Temporal relationship between proliferating and apoptotic hormone-producing and endothelial cells in the equine corpus luteum

J Aguilar¹, H M Fraser², H Wilson², E Clutton¹, D J Shaw¹ and E D Watson¹

¹Division of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin, Midlothian EH25 9RG, UK ²Medical Research Council Human Reproductive Sciences Unit, Centre for Reproductive Biology, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

Correspondence should be addressed to J Aguilar; Email: javier.aguilar@ed.ac.uk

Abstract

The temporal relationship between endothelial cell death, vascular regression and the death of hormone-producing cells in the mare has not been established. To determine the dynamics of cell proliferation and death throughout the luteal phase, corpora lutea were studied at the early, mid- and late luteal phase, and after treatment with cloprostenol in the mid-luteal phase to induce premature luteolysis. Changes in cell proliferation and apoptosis were investigated utilising specific markers (phosphorylated histone-3 and activated caspase-3 respectively). Histone-3 positive cells were most abundant during the early luteal phase, and were mainly present in endothelial cells. Histone-3 activity significantly increased in hormone-producing cells 36 h after cloprostenol treatment. Frequency of activated caspase-3 staining peaked on day 14, and was induced by 36 h after cloprostenol administration in mid-luteal phase. However, cell death occurred simultaneously in the endothelial and hormone-producing cells. These results show that a subset of hormone-producing cells enter the early stages of cell division around luteolysis, while the majority of cells are undergoing cell death. Natural and induced functional and structural luteal regression in the mare can be at least partially attributed to simultaneous apoptosis of endothelial and hormone-producing cells. However, there is no evidence that endothelial cell death is the trigger for naturally occurring luteolysis.

Reproduction (2006) **132** 111–118

Introduction

The corpus luteum (CL) is involved in regulating and controlling the oestrous cycle of the mare (Ginther 1992a). Luteal tissue is heterogeneous and consists of endothelial cells, steroidogenic large and small luteal cells, as well as fibroblasts, smooth muscle cells and immune cells (Reynolds *et al.* 1994). The life span and function of the CL is regulated by complex interactions between stimulatory (luteotrophic) and inhibitory (luteolytic) mediators (Bachelot & Binart 2005) and these mechanisms vary among species. In humans and other primates, the lifespan of the CL is absolutely dependent on support from pituitary-derived luteinising hormone (LH) (Devoto *et al.* 2002). However, in domestic ruminants and horses, although the primary luteotrophic hormone is LH (Ginther 1992b, Watson *et al.* 1995, Berisha & Schams 2005), the termination of luteal activity is caused by uterine prostaglandin factor (PGF)-2 α . At the end of the luteal phase, the release of PGF-2 α causes a dramatic decrease in plasma progesterone,

which is defined as functional luteolysis and precedes structural regression of the CL (Douglas & Ginther 1972, 1976, Henderson & McNatty 1975). A distinct sequence of events is associated with the demise of the CL, including changes in blood supply (Miyamoto *et al.* 2005), infiltration of leucocytes (Bukovsky *et al.* 1995, Gaytan *et al.* 1998), and death of steroidogenic and endothelial cells by both apoptotic (Juengel *et al.* 1993, McCormack *et al.* 1998) and non-apoptotic mechanisms (Fraser *et al.* 1999, Morales *et al.* 2000, Gaytan *et al.* 2002). However, the mechanisms involved in regression of luteal tissue in the mare are not fully understood.

PGF-2 α or its analogues are widely used clinically to manipulate the oestrous cycle of the mare (Douglas & Ginther 1972, Vanderwall *et al.* 2000, Nie *et al.* 2001). However, the early CL is refractory to PGF-2 α , limiting its use until after days 5–6 postovulation (Paccamonti *et al.* 1991). New methods of inducing luteolysis are under investigation in other species. In particular, the regulation of endothelial cell proliferation and function

may be targeted by inhibition of vascular endothelial growth factor (VEGF) using potent antagonists, such as the receptor-based inhibitor VEGF trap. Single injections have proved to be effective in suppressing luteal function at early and mid-luteal phases in the macaque (Fraser *et al.* 2005), while immunoneutralisation of the VEGF receptor-2 in pregnant mice resulted in premature loss of function and structural luteolysis (Pauli *et al.* 2005). Therefore, it seems that by reducing or avoiding neovascularisation in the early luteal tissue, luteolysis could be induced at a very early stage. An equivalent development in the mare could provide a therapy for suppression of luteal function and/or induction of luteolysis as early as the day of ovulation.

