DISSERTATION

FACTORS AFFECTING STEROIDOGENESIS AND LUTEOLYSIS IN OVINE AND EQUINE CORPORA LUTEA

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TERESA LEA SLOUGH ENTITLED FACTORS AFFECTING STEROIDOGENESIS AND LUTEOLYSIS IN OVINE AND EQUINE CORPORA LUTEA BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

FACTORS AFFECTING STEROIDOGENESIS AND LUTEOLYSIS IN THE OVINE AND EQUINE CORPUS LUTEUM

Repeated serial biopsies of luteal tissue were collected from mares (n=5) on days 2 and 5 following ovulation, and on alternating days from day 12 until luteolysis. Messenger RNA levels of steroidogenic acute regulatory protein (StAR); 3β-hydroxysteroid dehydrogenase, Δ5-Δ4 isomerase (3β-HSD); cyclooxygenase-2 (Cox-2); and caspase-3 were measured. The procedure did not affect progesterone production or luteal area. Messenger RNA expression of StAR ($p=0.10$) and 3β-HSD ($p=0.15$) decreased between samples obtained on days 12 and 14 and no significant differences in caspase-3 and Cox-2 mRNA levels were detected.

A second experiment was conducted to determine if blocking luteal PG production would inhibit luteolysis. An implant containing 0 mg, 3 mg, or 30 mg of indomethacin (indo) was injected into the CL of cycling mares (n=18) on day 9 following ovulation. Half of each treatment group received an i.m. injection of Lutalyse (+ PG) on day 12 (n=3). Decreases in serum concentrations of progesterone were detected in all groups between days 12-13 ($p<0.08$) and no treatment effect was detected. Thus, no evidence was obtained that inhibition of intraluteal $PGF_{2\alpha}$ synthesis affects luteolysis in the mare.

A final experiment was conducted to examine the role of intraluteal $PGF_{2\alpha}$ synthesis during luteolysis in the ewe. On day 9 following heat detection, the CL of ewes with unilateral ovulations (n=20) were treated with an implant containing 0 mg or 10 mg

A final experiment was conducted to examine the role of intraluteal $PGF_{2\alpha}$ synthesis during luteolysis in the ewe. On day 9 following heat detection, one of the following treatments was administered: in ewes with unilateral ovulations (n=20), a CL was treated with an implant containing 0 mg or 10 mg indo. In ewes with bilateral ovulations (n=10), one CL was treated with 0 mg indo and a CL on the opposite ovary was treated with 10 mg indo. On day 12, five ewes from each treatment group received a single i.m. injection of 10 mg Lutalyse. Blood was collected every 4 hours for 24 hours following PG injection. Lutalyse treatment caused a decline in serum concentrations of progesterone within 4 hours (p=0.02), but by 24 hours ewes receiving 10 mg indo + PG had serum concentrations of progesterone that were higher than those receiving 0 mg indo + PG (p=0.04). While CL exposed to PG had lower levels of mRNA encoding StAR and higher levels of mRNA encoding cox-2, indo treatment did not affect mRNA expression. Some results supported the hypothesis that luteal PG production plays a critical role in luteolysis, but the results were not definitive.

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Chapter 1 Review of the Literature

The survival of a specie depends on its ability to reproduce. In mammals, the female progresses through an estrous cycle that enables reproduction. One very important component of the estrous cycle is the formation, function, and demise of a transient endocrine gland known as the corpus luteum (CL).

Corpora (bodies) lutea (yellow) were named in the fifteenth century by Marcello Malpighi and described by Regnier de Graaf (Reviewed in Niswender et al., 2000). Prenant (1898) determined that CL were most likely endocrine glands, and shortly thereafter Frankel removed CL from pregnant rabbits, which resulted in the loss of pregnancy (1903). In the 1930's, four different groups crystallized and characterized the substance secreted by the CL (Allen and Wintersteiner, 1934; Butenandt et al., 1934; Hartmann and Wettstein, 1934; Slotta et al., 1934), and this hormone was named progesterone. (Reviewed in Niswender et al., 1994; Niswender et al., 2000; Niswender and Nett, 1994)

The primary function of the CL is the secretion of progesterone, which is essential for maintaining pregnancy. If the female does not become pregnant, the CL must stop producing progesterone to allow another ovulation. To reinitiate the estrous cycle and create another opportunity to conceive, the CL undergoes luteolysis, or luteal regression.

The Estrous Cycle

Estrus, as defined by *The New American Webster Handy College Dictionary* (1995), is "the period of greatest female sexual receptivity." The estrous cycle encompasses the period of time from one estrus to the next. While the estrous cycle can be divided into several stages, it is often divided into the follicular phase, during which estrus occurs, and the luteal phase, when the female is under the influence of progesterone. In the cow, the length of the estrous cycle averages 21-22 days, with estrus lasting just 18-19 hours. The cow will ovulate 10-11 hours after the onset of estrus (Reviewed in Hafez, 1975). The ewe's estrous cycle is 16-17 days in length, but estrus is slightly longer than in the cow: about 24-36 hours. The ewe ovulates during late estrus (Reviewed in Hafez, 1975). The mare is quite different. While her estrous cycle averages 19-25 days in length, she displays estrus for 4-8 days. Mares generally ovulate 1-2 days before the end of estrus (Reviewed in Hafez, 1975).

The Hormonal Environment Leading to Ovulation.

The hypothalamus plays a critical role in regulating the estrous cycle through the production and release of gonadotropin releasing hormone (GnRH). When first discovered, GnRH was called luteinizing hormone-releasing hormone (LH-RH) because it stimulated LH release in all species examined (Schally, 1978). More investigation revealed that GnRH is a decapeptide that travels through the hypothalamo-hypophysial portal vessels to the gonadotrophs of the anterior pituitary gland where GnRH stimulates the release of both LH and follicle stimulating hormone (FSH) (Schally, 1978). Many reports indicate a tight synchrony of GnRH and LH release, (Clarke and Cummins, 1985,

1982; Karsch et al., 1987) but the release of GnRH and LH are not always correlated (Nett et al., 1974; Sharp and Grubaugh, 1987).

Gondadotropin releasing hormone is secreted in picomolar concentrations, and once it enters systemic circulation, it becomes so dilute that it is virtually impossible to reliably measure (Irvine and Alexander, 1987). Techniques have been developed to sample portal blood which has allowed scientists to determine the pattern of secretion of GnRH from the hypothalamus in a variety of species, including the ewe (Clarke and Cummins, 1982; Porter and Smith, 1967). A push-pull perfusion system in the medial basal hypothalamus has been utilized to study GnRH secretion in horses (Sharp and Grubaugh, 1987). While C.H.G. Irvine and S.L. Alexander estimate the half-life of GnRH to be approximately 30 minutes in the horse (McKinnon and Voss, 1993), others report faster degradation of GnRH in equine blood than in the blood of other species (Nett and Adams, 1977). The half-life of GnRH is reported to be approximately 7 minutes in other species (Hafez, 1975).

In the mare, GnRH is secreted in irregular episodic patterns with periods lacking any detectable secretion in anestrus, transitional, and diestrus mares. As the mare enters the breeding season, the mean secretory rate of GnRH increases and is highest during estrus (Sharp and Grubaugh, 1987). Mares in estrus secrete episodic pulses of GnRH every two hours (Irvine and Alexander, 1988).

The secretory pattern of GnRH dictates which gonadotropins will be released. High GnRH pulse frequency in ewes, once per hour, precede a greater rise in LH than FSH (Clarke, 1984). In anestrous mares, GnRH secretion is inadequate to stimulate more than one gonadotropin pulse per day (Reviewed by C.H.G. Irvine and S.L. Alexander in

McKinnon and Voss, 1993). In cyclic mares, if GnRH release stimulates 2-4 gonadotropin releases per day, then FSH is the predominant hormone released from the anterior pituitary gland. If the size and frequency of GnRH pulses are sufficient to induce gonadotropin release every two hours, then LH is the primary gonadotropin released, and ovulation ensues (Alexander and Irvine, 1987).

Luteinizing hormone and FSH are both glycoproteins made up of two subunits: alpha and beta. Within a given specie, alpha is virtually identical for LH, FSH, and thyroid-stimulating hormone (TSH). In the mare, the amino acid sequence of alpha is identical for LH, FSH, TSH and equine chorionic gonadotropin (eCG). The beta subunit is what confers specificity to each of these hormones, except eCG. Equine chorionic gonadotropin differs from eLH only in that it contains more carbohydrate (Reviewed by W.R. Allen and M.J. Cooper in McKinnon and Voss, 1993). Luteinizing hormone and FSH in the horse each have a molecular weight of about 34,000 kDa (Braselton and McShan, 1970), and both are approximately 25% carbohydrate (Landefeld and McShan, 1974). In the equine, many of the carbohydrate side chains have a sialic acid on the end. Equine LH is much more heavily sialylated than LH of other species, giving it a much longer half-life (Reviewed by S.L. Alexander and C.H.G. Irvine in McKinnon and Voss, 1993). Equine LH has a half-life of 5 hours while ovine LH, which is not sialylated, has a half-life of only 25 minutes (Catchpole et al., 1935).

Both LH and FSH are secreted in pulsatile, rhythmic oscillations, and both are regulated by GnRH (Evans and Irvine, 1976). There is also evidence of constitutive release, particularly of FSH (McNeilly et al., 2003). Constitutive FSH release is not associated with GnRH stimulation or the storage of FSH (McNeilly et al., 2003). The

release of FSH in the ewe may be closely linked to synthesis of FSH (Chappel et al., 1983). The preovulatory prolonged LH surge is directly due to increased GnRH secretion and results from the release of LH stored in secretory granules (McNeilly et al., 2003). It is possible that high concentrations of LH stimulate further release of LH (Nett and Niswender, 1976).

Follicle stimulating hormone is released in a biphasic pattern in the mare. Broad peaks of FSH have been detected every 10-12 days. The first peak occurs during the midluteal phase, and the other occurs shortly after ovulation (Ginther, 1992; Reviewed by S.L. Alexander and C.H.G. Irvine in McKinnon and Voss, 1993). These two broad peaks may be associated with the emergence of follicular waves (Ginther, 1992). Serum concentrations of LH are very low in the mare during the midluteal phase of the estrous cycle, but as ovulation approaches, there is a prolonged rise in LH. In the mare, the preovulatory LH surge can persist more than a week, markedly longer than in other farm species (Reviewed by S.L. Alexander and C.H.G. Irvine in McKinnon and Voss, 1993).

Luteinizing hormone and FSH regulate ovarian function, and ovarian hormones feed back on the hypothalamus and anterior pituitary to influence LH and FSH secretion. High estradiol concentrations are inhibitory to LH secretion, but at times estradiol is also stimulatory and has been implicated in triggering the preovulatory LH surge in sheep (Clarke and Cummins, 1984). Estradiol acts at the level of the gonadotrope to suppress LH secretion (Clarke and Cummins, 1985). Estradiol treatment causes polarization of LH granules (McNeilly et al., 2003; Thomas and Clarke, 1997), enabling the preovulatory LH surge in ewes. Estradiol may also act at the level of the hypothalamus to regulate GnRH and thus gonadotropin secretion during the prolonged preovulatory LH surge.

Disconnection of the ovine hypothalamus from the anterior pituitary will eliminate an estradiol-induced prolonged LH surge (Clarke and Cummins, 1984) and the increase in GnRH concentration that is detectable in anestrous ewes treated with estradiol (Clarke, 1988). The mare does not respond to estradiol in the same manner reported in other species.

While estradiol prepares the gonadotropes for a prolonged LH surge, progesterone is essential for the completion of an preovulatory LH surge in most species (Reviewed in McNeilly et al., 2003). One exception to this is the ewe, in whom progesterone is not produced by the follicle before ovulation (Thomas et al., 1987).

Another follicular hormone that plays a role in regulating gonadotropin secretion is inhibin, a 32,000 kDa protein secreted by granulosa cells of the follicle. It consists of disulfide-linked heterodimers: a common α-subunit and one of two β-subunits (Hsueh et al., 1994). Inhibin reduces transcription (Clarke et al., 1993) and shortens the half-life of FSHβ mRNA (Attardi and Winters, 1993). Follicle stimulating hormone generally stimulates inhibin production, and inhibin generally inhibits FSH synthesis, thus providing a feedback mechanism to maintain homeostasis. There are instances in the ewe where inhibin and FSH concentrations decrease synchronously (Tsonis et al., 1988), indicating that inhibin is not the sole regulator of FSH levels.

After ovulation, a corpus luteum develops and begins to secrete progesterone. Concentrations of progesterone rise around the time of ovulation and remain elevated until approximately three days before the next estrus in the mare (S.L. Alexander and C.H.G. Irvine in McKinnon and Voss, 1993). Administration of progesterone inhibits secretion of LH during the breeding season in the mare (Garcia et al., 1979). In the ewe,

progesterone reduces GnRH pulse frequency rather than changing the pituitary response to GnRH (Karsch et al., 1987). Although progesterone negatively feeds back on secretion of LH, LH has a stimulatory effect on the CL to produce higher concentrations of progesterone (Watson et al., 2000a), providing another feedback mechanism to maintain homeostasis.

The Ovarian Environment Leading to Ovulation.

Not only does the follicle secrete hormones such as estradiol and inhibin, but it also releases an oocyte. The follicle contains several cell types and progresses through a marked series of changes as it matures. The cell types and stages of folliculogenesis are similar in most domestic species and are reviewed by R.A. Pierson in McKinnon and Voss (1993) and Campbell et al. (2003). The follicle begins as a primordial follicle with only a single layer of flattened pre-granulosa cells. Once a follicle begins to mature, it becomes a primary follicle and must go on to either ovulate or undergo atresia. A primary follicle is marked by the development of a zona pellucida around the oocyte and the transformation of granulosa cells into a layer of cuboidal-shaped cells. The theca interna, which surround the granulosa cells, also become more cuboidal in shape. As the follicle develops into a secondary follicle, multiple layers of granulosa cells develop. When the granulosa cells begin to secrete small amounts of fluid and an antrum forms inside the follicle, the follicle is termed tertiary. Greater than 99 percent of all follicles will undergo atresia (Hsueh et al., 1994), and only a select few will go on to ovulate.

Ovulation, particularly as is applies to the mare, has been reviewed by Ginther (Ginther, 1992). Proteolytic enzymes such as plasmin, serine proteases, and collagenolyases degrade collagen and decrease the tensile strength of the follicle wall.

Intrafollicular pressure decreases, which is illustrated by the softening of the follicle prior to ovulation. At the time of ovulation in mares, the oocyte travels through the ovulation fossa, where it exits the ovary and enters the oviduct. In other species, ovulation occurs on the outside of the ovary and the infundibulum is responsible for capturing the oocyte and drawing it into the oviduct. Once inside the oviduct, the oocyte is carried toward the uterus and can be fertilized.

The Corpus Luteum

Formation of the Corpus Luteum.

Following ovulation, the follicular reminants differentiate and become a corpus luteum (CL). Although much of the following description of luteinization is based on studies in the rat (Anderson and Little, 1985), the process is similar in the cow and ewe. Luteinization begins before ovulation occurs. After the ovulatory stimulus has been detected, the granulosa cells hypertrophy and nuclear activation begins. Once the oocyte has been released, the follicular basement membrane begins to disintegrate and blood vessels fill the cavity. The resulting structure is known as a corpus hemorragicum, which is the first grossly visible stage of luteinization (Anderson and Little, 1985). In cows, at the time of the preovulatory LH surge, there is an increase in blood flow to the follicle. From day 2 until day 5 after estrus, the blood flow continues to increase in parallel with CL volume (Acosta et al., 2003). The LH surge induces mitochondrial alterations and the formation of smooth endoplasmic reticulum (SER) within 30-40 hours of the ovulatory signal (Niswender and Nett, 1994). As luteinization proceeds, many gap junctions and cytoplasmic projections form on luteal cells (Anderson and Little, 1985).

Luteinization encompasses a time of very rapid growth and cellular proliferation. The CL of cows and ewes contain two distinct luteal cell types: small luteal cells (SLC) of thecal origin and large luteal cells (LLC) derived from granulosa cells (Fitz et al., 1982; Niswender and Nett, 1994). Along with luteal cells, the CL contains several other cell types including endothelial cells, fibroblasts, pericytes, and other cells that originate in the bloodstream (Reviewed in Niswender et al., 2000). While the number of LLC remains essentially unchanged during luteal growth, LLC hypertrophy to nearly double in diameter (Farin et al., 1989). Other cells types present during luteinization increase in number, and the net result in the ewe is growth from a 40 mg follicle to a 700 mg CL (Farin et al., 1989). The cellular proliferation of fibroblasts, endothelial cells, and SLC is similar to the growth rate observed in cancerous tumors (Niswender et al., 2000).

The mare is unique in that her steroidogenic luteal cells are derived almost exclusively from cells of granulosa origin. Van Niekerk et al. (1975) described the morphological changes associated with luteinization in the mare. Theca cells in the equine follicle begin degenerating just prior to ovulation and are virtually gone within 24 hours of ovulation. The granulosa cells are 10 μm in diameter two hours after ovulation, and are 15 μm within 24 hours of ovulation. Within three days, all granulosa cells are luteinized. Maximum hypertrophy is achieved by day 9, when the luteal cells average 37.5 μm in diameter. This size is maintained until day 12, at which point the luteal cells begin to shrink. These cells measure 20 μm on day 16, and by day 20 they are in a complete state of regression with condensed cytoplasm and shrunken nuclei (Harrison, 1946; Van Niekerk et al., 1975).

Morphology of the CL.

Although approximately 50% of luteal volume in ewes is comprised of endothelial cells (O'Shea et al., 1989), the most intensely investigated components of the CL in domestic ruminants are the LLC and SLC, which compose 30% - 57% of the total luteal volume of the CL (Braden et al., 1988; Nett et al., 1976; O'Shea et al., 1989). Large luteal cells are among the largest endocrine cells in the body, ranging from 20-40 μm, depending on specie (Enders, 1973). Large luteal cells can be distinguished by their polyhedral shape and lightly staining cytoplasm. Large luteal cells are also characterized by a distinct nucleolus in their large, centrally located nucleus (Niswender and Nett, 1994). These cells account for 25-37% of the total CL volume (Farin, 1987; Rodgers et al., 1984). In contrast, SLC are 22 μm or less in diameter. This cell type is spindle-shaped and has a darkly staining cytoplasm. Small luteal cells in ewes also contain large lipid droplets and an irregularly-shaped nucleus with what appear to be cytoplasmic inclusions (Fitz et al., 1982; Niswender and Nett, 1994). Only 12-21% of the CL volume is accounted for by SLC (Farin, 1987; Rodgers et al., 1984).

