoplasm. Fibroblasts were elongated with a long axis of about 2–6 μm . They had little cytoplasm and were present in trabecular connective tissue. Different types of cell were expressed as mean $\pm$ 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.

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 $\times 100$). Areas were analysed at $\times 200$ magnification using two sections from each animal and four fields per section. The results were expressed as percentage mean $\pm$ SEM per unit area.

MAC 387-positive cells. Positive cells in the cavity of the corpora lutea were counted at $\times 400$ magnification and expressed as mean $\pm$ SEM per field.

Statistical analysis

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

Results

vWF immunostaining

vWF immunostaining was localized to endothelial cells of microvessels and capillaries (Fig. 1). After ovulation, sprouting endothelial cells invaded the cavity of the corpus haemorrhagicum and began to form a

Fig. 3. Ki67 immunopositive cells ((a) proliferation index; (b) endothelial cells; (c) luteal cells; (d) other cells) in proliferating cells in the equine corpus luteum throughout 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 after i.m. administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ($n = 3$ each) analogue. Columns with different superscripts are significantly different ($P < 0.05$).

vascular bed between the luteal cells (Fig. 1a). The mid-luteal phase was characterized by a dense network of fully differentiated capillaries (Fig. 1b), and the density of the microvessels and the area of vWF immunostaining were 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 (Fig. 1c). This regression resulted in a marked decrease ($P < 0.01$) in microvessel density and area of vWF immunostaining. At 12 h after $PGF_{2\alpha}$ administration, microvessels underwent vasodilation (Fig. 1d), resulting in a significant increase in vessel density ($P < 0.01$). However, by 36 h, vasodilation was no longer apparent. At this time there was a sharp decline in the density of the microvessels and the area of vWF immunostaining ($P < 0.01$) compared with at 12 h after $PGF_{2\alpha}$ administration. No immunostaining was present in negative control sections.

Ki67 immunostaining

Ki67 immunostaining was observed in all sections examined. The proliferation index changed throughout the oestrous cycle and after $PGF_{2\alpha}$ -induced luteolysis (Fig. 3); the highest proliferation index was in the early

luteal phase; the proliferation index declined by $> 70\%$ in the mid-luteal phase and early regression phase ($P < 0.01$). During late regression and at 36 h after $PGF_{2\alpha}$ administration, a significant increase in the proliferation index was observed ($P < 0.05$) compared with early regression and at 12 h after $PGF_{2\alpha}$ administration. Endothelial cells showed the highest proliferation rate during the early luteal phase (Figs 3b and 4a,b); by the mid-luteal phase (Fig. 4b) and early regression (both natural and induced; Fig. 4c,e), the number of endothelial cells that stained positively for Ki67 decreased significantly ($P < 0.01$; Fig. 3b). During natural and induced regression, very few endothelial cells were undergoing proliferation. Luteal cells stained for Ki67 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 (Figs 3c and 4b,c,e). During late regression (Fig. 4d) and at 36 h after $PGF_{2\alpha}$ (Fig. 4f), some luteal cells showed immunostaining. Other types of cell (probably leucocytes and fibroblasts based on morphology), particularly in the regression phase, showed Ki67 positive staining (Fig. 4d,f). These cells increased significantly ($P < 0.01$) on day 17 and at 36 h after $PGF_{2\alpha}$ administration (Fig. 3d). No immunostaining was present in negative control sections (Fig. 4f, inset).

Fig. 4. Ki67 immunostaining of the equine corpus luteum during (a) the early luteal phase, days 3–4; (b) mid-luteal phase, day 10; (c) early regression, day 14; (d) late regression, day 17; (e) 12 h after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced regression and (f) 36 h after $PGF_{2\alpha}$ -induced regression. Inset in (f) shows a negative control section from tissue collected 36 h after $PGF_{2\alpha}$ administration, in which primary antibody was replaced by normal mouse serum. Proliferating cells show dark brown nuclear staining. Thick arrows show endothelial cells, arrowheads show luteal cells and thin arrows show inflammatory cells. Scale bar represents 25 μ m.