Development of such therapies for the mare requires more detailed knowledge of events surrounding luteal regression. For example, if a triggering event in luteolysis in the mare involves regression of the endothelium, leading to death of the hormone-producing cells, it may be possible to induce this event prematurely with a VEGF antagonist. In support of this hypothesis, the apoptotic endothelial cell death has been reported as an early event in luteolysis in the sheep and guinea pig (Azmi & O'Shea 1984, Sawyer *et al.* 1990). In the CL of the mare, Al-zi'abi *et al.* (2002) reported the presence of apoptotic changes around luteolysis. Since that study, more accurate markers are available to investigate cell proliferation and cell death by apoptosis. In particular, the detection of activated caspase-3 has become a reliable indicator of apoptotic cell death in several tissues, including the CL of various species (Boone & Tsang 1998, Rueda *et al.* 1999, 2000). Furthermore, Carambula *et al.* (2002) demonstrated that the presence of caspase-3 is a prerequisite for apoptosis to proceed normally during luteal regression in mice. Therefore, the detection of caspase-3 can be used to accurately identify apoptotic cells in the regressing equine CL.

As tissue growth and regression is a regulated balance of cell death and cell proliferation, studies on changes in cell death should also evaluate cell proliferation in the same samples. In a previous study, Ki67 was used as a marker of cell proliferation in the equine CL (Al-zi'abi *et al.* 2002). In addition to establishing that intense angiogenesis occurred during the early luteal phase, the Ki67 antigen was detected in hormone-producing cells during luteal regression (Al-zi'abi *et al.* 2003). In order to investigate this further, we have utilised a more recently validated marker, phosphorylated histone-3, which identifies cells only at the initial stages of mitosis (prophase and anaphase; Hendzel *et al.* 1997), and therefore, is more accurate in detecting dividing cells than Ki67. Thus, the aim of the present study was to use new tools to investigate temporal proliferative and apoptotic changes in tissue collected after natural and induced luteolysis. Specifically, the study set out to determine whether endothelial cell death occurred

before apoptotic changes were detected in hormone-producing cells.

Materials and Methods

Animals and tissue collection

Fifteen pony mares aged 4–12 years and between 250 and 450 kg body weight were used in this study. During oestrus, ovarian activity of the mares was examined daily by transrectal ultrasonography. The day on which ovulation was detected was considered to be day 0. After appropriate sedation and analgesia, a colpotomy incision was used to remove the ovary containing the CL (Lawler *et al.* 1999). This study was performed under the approval of the University of Edinburgh Ethical Review Committee and a project licence obtained under the Home Office Animals (Scientific Procedures) Act 1986.

Corpora lutea were obtained at different stages of the oestrous cycle: days 3–4 (early luteal phase; $n=3$), day 10 (mid-luteal phase; $n=4$), day 14 (early regression; $n=4$) and day 17 (late regression; $n=4$). In addition, corpora lutea were collected 12 h ($n=4$) and 36 h ($n=3$) after administration (i.m.) of the PGF-2 α analogue, cloprostenol (Estrumate, 263 μg 500 kg^{-1} , Schering-Plough Animal Health, Middlesex, UK) on day 10 of the oestrous cycle. After surgical removal, the ovaries were immediately transported to the laboratory on ice and the corpora lutea dissected free of connective tissue.

All the luteal tissue samples were fixed in 10% (v/v) neutral phosphate buffered formalin (pH 7.0) for 24 h at

Table 1 Area of 100 hormone-producing cells (μm^2) in corpora lutea (CL) at different stages of the luteal phase and after natural and induced luteolysis in the mare.

Stage of luteal phase	Number of CLs	Lutein cell area (μm^2) $\pm$ S.E.M.	Conversion factor ¹
Early	3	288.8 $\pm$ 6.81 ^c	0.44
Mid	4	643.5 $\pm$ 14.32 ^{a,x}	1
Early regression	4	550.3 $\pm$ 12.58 ^{a,b}	0.85
Late regression	4	350.1 $\pm$ 9.05 ^c	0.54
Mid + 12 h post-cloprostenol	4	533.9 $\pm$ 12.00 ^y	0.83
Mid + 36 h post-cloprostenol	3	401.1 $\pm$ 10.73 ^z	0.62

Different superscripts (a–c and x–z) indicate statistical differences $P<0.001$. Early, days 3–4 postovulation; Mid, day 10 postovulation; Early regression, day 14 postovulation; Late regression, day 17 postovulation.