Large luteal cells contain many mitochondria of varying sizes and shapes and have an abundance of SER, both key components for steroidogenic function. Large luteal cells also have extensive Golgi complexes which may be in direct communication with the SER. Large luteal cells contain electron-dense, membrane-bound secretory granules. These granules exocytose to release their contents, which may include oxtyocin (OT), relaxin, or other, as yet unidentified, components (Reviewed in Niswender and Nett, 1994). The effects of prostaglandin (PG) $F_{2\alpha}$ are mediated through LLC, as these cells contain $PGF_{2\alpha}$ receptors (Fitz et al., 1982; Hoyer, 1998; Juengel et al., 1996). Large luteal

cells in ewes also contain receptors for estradiol (Glass et al., 1984) and PGE_2 (Fitz et al., 1982).

Small luteal cells have moderate numbers of mitochondria that vary in size, and they contain large amounts of SER. The Golgi complex is less pronounced in SLC than in LLC, and SLC lack visible secretory granules. Small luteal cells contain large amounts of lipid, which is virtually absent in LLC of ewes (Reviewed in Niswender and Nett, 1994). Small luteal cells contain LH receptors that are linked to a cyclic adenosine monophosphate (cAMP) pathway. Thus secretion of progesterone in SLC is LH-responsive, which is in direct contrast to LLC which do not respond to LH (Fitz et al., 1982; Hoyer, 1998).

There are small cells present in the equine CL, although there are no steroidogenic cells of thecal origin. These small cells are eosinophilic and are believed to be in some sort of resting stage. These cells may be capable of differentiating into LLC, but knowledge in this area is still incomplete (G.D. Niswender and T.M. Nett in McKinnon and Voss, 1993). Although there are no SLC of thecal origin, the equine CL does contain a single class of high affinity LH receptors (Broadley et al., 1994). The number of LH receptors and receptor affinity both increase significantly from day 1 to day 13 of the mare's estrous cycle, and this is positively correlated with serum concentrations of progesterone (Roser and Evans, 1983). Luteinizing hormone provides trophic support to the equine CL; antibodies against equine gonadotropins induce luteolysis (Pineda et al., 1972) and treatment with human chorionic gonadotropin (hCG, with LH-like activity) extends luteal function (Ginther, 1992).

The CL consumes significantly more oxygen per unit weight than most other tissues; consequently, the CL is highly vascularized to deliver the necessary oxygen to meet the extremely high metabolic demands of the tissue. Capillary lumina accounts for $10 \pm 0.4\%$ of the total luteal volume in the cyclic ewe (Farin, 1987). Most luteal cell membranes are either in direct contact with a capillary wall or in close proximity to one (Dharmarajan et al., 1985). (Reviewed in Niswender et al., 2000; Swann and Bruce, 1987).

The Role of Progesterone.

Progesterone is critical for the maintenance of pregnancy. There have been reports that a minimum of 2.5 ng/ml (Douglas et al., 1985) up to 4.0 ng/ml (Shideler et al., 1982) serum concentrations of progesterone are necessary to maintain pregnancy in mares. Higher serum concentrations of progesterone, up to 25 ng/ml, do not increase pregnancy rates as long as the minimum serum concentration of progesterone is maintained (Knowles et al., 1993). There have been similar findings in the ewe: exceeding normal serum concentrations of progesterone does not improve pregnancy rates in ewes already demonstrating adequate fertility (Diskin and Niswender, 1989).

Progesterone prevents uterine contractions (Lofgren et al., 1992; Lye and Porter, 1978; Pashen, 1984), and it inhibits ovulation by suppressing gonadotropin secretion from the anterior pituitary (Allen, 2001b; McCracken et al., 1996). In addition to promoting endometrial cell proliferation and differentiation, progesterone protects endometrial cells from apoptosis (Rotello et al., 1992).

During the initial stages of pregnancy, the CL is responsible for all progesterone production. Luteal progesterone is no longer required after day 50 of pregnancy in sheep

(Diskin and Niswender, 1989). By day 50, the placenta is secreting enough progesterone to maintain pregnancy without luteal progesterone support.

Until day 40 of pregnancy in the mare, the primary CL is responsible for secreting enough progesterone to maintain pregnancy (Allen, 2001b). Endometrial cups begin to secrete equine chorionic gonadotropin (eCG, otherwise known as pregnant mare serum gonaodotropin, or PMSG) around days 38-40 (Allen and Moor, 1972). Equine chorionic gonadotropin, a heavily glycosylated 70,000 kDa protein, has FSH- and LH- like activity. It stimulates the formation of secondary CL from days 40-140 (Knowles et al., 1993; Squires et al., 1974). These secondary CL produce progesterone to supplement the progesterone being secreted by the primary CL (Knowles et al., 1993). Around days 90-100, the placenta begins to secrete significant amounts of progesterone (Knowles et al., 1993) and by days 180-220 all CL regress and pregnancy is maintained by placental secretion of progestgins (Holtan et al., 1991; Squires and Ginther, 1975). During late gestation in the mare, progesterone levels are undetectable. Instead, progestagens such as pregnenolone and progesterone metabolites are present (Holtan et al., 1991) and required to maintain a viable pregnancy (Rossdale et al., 1991).

An increase, followed by a decrease in progestins may be critical in timing the onset of parturition in the equine. There is a dramatic rise in progestagen concentrations toward end of gestation, and then levels fall within 6 days of the onset of parturition (Chavatte et al., 1997).

Exogenous progesterone is routinely administered to pregnant animals to prevent early embryonic losses. In the absence of endogenous progesterone, exogenous progestins are capable of supporting pregnancy in mares and ewes (Knowles et al., 1993;

Parr et al., 1982), but the clinical value of this treatment has been questioned. Some researchers have found increased pregnancy rates with progesterone supplementation in sheep (McMillan, 1987; Peterson et al., 1984). Others report that in ewes with high fertility, exogenous progesterone treatment does not increase embryo survival rates (Diskin and Niswender, 1989). Many early embryonic losses in the mare are due to factors other than low progesterone values (Allen, 2001b), and McKinnon et al. (2000) have suggested that many synthetic progestagens administered to mares have no biological activity.

Steroidogenesis in the Corpus Luteum.

Progesterone is derived from cholesterol, which is primarily synthesized in the liver (Krisans, 1996). The CL can produce progesterone from high-density lipoprotein (HDL) or low-density lipoprotein (LDL) (Pate and Condon, 1982), and when necessary, the CL can synthesize cholesterol from acetate (Cook et al., 1967). Low-density lipoprotein is taken into the cell through receptor-mediated endocytosis (Brown and Goldstein, 1986), while the mechanism of HDL uptake is not fully understood. There is an HDL receptor known as scavenger receptor class B, type I (SR-B1) that likely plays a role in HDL uptake in rodent CL (Rao et al., 2003). In ruminants, HDL is the most important cholesterol source for progesterone production (Wiltbank et al., 1990). Cholesterol ester synthetase in the CL can esterify cholesterol with fatty acids and store cholesterol esters in lipid droplets. Cholesterol esterase can then hydrolyze the cholesterol esters to release free cholesterol when required (Niswender et al., 2000). Protein kinase A (PKA) activates cholesterol esterase through phosphorylation, thus providing a point for

second messenger regulation (Reviews: Juengel and Niswender, 1999; Niswender et al., 2000; Stocco and Clark, 1996a; Stocco and Clark, 1996b).

Transport of cholesterol to mitochondria is essential for steroidogenesis. Cholesterol transport through cytosol requires an intact cytoskeleton (Rodgers et al., 1985), and sterol binding proteins play a role in this transport (Ikonen, 1997). Altering the cytoskeleton clearly affects a cell's ability to synthesize steroids. Some cells increase and others decrease steroid output in response to microtubule disruption (Stocco and Clark, 1996a). Sterol carrier protein 2 (SCP2) is a 13 kDa protein that plays a role in cholesterol transport. In a 1:1 ratio, SCP2 transports cholesterol from lipid droplets to mitochondria, and it can stimulate steroid production in isolated mitochondria. (Reviewed in Stocco and Clark, 1996a)

The most acutely regulated and rate-limiting step of steroidogenesis is the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Stocco and Clark, 1996a). A protein critical to this process is the steroidogenic acute regulatory protein (StAR) (Clark et al., 1994; Petrescu et al., 2001; Stocco and Clark, 1996a; Sugawara et al., 1995). This 37 kDa protein contains a mitochondrial targeting sequence, and after inserting into the mitochondria, it is cleaved to 30 and 32 kDa forms (Stocco and Sodeman, 1991). The cleaved StAR is then rapidly degraded by proteosome activity (Tajima et al., 2001), with a half-life of 4-5 hours in the mitochondria (Granot et al., 2002).

Once at the mitochondria, StAR is thought to transfer cholesterol to a pore in the mitochondrial membrane consisting of peripheral-type benzodiazepine receptor (PBR). Although PBR is located in the outer mitochondrial membrane (Anholt et al., 1986), this

18 kDa protein lacks a mitochondrial targeting sequence. Modeling indicates that PBR has five transmembrane domains that may protect hydrophobic cholesterol from the mitochondrial medium (Papadopoulos, 1998). Peripheral-type benzodiazepine receptor is thought to facilitate cholesterol transport to the inner mitochondrial membrane by serving as a cholesterol channel. Peripheral-type benzodiazepine receptor is likely complexed with the mitochondrial voltage-dependent anion carrier (VDAC) in a 5:1 ratio, and studies indicate that 4-6 PBR may form each pore. These pores are believed to be located at contact sites where the inner mitochondrial and outer mitochondrial membranes come into contact with each other (Reviewed in Papadopoulos et al., 1994).

Removing either StAR or PBR causes a dramatic reduction in steroidogenesis, indicating both proteins are required for normal progesterone synthesis (Caron et al., 1997; Culty et al., 1999; Hauet et al., 2002; Papadopoulos, 1998; Papadopoulos et al., 1997a; Papadopoulos et al., 1997b). Fluorescence energy transfer (FRET) experiments have shown that StAR and PBR interact with each other, supporting the hypothesis that the two proteins work together to transport cholesterol from the cytosol to the inner mitochondrial membrane (West et al., 2001).

Peripheral-type benzodiazepine receptor ligands stimulate steroidogenesis in isolated mitochondria (Papadopoulos et al., 1990). The natural ligand for PBR is an 8.2 kDa protein named endozepine (also known as diazepam-binding inhibitor). Endozepine appears to play a role in steroidogenesis, but its role is not clearly defined. Endozepine stimulates pregnenolone formation in isolated mitochondria from adrenocortical and Leydig cells (Papadopoulos, 1998). An endozepine agonist has been shown to increase progesterone production in SLC (Fitz et al., 1982). Targeted deletion of endozepine

inhibits steroidogenesis in MA-10 Leydig tumor cells and R2C cells (Boujrad et al., 1993; Garnier et al., 1994; Papadopoulos et al., 1997a). Peripheral-type benzodiazepine ligand binding may affect the opening (or release state) of the channel, thus affecting cholesterol movement (Papadopoulos, 1998). Large luteal cells, which constitutively secrete more progesterone than SLC, have 3.5 times as much endozepine as SLC (Niswender, 2002; Niswender et al., 2000).

Once cholesterol has been transported inside the mitochondrial matrix, cytochrome P-450 side chain cleavage enzyme ($P\text{-}450_{scc}$) cleaves the side chain from cholesterol, thereby producing pregnenolone (Stone and Hechter, 1954). Cytochrome $P450_{scc}$ mRNA increases from early to mid-luteal stages (Belfiore et al., 1994; Juengel and Niswender, 1999), but it does not appear to be acutely regulated by PKA or PKC second messenger systems (Belfiore et al., 1994). Pregnenolone is then transported to the SER where 3β-hydroxysteroid dehydrogenase/Δ^5,Δ^4 isomerase (3β-HSD) converts pregnenolone to progesterone. Although mRNA levels for 3β-HSD changes throughout the estrous cycle (Juengel et al., 1998; Juengel and Niswender, 1999), there is a great excess of 3β-HSD activity in the CL and this is not a rate-limiting aspect of steroidogenesis (Juengel and Niswender, 1999). Progesterone is not stored within luteal cells; it simply diffuses out of the cell and into circulation (Reviewed in: Niswender et al., 2000).

The production of progestins is both positively and negatively regulated by hormones. Luteinizing hormone enhances steroidogenesis in the CL of many species. The effects of LH on steroidogenesis in the ruminant CL are mediated primarily through its actions on SLC (Alila et al., 1988; Hoyer et al., 1984). Luteinizing hormone binds to its

receptor, interacts with its stimulatory GTP-binding protein, activates adenylate cyclase, and consequently causes an increase in intracellular cAMP (Hoyer et al., 1984). Increased cAMP can cause a number of cellular responses including: changes in phosphorylation state in a number of proteins (including StAR and PBR), an increase in protein synthesis, and synthesis of phospholipid. All of these effects can cause increased transfer of cholesterol to the inner mitochondrial membrane (Papadopoulos, 1998) and thus increased steroidogenesis. Without continuing stimulation by LH, levels of mRNA for 3β–HSD, $P450_{scc}$, and StAR all decrease (Niswender, 2002). Luteinizing hormone may also stimulate SLC to differentiate into LLC (Donaldson and Hansel, 1965; Farin et al., 1988) which could increase basal rates of secretion of progesterone.

There are differences in the rate and regulation of progesterone production between SLC and LLC. Small luteal cells are LH- and cAMP-responsive, while LLC are not (Alila et al., 1988; Belfiore et al., 1994; Fitz et al., 1982). Small luteal cells contain receptors for LH and respond to LH stimulation with a 5-15 fold increase in output of progesterone. Large luteal cells also have LH receptors, but they are not stimulated by LH to increase production of progesterone in the ruminant (Fitz et al., 1982). The number of luteal LH receptors peaks on approximately day 10 of the estrous cycle, and the number of LH receptors is positively correlated to serum and luteal concentrations of progesterone in ewes (Diekman et al., 1978b).

Large luteal cells are responsible for more than 80 percent of the progesterone produced by the CL (Niswender et al., 1985). There appears to be a constitutive mechanism for the secretion of progesterone in LLC (Diaz et al., 2002; Hoyer et al.,

1984), which is necessary to sustain adequate blood levels of progesterone to maintain pregnancy.

The bovine CL has membrane receptors for progesterone. The role of this progesterone receptor in the CL is under investigation (Rae et al., 1998). Other local mechanisms can also affect secretion of progesterone, with insulin-like growth factors (IGF) –I and –II both causing an increase in luteal production of progesterone (Webb et al., 2002).

Luteolysis.

Ovulation is suppressed as long as the CL is secreting progesterone (Allen, 2001b; McCracken et al., 1996). If pregnancy does not occur, the CL must regress for the estrous cycle to be reinitiated. Luteal regression occurs between days 12-15 following estrus in ewes (McCracken et al., 1972) and around day 14 following ovulation in the mare (Ginther, 1992). Often luteolysis is further broken down into two categories: 1. functional regression, which includes a decrease in progesterone production and 2. structural luteolysis, which includes the loss of cells that form the CL (Hoyer, 1998).

The conventional model of luteolysis states that increasing levels of progesterone from the CL prime the uterus to produce prostaglandins (PG). High concentrations of progesterone cause endometrial lipid stores and cyclooxygenase (Cox) protein to increase (Brinsfield and Hawk, 1973; Hansel et al., 1975). Estrogen from the growing dominant follicle causes an upregulation of oxytocin (OT) receptors in the endometrium, but this effect is inhibited by progesterone during the luteal phase. High levels of progesterone eventually lead to the destruction of the progesterone receptor within the uterus, which allows an increase in OT receptors and the uterus becomes responsive to OT (McCracken

et al., 1996). Increasing estrogen from preovulatory follicles is thought to cause the neurohypophysis to release OT (Hooper et al., 1986). There is more recent evidence that up to 90% of the initial OT pulse is generated from intraluteal sources in the ewe (McCracken et al., 1996). The uterus responds to high concentrations of OT by producing and releasing $PGF_{2\alpha}$. Uterine $PGF_{2\alpha}$ stimulates the CL to release OT and a positive-feedback loop occurs between uterine $PGF_{2\alpha}$ and luteal OT. Luteal exposure to elevated $PGF_{2\alpha}$ causes luteolysis (Reviewed in Silvia et al., 1991).

It has been known for some time that $PGF_{2\alpha}$ is responsible for causing luteolysis in domestic livestock (Fitz et al., 1984; Hansel et al., 1973; McCracken et al., 1970). By measuring 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM), the primary metabolite of $PGF_{2\alpha}$, it has been determined that there are a series of episodic releases of $PGF_{2\alpha}$ just prior to luteolysis (Kindahl et al., 1976). According to McCracken et al. (McCracken et al., 1996) pulses of $PGF_{2\alpha}$ in the ewe are about an hour in duration and occur at 6-9 hour intervals. It has been long accepted that this $PGF_{2\alpha}$ is of uterine origin because hysterectomy in many species delays or prevents luteolysis (Anderson et al., 1961; Anderson et al., 1966; Ginther and First, 1971; Malven and Hansel, 1964; Wiltbank and Casida, 1956).

Prostaglandin $F_{2\alpha}$ is metabolized very rapidly in the lung of most species (Piper et al., 1970), and so it is important that uterine $PGF_{2\alpha}$ be transferred to the CL without first passing through the lungs. Counter-current exchange from the utero-ovarian vein to the ovarian artery allows the transfer of $PGF_{2\alpha}$ from the uterine horn to the ipsilateral ovary (Del Campo and Ginther, 1973). This exchange mechanism allows about 1% of uterine $PGF_{2\alpha}$ to reach the ovary without first passing through general circulation (McCracken et al., 1996). There is no uterine-ovarian counter-current exchange mechanism in the mare.

The equine vascular architecture does not support a local transfer mechanism (Douglas and Ginther, 1975) and partial hysterectomy experiments do not indicate a local transfer of $PGF_{2\alpha}$ from the uterus to the CL in mares (Ginther and First, 1971). To compensate for the lack of a local transfer mechanism for $PGF_{2\alpha}$, the mare's lung is less efficient at metabolizing $PGF_{2\alpha}$ and the mare's luteal $PGF_{2\alpha}$ receptors have a higher affinity for the hormone than luteal $PGF_{2\alpha}$ receptors in ruminants (Schramm et al., 1983; Watson and Sertich, 1990).

The signal that initiates the release of $PGF_{2\alpha}$ from the uterus is still under investigation. One theory is that the hypothalamus releases OT in response to increasing estradiol from the growing preovulatory follicle (McCracken et al., 1996). Follicular abolishment and a lack of estradiol prevents luteolysis and prolongs the period between PGFM peaks (Zhang et al., 1991), and exogenous estradiol stimulates oxytocin and $PGF_{2\alpha}$ release in the ewe (Al-Matubsi et al., 1997).

Estradiol from the follicle may stimulate the release of OT from the hypothalamus which in turn causes the release of a small amount of $PGF_{2\alpha}$ from the uterus (Fairclough et al., 1980; King and Evans, 1984; Lafrance and Goff, 1990; Roberts et al., 1976) and an increase in uterine expression of protein and mRNA encoding Cox-2 (Xiao et al., 1999). The CL responds to uterine $PGF_{2\alpha}$ with its own production of $PGF_{2\alpha}$ (Tsai and Wiltbank, 1997) and OT (Al-Matubsi et al., 1997; Flint and Sheldrick, 1983a; Moore et al., 1986; Tsai and Wiltbank, 1997). A positive feedback loop exists between the uterus and CL, with luteal OT stimulating further uterine release of $PGF_{2\alpha}$, which stimulates more luteal production of $PGF_{2\alpha}$ and OT release (Reviewed in Niswender et al., 2000).