Fig. 5. *In situ* hybridization for vascular endothelial growth factor (VEGF) mRNA under dark field microscopy. Sections of equine corpora lutea with the cellular luteal cavity on the left-hand side are shown during (a) the early luteal phase, (b) mid-luteal phase, (c) early regression and (d) 36 h after $\text{PGF}_{2\alpha}$ administration during the mid-luteal phase (day 10). Cells expressing VEGF mRNA are associated with grain clusters (white grains) whereas amorphous refraction from tissue is grey. Note the large number of grain clusters in the early and mid-luteal phase with a marked reduction during early regression and 36 h after $\text{PGF}_{2\alpha}$ administration. In the corpora lutea shown in (c and d), the cells producing VEGF mRNA were also restricted to the peripheral or central areas of the tissue. Scale bar represents 50 μm .

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 luteal cells in the early (Fig. 5a) and mid-luteal phases (Figs 5b and 6a). By early and late regression, and in the $\text{PGF}_{2\alpha}$ -treated animals, grain density representing VEGF mRNA in the luteal cells had decreased markedly (Fig. 5c,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 corpus luteum (Fig. 6b). These cells were identified as macrophages using MAC 387 immunostaining (Fig. 6c,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.

VEGF immunostaining

Immunostaining was located mainly in the cytoplasm of luteal cells. Moderate to intense immunostaining was found in most of the luteal cells during the early luteal phase (Fig. 7a). At this stage, some endothelial cells also showed 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. 7b). By early luteal regression, only a few luteal cells showed pale immunostaining (Fig. 7c). During

Table 1. Density of vascular endothelial growth factor (VEGF) mRNA grains in equine luteal cells and the expression of VEGF mRNA in the central cavity throughout 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 ($n = 3$ each) after i.m. administration of the $\text{PGF}_{2\alpha}$ analogue

Stage of the luteal phase	Days 3–4	Day 10	Day 14	Day 17	12 h after $\text{PGF}_{2\alpha}$ administration	36 h after $\text{PGF}_{2\alpha}$ administration
VEGF mRNA grains in luteal cells	+++	++/+++	+	–/+	–/+	–/+
Number of non-luteal cells in the corpus luteum cavity expressing VEGF mRNA per field	1.6 ± 0.4	1.4 ± 0.2	6.8 ± 1.3^a	No cavity	7.1 ± 6.0^a	5.9 ± 1.5^a
Number of MAC 387 positive cells in the cavity of the corpus luteum per field	1.1 ± 0.3	0.7 ± 0.4	5.3 ± 1.4^a	No cavity	6.5 ± 1.2^a	7.1 ± 1.3^a

^aSignificantly ($P < 0.05$) higher than early and mid-luteal phase.

Fig. 6. *In situ* hybridization for vascular endothelial growth factor (VEGF) mRNA under light field microscopy for luteal and non-luteal cells, and immunostaining for MAC 387 positive cells in the equine corpus luteum. (a) A section showing VEGF mRNA counterstained with haematoxylin-eosin showing black grains representative of VEGF expression in the luteal cells in mid-luteal phase (arrows). (b) Non-luteal cells (macrophages; arrowheads) in the cavity of a corpus luteum collected 12 h after $\text{PGF}_{2\alpha}$ administration expressing specific VEGF mRNA. (c) Corpus luteum cavity in the early luteal phase showing some macrophages immunostained with MAC 387. (d) Immunostaining of macrophages in the cavity of corpus luteum at early regression using MAC 387. Scale bar represents 20 μm .

Fig. 7. Vascular endothelial growth factor (VEGF) protein immunostaining in equine corpus luteum during (a) the early luteal phase (days 3–4), (b) mid-luteal phase (day 10), (c) early regression (day 14), (d) 36 h after $\text{PGF}_{2\alpha}$ administration, (e) 36 h after $\text{PGF}_{2\alpha}$ administration showing neutrophils in luteal tissue and blood vessels (arrows) and (f) negative control. Scale bar represents (a–d,f) 50 μm and (e) 25 μm .

late regression, strong immunostaining was observed in some luteal cells scattered throughout the corpus luteum and in some sections the staining was diffuse, and the trabeculae were also stained. At 12 and 36 h after $\text{PGF}_{2\alpha}$

administration, some luteal cells and many neutrophils were immunostained (Fig. 7d). The neutrophils were frequently found clustered around blood vessels. Some of these vessels also contained strongly stained neutrophils