¹Conversion factor. Because of the hypertrophy of the hormone-producing cells during mid-luteal phase, the number of cells per microscopic field is reduced. The conversion factor was calculated by dividing the cell area at each stage by the value of the stage with the largest area (mid-luteal). This conversion factor represents the proportional change in size related to the size of the cells in the mid-luteal phase. To compensate for this change, the conversion factor shown in the table was multiplied by the number of cells labelled by histone-3 and caspase-3 immunostaining.

Figure 1 Immunostaining of histone-3 in equine luteal tissue of (a) days 3–4, (b) day 14, (c) day 17 and (d) 36 h of cloprostenol administration. Red arrows indicate immunopositive nuclei of luteal cells and black arrows show immunopositive nuclei of endothelial cells. Scale bar represents 40 μ m.

room temperature and then embedded in paraffin wax. Serial sections of 4 μ m were mounted on to glass microscope slides coated with poly-L-lysine (Sigma Chemical Co.).

Immunohistochemistry

For immunocytochemistry, sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol and placed in water. Antigen retrieval was performed by pressure-cooking (Tefal Clypso Pressure Cooker, Tefal, Essex, UK) sections in 0.01 M citrate buffer, pH 6, for 5 min at high-pressure setting 2. Sections were left for 20 min in hot buffer and washed in Tris-buffered saline (TBS) (0.05 mol/l Tris and 9 g/l NaCl). To determine the localisation and changes in the

number of dying cells, an antibody to activated caspase-3 (Asp175) (New England Biolabs, Hitchin, UK) was used. After pressure-cooking, endogenous peroxidase activity was quenched by 5-min incubation in peroxidase block (EnVision horse radish peroxidase (HRP) kit, Dako, Cambridgeshire, UK) at room temperature, then the slides were washed and blocked with normal goat serum (NGS, diluted 1:5 in TBS containing 5% BSA for 30 min at room temperature). Sections were then incubated overnight at 4 °C with cleaved caspase-3 antibody at a 1:100 dilution in NGS. Slides were then washed in TBS and incubated with labelled polymer-HRP as secondary antibody (EnVision kit) for 30 min. Visualisation was achieved by DAB Substrate (EnVision kit): sections were then counterstained with haematoxylin, dehydrated and mounted in Pertex.

Figure 2 Number of histone-3 immunopositive endothelial (a) and hormone-producing cells (b) per ten fields ($\times 250$) in equine corpus luteum (CL) throughout the early phase (days 3–4; $n=3$); mid-luteal phase (day 10; $n=4$); early regression (day 14; $n=4$); late regression (day 17; $n=4$) and 12 h ($n=4$) and 36 h ($n=3$) after i.m. administration of cloprostenol. Values are mean $\pm$ S.E.M. *Indicate values significantly higher ($P < 0.05$) than day 10.

Phosphorylated histone-3 localisation was performed as above using anti-phospho histone H3 antibody (Upstate Ltd, Milton Keynes, UK) diluted 1:6000 in normal porcine serum (1:5 dilution in TBS containing 2.5% BSA). Sections lacking the primary antibody were used as negative controls.

Quantification

For both histone-3 and caspase-3 immunostaining, ten fields per section were randomly selected and examined blind at $\times 250$ magnification. The numbers of labelled cells were recorded. Each labelled cell was classified as either hormone-producing cell or endothelial cell. It was considered that the use of double immunostaining to confirm the identity of hormone-producing cells would not be feasible due to the rapid decline in expression of steroidogenic enzymes in the equine CL, after PG treatment (Beg *et al.* 2005); therefore, hormone-producing cells were identified based on their characteristic morphology of copious cytoplasm, their polyhedral shape and spherical nucleus, which usually contained a well-defined nucleolus. Endothelial cells had sparse cytoplasm of elongated or variable shape, and a spindle or round nucleus that occupied most of the cell. Changes

in cell size during the luteal phase influence the proportion of cells per unit area of tissue. To adjust for this effect, a conversion factor from the measurements of cell area throughout the cycle was used to quantify immunostained cells as described above (Wulff *et al.* 2001). Sections were examined at $\times 40$ magnification and images captured (Image Pro Plus, Media cybernetics, Silver Spring Inc., MD, USA Windows). Hormone-producing luteal cells were identified according to their morphological appearance as described above. Ten fields were randomly chosen in each section and the areas of ten cells were measured in each field. The cell areas of hormone-producing cells of corpora lutea collected on day 3 (early), day 10 (mid), day 14 (early regression), day 17 (late regression), and 12 and 36 h after cloprostenol treatment on day 10 were compared.