Immunization against OT delays luteolysis (Sheldrick et al., 1980) and administration of OT initiates luteolysis in the ewe (Milvae and Hansel, 1980a), providing evidence that OT is an important component of the luteolytic cascade in the ovine. Others have found no requirement for oxytocin to maintain normal ovine luteal function (Kotwica and Skarzynski, 1993; Kotwica et al., 1997; Milvae et al., 1991). Still others have reported shorter estrous cycles in response to OT treatment, but they attributed the shortened diestrous phase to inhibition of CL formation (Armstrong and Hansel, 1959).

Serum concentrations of OT increase as the mare nears the end of diestrus (Tetzke et al., 1987), but episodic pulses of OT from the posterior pituitary occur following episodic pulses of $PGF_{2\alpha}$, rather than secretion of OT preceding the release of PG (Vanderwall et al., 1998). Others report pulses of oxytocin precede pulses of PGFM in response to administration of exogenous PG in the mare (Shand et al., 2000). Exogenous treatment with OT in the mare has failed to induce luteolysis (Neely et al., 1979), but the timing of OT treatment is likely critical in determining the biological response. Stout et al. (1999) reported that treatment with OT initiated before day 8 of the estrous cycle prolongs luteal function, but treatment initiated on day 10 shortened the luteal phase in 2 of 5 mares. Single treatments of OT lead to increased PGFM concentrations without inducing luteolysis, but pulsatile treatment with OT stimulates a decline in progesterone release and an earlier return to estrus in cyclic mares (Goff et al., 1987).

Production of $PGF_{2\alpha}$ is the result of a three-step enzymatic process. First, phospholipases A_2 and C hydrolyze membrane phospholipids. This hydrolysis releases arachidonic acid (Kawai and Clark, 1986). Secondly, Cox-1 and -2 convert arachidonic

acid to PGG_2. Prostaglandin G_2 then undergoes a peroxidase reaction to reduce it to PGH_2 (Wiltbank and Ottobre, 2003). The conversion of arachidonic acid to PGH_2 are the rate-limiting steps in PG production (Watanabe et al., 1985). Cyclooxygenase-1 is constitutively active; thus most research has focused on the inducible, highly-regulated Cox-2 (Herschman, 1994). Some researchers have reported that Cox-1 is undetectable in bovine endometrial tissue, but Cox-2 increases during late diestrus (Arosh, 2002). In ovine endometrial tissue, Cox-1 levels do not change with stage of the estrous cycle. Cyclooxygenase-2, on the other hand, reaches its highest values from days 12-15 after ovulation and can be induced by steroid treatment (Charpigny et al., 1997). A third isoform, Cox-3, has recently been identified (Chandrasekharan et al., 2002), but its role in reproduction has not been investigated. Finally, prostaglandin F synthase (PGFS) then rapidly converts PGH_2 to $PGF_{2\alpha}$ (Watanabe et al., 1985). In the endometrium of cattle, PGFS is not significantly expressed at the time of luteolysis. Instead, a newly identified 20α-hydroxysteroid dehydrogenase gene is strongly expressed that has high PGFS activity (Madore et al., 2003).

Uterine involvement is not critical to luteolysis in primates (Beling et al., 1970; Neill et al., 1969). It has been suggested that intraluteal production of PG may, through paracrine or autocrine routes, initiate luteolysis in primates (Auletta and Flint, 1988; Sargent et al., 1988). The role of intraluteal production of PG may be important in domestic livestock, as well. Administration of $PGF_{2\alpha}$ stimulates up to a 40-fold increase in intraluteal production of PG (Wu and Wiltbank, 2001a). Exogenous treatment with $PGF_{2\alpha}$ increases secretion of $PGF_{2\alpha}$ from the utero-ovarian unit into circulation (Wade and Lewis, 1996). Exposure of ovine luteal cells to $PGF_{2\alpha}$ leads to further production of

PG (Rexroad and Guthrie, 1979; Tsai and Wiltbank, 1997), and mRNA for Cox-2 increases in the ovine CL in response to treatment with $PGF_{2\alpha}$ (Tsai and Wiltbank, 1997). An E-box region about 50 base pairs upstream of the Cox-2 transcription initiation start site appears to be critical in the upregulation of Cox-2 in response to PG treatment (Diaz et al., 2002; Wu and Wiltbank, 2001b).

Binding of $PGF_{2\alpha}$ to its receptor causes an increase in free intracellular calcium, which has been shown to activate PLA_2 which in turn causes the release of arachidonic acid (Rosenthal et al., 1995; Wiltbank and Ottobre, 2003). There is a positive feed-forward mechanism with binding of PG causing increased synthesis of PG (Reviewed in Wiltbank and Ottobre, 2003). Ovariectomized ewes respond to increasing estrogen and decreasing progesterone with 75% less PGFM than intact animals. The authors concluded this was likely due to a lack of luteal release of OT (Silvia and Raw, 1993), but it is possible that the CL itself would contribute the additional $PGF_{2\alpha}$ normally secreted.

During approximately the first 5 days following ovulation, the ovine, bovine and equine CL are refractory to $PGF_{2\alpha}$-induced luteolysis. Normal clinical doses of exogenous PG do not result in luteolysis, instead there is a decline in expression of mRNA encoding Cox-2 in response to administration of PG (Tsai and Wiltbank, 1998). Perhaps this refractory state occurs because the tissue does not respond to PG with an increase in Cox-2 and so an increase in intraluteal PG does not occur. Tsai and Wiltbank (1998) suggest that the failure of early CL to undergo luteolysis in response to PG injection is due to an inability to produce intraluteal PG. The refractoriness of the early CL to exogenous PG may be enhanced by increased 15-hydroxyprostagladin dehydrogenase (PGDH) activity (Silva et al., 2000). Prostaglandin dehydrogenase is the

enzyme that performs the rate-limiting step in the inactivation of $PGF_{2\alpha}$ (Reviewed in Okita and Okita, 1996). Increased PGDH activity increases the catabolism of exogenous $PGF_{2\alpha}$ and possibly prevents the up-regulation of Cox-2, thus preventing an increase in intraluteal production of PG (Silva et al., 2000).

Other factors known to be involved in the luteolytic cascade induce intraluteal PG production: cytokines, endothelin-1, estradiol-17β, OT, noradrenaline, nitric oxide, and the removal of progesterone (Diaz et al., 2002; Wiltbank and Ottobre, 2003). Short-lived CL produce more PG than normal CL, and the relative quantity of $PGF_{2\alpha}$ is higher in the subnormal CL (Hu et al., 1990). In cultured equine luteal cells, the ratio of intraluteal PGF:PGE_2 is at its highest during the luteolytic phase of the estrous cycle (Watson and Sertich, 1990). Some believe the ratio of PGs may be more important in luteolysis than total amount of $PGF_{2\alpha}$ (Milvae and Hansel, 1983).

Receptors for $PGF_{2\alpha}$ are found on LLC and are seven-transmembrane G protein-coupled receptors. Upon stimulation of the receptor for $PGF_{2\alpha}$, membrane-bound phospholipase C (PLC) is activated via a stimulatory G protein (Berridge and Irvine, 1984). This G protein catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Berridge and Irvine, 1984; Davis et al., 1987). The resulting increase in IP_3 leads to the release of free Ca^{2+} from the SER and a Ca^{2+} influx from extracellular sources. Increased free Ca^{2+} and DAG together cause the activation of protein kinase C (PKC) (Nishizuka, 1988).

Accordingly, $PGF_{2\alpha}$ treatment of LLC results in increased intracellular Ca^{2+} (Wiltbank et al., 1991; Wiltbank et al., 1989) and enhances the activity of PKC (Wiltbank et al., 1991). There are contradictory resports regarding the source of increased

intracellular Ca^{2+}. According to Wiltbank et al. (Wiltbank et al., 1989) the increased Ca^{2+} in LLC comes primarily from extracellular sources, and treatment of SLC with $PGF_{2\alpha}$ has no effect on levels of free intracellular Ca^{++}. According to Wegner et al. (1994), the increased cytosolic Ca^{2+} in ovine LLC in response to PG is primarily derived from intracellular stores.

Activation of PKC by $PGF_{2\alpha}$ leads to decreased luteal steroidogenesis, but it does not cause apoptosis in luteal cells (McGuire et al., 1994; Wiltbank et al., 1991). The calcium influx triggered by $PGF_{2\alpha}$ is responsible for the cytotoxic effects (Wiltbank et al., 1991). Calcium influxes are maximal within 30 seconds of $PGF_{2\alpha}$ treatment, and elevated intracellular Ca^{2+} is evident for 8-10 minutes in mixed bovine luteal cells (Davis et al., 1987). Prostaglandin $F_{2\alpha}$ reduces the activity of select ion pumps within the cell (Albert et al., 1984; Kim and Yeoun, 1983), which may contribute to sustained elevations in intracellular Ca^{2+} concentrations (Knickerbocker et al., 1988).

The first detectible sign of luteolysis is a decrease in serum concentrations of progesterone. Declining progesterone occurs prior to a decrease in luteal cell numbers (Braden et al., 1988) or an increase in apoptosis (Juengel et al., 1993), so a decline in the number of steroidogenic cells is not responsible for the decline in secretion of progesterone. Decreased secretion of progesterone occurs simultaneously with a decrease in blood flow to the CL (Nett et al., 1976), but Ohtani et al. (2004) found that secretion of progesterone decreased in CL independently of reduced blood flow. A decrease in secretion of progesterone from cultured LLC treated with $PGF_{2\alpha}$ provides strong evidence that the hormone acts directly on luteal cells to cause reduced steroidogenesis (Wiltbank et al., 1991).

In a review, Niswender et al. (2000) suggest several possible mechanisms that could be involved in the reduction of serum concentrations of progesterone. In response to $PGF_{2\alpha}$ exposure, there could be a down regulation of luteotropic hormone receptors, there could be reduced cholesterol uptake in luteal cells, receptor stimulation could affect intracellular transport of cholesterol, and there could be inhibition of steroidogenic enzymes within the cells.

The number of LH receptors in luteal tissue of ruminants decreases in response to exposure to $PGF_{2\alpha}$ (Guy et al., 1995), but this decrease is not detected until after a decrease in progesterone has already been observed (Diekman et al., 1978a; Neuvians et al., 2004b). In response to $PGF_{2\alpha}$, the mare also responds with a decrease in progesterone that precedes a decrease in number of LH receptors (Roser et al., 1982). Considering LH receptors are primarily located on SLC in ruminants, and most production of progesterone in the CL is due to activity in LLC, it is unlikely that this is a major mechanism responsible for the decline in steroidogenesis. Prostaglandin $F_{2\alpha}$ stimulates the PKC pathway (Wiltbank et al., 1991), and stimulators of PKC infused into the CL cause a decrease in steroidogenesis (Ohtani et al., 2004), so $PGF_{2\alpha}$ likely causes decreased production of progesterone by activating the PKC pathway. Administration of $PGF_{2\alpha}$ also leads to decreased concentrations of mRNA and protein for StAR and 3β-HSD, further inhibiting steroidogenesis (Juengel and Niswender, 1999).

Prostaglandin $F_{2\alpha}$ inhibits the PKA pathway, which is stimulatory to steroidogenesis in luteal cells. Prostaglandin $F_{2\alpha}$ may cause an increase in phosphodiesterase activity, which leads to increased cAMP degradation and inhibition of the PKA pathway (Agudo et al., 1984). It has been suggested that constitutive secretion

of progesterone from LLC is due to constitutive activity of PKA (Diaz et al., 2002), so inhibiting PKA would decrease steroidogenesis. Cell-to-cell communication between LLC and SLC may also serve to inhibit the PKA pathway and thus inhibit LH-induced steroidogenesis (Knickerbocker et al., 1988).

It is unlikely that $PGF_{2\alpha}$ decreases luteal production of progesterone by inhibiting the uptake and release of cholesterol. During the ovine estrous cycle, mRNA for LDL receptor increases from days 12 to 15 (Juengel and Niswender, 1999), but there is a reduction in the number of LDL receptors in ovine luteal cells in response to treatment with $PGF_{2\alpha}$ (Rodgers et al., 1987). Ovine luteal tissue preferentially uses HDL as a substrate for the production of progesterone (Wiltbank et al., 1990), so it is unlikely that $PGF_{2\alpha}$ negatively affects the cell's ability to acquire cholesterol. Cholesterol esterase activity is not affected by treatment with $PGF_{2\alpha}$ in LLC (Wiltbank et al., 1993).

It is more likely that the $PGF_{2\alpha}$-induced decrease in the secretion of progesterone is due to a disruption in transport of cholesterol through the cytosol to the inner mitochondrial membrane. Disrupting this transport system would adversely affect cholesterol transport to the mitochondria, but it is not clear whether physical disruption or another mechanism causes the decreased steroidogenesis (Carnegie et al., 1987; Carnegie and Tsang, 1988; Sawyer et al., 1990a; Silavin et al., 1980). Exposure to $PGF_{2\alpha}$ causes the microtubule network in luteal cells to disintegrate. In ewe CL, treatment with $PGF_{2\alpha}$ dramatically decreases staining for tubulin, a major component of the cytoskeletal system, before a decline in tissue progesterone can be detected (Murdoch, 1996). Treatment with $PGF_{2\alpha}$ in rats leads to a decrease in sterol carrier protein (SCP-2) (McLean et al., 1995), which interacts with the cell cytoskeleton to transport cholesterol.

Transport of cholesterol from the outer to the inner mitochondrial membrane is also likely affected by treatment with $PGF_{2\alpha}$. Steroidogenic acute regulatory protein and its mRNA in ovine and bovine luteal tissue decreases in response to $PGF_{2\alpha}$ (Juengel et al., 1995; Pescador et al., 1996). The orphan nuclear receptor DAX-1 may bind to hairpin structures present in the StAR promoter in response to $PGF_{2\alpha}$, and this may be the mediator for the decline in levels of mRNA for StAR (Zazopoulos et al., 1997). Steroidogenic acute regulatory protein contains multiple PKC phosphorylation sites, so $PGF_{2\alpha}$ may inhibit protein function of StAR through PKC phosphorylation. When cholesterol analogues that freely diffuse across the mitochondrial membrane are present in luteal cells treated with $PGF_{2\alpha}$, a decrease in steroidogenesis is not observed (Grusenmeyer and Pate, 1992). This provides evidence that cholesterol transport is involved in decreased secretion of progesterone.

The conversion of cholesterol to progesterone is not acutely affected by treatment with $PGF_{2\alpha}$, as neither enzyme involved in this conversion is down-regulated in response to treatment. In most reports, neither mRNA, protein, or activity of cytochrome P450 side-chain cleavage enzyme ($P\text{-}450_{scc}$) is decreased in response to $PGF_{2\alpha}$ treatment (Belfiore et al., 1994; Rodgers et al., 1995; Wiltbank et al., 1993). In one case, the mRNA for $P450_{scc}$ did decrease in response to administration of PG in ewes (Juengel et al., 2000) and there is a similar report of reduced expression of mRNA encoding $P450_{scc}$ following treatment with PG in bovine CL (Neuvians et al., 2004b). During luteolysis, 3β-HSD activity also remains normal (Juengel et al., 1998) despite a decrease in expression of mRNA for 3β-HSD (Juengel et al., 1998; McGuire et al., 1994; Tian et al., 1994).

Secretion of progesterone is decreased in both LLC and SLC during luteolysis, but because SLC do not have high-affinity receptors for $PGF_{2\alpha}$ (Balapure et al., 1989; Fitz et al., 1982), SLC cannot be responding directly to $PGF_{2\alpha}$. Decreased progesterone production in SLC may be mediated through OT released by LLC in response to $PGF_{2\alpha}$. Small luteal cells have significantly more OT receptors than LLC in the pig (Pitzel et al., 1993a). The OT receptor responds to ligand binding with an increase in inositol phosphate turnover (Flint et al., 1995), which leads to activation of PKC, and activation of PKC reduces steroidogenesis. Some report no effect of OT on secretion of progesterone in isolated ovine LLC or SLC (Rodgers et al., 1985).

Ovine CL respond to $PGF_{2\alpha}$ with a dose-dependent increase in secretion of OT (Ohtani et al., 2004), with up to a 950% increase in concentration of OT within 4 hours of injection of PG (Ohtani et al., 1998). Cows also respond to administration of PG with a dramatic increase in secretion of OT, but the same pattern is not detected during natural luteolysis (Shaw and Britt, 2000). Instead, 2-3 distinct pulses of OT are detectable in a 12-hour sampling period prior to natural luteolysis. All pulses of OT cease more than 72 hours before a fall in progesterone (Shaw and Britt, 2000). *In vitro* culture of bovine luteal cells has shown that these cells increase production of PG in response to treatment with OT, but treatment with PG does not induce the release of OT (Grazul et al., 1989).

Whether OT is essential to luteolysis remains debatable. Administration of the OT antagonist L-368,899 to cyclic ewes results in decreased frequency and amplitude of pulses of PGFM and a delay in luteolysis (Mann et al., 2003), but ewes with depleted luteal stores of OT undergo luteolysis in response to exogenous PG (Sheldrick and Flint, 1983). Treating cows with noradrenaline to deplete luteal stores of OT by 75% does not

prolong the luteal phase (Kotwica and Skarzynski, 1993), nor does treatment of cows with CAP-527, an OT antagonist, prolong the luteal phase (Kotwica et al., 1997).

The decline in blood flow to CL undergoing luteolysis is initiated directly by $PGF_{2\alpha}$ and affected by intraluteal production of potent vasoconstrictors, such as endothelin-1 (ET-1) (Ohtani et al., 1998). Endothelin-1, a 21-amino acid peptide, is produced by endothelial cells in regressing ovine CL in response to $PGF_{2\alpha}$ (Ohtani et al., 2004). Both large and small bovine luteal cells contain ET-1 receptors (Girsh et al., 1996). Endothelin-1 increases in two distinct peaks in response to injection of PG in cows. The first peak occurs at the time of reduced steroidogenesis, and the second peak occurs at the time of structural luteolysis in the cow (Ohtani et al., 1998). Endothelin-1 may also inhibit steroidogenesis in luteal cells directly (Girsh et al., 1996 ; Hinckley and Milvae, 2001; Ohtani et al., 2004). There has been some suggestion that the effects of $PGF_{2\alpha}$ can be abolished, or at least attenuated, by the presence of an ET-1 receptor antagonist in a variety of species (Girsh and Dekel, 2002; Girsh et al., 1996; Hinckley and Milvae, 2001). Endothelial cells in the bovine CL have receptors for $PGF_{2\alpha}$ (Mamluk et al., 1998), so $PGF_{2\alpha}$ may also be acting directly on these cells to cause cellular degeneration and capillary loss (O'Shea et al., 1977).