Fig. 8. The percentage area of vascular endothelial growth factor (VEGF) immunostaining in the equine corpus luteum throughout 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 ($n = 3$ each) after i.m. administration of the $\text{PGF}_{2\alpha}$ analogue. Data are expressed as mean $\pm$ SEM. ^aValue significantly ($P < 0.05$) higher than all other stages; ^bvalue significantly ($P < 0.05$) higher than early regression (day 14) and 12 h after $\text{PGF}_{2\alpha}$; ^cvalue significantly ($P < 0.05$) higher than early regression (day 14); ^dvalue significantly ($P < 0.05$) higher than early regression (day 14) and 12 h after $\text{PGF}_{2\alpha}$.

(Fig. 7e). At all stages, macrophages and other non-luteal cells showed immunostaining for VEGF both in the central cavity of the corpus luteum and in the adjacent capsule. The percentage area of immunostaining was high during the early and mid-luteal phases (Fig. 8) and then decreased significantly ($P < 0.05$) during early regression. By late regression, immunostaining increased significantly ($P < 0.05$) compared with early regression. At 12 h after $\text{PGF}_{2\alpha}$ administration, the immunostaining had decreased significantly ($P < 0.05$) compared with the mid-luteal phase (day 10). The area of immunostaining then increased by 36 h after $\text{PGF}_{2\alpha}$ administration. No immunostaining was present in the negative control sections (Fig. 7f).

Discussion

The present study describes angiogenesis and cell proliferation throughout the oestrous cycle and after $\text{PGF}_{2\alpha}$ -induced luteolysis in the equine corpus luteum. The marked endothelial cell proliferation during the early and mid-luteal phases demonstrates intense angiogenesis. The high expression of VEGF mRNA and protein in the luteal cells during this period and the decrease in VEGF mRNA and reduction in endothelial cell proliferation during natural and induced luteolysis were strongly indicative of a paracrine role for VEGF in regulation of blood vessel growth and development in the corpora lutea of mares.

The high proliferation of endothelial cells in the early luteal phase led to increased vascularity as the corpus luteum matured. The presence of a dense capillary network by the mid-luteal phase is required for optimal delivery of progesterone precursors to, and progesterone from, the luteal cells, at a time when progesterone concentrations are at a maximum (Al-zi'abi *et al.*, 2002). These findings are in agreement with reports in other species (Niswender *et al.*, 1979; Gaytan *et al.*, 1999; Dickson and Fraser, 2000). In the present study, a decrease in the endothelial cell proliferation during late regression, and at 36 h after $\text{PGF}_{2\alpha}$ administration, coincided with an observed decrease 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 phases in the present study showed a direct temporal association with high endothelial cell proliferation and the presence of a dense capillary network. Furthermore, the location of the protein, as visualized by immunohistochemistry, also indicated a close association with angiogenesis. In the early luteal phase, when angiogenesis was at its peak, there was diffuse immunostaining throughout the corpus luteum, including immunostaining in the endothelial cells. In the mature corpus luteum, 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 corpus luteum (Dickson *et al.*, 2001). Inhibition of VEGF *in vivo* during the luteal phase in monkeys has established a physiological role for VEGF in luteal angiogenesis and function (Fraser *et al.*, 2000). Therefore, the findings of the present study strongly indicate a role for VEGF in regulating angiogenesis in the equine corpus luteum. During early regression (day 14), VEGF mRNA expression and immunostaining decreased in the equine corpus luteum, and were associated with a marked decrease in endothelial cell proliferation. The time course of these events was also confirmed by our observations after $\text{PGF}_{2\alpha}$ -induced luteolysis. At 12 h after $\text{PGF}_{2\alpha}$ administration, there was a sharp decrease in VEGF mRNA and protein accompanied by low endothelial cell proliferation rates. These findings indicate that either $\text{PGF}_{2\alpha}$ has a direct inhibitory effect on proliferating endothelial cells and this may coincide with the decrease in VEGF production, or that $\text{PGF}_{2\alpha}$ inhibits endothelial cell proliferation by decreasing VEGF expression.