Statistical analysis

All analyses were carried out using S-PLUS (Insightful, Seattle, WA, USA). Overall differences in cell area at different stages of the luteal cycle in the horses were compared using linear mixed effect models. Which mare the cells came from was entered as a random effect to account for the repeated sampling from the same mare and stage of luteal cycle was entered as a fixed effect (Pinheiro & Bates 2000). Preliminary analysis revealed that a quadratic function was required to describe the changes in cell area from early, through mid to late regression. *Post hoc* analyses were then carried out to ascertain between which phases any significant changes had occurred. Linear mixed effect models were also used to look at the changes in cells area 12 and 36 h after cloprostenol treatment on day 10. The total number of labelled cells in the ten sections examined was compared (after application of the conversion factor described above) independently between mid-luteal (day 10) and all the other stages using Mann–Whitney tests due to the distribution of the data. All results were considered to be statistically significant when $P < 0.05$.

Results

Progesterone concentrations and histological appearance of luteal tissue reported elsewhere (Al-zi'abi *et al.* 2002) confirmed that the functional luteolysis occurred by day 14 (mean plasma progesterone concentration = 0.67 ± 0.17 ng/ml) and was induced by 12 h after PG administration at day 10 of the oestrous cycle (mean plasma progesterone concentration = 1.0 ± 0.01 ng/ml) in all the mares in the group.

Cell area

The cell area of hormone-producing cells underwent significant changes during the luteal phase. Cell areas

Figure 3 Immunostaining of activated caspase-3 in equine luteal tissue of (a) day 10, (b) day 14, (c) day 17 and (d) 36 h of cloprostenol administration. Red arrows indicate immunopositive luteal cells and black arrows show positive endothelial cells. Scale bar represents 40 µm.

were smallest in the early luteal phase and by the mid-luteal phase the cell area had increased to its maximum size before decreasing progressively during natural regression (Table 1). Cell area size also decreased in a linear way following induced luteolysis, and did so within 12 h of administration (Table 1). These changes in cell size were taken into consideration, when evaluating the number of cells labelled by both markers, phosphorylated histone-3 and activated caspase-3. By multiplying with the conversion factor shown in Table 1, overestimation of the number of labelled cells in those stages with smaller cell sizes (e.g., early luteal phase, late regression) was avoided.

Histone-3 immunostaining

Phosphorylated histone-3 immunostaining was localised to the nuclei of endothelial and hormone-producing

cells throughout the different CL stages analysed (Fig. 1). The number of labelled endothelial cells (Fig. 1a) was significantly higher at days 3–4 compared with day 10 (Fig. 2a), indicating mitotic activity in the early CL associated with neovascularisation. In all the other stages (days 10, 14 and 17) and 12 and 36 h after cloprostenol administration, the number of labelled endothelial nuclei remained low, although a dense network of capillaries was present among luteal cells at all stages. The number of labelled hormone-producing cells was low in the early luteal phase and labelled cells were rare in mid-luteal phase, and 12 h after cloprostenol. However, during regression on days 14 and 17 and 36 h post-cloprostenol, hormone-producing cells staining for histone-3 were clearly evident (Fig. 1b–d). The incidence of such cells was significantly higher 36 h postcloprostenol when compared with day 10 (Fig. 2b).

Figure 4 Number of caspase-3 immunopositive endothelial (a) and hormone-producing cells (b) per ten fields ($\times 250$) in the equine CL throughout the early phase (days 3–4; $n=3$); mid-luteal phase (day 10; $n=4$); early regression (day 14; $n=4$); late regression (day 17; $n=4$) and 12 h ($n=4$) and 36 h ($n=3$) after i.m. administration of cloprostenol. Values are mean $\pm$ S.E.M. *Indicate values significantly higher ($P < 0.05$) than day 10.