Angiotensin II (Ang II) may also play a role in local vasoconstriction. Angiotensin II is released from bovine luteal tissue in response to treatment with $PGF_{2\alpha}$ (Hayashi and Miyamoto, 1999), and Ang II and ET-1 peak at the time of luteolysis in the cow (Acosta et al., 2003). Within 2 hours of administration of PG, staining of Ang II is visible in some LLC. The content of Angiotensin II and ET-1 peptide in bovine CL peaks at 24 hours, and by 48 hours staining for Ang II and ET-1 is intense in apoptotic luteal

cells (Schams et al., 2003). In cattle, Ang II alone can suppress secretion of progesterone (Hayashi and Miyamoto, 1999), but blocking angiotensin converting enzyme and thus production of Ang II does not prevent luteolysis in CL treated with PG (Schams et al., 2003). Expression of vascular endothelial growth factor (VEGF) is also reduced in luteolytic CL in mares (Al-zi'abi et al., 2003) and cattle (Neuvians et al., 2004a).

Secretion of progesterone decreases prior to observable morphological changes in luteal tissue, which do not become apparent until 24-36 hours after treatment with $PGF_{2\alpha}$ in sheep (Sawyer et al., 1990b). One of the first morphological changes detectable during luteolysis is evidence of apoptosis in endothelial cells of luteal capillaries (Kerr et al., 1972; Sawyer et al., 1990b). The size of LLC and number of SLC decreases during luteolysis in ovine CL (Braden et al., 1988; Schwall et al., 1986). Apoptosis is marked by shrinkage of the cytoplasm (Schwartzman and Cidlowski, 1993), which may explain the decreasing size of LLC. Luteal tissue in ruminants treated with $PGF_{2\alpha}$ accumulates lipid droplets, and the number of granules decline (Heath et al., 1980; Nett et al., 1976).

The immune system is also involved in luteolysis. Leukocytes invade the CL during luteolysis (Brannstrom and Norman, 1993). Eosinophils (such as macrophages) accumulate in the regressing CL, particularly in the parenchyma and blood vessels, before a decrease in secretion of progesterone is detected (Hehnke et al., 1994; Komatsu et al., 2003; Murdoch, 1987; Murdoch and Steadman, 1991). Macrophages infiltrate and phagocytose degenerating luteal cells (Adams and Hertig, 1969; Paavola, 1979), and they degrade the extracellular matrix (Parker, 1991; Tscheshe et al., 1986).

With the influx of macrophages (Komatsu et al., 2003) there is an increase in tumor necrosis factor-α (TNF-α) (Bagavandoss et al., 1988; Fairchild-Benyo and Pate,

1992) and T lyphocytes (Komatsu et al., 2003) in the regressing CL. T lymphocytes accumulate and produce interferon-γ (IFN-γ), which induce bovine luteal cells to produce major histocompatibility complex antigens (Fairchild and Pate, 1989). Luteal cells respond to T lymphocytes by becoming sensitive to Fas-ligand (FasL) due to increased TNF-α and IFN-γ. Macrophages, fibroblasts, and endothelial cells all produce interleukin-1 (IL-1), which stimulates secretion of $PGF_{2\alpha}$ in the cow (Nothnick and Pate, 1990). Tumor necrosis factor-α also stimulates production of $PGF_{2\alpha}$ in the cow (Fairchild-Benyo and Pate, 1992). Both IL-1 and TNF-α inhibit production of progesterone (Pitzel et al., 1993b), but TNF-α is not produced until after progesterone has already decreased (Ji et al., 1991). These reports of increased cytokine expression were confirmed by Neuvians (2004b) who reported increases in TNF-α, IL-1β, and IFNγ after treatment with $PGF_{2\alpha}$ in bovine CL. Boiti et al. (2004) have suggested that increased cytokines may lead to the upregulation of nitric oxide synthase (NOS). Nitric oxide (NO) then inhibits steroidogenesis and induces p53 expression.

Apoptosis is an integral aspect of luteolysis (Al-zi'abi et al., 2002; Bacci et al., 1996; Boone and Tsang, 1998; Juengel et al., 1993; Martin et al., 1994; Matsuyama et al., 1996; Vaskivuo et al., 2002; Yadav et al., 2002). Bcl-2 family members determine whether apoptosis will be initiated through the activation of caspases (Green, 1998; Gross et al., 1999), which are a family of intracellular cysteine proteases (Green and Reed, 1998). Activation of caspase-3 causes rapid cleavage of a number of key functional and structural proteins within a cell (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998), and experiments with caspase-3 null mice suggest that its expression is critical to structural luteolysis. Caspase-3 null mice, at the time of expected luteolysis, have

decreased serum concentrations of progesterone, but there is no detectable evidence of apoptosis and many CL are retained (Carambula et al., 2002). Messenger RNA encoding caspase-3 and activity of caspase-3 both increase in ovine CL subjected to treatment with $PGF_{2\alpha}$ (Rueda et al., 1999).

In bovine CL undergoing luteolysis, mRNA encoding bax increases and mRNA for bcl-2 is constant (Rueda et al., 1997). The ratio of these two proteins is critical in initiating apoptosis. Bcl-2 promotes cell survival, and bax prevents bcl-2 from functioning normally (Oltvai et al., 1993), thus promoting cell death. Upon receiving a death signal, apoptotic bcl-2 family proteins integrate into membranes, namely the mitochondrial outer membrane (Gross et al., 1999). This leads to an opening of the mitochondrial permeability transition pore (MPTP), which contains VDAC and PBR (Casellas et al., 2002). The opening of the MPTP causes a change in the mitochondrial membrane potential. This can be accompanied by mitochondrial swelling and release of cytochrome c. Cytochrome c activates Apaf-1 which goes on to activate caspases –9 and –3. The opening of the MPTP is almost always triggered by high levels of calcium (Reviews: Casellas et al., 2002; Green and Reed, 1998; Gross et al., 1999), and high intracellular Ca^{2+} is responsible for initiating structural regression of the CL (Wiltbank et al., 1991).

The first visible sign of apoptosis in ovine luteal tissue is the presence of nuclear fragments of degenerate chromatin (Sawyer et al., 1990b). Apoptotic cells shrink and cytoplasmic fractions become membrane-bound (Kerr et al., 1972). These are known as apoptotic bodies and are targeted by the immune system for phagocytosis by macrophages (Gemmell et al., 1976). The activation of Ca^{2+}-dependent endonucleases

causes internucleosomal cleavage of genomic DNA. This results in "laddering" of DNA into 185-bp fragments when run on an agarose gel, which is equal to the length of DNA wrapped around a histone in a nucleosome (Arends et al., 1990; Rueda et al., 1995; Schwartzman and Cidlowski, 1993).

There is evidence that progesterone may have anti-apoptotic properties (Luciano et al., 1994; Peluso and Pappalardo, 1994; Rotello et al., 1992). Activation of PKC and an influx of Ca^{2+} both occur in response to $PGF_{2\alpha}$ exposure in LLC (Davis et al., 1987; Wiltbank et al., 1991). Activation of PKC causes decreased steroidogenesis (McGuire et al., 1994) and the Ca^{2+} influx is responsible for initiating the apoptotic cascade (Wiltbank et al., 1991). High concentrations of progesterone block Ca^{2+} influxes in a number of tissues (Barbagallo et al., 2001; Crews and Khalil, 1999; Fomin et al., 1999; Luciano et al., 1994), and so it is possible that it also blocks Ca^{2+} influx into luteal cells. Blocking production of progesterone and/or treatment with a progesterone receptor antagonist will induce apoptosis in bovine luteal cells (Rueda et al., 2000). The opposite is also true: supplementation with progesterone can inhibit luteolysis in the rat (Goyeneche et al., 2003; Kuranaga et al., 2000). If progesterone must decline before apoptosis can occur, this may explain why two injections of PG are more efficient at inducing luteolysis than single injections, even when smaller dosages are utilized (Hackett and Robertson, 1980; Irvine et al., 2002; Wade and Lewis, 1996). Handler et al. (2004) concluded that serum concentration of progesterone at the time of treatment with $PGF_{2\alpha}$ was more important than dosage of $PGF_{2\alpha}$ in initiating luteolysis in ponies.

Maternal Recognition of Pregnancy

If a female becomes pregnant, then luteolysis must be prevented. The CL must remain functional and continue to produce progesterone for the embryo to survive. The bovine embryo produces a luteotropic substance that increases secretion of progesterone from the CL as early as day 10 of pregnancy, and concentrations of PGF are not decreased in pregnant animals compared to cyclic heifers (Lukaszewska and Hansel, 1980). Uterine-ovarian venous concentrations of $PGF_{2\alpha}$ are also no different in pregnant ewes compared to cyclic ewes, although uterine production of PGE_2 is elevated (Nett and Niswender, 1981; Silvia et al., 1984). While researchers had speculated that PGE_2 may be involved in maternal recognition of pregnancy in ruminants (Fitz et al., 1984; Rawlings and Hyland, 1985), maternal recognition of pregnancy is now known to occur in response to interferon tau (IFN-τ) secreted by the embryo. The embryo trophectoderm produces IFN-τ for a limited time in early gestation; in sheep this protein is produced by day 13-21 embryos (Flint and Sheldrick, 1986; Martal et al., 1979; Moor and Rowson, 1966). Days 12-13 following ovulation in the ewe seem to be critical for the determination of whether to maintain or abolish the CL (Moor and Rowson, 1966; Silvia and Niswender, 1986). During this period, the CL of pregnant ewes are resistant to exogenous treatment with PG, with progesterone declining and then returning to pretreatment values in response to PG (Silvia and Niswender, 1986).

Interferon tau stimulates endometrial production of PGE_2 and Cox-2. At high concentrations, IFN-τ causes an increase in the ratio of PGE_2: $PGF_{2\alpha}$ in endometrial tissue (Asselin and Fortier, 2000), although others report this effect only in stromal cells (Xiao et al., 1998). Treatment of endometrial cells with IFN-τ causes an increase in

mRNA encoding Cox-2 and PGES, which likely contributes to increased total production of PG, and an increase in the PGE:PGF ratio in cattle (Parent et al., 2002). Interferon tau inhibits the development of uterine OT and estrogen receptors, which effectively prevents uterine pulses of $PGF_{2\alpha}$, although basal production of PG is elevated in pregnant ewes compared to cyclic ewes (Kim et al., 2003). Interferon τ effectively prevents luteolysis, the CL is maintained, and the pregnancy progresses (Mann et al., 2003; Spencer and Bazer, 2002).

In the mare, the factor responsible for maternal recognition of pregnancy is still unidentified. The mare must recognize she is pregnant during the oviductal transport stage and at the time of luteolysis. An oocyte that is not fertilized is not transported out of the oviduct in the mare; oviductal transport normally takes place only in the case of successful fertilization (Betteridge and Mitchell, 1974). Early equine embryos (day 5-6) secrete significant quantities of PGE_2, and this secretion is temporally associated with the time of oviductal transport (Weber et al., 1991b). Infusion of PGE_2 hastens oviductal transport, so it is plausible that PGE_2 from the embryo stimulates specific receptors and leads to embryonic transport into the uterus (Weber et al., 1991a). Prostaglandin E_2 may act directly on the oviduct to relax the circular smooth muscle and enable the passage of the embryo (Allen, 2001a).

The second component of maternal recognition of pregnancy in the mare occurs as the CL is maintained past the time of normal luteolysis (days 14-16). Uterine infusion of pharmacological doses of PGE_2 prolongs luteal function in cyclic mares (Vanderwall et al., 1994). At the time of maternal recognition in the mare, the embryo is spherical (Van Niekerk, 1965) and mobile (Ginther, 1983). This mobility may ensure that a large

percentage of the endometrium is exposed to the factor responsible for maternal recognition of pregnancy. When comparing uterine flushings between pregnant and cyclic mares on days 14-16 after ovulation, pregnant mares have significantly lower concentrations of $PGF_{2\alpha}$ (Stout and Allen, 2002; Watson and Sertich, 1989). Early work had shown that co-culture of an embryo with endometrial tissue decreased PGF secretion compared to endometrial tissue cultured without an embryo (Sharp et al., 1984; Watson and Sertich, 1989).

Pregnant mares have a reduced $PGF_{2\alpha}$ response to treatment with oxytocin compared to cyclic controls (Goff et al., 1987). During maternal recognition of pregnancy in the mare, there is a decrease in uterine OT receptors (Stout et al., 2000) which could inhibit the release of $PGF_{2\alpha}$. Others report that while numbers of OT receptor are decreased in day 14 pregnant mares compared to nonpregnant mares, numbers of OT receptor on day 12 are no different between the two groups (Sharp et al., 1997). Uterine biopsy on day 12 caused similar release of OT between the two groups, but the pregnant mares had significantly lower serum concentrations of PGFM. This led the authors to conclude that there was another mechanism suppressing secretion of $PGF_{2\alpha}$ rather than decreased numbers of OT receptor. The same study found that there was a decrease in affinity of OT for its receptor in the early stages of pregnancy (Sharp et al., 1997). Boerboom et al. (2004) report that early during the equine pregnancy, there is decreased expression of mRNA for Cox-2. Decreased Cox-2 would cause decreased ability to synthesize $PGF_{2\alpha}$, and it is a more likely explanation for decreased secretion of $PGF_{2\alpha}$ than changing levels of PGE_2 synthase or $PGF_{2\alpha}$ synthase, both genes having constant levels of mRNA that do not change in early pregnancy (Boerboom et al., 2004).

Seasonality of Reproduction in the Ewe and Mare

The reproductive cycle of both the mare and ewe are influenced by day length, with the mare being a long-day breeder (Hughes et al., 1975) and the sheep a short-day breeder (Malpaux et al., 2002). Both reproductive schemes allow the female to become pregnant at the appropriate time of year to promote spring parturition, giving the young the maximum chance of survival because weather and forage conditions are typically most favorable in the spring.

The most reliable environmental cue associated with season is photoperiod, and the reproductive patterns of both mares and ewes are strongly affected by day length. Darkness stimulates the release of melatonin (Rollag and Niswender, 1976; Rollag et al., 1978b) from the pineal gland into the surrounding vasculature (Rollag et al., 1978a). The retina of the eye detects photoperiodic information and this information is transmitted to the pineal gland through a series of neuronal relays (Reviewed in Malpaux et al., 2002). The pineal gland responds with high secretion of melatonin at night, and low secretion during the day (Karsch et al., 1984). Melatonin then acts upon other systems to affect seasonal reproductive patterns.

In ewes, who are reproductively active during the short days of fall and winter, melatonin stimulates the secretion of GnRH and LH (Viguie et al., 1995). Administration of melatonin to anestrous ewes will reinitiate ovulation after 40-50 days (Kennaway, 1988). Suppression of LH during long days, in part due to increased sensitivity to the negative feedback effect of estradiol (Gallegos-Sanchez et al., 1997), essentially eliminates ovarian activity and is a key component of the anestrous period in ewes. Dopamine, noradrenaline, seratonin, and specific amino acids may also have an effect on

seasonality. In fact, the negative feedback effect of estradiol on secretion of LH may be mediated by dopaminergic systems (Bertrand et al., 1998). Thyroidectomy suppresses the anestrous period in ewes; thus, the thyroid is also a critical component of the seasonal reproductive pattern of the ewe (Reviewed in Karsch et al., 1995). If maintained under constant light regimes, ewes will eventually become refractory to the prevailing day length. This has caused some to speculate that photoperiod simply serves to synchronize the annual reproductive rhythm (Reviewed in Malpaux et al., 2002; Thiery et al., 2002; Thiery and Malpaux, 2003).

The mare responds to increased melatonin with the opposite response as is observed in ewes. In mares, it is long days and thus low melatonin that allows increased secretion of gonadotropin. Mean monthly plasma concentrations of LH are higher during the summer than during the winter in ovariectomized mares (Garcia et al., 1979). In the equine, melatonin influences the hypothalamus through a network of interneurons (Reviewed in Malpaux et al., 1999). This communication involves a number of neurotransmitters such as opioids, catecholamines, and dopamine (Nagy et al., 2000). While levels of thyroid hormone are decreased in anestrous mares, thyroxine does not appear to be as critical in regulating seasonal reproduction in mares as it is in sheep (Porter et al., 1995). To hasten the onset of the breeding season in transitional mares, many mares are kept "under lights." Simulated long days will significantly hasten the onset of the first ovulation in the spring (Burkhardt, 1947). A similar response is detected in mares exposed to a 1-2 hour pulse of light 9.5-10 hours after the sudden onset of darkness (Palmer et al., 1982). This may reflect a photosensitive phase during the period of darkness that is critical to regulating ovarian activity. While photoperiod is

undoubtedly the most important factor in regulating annual cyclic activity in mares, warmer temperatures and higher planes of nutrition have some effect in hastening the onset of ovulation in the spring (Reviewed in Nagy et al., 2000).

The regulation of the female reproductive cycle is critical to the survival of a specie. It involves careful regulation of follicular development, ovulation, the formation of a CL, progesterone secretion, and then either the maintenance of pregnancy or demise of the CL.

Despite the large body of work that has been done in the area of luteal research, there are still deficiencies in our knowledge. We do not know what changes in expression of mRNA occur in the normally cycling mare. We do not know exactly how important luteal production of PG is to the luteolytic process. We do not know what causes SLC to regress during luteolysis. And we do not fully understand the molecular mechanisms involved in the two phases of luteal regression. These deficiencies led to the proposal of the following experiments.

Chapter 2 Temporal Gene Expression in the Corpora Lutea of Cyclic Mares

The corpus luteum (CL) is a transitory endocrine gland that forms on the ovary following ovulation. The CL produces progesterone, which is necessary for the maintenance of pregnancy. If the female does not become pregnant, the CL must regress before another ovulation will occur (Reviewed in Niswender et al., 2000).

Progesterone is synthesized from the precursor cholesterol. In order for steroidogenesis to occur, cholesterol must be transported to the inner mitochondrial membrane where the side chain is cleaved by cytochrome P450 side-chain cleavage enzyme to yield pregnenolone. Pregnenolone is then transported to the smooth endoplasmic reticulum and converted to progesterone (Reviewed in Juengel and Niswender, 1999.) Steroidogenic acute regulatory protein (StAR) is involved in the transportation of cholesterol to the inner mitochondrial membrane, which is the rate-limiting step of steroidogenesis (Stocco and Clark, 1996a). Levels of mRNA and protein for StAR are highest early in the estrous cycle and decrease in ovine and bovine luteal tissue during luteolysis (Juengel et al., 1995; Juengel and Niswender, 1999; Pescador et al., 1996).