In an earlier report, neutrophils were identified by Periodic acid–Schiff reaction and showed massive infiltration of the luteal tissue by neutrophils at 12 and 36 h after $\text{PGF}_{2\alpha}$ administration (Al-zi'abi *et al.*, 2002). In the present study, many of these neutrophils showed

positive immunostaining for VEGF protein, although mRNA encoding VEGF was not detected. This is the first report to our knowledge of VEGF protein in neutrophils in the corpus luteum. Recent studies on the human endometrium have demonstrated the presence of VEGF protein in neutrophils (Mueller *et al.*, 2000; Gargett *et al.*, 2001). Furthermore, stimulated neutrophils contain mRNA encoding VEGF and secrete VEGF *in vitro* (Scapini *et al.*, 1999). In the present study, VEGF immunostaining in neutrophils was not associated with an increase in proliferating endothelial cells. Therefore, it is possible that after PGF_{2α} administration, VEGF protein may play a role in non-angiogenic functions in the corpus luteum, such as the regulation of vascular permeability, vasodilation or mediation of endothelial cell survival (Goede *et al.*, 1998; Berisha *et al.*, 2000). Furthermore, VEGF may play an indirect role in neutrophil migration by stimulating release of chemokines (Lee *et al.*, 2002) that will contribute to neutrophil infiltration into the regressing corpus luteum.

Non-luteal cells, which were identified as macrophages, showed expression of VEGF mRNA and protein in the cavity of the corpus luteum throughout the luteal phase. Human macrophages can produce VEGF protein and express VEGF mRNA (Cejudo-Martin *et al.*, 2001; Cho *et al.*, 2001). The number of these macrophages expressing VEGF mRNA increased during regression, whereas there was a decrease in VEGF expression in luteal cells within the same corpus luteum. It is possible that macrophages trapped in the central clot experienced hypoxia which was responsible for increasing the expression of VEGF mRNA.

In late regression, there was intense immunostaining for VEGF in degenerated luteal cells. However, VEGF mRNA 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 and Fraser (2000) in a study that used the same antibody.

Vasodilation and infiltration by neutrophils was observed 12 h after PGF_{2α} administration. PGF_{2α} is generally regarded as a vasoconstrictor. Mares are particularly sensitive to the effects of administered PGF_{2α} (Douglas and Ginther, 1975; Oxender *et al.*, 1975). Therefore, it is possible that the combination of the pharmacological dose of PGF_{2α} and the sensitivity of mares to PGF_{2α} resulted in this unexpected observation at the ovary.

Other types of cell with the morphology of leucocytes and fibroblasts stained positively for Ki67 during late regression and at 36 h after the administration of PGF_{2α}. These findings support recent data, which showed increased numbers of proliferating T lymphocytes and macrophages during regression in the bovine corpus luteum (Petroff *et al.*, 1997), and in the mare increased

numbers of lymphocytes have been reported during both induced and natural luteal regression (Lawler *et al.*, 1999). The role of immune cells in luteolysis has been investigated in many species, including horses (Lawler *et al.*, 1999). Immune cells, primarily macrophages and T lymphocytes, are present in the corpus luteum, and their numbers increase significantly at the time of luteolysis. Invading macrophages will assist in clearing the degenerating luteal cells (Al-zi'abi *et al.*, 2002). It has also been suggested that immune cells are involved directly in the destruction of luteal cells (Petroff *et al.*, 1997, 1999) as well as in the loss of steroidogenic function. Fibroblasts are known to increase in number in the regressing corpus luteum in many species, including horses (Van Niekerk *et al.*, 1975). In the present study, a proportion of cells with the morphology of luteal cells stained positive for Ki67 during late regression and 36 h after PGF_{2α} administration. This finding was unexpected, as 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). In addition, this is a time of cell death in the equine corpus luteum (Al-zi'abi *et al.*, 2002). As luteolysis is associated with both apoptosis and a form of cell death that remains to be fully defined (Al-zi'abi *et al.*, 2002), it may be that this paradoxical labelling represents staining of degenerated protein in these dying cells.

In conclusion, luteal regression is associated with a marked decrease in proliferating endothelial cells and an increase in proliferating cells of other types, probably leucocytes and fibroblasts. The pattern of VEGF expression is strongly indicative of a paracrine role in regulation of blood vessel development, growth and regression in the equine corpus luteum. Manipulation of angiogenesis in the equine corpus luteum may provide a useful alternative method for controlling the oestrous cycle in mares.

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