Caspase-3 immunostaining

Activated caspase-3 immunostaining was observed mainly in the cytoplasm of the cells, but occasionally included the nuclei (Fig. 3). In the early and mid-luteal phases, few hormone-producing cells or endothelial cells were labelled (Fig. 3a). Staining of endothelial and hormone-producing cells was clearly evident on days 14 and 17 and 36 h after cloprostenol treatment (Fig. 3b–d). The number of endothelial cells showing activated caspase-3 labelling increased significantly in early regression (day 14; Fig. 3b) and 36 h postcloprostenol compared with mid-luteal phase (day 10) (Fig. 4a). Similarly, at early regression (day 14) and 36 h postcloprostenol, the number of hormone-producing cells showing caspase-3 labelling increased significantly compared with mid-luteal phase (day 10) (Fig. 4b).

Discussion

This is the first study to analyse the dynamics of luteal tissue at the time of regression in the mare using sensitive

markers of cell proliferation and apoptosis. Detection of activated caspase-3 by immunostaining confirms that in the mare, apoptosis occurs in both types of cells: hormone-producing and endothelial cells, during the natural and induced process of luteolysis. Contrary to our expectations, an increase in activated caspase-3-labelled cells was detected in both endothelial and hormone-producing cells at the same time points; day 14 of the natural luteal phase and 36 h after cloprostenol injection. Despite inducing luteolysis with cloprostenol and examining the early time point (12 h), when the earliest structural changes associated with luteolysis are taking place (Al-zi'abi *et al.* 2002), there was no evidence that the endothelial cell death precedes that of hormone-producing cells. Therefore, the hypothesis of endothelial cells dying by apoptosis before the luteal cells could not be supported.

The onset of endothelial cell death, as indicated by the appearance of activated caspase-3 staining, at day 14 and after cloprostenol-induced luteolysis coincides with the disappearance of VEGF mRNA and protein from the hormone-producing cells of the equine CL (Al-zi'abi *et al.* 2003). This supports the idea that VEGF acts not only as an endothelial cell proliferation factor, but also as a survival factor for recently formed endothelium, and that its downregulation is associated with endothelial cell death in this species. In contrast to the current results in the mare, studies in our laboratory on the regressing human and marmoset CL have revealed that the localisation of activated caspase-3 to endothelial cells to be a very rare event. In addition, in a recent report on the localisation of activated caspase-3 in the regressing CL of the macaque, the specific localisation to endothelial cells was not mentioned (Peluffo *et al.* 2005). This suggests that in domestic species, in which structural luteolysis is brought about by endogenous uterine PG, the endothelial cell death plays a major role in the luteolytic cascade. By contrast in primates, where there is no endogenous luteolysis, luteolysis is a relatively protracted event involving predominantly non-apoptotic mechanisms (Fraser *et al.* 1999) or necrosis of other luteal cells (Gaytan *et al.* 2002), and in which apoptosis of endothelial cells is less apparent.

Although dividing cells have been detected in the regressing CL, their frequency was relatively low (Al-zi'abi *et al.* 2003), and it was thought to be unlikely that quantitative PCR would be sensitive enough to detect proliferation in the tissue. Immunolocalisation of phosphorylated histone-3 proved an excellent marker of cell proliferation in the equine CL and confirmed that intense angiogenesis occurs in the early luteal phase of the mare as in other species studied to date (Jablonska-Shariff *et al.* 1993, Christenson & Stouffer 1996, Dickson & Fraser 2000). A surprising finding in our previous study (Al-zi'abi *et al.* 2003) was that cell proliferation, based upon incorporation of Ki67, also occurs in presumptive hormone-producing cells in the regressing luteal tissue

during the late luteal phase and after PG administration. Since proliferation was detected in the same specimens using a marker that is considered the most stringent indicator of cell proliferation, we provided strong evidence that a subset of hormone-producing cells enter the early stages of cell division, while the majority of cells are undergoing cell death. However, the reason for this phenomenon remains to be determined.

In conclusion, although luteolysis is associated with early deletion of the endothelium in some species (Azmi & O'Shea 1984, Sawyer *et al.* 1990, Modlich *et al.* 1996, Gaytan *et al.* 2002), there is no evidence that endothelial cell death is the trigger for naturally occurring luteolysis in the mare, because we have established that the death of both endothelial and hormone-producing cells is temporally associated. However, this does not exclude the possibility that inducing selective regression of the immature vasculature could trigger functional and structural luteolysis as a result of decreased blood supply.