The enzyme 3β-hydroxysteroid dehydrogenase, Δ 5-Δ 4 isomerase (3β-HSD) is responsible for the conversion of pregnenolone to progesterone in the smooth endoplasmic reticulum. The conversion rate of pregnenolone to progesterone indicates

that enzyme activity of 3β-HSD is present in luteal tissue in excess (Wiltbank et al., 1993). While 3β-HSD is not rate-limiting in steroidogenesis, expression of its mRNA in CL is known to remain steady from days 3-12 during the ovine estrous cycle (Hawkins et al., 1993), while levels of mRNA encoding 3β-HSD decrease dramatically in the ruminant CL during luteolysis induced by exogenous prostaglandin (PG) (Juengel et al., 1998; McGuire et al., 1994; Tian et al., 1994).

The CL also produces PG, particularly during the early stages of the estrous cycle and during luteolysis (Reviewed in Wiltbank and Ottobre, 2003). The rate-limiting step in PG production is the conversion of arachidonic acid to PGG_2 by cyclooxygenase-2 (Cox-2), the inducible, highly-regulated form of Cox (Wiltbank and Ottobre, 2003). Levels of mRNA encoding Cox-2 and total content of PG are known to be high in the developing CL of cows and mares (Kobayashi et al., 2002; Patek and Watson, 1983; Watson and Sertich, 1990). Mature CL in ruminants respond to exogenous PG with an increase in mRNA encoding Cox-2 (Diaz et al., 2002; Tsai and Wiltbank, 1997, 1998).

Apoptosis is the mechanism by which luteolytic cells regress. Caspase-3 is a mediator of the final stages of apoptosis. Its activation leads to the cleavage of key proteins, such as actin, in the cell (Caciola-Rosen et al., 1996), and mRNA levels for caspase-3 have been shown to increase three-fold during luteolysis in the ewe (Rueda et al., 1999).

Previous work describing luteal gene expression required removal of CL at predetermined stages of the estrous cycle. The limitation of this technique was that the same CL could not be sampled at more than one time point. For each time point, a different set of animals was required and different CL were collected. The ultrasound-

guided serial biopsy technique developed for the current experiment overcomes this limitation and allows unique insight into intraluteal changes within a single CL. Ultrasound-guided biopsy has been used to sample the gastrointestinal tract in small animals (Penninck et al., 1993), the liver and kidney of dogs (Hoppe et al., 1986), and luteal tissue in cattle (Kot et al., 1999; Tsai et al., 2001).

While many studies have characterized levels of mRNA for StAR, 3βHSD, Cox-2, and caspase-3 in the luteal tissue of ruminants, very litte information is available on normal luteal function in the mare. The following investigation was designed to characterize mRNA levels for StAR, 3βHSD, Cox-2 and caspase-3 in the equine CL over the course of the estrous cycle.

Materials and Methods

Steroidogenesis requires StAR and 3βHSD, and luteolysis involves Cox-2 and caspase-3. Both steroidogenesis and luteolysis are integral aspects of luteal function; therefore, concentrations of mRNA encoding these four genes were examined in the cycling mare. The procedure described for sampling the CL of cows (Kot et al., 1999; Tsai et al., 2001) was modified in the current experiment for use in the mare.

Samples of equine luteal tissue were collected on days 2 and 5 of the estrous cycle (with ovulation occurring on day 0) and on alternating days from day 12 until luteal regression. Day 2 of the estrous cycle was selected to represent an early CL. Day 5 was sampled because it is only after day 5 that luteolysis can reliably be induced with exogenous PG. Finally, the CL were sampled more intensely from day 12 until the CL were too small for further sampling. On day 12, steroidogenesis should still be high, and thereafter the CL should undergo luteolysis.

The ovaries of 5 cyclic mares were examined daily by rectal ultrasound evaluation to determine date of ovulation. Three mares were assigned to a "biopsy" cycle and two mares were assigned to a "control" cycle. Following the initial cycle for each mare, she was transferred to the opposite group during the subsequent estrous cycle. This design allowed each mare to serve as her own control. The first mare to ovulate was assigned to the biopsy treatment group, the second mare to ovulate was designated as a control, and the treatments were alternated according to ovulation date in an effort to reduce any seasonal effects. These experiments began on June 13 and concluded on September 2, 2003.

During each control cycle, the area of each CL was monitored by ultrasound evaluation using an Aloka SSD-500v machine with a 5MHz probe. The CL were measured on alternating days from day 2 until the subsequent ovulation, with the addition of an examination on day 5. These measurements allowed the formation and regression of each CL to be monitored throughout the cycle. Ten ml of blood were collected daily via jugular venapuncture for quantification of serum concentration of progesterone. The blood was allowed to clot at room temperature for 1-2 hours or at 4°C overnight. The blood was centrifuged at 3000 x g for 15 minutes, serum removed and stored at -20°C until measurement of progesterone concentration by radioimmunoassay (RIA) (Niswender, 1973). Three of the mares had abnormal control cycles characterized by retained CL or luteinized follicles. Data collected from these cycles were excluded from analysis. A retained CL was defined as serum progesterone above 1 ng/ml and a luteal structure visible upon ultrasound evaluation past day 25 of the estrous cycle. Because of the number of abnormal, and therefore excluded, cycles, an additional mare was

monitored for a control cycle. This provided data from a total of 3 control cycles suitable for analysis.

The mares assigned to biopsy cycles were subjected to the same palpation and blood collection regimen as the control mares. Additionally, luteal biopsies were collected on days 2 and 5 of the estrous cycle, and then on alternating days from day 12 until the CL was too small for a biopsy to be possible. During the biopsy procedure, mares were restrained in palpation stocks and administered 0.5 mg/lb Banamine approximately 10-15 minutes before the procedure. Mares were also administered preanesthetic medication (0.44 mg/kg xylazine, 0.016 mg/kg butorphanol and 0.044 mg/kg propanthaline bromide) 3-5 minutes prior to the procedure. Each mare was twitched and the tail wrapped in a fingerless plastic sleeve to prevent contamination of the ultrasound probe/needle apparatus (5MHz ultrasound transducer, plastic handle, and 60 cm 18 gauge spring-loaded biopsy needle from U.S. Biopsy (SABD-1860-15-T)). The rectum was evacuated and the perineal area washed using betadine soap. After drying the perineal area, the ultrasound probe/needle apparatus was introduced into the vagina using a sterile sleeve and sterile lubricant. The apparatus was maneuvered through the vagina until it reached the vaginal fornix. The technician grasped the mare's ovary containing the CL to be biopsied, per rectum, and manipulated it to align the biopsy device with the CL. The needle was inserted through the vaginal wall and into the CL. The spring-loaded attachment was released and a luteal biopsy acquired. The biopsy procedure required approximately 3-5 minutes. Four mares demonstrated normal luteal regression during their biopsy cycles and tissue samples were collected until days 14-16; the fifth mare had a retained CL. Luteal biopsies were collected from her until day 20 of the estrous cycle,

but data collected from this abnormal estrous cycle were not included in the statistical analysis.

Immediately following the acquisition of luteal tissue, the sample was removed from the biopsy device with H_2O_2-treated forceps and placed in diethyl pyrocarbonate (Depc)-treated phosphate buffered saline (PBS). The biopsy tissue was examined under a dissecting microscope and ovarian stromal tissue was removed. The luteal tissue was then placed into a 1.7 ml Eppendorf tube and weighed. The Eppendorf was plunged into liquid nitrogen to snap freeze the tissue and stored at -80°C until further analysis.

PCR Analysis.

Ribonucleic acid extracted from the equine luteal tissue samples was subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) to quantify steady state levels of mRNA encoding StAR, 3β-HSD, Cox-2, and caspase-3. Each sample was homogenized with a Virtis Handishear tissue homogenizer, and RNA isolated following the manufacturer's directions for TriReagent (Sigma Aldrich). Immediately following RNA isolation, the RNA was treated with TURBO DNAse reagent (Ambion) for 30 minutes at 37°C to remove any genomic DNA from the sample. The RNA was then quantified by measuring the OD A260 with a spectrophotometer. Following RNA isolation, DNAse treatment, and quantification, the RNA was stored at -80°C.

The isolated RNA was converted to cDNA using reverse transcription (RT). One μg of RNA was used in each 40 μl RT reaction if possible (as recommended by Dr. Lane K. Christenson, personal communication). Of the 30 biopsy tissue samples, less than 1 μg of RNA was extracted from six samples; in these cases, all RNA obtained from the sample was included in a 40 μl RT reaction. Depc-treated water was added to each RNA

sample to bring the total volume to 25 µl. The RNA and water were heated to 65°C for 10 minutes to remove any secondary structure. Reverse transcription reaction components were then added: 300 units of MMLV (Moloney murine leukemia virus) enzyme, 1X MMLV buffer, 1 mM dNTP's, 37 units RNAsin, 0.375 µg random primers, and 0.001 M DTT. Each RT reaction was incubated at 42°C for one hour. The MMLV enzyme was then heat inactivated at 95°C for 10 minutes. Sixty µl of depc-treated water were added to the final product to bring the total volume to 100 µl. The resulting cDNA was stored at -80°C until PCR analysis.

Standards for each gene were generated from DNA cloned into a plasmid (PGEM-T or pCR 3.1). The equine StAR vector was kindly donated by Dr. Jean Sirois (Universite de Montreal). Plasmids containing the remaining genes of interest were created. The sequences for equine 3β-HSD (Accession number D89666 D89665) and Cox-2 (Accession number AB041771) were available in GenBank, and primers were designed based on the reported sequences. These primers were used in PCR with equine cDNA to yield a DNA product. The DNA product was subcloned into the PGEM-T plasmid (Promega #A3600). Competent *E. coli* cells were transformed with the plasmid. A midi-culture of the transformed cells was grown and the plasmid isolated (Qiagen Plasmid Midi Kit #12143). The plasmid DNA was sequenced (Davis Sequencing, Davis, CA) to ensure the DNA obtained corresponded with the known sequence for the gene of interest. The plasmid was then subjected to an *in vitro* transcription reaction (Ambion's MegaScript T7 kit #1333) to generate cRNA. The cRNA was quantified using the OD A260 reading. Known amounts of each cRNA were placed into two separate RT reactions.

The same process was used to generate a caspase-3 standard, with one modification. The equine caspase-3 sequence had not been reported. Thus degenerate primers were designed based on the sequences known for other species. The primer sequences 5'GTGMTTCTAAGYCATGGDGAWGAAGG3' and 5'CCYCGGCAGGCC TGAATRATGAARAG3' yielded a product. This product was then subcloned into PGEM-T, sequenced, and standards were created using the previously described methods.

Ribosomal protein S15 was used to correct for differences in starting quantities of total RNA. S15 is a protein that makes up part of the small ribosomal subunit, appears to be relatively stable under a number of conditions, and is expressed at a level similar to that of many genes of interest (Dr. Russell Anthony, personal communication). The bacterial homologue, S19, appears to be involved in the initiation and elongation elements of translation (Hirano et al., 1987). The equine S15 sequence had not been reported, so ovine S15 primers were used in PCR with equine cDNA to generate an equine S15 DNA product. This product was submitted for sequencing and was highly homologous to other known S15 sequences. This product was then used to generate equine S15 standards.

Gene	Primer Sequence
StAR	5'-GGGTCTAATGTCTACCTGAAAACA-3'
	5'-GGTCCCAGTCCTTGTATTTAGC-3'
3βHSD	5'-TGGAGGAGAAGGAGGTACAGGAG-3'
	5'-CTTCAGGAACTGCTCATCCAGAATG-3'
Cox-2	5'-CGCAAACGCTTTCGGCTGAC-3'
	5'-CCACCAGAAGGGCAGGATACAG-3'
Caspase-3	5'-GTGATTCTAAGCCATGGGGATGA AGG-3'
	5'-CCCCGGCAGGCCTGAATGATGAAGAG-3'
S15	5'-ATCATTCTGCCCGAGATGGTG-3'
	5'-TGCTTTACGGGCTTGTAGGTG-3'

Table 2.1. Primer sequences utilized with equine cDNA for real time RT-PCR analysis.

To quantify steady state mRNA levels in the luteal samples, real-time PCR was utilized to measure the amount of cDNA in each sample corresponding to each target gene. Each PCR reaction included 5 μl of the RT product, 1X BioRad iQ SYBR Green Supermix (Catalog #170-8882), 0.2 μM each of the forward and reverse primers, and water to bring the total volume to 25 μl. Each sample was loaded in triplicate into a 96-well plate. A BioRad iCycler (Hercules, CA) was used to carry out the thermal cycling and measure fluorescence emitted from the SYBR green. SYBR green binds nonspecifically to double-stranded DNA, so as the cDNA is amplified, there is a corresponding increase in fluorescence.

Each PCR began with 3 min at 95°C. Then there were 50 cycles of 15 seconds at 95°C (melting), 45 seconds at 58 °C (annealing), and 30 seconds at 72 °C (extension). Fluorescence data were collected while the samples were at 72 °C during each cycle. Following 50 cycles, a melt curve was generated. The melt curve began at 55 °C and the temperature was increased by 0.5 °C every 10 seconds. The temperature was increased 80

times and fluorescence data collected at each temperature. The melt curve identified the temperature at which the PCR products melted apart, thus giving an indication of the size and number of products obtained in each well. Any wells that produced aberrant melt curves (compared to the other samples in the same plate for the same gene) were excluded from analysis.

In each 96-well PCR plate, the serially diluted points from both sets of standards were run in duplicate. An arbitrary fluorescence threshold was determined for each plate, and the cycle number at which the fluorescence in each individual well reached that threshold was designated as the Ct value for that sample. Starting quantities of mRNA in unknown samples were calculated by the BioRad software by comparing the Ct value for unknown samples against the standard curve. The calculated starting quantities for triplicate wells were averaged and normalized to S15 to determine relative quantities of the target mRNA.

Progesterone Assay.

Concentrations of progesterone were determined in the daily serum samples for each mare using radioimmunoassay (Niswender, 1973). Samples were run in one assay and the intra-assay coefficient of variation was 18.4%

Statistical Analysis.

A Fisher's protected LSD was used to compare serum concentrations of progesterone and CL area between treatment groups and between days. Messenger RNA values from the real time RT-PCR analysis were normalized to S15, log transformed, and compared with Fisher's protected LSD using repeated measures in SAS.

Results

Mares in their control cycles and mares in their biopsy cycles had similar patterns of progesterone secretion (Figure 2.1), and there were no differences in average serum concentrations of progesterone between groups from days 3-12, except on day 9 when the biopsy mares had lower progesterone than control mares ($p = 0.02$). Mares in both groups had average concentrations of progesterone above 4 ng/ml throughout the luteal phase. Serum concentrations of progesterone declined in both groups between days 12 and 13 (biopsy $p = 0.01$, control $p < 0.01$), at the time of expected luteolysis.

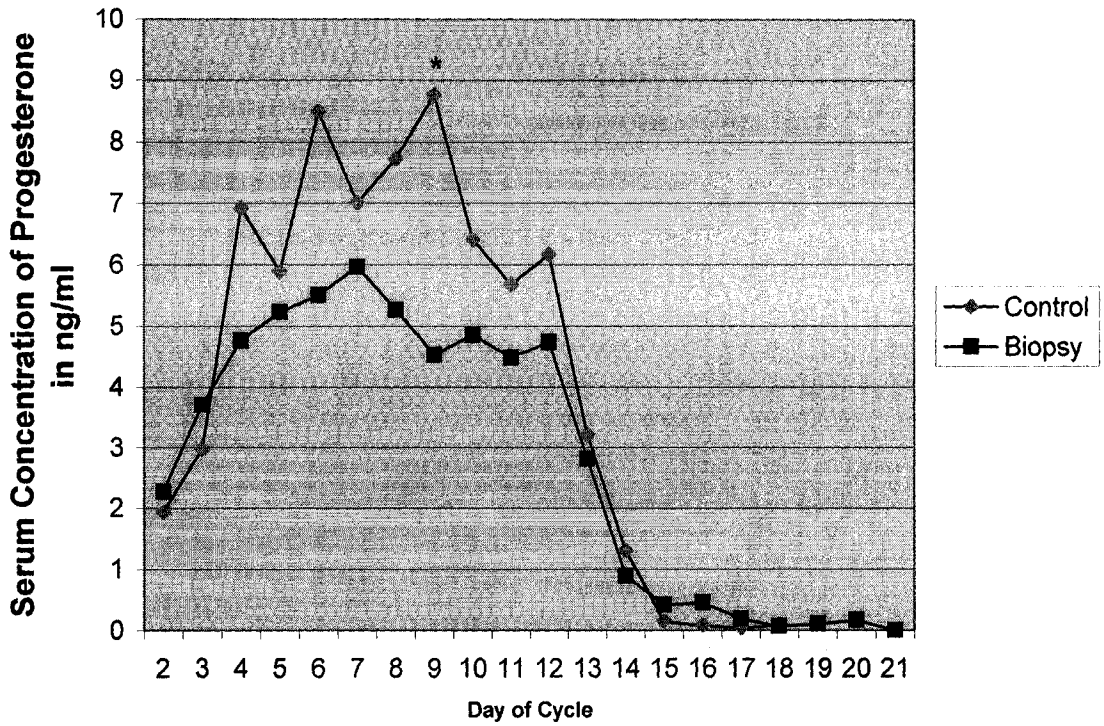

Figure 2.1. Serum concentrations of progesterone in mares during their control (n = 3) and biopsy (n = 4) cycles. (Data collected from mares with retained CL were excluded.) The only difference between groups was detected on day 9 ($p = 0.02$). Serum concentrations of progesterone declined between days 12 and 13 in mares during the control ($p < 0.01$) and biopsy cycles ($p = 0.01$).

Analysis of CL area revealed no differences in size between the CL of mares in biopsy and control cycles ($p = 0.16 - 0.69$) on any day (Figure 2.2). Corpora lutea measured during the mares' control cycles first demonstrated a significant reduction in CL area between days 10-12 ($p = 0.01$), while the biopsied CL were delayed by one measurement period with the first significant reduction in area detected between days 12-14 ($p = 0.01$).