References

- Al-zi'abi MO, Fraser HM & Watson ED 2002 Cell death during natural and induced luteal regression in mares. *Reproduction* **123** 67–77.
- Al-zi'abi MO, Watson ED & Fraser HM 2003 Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum. *Reproduction* **125** 259–270.
- Azmi TI & O'Shea JD 1984 Mechanism of deletion of endothelial cells during regression of the corpus luteum. *Laboratory Investigations* **51** 206–217.
- Bachelot A & Binart N 2005 Corpus luteum development: lessons from genetic models in mice. *Current Topics in Developmental Biology* **68** 49–84.
- Beg MA, Gastal EL, Gastal MO, Ji S, Wiltbank MC & Ginther OJ 2005 Changes in steady-state concentrations of messenger ribonucleic acids in luteal tissue during prostaglandin F_{2α} induced luteolysis in mares. *Animal Reproduction Science* **90** 273–285.
- Berisha B & Schams D 2005 Ovarian function in ruminants. *Domestic Animal Endocrinology* **29** 305–317.
- Boone DL & Tsang BK 1998 Caspase-3 in the rat ovary: localization and possible role in follicular atresia and luteal regression. *Biology of Reproduction* **58** 1533–1539.
- Bukovsky A, Caudle MR, Keenan JA, Wimalasena J, Upadhyaya NB & Van Meter SE 1995 Is corpus luteum regression an immune-mediated event? Localization of immune system components and luteinizing hormone receptor in human corpora lutea. *Biology of Reproduction* **53** 1373–1384.
- Carambula F, Matikainen T, Lynch MP, Flavell RA, Dias Goncalves PB, Tilly J & Rueda BR 2002 Caspase-3 is a pivotal mediator of apoptosis during regression of the ovarian corpus luteum. *Endocrinology* **143** 1495–1501.
- Christenson LK & Stouffer RL 1996 Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and simulated early pregnancy. *Endocrinology* **137** 367–374.
- Devoto L, Kohen P, Vega M, Castro O, Gonzalez RR, Retamales I, Carvallo P, Christenson LK & Strauss JF 2002 Control of human luteal steroidogenesis. *Molecular Cell Endocrinology* **186** 137–141.
- Dickson SE & 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.
- Douglas RH & Ginther OJ 1972 Effect of prostaglandin F_{2α} on length of diestrus in mares. *Prostaglandins* **2** 265–268.
- Douglas RH & Ginther OJ 1976 Concentrations of PGF_{2α} in uterine venous plasma of mares during the estrous cycle and early pregnancy. *Prostaglandins* **11** 251–259.
- Fraser HM, Lunn SF, Harrison DJ & Kerr JB 1999 Luteal regression in the primate: different forms of cell death during natural and releasing-releasing hormone antagonist or prostaglandin analogue-induced luteolysis. *Biology of Reproduction* **61** 1468–1479.
- Fraser HM, Wilson H, Morris KD, Swanston I & Wiegand SJ 2005 Vascular endothelial growth factor trap suppresses ovarian function at all stages of the luteal phase in the macaque. *Journal of Clinical Endocrinology and Metabolism* **90** 5811–5818.
- Gaytan F, Morales C, Garcia-Pardo L, Reymundo C, Bellido C & Sanchez-Criado JE 1998 Macrophages, cell proliferation, and cell death in the human menstrual corpus luteum. *Biology of Reproduction* **59** 417–425.
- Gaytan F, Morales C, Bellido C & Sanchez-Criado JE 2002 Selective apoptosis of luteal endothelial cells in dexamethasone-treated rats leads to ischemic necrosis of luteal tissue. *Biology of Reproduction* **66** 232–240.
- Ginther OJ 1992a *Characteristics of the ovulatory season, Reproductive Biology of the Mare*, 2 edn Cross Plains, Wisconsin, USA: Equiservices, p. 199.
- Ginther OJ 1992b *Endocrinology of the ovulatory season, Reproductive Biology of the Mare*, 2 edn Cross Plains, Wisconsin, USA: Equiservices, p. 266.
- Henderson KM & McNatty KP 1975 A biochemical hypothesis to explain the mechanism of luteal regression. *Prostaglandins* **19** 779–797.
- Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP & Allis CD 1997 Mitosis-specific phosphorylation of Histone 3 (H3) initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106** 348–360.
- Jablonska-Sharif A, Grazul-Bilska AT, Redmer DA & Reynolds LP 1993 Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle. *Endocrinology* **133** 1871–1879.
- Juengel JL, Garverick HA, Johnson AL, Youngquist RS & Smith MF 1993 Apoptosis during luteal regression in cattle. *Endocrinology* **132** 249–254.
- Lawler DF, Hopkins J & Watson ED 1999 Immune cell populations in the equine corpus luteum throughout the oestrous cycle and early pregnancy: an immunohistochemical and flow cytometric study. *Journal of Reproduction and Fertility* **117** 281–290.
- McCormack JT, Friederichs MG, Limback SD & Greenwald GS 1998 Apoptosis during spontaneous luteolysis in the cyclic golden hamster: biochemical and morphological evidence. *Biology of Reproduction* **58** 255–260.
- Miyamoto A, Shirasuna K, Wijayagunawardane MP, Watanabe S, Hayashi M, Yamamoto D, Matsui M & Acosta TJ 2005 Blood flow: a key regulatory component of corpus luteum function in the cow. *Domestic Animal Endocrinology* **29** 329–339.
- Modlich U, Kaup FJ & Augustin HG 1996 Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involves endothelial cell detachment and vessel occlusion. *Laboratory Investigations* **74** 771–780.
- Morales C, Garcia-Pardo L, Reymundo C, Bellido C, Sanchez-Criado JE & Gaytan F 2000 Different patterns of structural luteolysis in the human corpus luteum of menstruation. *Human Reproduction* **15** 2119–2128.
- Nie GJ, Goodin AN, Braden TD & Wenzel JG 2001 Luteal and clinical response following administration of dinoprost tromethamine or cloprostenol at standard intramuscular sites or at the lumbosacral acupuncture point in mares. *American Journal of Veterinary Research* **62** 1285–1289.
- Paccamonti DL, Rodriguez HF, Myers MW & Godke RA 1991 Effect of PGF or hCG administration during the early luteal phase on progesterone secretion in the mare. *Proceedings of the American Association of Equine Practitioners* **37** 151–159.