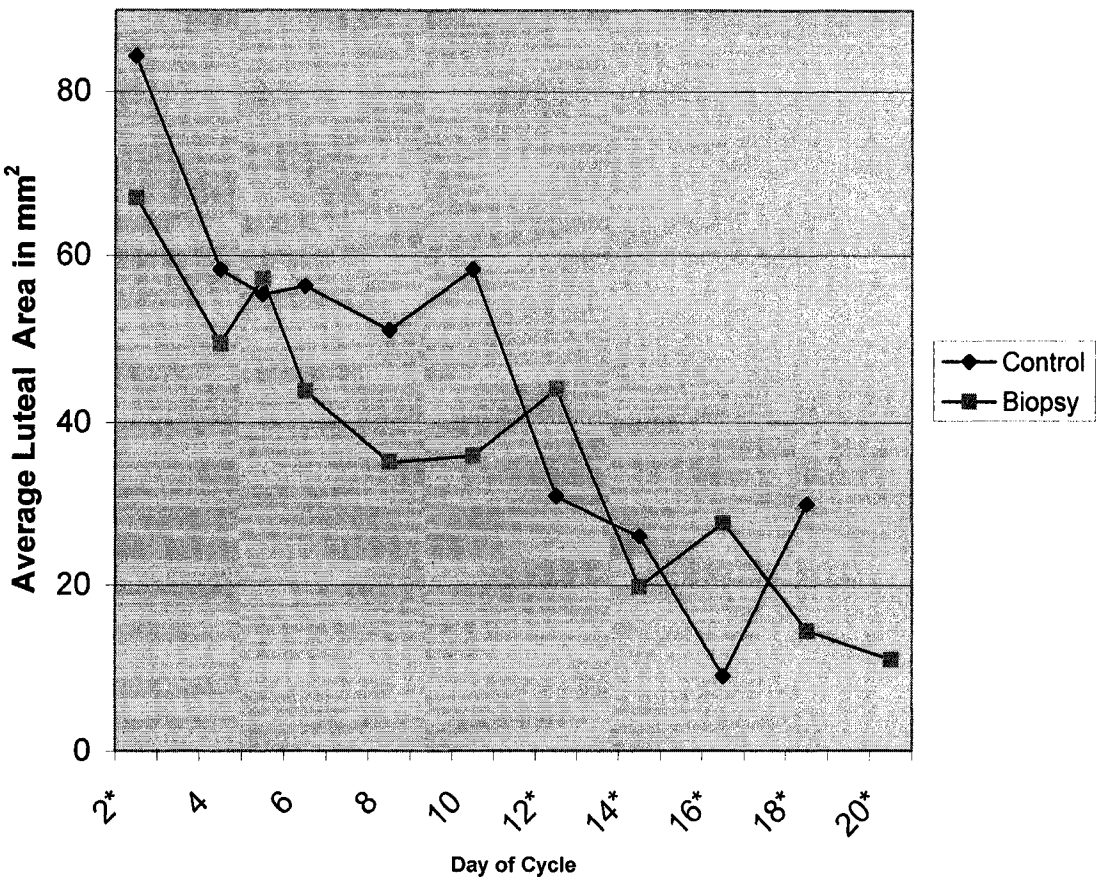

Figure 2.2. Average area of CL in mares during control (n = 3) and biopsied (n = 4) cycles. There were no differences between groups on any day. An * next to the day of the cycle indicates biopsies were performed on that day in the biopsy cycle group. A reduction in CL area was detected between days 10-12 in the control mares (p = 0.01) and days 12-14 in biopsy cycle mares (p = 0.01).

Messenger RNA.

The mRNA data collected for each gene of interest were normalized by dividing fmol of the gene of interest by fmol S15 (housekeeping gene) within the same sample. This enabled each gene of interest to be expressed relative to total mRNA collected from each tissue sample. After collecting the PCR data and quantifying levels of S15 mRNA, it became apparent that S15 mRNA levels were only consistent until day 14, after which point they decreased (Figure 2.3). Due to the variability among samples, this decline in mRNA encoding S15 was not statistically significant ($p > 0.20$), but it was of high enough magnitude to be of concern. Because levels of S15 mRNA were consistent only through day 14, data collected from biopsies taken after day 14 were excluded from analysis.

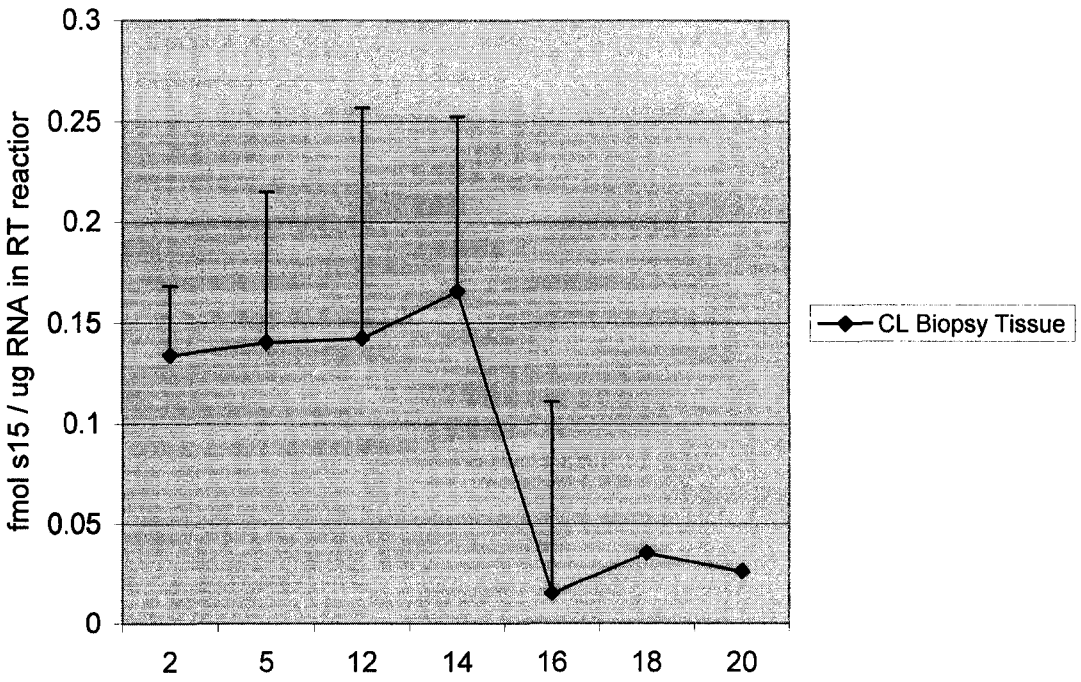

Figure 2.3. Levels of S15 mRNA in equine CL biopsy samples as measured by real time RT-PCR and normalized against starting amount of RNA as determined by the Ribogreen RNA quantification kit.

Mean levels of StAR mRNA were not different on any of the sampling dates (Table 2.2), but there was a trend for mRNA encoding StAR to decrease between days 12 and 14 ($p = 0.10$). Values for mRNA encoding StAR were positively and significantly correlated to serum concentrations of progesterone on days 5 ($R = 0.95$; $p = 0.05$) and 14 ($R > 0.99$; $p < 0.01$). The same trend was apparent with 3β-HSD: levels of mRNA for 3β-HSD declined between days 12 and 14 ($p = 0.15$). There were positive correlations between mRNA for 3β-HSD and concentrations of progesterone on days 5 ($R = 0.94$; $p = 0.06$) and 12 ($R > 0.99$; $p = 0.05$). There were no differences detected in levels of mRNA encoding Cox-2 or caspase-3 between any of the sampling dates (Table 2.2).

In the retained CL, while only representing a $n = 1$, levels of mRNA for StAR and 3β-HSD peaked earlier than in normal CL and Cox-2 expression appeared lower in the retained CL on days 5 and 12. Otherwise there were no obvious differences between the retained CL and the normal CL in the levels of mRNA for any of the genes evaluated.

Normal CL	**fmol relative to fmol S15**			
Day of Estrous Cycle	**StAR**	**3β-HSD**	**Cox-2 (x 10^{-3})**	**Caspase-3 (x 10^{-3})**
2	0.017 ± 0.006	0.92 ± 0.50	1.4 ± 1.3	5.6 ± 1.7
5	0.030 ± 0.016	1.6 ± 0.90	0.23 ± 0.091	4.5 ± 0.55
12	0.046 ± 0.018	2.1 ± 1.1	0.30 ± 0.062	5.2 ± 1.8
14	0.021 ± 0.016	0.85 ± 0.52	0.18 ± 0.10	5.5 ± 0.75
Retained CL	**fmol relative to fmol S15**			
Day of Estrous Cycle	**StAR**	**3β-HSD**	**Cox-2 (x 10^{-3})**	**Caspase-3 (x 10^{-3})**
2	0.005	0.28	0.14	6.0
5	0.047	2.7	0.09	5.9
12	0.00024	0.012	0.085	7.8
14	0.00023	0.0066	0.33	5.7

Mean ± SEM

Table 2.2. Levels of mRNA encoding StAR, 3β-HSD, Cox-2, and caspase-3 in biopsy tissue taken from the CL of normally cyling mares and one mare with a retained CL. All values were normalized against fmol of S15.

Discussion

Although there was considerable variability in the amount of tissue collected per biopsy (2.7 – 27.5 mg), each yielded enough tissue to quantify steady state levels of mRNA encoding 5 different genes using real time RT-PCR. All CL were sampled until day 14 and only two CL were sampled on day 16 (excluding the retained CL). In eight of the 26 biopsies (31%), a first biopsy procedure yielded primarily stroma, so an additional biopsy was required to obtain luteal tissue. No mares demonstrated any sign of discomfort after the procedure, and no luteal or ovarian trauma was visible with ultrasound following any of the procedures. This is in contrast to the effects of intraluteal injection reported by Weber et al. (2001); they found that in response to intraluteal injections of $PGF_{2\alpha}$, 3 of 24 (12.5%) mares developed ovarian abscesses.

Because the mean serum concentrations of progesterone and luteal areas between the two groups of mares were similar, the biopsy procedure did not appear to adversely affect luteal function. Levels of progesterone in serum were above 4 ng/ml throughout diestrus in both groups of mares. Concentrations of progesterone above 4 ng/ml are adequate to maintain pregnancy in the mare (Ginther, 1992). Progesterone values declined in both groups between days 12-13, indicating the beginning of luteolysis. Mares typically initiate luteolysis around day 14 of the estrous cycle (Ginther, 1992).

Due to a large amount of variability, there were no significant differences in CL area throughout the estrous cycle between control and biopsied CL. A decrease in area was detected between days 10-12 for the control CL and between days 12-14 for the biopsied CL. In both cases, a decline in CL area was observed at approximately the same time or slightly after a decline in serum concentration of progesterone was detected.

Messenger RNA Levels.

While S15 has been reported to have the features characteristic of a housekeeping gene (Kitagawa et al., 1991) and has been reported to remain at constant levels across a variety of conditions and treatments (Dr. Russell Anthony, personal communication; Slough, Chapter 3), its mRNA was not consistent in the samples collected late in the luteal phase of the cycle. The declining concentrations of progesterone and CL area after day 14 indicate that the CL were undergoing luteolysis. It is possible that the cells were undergoing apoptosis and levels of most mRNA were declining at this stage of the estrous cycle. Levels of S15 were consistent from days 2 – 14 and so S15 mRNA values were used to normalize the mRNA data from all samples collected prior to day 16. All mRNA data collected from day 16 CL were removed from analysis.

Steroidogenic acute regulatory protein in the equine CL is localized to large luteal cells (LLC) (Watson et al., 2000b), which are the only steroidogenic cell type in the equine CL (G.D. Niswender and T.M. Nett in McKinnon and Voss, 1993). Levels of StAR protein have been investigated by Watson et al. (2000b) in equine follicles, early CL, and pregnant vs. non-pregnant CL. There has not been a description of normal StAR expression throughout the estrous cycle of a cyclic mare. In ewes, concentrations of mRNA encoding StAR peak early in the estrous cycle (Juengel et al., 1995) and decline steadily from day 3 to day 15 (Juengel and Niswender, 1999). In the mare, levels of mRNA encoding StAR decrease after day 12. The observed decline in mRNA for StAR occurred at the same time that a decrease in serum progesterone was detected. In this experiment, biopsy samples were not taken between days 5 – 12, but in general, as progesterone values increased, so did levels of mRNA for StAR. Values for mRNA

encoding StAR were positively and significantly correlated to serum concentrations of progesterone on days 5 and 14. The data correspond to information collected in other species that indicates that StAR plays a rate-limiting role in steroidogenesis (Stocco and Clark, 1996b).

Concentrations of mRNA for 3β-HSD have not been reported in the equine CL. In the ewe, luteal mRNA encoding 3β-HSD increases from days 3 to 9 and then declines thereafter until day 15 (Juengel and Niswender, 1999). A similar trend was observed in equine luteal tissue. While there were no differences in level of mRNA for 3β-HSD from day 2 until day 12, there was a decline by day 14. There were also positive correlations between mRNA for 3β-HSD and concentrations of progesterone on days 5 and 12. Serum concentration of progesterone is highly correlated to levels of mRNA encoding StAR and 3β-HSD in ewes following PG injection (Juengel et al., 2000). In other species, 3β-HSD is not rate-limiting in progesterone production and 3β-HSD activity appears to be in great excess (Couet et al., 1990; Juengel et al., 1994), but because both StAR and 3β-HSD are involved in steroidogenesis, it seems logical that the corresponding levels of mRNA would correlate with serum concentrations of progesterone. In response to exogenous PG, it has been reported that mRNA for 3β-HSD decreases significantly in ewes (Juengel et al., 1998) and cows (Tian et al., 1994). There was a trend toward a decline in level of mRNA for 3β-HSD at the time of luteolysis ($p=0.15$) in these equine luteal tissue samples.

While it appeared that the highest level of mRNA for Cox-2 in the biopsy samples occurred on day 2, this difference was not significant, likely due to the variability in measurements of both Cox-2 and S15. Elevated levels of mRNA encoding Cox-2 in the

early CL corresponds with what has been reported in the literature for other species. In cattle, secretion of PG and concentrations of mRNA for Cox-2 are highest in the early luteal phase (Kobayashi et al., 2002). Treatment with indomethacin, which blocks production of PG, can block the formation of a CL in the cow (Milvae and Hansel, 1985), which illustrates the importance of PGs in the formation and early function of CL. There have been reports of mRNA encoding Cox-2 increasing in mature bovine CL after a luteolytic dose of exogenous PG (Diaz et al., 2000; Narayansingh et al., 2002; Tsai and Wiltbank, 1997, 1998), but this increase may be transient (Tsai et al., 2001). An increase in mRNA for Cox-2 might also have been expected at the time of luteolysis in these mares, but the sampling frequency may not have been adequate to detect increased mRNA. Equine mRNA encoding Cox-2 contains numerous repeats of the Shaw-Kamen's sequence, which is typically present in mRNA with short half-lives (Boerboom and Sirois, 1998); thus increases in the level of this mRNA could be difficult to detect.

Finally, there were no differences in level of mRNA encoding caspase-3 between any of the days sampled. Expression of caspase-3 in the equine CL has not been investigated, and it has only been described in the CL of other species in relation to luteolysis. Caspase-3 deficient mice undergo functional regression of their CL, but structural regression fails to occur (Carambula et al., 2002). In ewes given exogenous PG, levels of mRNA for caspase-3 are increased at 12 and 24 h post-injection (Rueda et al., 1999). Levels of mRNA encoding caspase-3 have not been described in natural luteolysis and it is entirely possible that any increases were so subtle or transient that they were not detected in the current experiment.

Collecting serial biopsies of luteal tissue from cyclic mares has no detrimental effects on CL function or size. It provides the first description of luteal levels of mRNA encoding StAR, 3β-HSD, Cox-2, and caspase-3 in the cyclic mare. This is also the first report of luteal levels of mRNA measured in the same female over the course of her estrous cycle in any specie.

Chapter 3 The Effect of Intraluteal Indomethacin Treatment on Luteolysis in Mares

The currently accepted model of luteolysis in domestic livestock states that prostaglandin (PG) of uterine origin is responsible for lysis of the corpus luteum (CL) (Silvia et al., 1991). There is little doubt that luteal tissue produces PG in cows and mares (Diaz et al., 2002; Hu et al., 1990; Watson and Sertich, 1990), and as more evidence accumulates, it appears that PG of luteal origin may be a critical component of the luteolytic cascade (Wiltbank and Ottobre, 2003).

Cyclooxygenase (Cox) is responsible for conversion of arachidonic acid to PGG_2, the rate-limiting step in the production of PG. While Cox-1 appears to be constitutively active, the inducible Cox-2 is a more likely target for cellular regulation (Moses and Bertone, 2002; Wiltbank and Ottobre, 2003). Exogenous PG stimulates an increase in intraluteal expression of mRNA encoding Cox-2 in the CL of pigs, rats and cows (Diaz et al., 2000; Narayansingh et al., 2002; Tsai and Wiltbank, 1997). Treatment with $PGF_{2\alpha}$ also activates protein kinase C (PKC) in luteal cells (Tsai and Wiltbank, 1997, 1998; Wu and Wiltbank, 2001b), and there is evidence that PKC regulates transcriptional factors that interact with an E-box in the 5' flanking region of the Cox-2 gene (Diaz et al., 2002; Wu and Wiltbank, 2001b).

To investigate the importance of intraluteal production of PG during luteolysis in the mare, an experiment was performed utilizing indomethacin to block the activity of both Cox-1 and Cox-2 (Brideau et al., 2001; Laneuville et al., 1994). By blocking the

activity of both enzymes, a critical component of the PG synthesis pathway was inhibited. While local production of PG was not measured in the current experiment, others have shown indomethacin to be effective in inhibiting production of PG in equine luteal cells (Watson and Sertich, 1990). Continuous indomethacin delivery was achieved by mixing indomethacin into Atrigel (Atrix Laboratories, Ft. Collins, CO), a viscous liquid polymer that solidifies upon exposure to an aqueous environment. Upon injection into luteal tissue, Atrigel solidifies and is slowly biodegraded over a period of 10-30 days, depending on the formulation.

Previous research by Griffeth (2002) utilizing Atigel delivery of indomethacin into the CL of ewes showed that while indomethacin treatment did not prevent a decline in steroidogenesis, the weights of indomethacin-treated CL removed on day 18 of the estrous cycle were significantly heavier than control CL. This provided the first evidence that intraluteal production of PG may be a requirement for structural regression of the CL to occur. The following experiment was designed to test the hypothesis that inhibiting intraluteal production of PG would prevent structural luteolysis in mares exposed to exogenous PG.

Materials and Methods

Eighteen mares and three dosages of indomethacin (0 mg, 3 mg, and 30 mg; n = 6 mares per dosage of indomethacin) were utilized for this experiment. Indomethacin was suspended in 300 μl of Atrigel and injected into CL 9 days following ovulation via an ultrasound-guided transvaginal technique. Ten minutes prior to the procedure, mares received an intravenous injection of analgesic (Flunixin Meglumine, 1.1 mg/kg). Mares were restrained in palpation stocks and administered preanesthetic medication (0.44

mg/kg xylazine, 0.016 mg/kg butorphanol and 0.044 mg/kg propanthaline bromide) 3-5 minutes prior to the procedure. The tail was wrapped in a fingerless plastic sleeve and secured to prevent contamination of the ultrasound probe/needle apparatus [5MHz ultrasound transducer, plastic handle, and implantation device (Cook Australia V-OPAA-1855 18 gauge 55 cm ovum pick-up aspiration needle)]. The rectum was evacuated and perineal area washed using a betadine soap. After drying the perineal area, the investigator introduced the ultrasound probe/needle apparatus into the vagina using a sterile sleeve and sterile KY jelly as a lubricant. The apparatus was maneuvered through the vagina until it reached the vaginal fornix. The investigator grasped the mare's ovary containing the CL to be implanted, per rectum, and manipulated it to align the CL with the injection needle. The needle was inserted through the vaginal wall and into the CL. The 300 μl Atrigel suspension was then deposited into the CL using a stylet to push the polymer through the needle. The procedure required approximately 3 to 5 minutes to complete. Mares were removed from the stocks and observed until they were fully alert and no signs of discomfort were observed.

On day 12 of the estrous cycle, 5 mg Lutalyse (Pharmacia & Upjohn Company, Kalamazoo, MI) were administered i.m. to half the mares in each group (n=3; + PG). Beginning on the day of indomethacin injection (day 9 after ovulation), 10 ml of blood was collected daily by jugular venapuncture. Area and diameter of all CL were measured daily via ultrasound evaluation. Once each mare developed a 35 mm follicle, blood was collected and the CL measured twice daily until the next ovulation. The first intraluteal injection took place on August 24, 2003.

Serum Concentrations of Progesterone.

After collection, blood was allowed to clot at room temperature for 1-2 hours or at 4°C overnight. The blood was centrifuged at 3000 x g for 15 minutes. Serum was removed and stored at -20°C until progesterone was measured by radioimmunoassay (Niswender, 1973). Samples were run in 3 assays and the intra-assay coefficients of variation were 2.7, 7.8 and 18.3%. The interassay coefficient of variation was 9.0%.

Statistical Analysis.

All data collected from mares with retained CL (defined as serum progesterone >1 ng/ml through day 25) were omitted from analysis. One mare in each of the treatment groups not receiving Lutalyse had a retained CL. The remaining data for serum progesterone concentrations, CL area, and CL diameter were analyzed using repeated measures in SAS. The data collected on days 9-11 (before Lutalyse treatment) was averaged and used for covariance analysis. A Fisher's protected LSD was then performed to make selected pairwise comparisons.

Results

Serum Concentrations of Progesterone.

All groups of mares, regardless of treatment, had decreases in serum concentration of progesterone between days 12 and 13 ($p = 0.08$ for controls (0 mg indomethacin), and $p < 0.01$ for all other groups (Figure 3.1)). By day 13, the 0 mg + PG ($p=0.01$), 3 mg + PG ($p<0.01$) and 30 mg + PG ($p=0.01$) treatment groups had lower serum concentrations of progesterone than controls (0 mg indomethacin). On and after day 14, there were no differences in serum concentration of progesterone between any of

the groups. Indomethacin treatment had no effect on serum concentration of progesterone at any of the dosages tested.

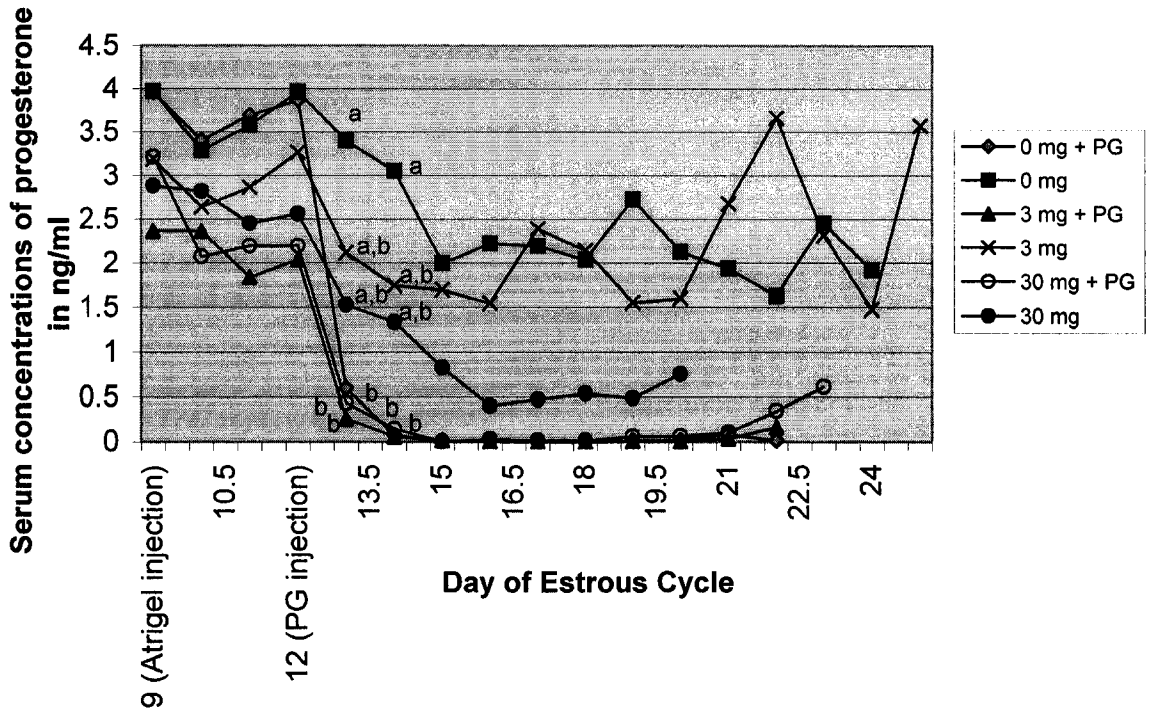

Figure 3.1. Average concentration of serum progesterone in mares according to treatment group. Different letters indicate significant differences on that day of the estrous cycle ($p<0.10$). All mares in each treatment group included (n=3 per group).

Luteal Measurements.

Ultrasound measurements of luteal area were highly variable. Even in the same mare from one measurement to the next, the data did not appear to be repeatable or reliable. Because of this variability, diameter, which should be less sensitive to measurement error than area, was also analyzed. When analyzing luteal area, a visual assessment of the data indicated that all groups demonstrated a decrease in size on day 13 (Figure 3.2). Compared to day 12 within group, the decrease in luteal area was significant by day 13 in controls (0 mg indomethacin; $p = 0.07$) and 30 mg indomethacin + PG-

treated CL (p = 0.03), and day 14 in the 0 mg indomethacin + PG-treated CL (p = 0.01) and 3 mg indomethacin + PG-treated CL (p = 0.03). Interestingly, the luteal areas in mares receiving 3 mg and 30 mg indomethacin were never different than on day 12.

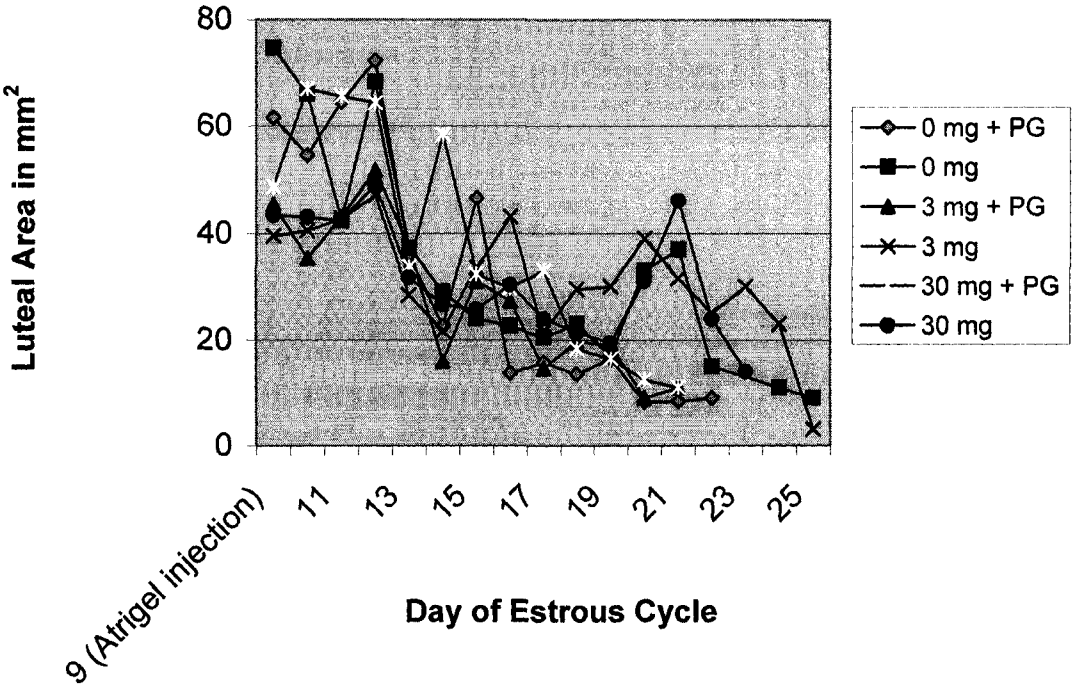

Figure 3.2. Average luteal area in mares grouped by treatment.

Compared to measurements on day 12 for each group, decreases in luteal diameter were significant on day 13 for the 30 mg indomethacin (p = 0.08) and 30 mg indomethacin + PG treatment groups (p = 0.02). Decreases in luteal diameter were significant by day 14 for the 0 mg indomethacin (p = 0.04), 0 mg indomethacin + PG-treated mares (p = 0.01), and 3 mg indomethacin + PG-treated mares (p = 0.10). The decrease in diameter did not become significant until day 17 in the 3 mg indomethacin

treatment group (p = 0.08). Whether measuring luteal area or diameter, decreases were observed on the same day in most cases. Comparing each group to controls, the only differences detected were on day 20 when all three PG-treated groups were different than controls (p = 0.07 – 0.09).

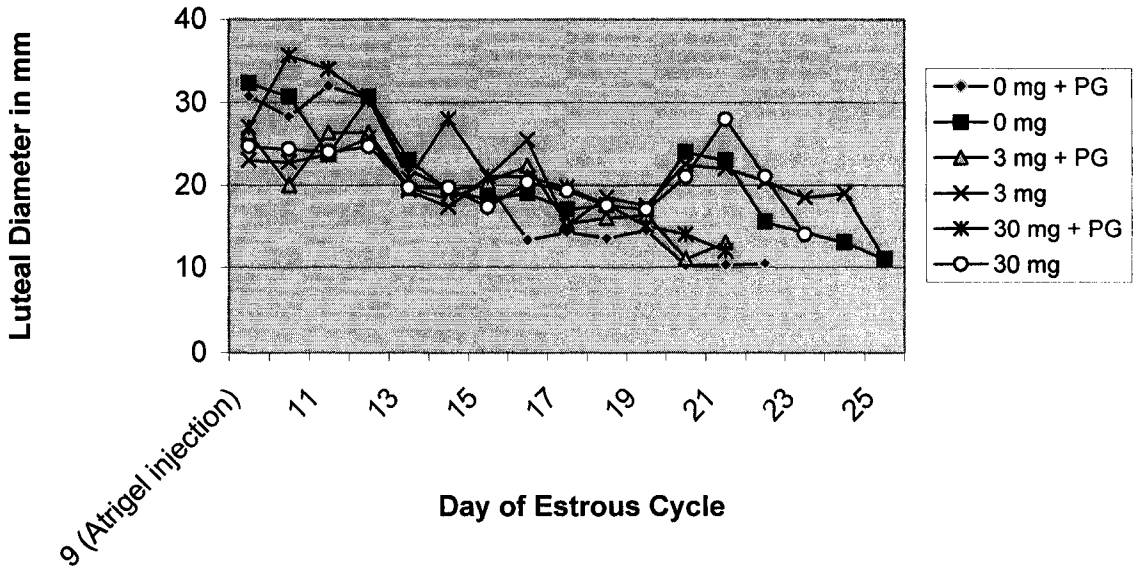

Figure 3.3. Average luteal diameter of mares grouped by treatment.

Discussion

A decrease in serum concentration of progesterone is expected within 24 hours of PG injection in mares (Al-zi'abi et al., 2002), which is what was observed in this study. The decrease in serum concentration of progesterone was earlier than expected in mares not receiving exogenous PG. Mares undergoing natural luteolysis typically experience decreasing progesterone around day 14 of the estrous cycle (Ginther, 1992). Because all groups of mares experienced a decline in secretion of progesterone on the same day, the Lutalyse treatment did not hasten a decline in steroidogenesis. Indomethacin treatment did not prevent a decrease in production of progesterone in any treatment group.

These results agree with data from equine luteal cells where indomethacin treatment had no effect on production of progesterone, even though production of PG was attenuated (Watson and Sertich, 1990). Griffeth (2002) also reported a decline in steroidogenesis despite indomethacin treatment in ovine luteal tissue. The current results do not agree with the other findings that indomethacin attenuated a decline in steroidogenesis in ovine luteal tissue exposed to exogenous PG (Slough, Chapter 4).

Corpora lutea treated with 3 or 30 mg of indomethacin were never smaller in area than they were on day 12. This supports the hypothesis that blocking intraluteal PG blocks structural luteolysis, but these data must be interpreted with care due to the variability in measurements and limited number of mares. Using diameter, a significant decrease in luteal size was detected in the 3 mg indomethacin and 30 mg indomethacin treatment groups. This is likely due to decreased variability in diameter measurements compared to area measurements, thus making the statistical analysis better able to define differences. The luteal diameter data indicates that CL in all groups decreased in size around days 13-14 and there probably were not any significant treatment effects.

While all mares in the study that received Lutalyse ovulated normally (around days 20-23 of the estrous cycle) following injection, one mare in each group that did not receive Lutalyse retained her CL and her serum progesterone concentrations remained > 1 ng/ml for more than 25 days. There were two mares that underwent luteolysis but failed to ovulate following the injection. Five mares were monitored for more than 45 days without ovulation being detected. Data from all mares in which luteolysis was detected were included in the statistical analysis. In total, 3 mares in each treatment group receiving Lutalyse were included in the data analysis, but only 2 mares were retained for

data analysis in each of the treatment groups not receiving exogenous PG. Because of the low number of mares in each group, the transitional ovarian activity of many of the mares, and the variability in ultrasound measurements, it is difficult to draw meaningful conclusions from this experiment. Indomethacin treatment did not cause the CL to maintain diestrous levels of progesterone production at the time of expected luteolysis. This experiment failed to prove that intraluteal production of PG is absolutely required for luteolysis to occur in the mare.

Chapter 4 The Effect of Intraluteal Indomethacin Treatment on Luteolysis in Ewes

Current dogma is that prostaglandin (PG) from the uterus is responsible for lysing the corpus luteum (CL) in domestic ruminants (Silvia et al., 1991). However, luteal tissue produces PG in cows (Diaz et al., 2002; Hu et al., 1990), and PG of luteal origin may also be a critical component of the luteolytic cascade (Wiltbank and Ottobre, 2003).

The rate-limiting step in the production of PG is the conversion of arachidonic acid to PGG_2, a reaction catalyzed by cyclooxygenase (Cox). Because Cox-1 is constitutively active, the more likely target for cellular regulation is the inducible form: Cox-2 (Moses and Bertone, 2002; Wiltbank and Ottobre, 2003). Mature CL respond to exogenous $PGF_{2\alpha}$ with an increase in intraluteal mRNA encoding Cox-2 and local production of PG (Diaz et al., 2000; Narayansingh et al., 2002; Tsai and Wiltbank, 1997). Treatment with $PGF_{2\alpha}$ leads to the activation of protein kinase C (PKC) in luteal cells (McGuire et al., 1994), and there is evidence that PKC regulates transcriptional factors that interact with an E-box in the 5' flanking region of the Cox-2 gene (Diaz et al., 2002; Wu and Wiltbank, 2001b). This is one pathway through which uterine or exogenous $PGF_{2\alpha}$ could lead to increased intraluteal production of PG.

To investigate the role of intraluteal production of PG in luteolysis, an experiment was performed utilizing indomethacin to block the activity of both Cox-1 and Cox-2 (Brideau et al., 2001; Laneuville et al., 1994). By blocking the activity of these enzymes, a critical component of the PG synthesis pathway was inhibited. While local production

of PG was not measured in the current experiments, others have shown indomethacin to be effective in inhibiting PG production in luteal cells (Milvae and Hansel, 1985; Skarzynski and Okuda, 1999; Watson and Sertich, 1990). Continuous indomethacin delivery was achieved by mixing indomethacin into Atrigel (Atrix Laboratories, Ft. Collins, CO), a viscous liquid polymer that solidifies upon exposure to an aqueous environment and is slowly biodegraded over a period of approximately 14 days. This degradation allows a continuous release of indomethacin.

Previous research by Griffeth (2002) utilizing an Atigel implant for delivery of indomethacin into the CL of ewes showed that while indomethacin treatment did not prevent a decline in serum concentrations of progesterone at the time of expected luteolysis, indomethacin-treated CL removed on day 18 of the estrous cycle were significantly heavier than control CL. Griffeth provided the first evidence that preventing intraluteal production of PG may inhibit structural regression of the CL. The following experiment was designed to test if inhibiting intraluteal production of PG in the CL of ewes would prevent natural or induced luteolysis.

Materials and Methods

Thirty ewes were synchronized for estrus, which was detected by twice daily observation in the presence of a vasectomized ram. Nine days following observed estrus, the ovaries were exteriorized and number and location of CL were determined. Ewes were classified as having unilateral (CL on one ovary; n = 20) or bilateral (CL on both ovaries, n = 10) ovulations. To ensure that individual CL were monitored and identified, only one CL on each ovary was retained. Any additional CL were removed at the time of surgery, with 16 of the unilaterally ovulating ewes having at least one CL removed, and

seven of the bilaterally ovulating ewes having at least one CL removed. Each ewe received one of the following treatments: 100 μl Atrigel injected into a CL (0 mg, n = 10), Atrigel + 10 mg indomethacin injected into a CL (10 mg, n = 10), or Atrigel into the left CL and Atrigel + 10 mg indomethacin into the right CL in bilaterally ovulating ewes (bilateral, n = 10).

The surgical procedure was as follows: Feed and water were withheld overnight to alleviate regurgitation and aspiration of rumen contents. Ewes were anesthetized using pentobarbital (25 mg/kg body weight) via the jugular vein and intubated with a tracheal tube to prevent aspiration of rumen fluid in the case of regurgitation. The abdominal area was shorn and scrubbed with betadine solution and zephrin and 70% ethyl alcohol applied. Intraluteal injections were performed after mid-ventral laparotomy and exposure of the ovaries. The procedure required 10-12 min per animal. The incision was closed using a continuous suture and the skin closed with interrupted mattress stitches utilizing Braunamid 2 USP. Animals were observed repeatedly during recovery from anesthesia. Once animals had recovered from the anesthetic, they were offered food and water.

Banamine, another Cox inhibitor, was administered via i.m. injection prior to surgery and on the morning following surgery to alleviate any discomfort the ewes might experience and to prevent endogenous production of PG in response to surgery. Beginning at 7:00 pm on the day of surgery (day 9 after estrus), 5 ml of blood were collected from each ewe via jugular venapuncture every 12 hours. On day 12, half the ewes in each treatment group (n = 5 per group) received 10 mg of Lutalyse via a single i.m. injection (+PG). Blood was then collected from all ewes every 4 hours for 24 hours, at which time CL were collected. Ewes were anesthetized by injection of pentobarbital

(25 mg/kg body weight) into the jugular vein. The reproductive tract was exposed via a mid-ventral laparotomy and CL were collected and decapsulated, Atrigel implants extracted, tissue weighed, and CL tissue frozen on dry ice for later analysis. The anesthetized ewes were then euthanized by exsanguination or injection of 60 mL of a saturated KCl solution as a single bolus into the jugular vein.