- Pauli SA, Tang H, Wang J, Bohlem P, Posser R, Hartman T, Sauer MV, Kitajewski J & Zimmermann RC 2005 The vascular endothelial growth factor (VEGF)/VEGF receptor pathway is critical for blood vessel survival in corpora lutea of pregnancy in the rodent. *Endocrinology* **146** 1301–1311.
- Peluffo MC, Young KA & Stouffer RL 2005 Dynamic expression of caspase-2, -3, -8, and -9 proteins and enzyme activity, but not messenger ribonucleic acid, in the monkey corpus luteum during the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **90** 2327–2335.
- Pinheiro JC & Bates DM 2000 *Mixed-effects models in S and S-plus*. Springer-Verlag, New York.
- Reynolds LP, Grazul-Bilska AT, Killilea SD & Redmer DA 1994 Mitogenic factors of corpora lutea. *Progress in Growth Factor Research* **5** 159–175.
- Rueda BR, Hendry IR, Tilly JL & Hamernik DL 1999 Accumulation of caspase-3 messenger ribonucleic acid and induction of caspase activity in ovine corpus luteum following prostaglandin F₂ treatment *in vivo*. *Biology of Reproduction* **60** 1087–1092.
- Rueda BR, Hendry IR, Ndjountche L, Suter J & Davis JS 2000 Stress-induced mitogen-activated protein kinase signalling in the corpus luteum. *Molecular and Cellular Endocrinology* **164** 59–67.
- Sawyer HR, Niswender KD, Braden TD & Niswender GD 1990 Nuclear changes in ovine luteal cells in response to PGF₂ alpha. *Domestic Animal Endocrinology* **72** 229–238.
- Vanderwall DK, Betschart RW & Squires EL 2000 Effect of PGF₂alpha and 13,14-dihydro-15-keto-PGF₂alpha (PGFM) on corpora luteal function in nonpregnant mares. *Theriogenology* **53** 1263–1271.
- Watson ED, Colston M & Broadley C 1995 LH and progesterone concentrations during diestrus in the mare and the effect of hCG. *Theriogenology* **43** 1325–1337.
- Wulff C, Dickson SE, Duncan WC & Fraser HM 2001 Angiogenesis in the human corpus luteum: simulated early pregnancy by HCG treatment is associated with both angiogenesis and vessel stabilization. *Human Reproduction* **16** 2515–2524.

Received 21 November 2005

First decision 13 February 2006

Revised manuscript received 28 March 2006

Accepted 9 May 2006