Serum Concentrations of Progesterone.

Blood samples were stored at 4°C for 4-28 hours and allowed to clot. The blood was then centrifuged at 3000 x g for 15 minutes. Serum was removed and stored at -20 °C until progesterone measurement. Radioimmunoassay (RIA) (Niswender, 1973) was utilized to measure serum concentrations of progesterone in each sample of blood from day 11 through the last sample obtained prior to euthanasia. Progesterone concentrations were also measured in the luteal tissue. Approximately 30 mg of each CL were homogenized in 1 ml of phosphate buffered saline (PBS). Progesterone was extracted from the homogenate using petroleum ether and concentrations of progesterone were measured using RIA (Niswender, 1973).

Messenger RNA in Luteal Tissue.

Luteal tissue from each CL was analyzed using real-time reverse transcription polymerase chain reaction (RT-PCR) to measure steady state levels of mRNA encoding Steroidogenic Acute Regulatory Protein (StAR); 3β-hydroxysteroid dehydrogenase, Δ 5-Δ 4 isomerase (3β-HSD); cyclooxygenase-2 (Cox-2); and caspase-3. Approximately 30 mg of each CL were homogenized with a Virtis Handishear tissue homogenizer and RNA isolated following the manufacturer's directions for TriReagent (Sigma Aldrich, St. Louis, MO). Immediately following RNA isolation and resuspension in 25 μl diethyl

pyrocarbonate (depc)-treated water, the RNA was treated with Ambion's TURBO DNAse reagent for 30 minutes at 37°C to remove any genomic DNA from the sample. The RNA was quantified by measuring the OD A260 and stored at -80°C.

After isolation and DNAse treatment of the RNA, samples were checked for RNA quality and genomic DNA contamination. Approximately 5 μg of total RNA from each sample was run on a formaldehyde gel to assess RNA quality. If both ribosomal bands were clearly visible, the RNA quality was considered acceptable. If degradation was apparent, fresh RNA was isolated from the remaining luteal tissue and quality was again assessed until high quality RNA was obtained. To determine if there was genomic DNA contamination, each sample of RNA was directly included in real-time PCR with actin primers—without first performing a RT reaction. Without first performing a RT reaction, there should be no DNA in the sample and the PCR should yield no product. If any product was generated in this reaction, then DNA contamination had occurred. If any of these PCR crossed threshold before cycle 35, genomic DNA contamination was suspected. Contaminated RNA was DNAse treated again and rechecked for DNA quality and genomic contamination.

Once the quality and purity of the RNA had been assured, the isolated RNA was converted to cDNA using reverse transcription (RT) for each sample. Duplicate RT was carried out for each RNA sample. Five μg of RNA were used in each 100 μl RT reaction. Five μg RNA from each sample were added to depc-treated water to bring the total volume to 67 μl. The RNA and water were heated to 65°C for 10 minutes to remove any secondary structure. The RT was then carried out with approximately 800 units of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV) enzyme, 1X MMLV

buffer, 1 mM dNTP's, 100 units RNAsin, 1.0 μg random primers, and 1 mM DL-Dithiothreitol (DTT). These components were added to the RNA and incubated at 42°C for one hour. The MMLV enzyme was then heat inactivated at 95°C for 10 minutes. The RT product (cDNA) was stored at -80°C until PCR.

Real-time PCR was utilized to quantify mRNA in each sample corresponding to the genes of interest. Each PCR included 5 μl of the RT product, 1X BioRad iQ SYBR Green Supermix (170-8882), 0.2 μM each of the forward and reverse primers designed specifically for the gene of interest, and water to bring the total volume to 25 μl. Duplicate samples from each reverse transcription reaction were measured in each CL, giving a total of 4 wells for each gene from each CL. SYBR green binds nonspecifically to double-stranded DNA, so as the cDNA from the gene of interest was amplified, there was a corresponding increase in fluorescence. A BioRad iCycler (Hercules, CA) was used for the thermal cycling and to measure fluorescence emitted, thus measuring the amount of DNA in each well.

The PCR conditions for each gene began with 3 min at 95°C, followed by 50 cycles of 15 s at 95°C (melting), 45 s at 58 °C (annealing), and 30 s at 72 °C (extension). Fluorescence data were collected at 72 °C during each cycle. Following the 50 cycles, a melt curve was generated by taking the samples to 55 °C and increasing temperature by 0.5 °C every 10 seconds. The temperature was increased 80 times. The data generated by the melt curve identified the temperature at which the PCR products melted apart, thus giving an indication of the size and number of products obtained in each well. Larger products melt apart at higher temperatures, and multiple products produce multiple

melting temperatures. Any wells with aberrant melt curves, compared to other samples or standards run with the same primers, were excluded from analysis.

Standards for each gene were generated from DNA cloned into a plasmid. Our laboratory already had the ovine StAR, ovine 3β-HSD, and bovine Cox-2 plasmids. The sequence for ovine caspase-3 had been reported in GenBank (Accession AF068837) but no plasmid was readily available that contained this sequence.

Primers were designed based on the known sequence for ovine StAR (Accession S80098) and the available plasmid was used to create standards. While a plasmid with ovine 3β-HSD was available, the sequence had not been published. To determine the sequence, the plasmid was transformed into competent *E. coli* and a midi-culture was grown. The plasmid was isolated (Qiagen Plasmid Midi Kit #12143) and sequenced (Davis Sequencing, Davis, CA). Once the sequence had been determined, primers were designed and the plasmid was used to create standards.

The bovine Cox-2 plasmid was available, and both the bovine (Accession AF031698) and ovine (Accession U68486) sequences have been reported. Primers were designed based on regions that were homologous for both species. Targeting a homologous region would allow the use of the same standard and primers for either bovine or ovine tissues.

Primers were designed based on the reported sequence for ovine caspase-3 (Accession AF068837). These primers were used in a PCR containing cDNA created from ovine RNA. The product was subcloned into PGEM-T plasmid (Promega) and sequenced. This plasmid was then utilized in generating ovine caspase-3 standards.

Gene	Primer Sequence
StAR	5'-GCTGCGTGGATTTATCAGGT-3'
	5'-5'CTCCTGGTCTTTGAGGATGC -3'
3βHSD	5'-CGGCACCTTGTACACTTGTG -3'
	5'- CTGGAGAACTTGCAGTAAT -3'
Cox-2	5'- CTCTTTCCTCCCTTTCACACCCATG -3'
	5'-5'GCTCTTCCTCCTGTGCCTGATG -3'
Caspase-3	5'- GTGAAGGTTTCCCTGAGGTTTGC -3'
	5'-5'AACTTCCACGAAAATACTGGCATGG -3'
S15	5'-ATCATTCTGCCCGAGATGGTG-3'
	5'-TGCTTTACGGGCTTGTAGGTG-3'

Table 4.1. Primer sequences utilized with ovine cDNA and standards for real time RT-PCR analysis.

Once all plasmids had been obtained or created, midi-cultures were grown for each gene and the plasmids isolated (Qiagen Plasmid Midi Kit #12143). The plasmids were subjected to an *in vitro* transcription (Ambion's MegaScript T7 kit #1333) to generate cRNA for the genes of interest. This cRNA was quantified with a spectrophotometer using the OD A260 reading and known amounts of cRNA were placed into two separate RT reactions. The RT products were serially diluted and used to create standard curves for each gene. The standards, therefore, were based on the known amount of cRNA that went into each RT rather than amount of DNA. This allowed the direct comparison of Ct values of unknown samples to Ct values of standards to determine levels of mRNA in the ovine tissue.

Ribosomal protein S15 was used as a housekeeping gene to correct for any variation in total amount of RNA in each sample. S15 is a protein that makes up part of the small ribosomal subunit, appears to be relatively stable under a number of conditions, and is expressed at a level similar to that of many genes of interest (Dr. Russell Anthony,

personal communication). Primer sequences were obtained from Dr. Russell Anthony. These primers were used to generate a PCR product that was subcloned into PGEM-T (Promega) and used to make standards as described above, with duplicate RT reactions performed to create duplicate standard curves.

In each PCR, duplicates of each serially diluted point from each RT reaction were included. An eight point standard curve was generated from four wells for each point. Each biological sample was also run with duplicate samples of each duplicate RT reaction. An arbitrary fluorescence threshold was determined for each plate based on the logarithmic amplification curves. The cycle number at which the fluorescence detected in each well reached the arbitrary threshold was designated as the Ct value for that sample. The Ct values of unknown samples were compared against the Ct values of known standards to determine how much mRNA was in the initial unknown samples. The starting quantities of mRNA calculated in each of the four wells for each sample were averaged and normalized against S15 to determine relative quantities of the target gene.

Statistical Analysis.

The serum concentrations of progesterone in the three samples just prior to Lutalyse injection were averaged to create a baseline value for each ewe, and this baseline was used for covariance analysis. Selected pairwise comparisons were made with a Fisher's protected LSD using repeated measures in proc mixed of SAS to compare serum progesterone, tissue progesterone, CL weights, and mRNA values. The mRNA data was first log transformed to normalize the data. Data from ewes with bilateral CL were evaluated separately from those with unilateral CL, and bilateral ewes were treated as a split plot.

Results

Serum Concentrations of Progesterone.

Within four hours of Lutalyse injection, decreased serum progesterone concentrations were detectable in both treatment groups receiving PG (0 mg indomethacin + PG, $p = 0.02$; 10 mg indomethacin + PG, $p = 0.02$) compared to controls (0 mg indomethacin; Figure 4.1). This difference was still evident 24 hours after Lutalyse injection with ewes receiving 0 mg indomethacin + PG ($p < 0.01$) and 10 mg indomethacin + PG ($p = 0.07$) having decreased serum progesterone compared to controls (0 mg indomethacin). While serum concentrations of progesterone in the 10 mg indomethacin treatment groups were different at 16 ($p = 0.09$) and 20 hours post-PG ($p = 0.02$), they were not different from each other at any other time point (10 mg indomethacin vs. 10 mg indomethacin + PG). Twenty-four hours after Lutalyse injection, ewes receiving 10 mg indomethacin + PG had significantly higher serum concentrations of progesterone than ewes receiving 0 mg indomethacin + PG ($p = 0.04$). Treatment with indomethacin (10 mg) also led to a decrease in serum progesterone compared to controls (0 mg indomethacin). Twelve hours after injection of PG, the ewes whose CL were treated with 10 mg indomethacin had lower serum concentrations of progesterone than controls (0 mg indomethacin; $p = 0.01$), and this difference was evident 24 hours after PG injection ($p = 0.08$).

Prostaglandin-treated ewes with bilateral CL had decreased serum concentrations of progesterone compared to those not receiving PG, which was evident by 12 hours after injection ($p = 0.04$; Figure 4.2). This decrease was apparent throughout the remainder of the sampling period. By 24 hours, the difference was dramatic ($p < 0.0001$).

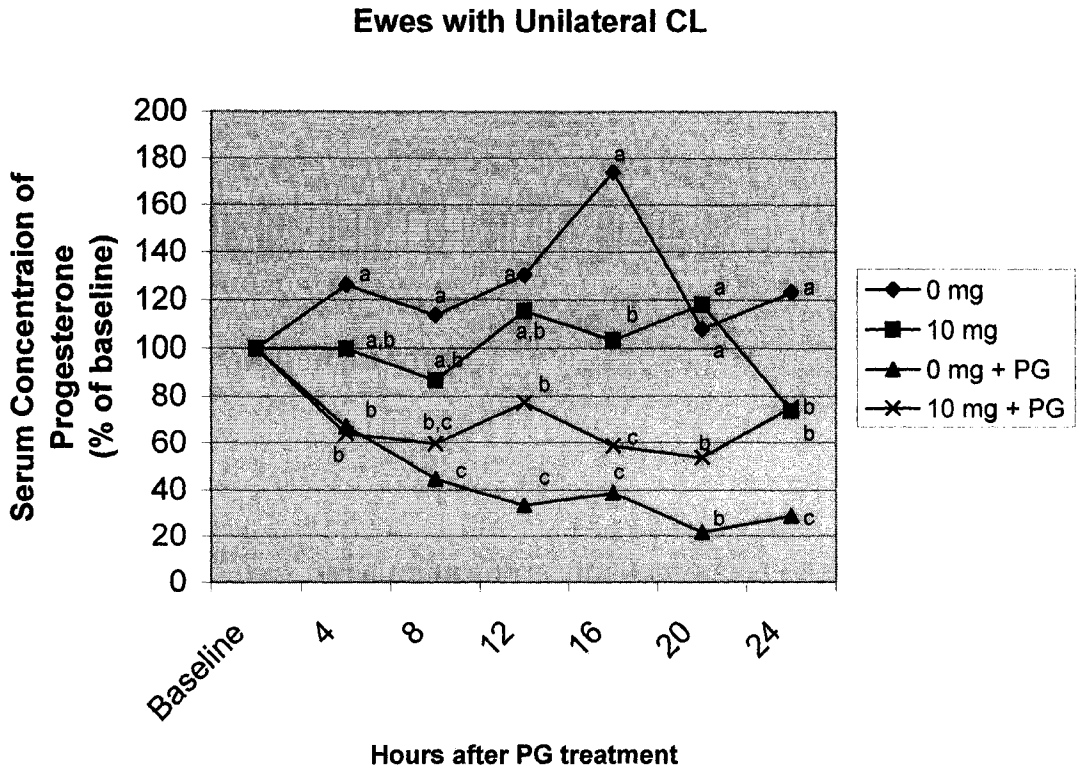

Figure 4.1. Mean serum concentrations of progesterone in ewes with unilateral CL as a percentage of baseline progesterone values for each group. Different letters indicate differences between treatments at each individual time point ($p < 0.10$).

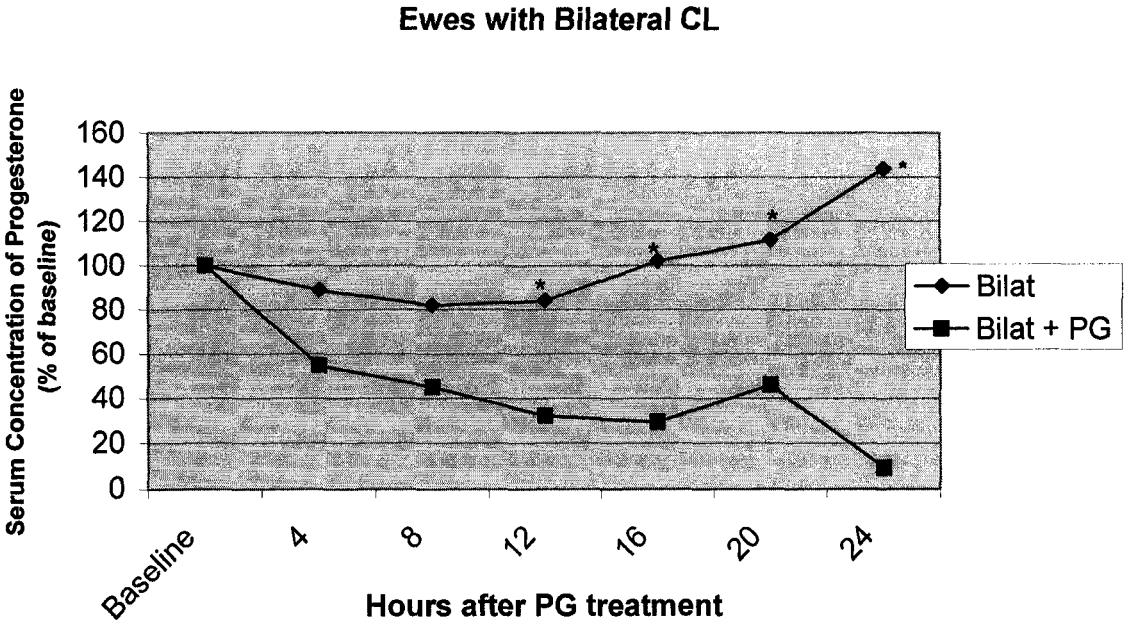

Figure 4.2. Mean serum concentrations of progesterone in ewes with bilateral CL. Progesterone concentrations given as a percentage of baseline for each group. An * indicates differences between the two groups of ewes at each individual time point ($p < 0.10$).

Tissue Concentrations of Progesterone.

There were no differences in the tissue concentrations of progesterone between groups with unilateral CL (Table 4.2). Prostaglandin administration caused a significant decrease in tissue concentration of progesterone in bilateral CL, with CL receiving 10 mg indomethacin + PG ($p = 0.01$) and 0 mg indomethacin + PG ($p = 0.02$) having lower tissue progesterone concentrations compared to controls (0 mg indomethacin). In ewes with bilateral CL, CL receiving 10 mg indomethacin had higher concentrations of progesterone than controls (0 mg indomethacin; $p = 0.06$) and all PG-treated CL ($p < 0.01$ in 0 mg indomethacin + PG; $p < 0.01$ in 10 mg indomethacin + PG). The two groups of PG-treated CL were not different from each other ($p = 0.56$).

0.01 in 0 mg indomethacin + PG; $p < 0.01$ in 10 mg indomethacin + PG). The two groups of PG-treated CL were not different from each other ($p = 0.56$).

Mean Luteal Concentrations of Progesterone and Luteal Weights 24 Hours after Treatment with Lutalyse.

	Luteal		
Treatment	Weight (mg)	P4 Concentration (ng/mg)	Total P4 Content (mg/CL)
Unilateral CL			
0 mg	828±49.8	10.6±0.598	8.74±0.523[a]
0 mg + PG	635±82.6	8.53±1.47	5.32±1.19[b]
10 mg	819±125	11.7±2.22	9.78±2.11[a]
10 mg + PG	763±77.5	8.62±1.18	6.93±1.40[a,b]
Bilateral CL			
0 mg	737±30.9[a]	8.94±1.15[a]	6.66±1.04[c]
0 mg + PG	505±50.6[b]	4.25±0.780[b]	2.14±0.498[d]
10 mg	603±86.4[b]	11.1±2.02[c]	6.59±1.41[c]
10 mg + PG	467±36.3[b]	3.63±0.644[b]	1.64±0.222[d]

Mean ± SEM
[a,b]Means within a column with different supersripts are different ($p < 0.10$)
Unilateral data analyzed separately from bilateral data.

Table 4.2. Luteal weights, tissue concentrations of progesterone and total content of progesterone in CL collected 24 hours following treatment with PG.

Luteal Weights.

There were no differences in luteal weight among any of the treatment groups in ewes with unilateral CL (Table 4.2). All CL treated with indomethacin or PG (10 mg indomethacin, $p = 0.07$; 10 mg indomethacin + PG, $p = 0.01$; 0 mg indomethacin + PG, $p = 0.02$) in the bilaterally ovulating ewes were lighter weight than control CL (0 mg indomethacin) in the same group of ewes. Corpora lutea treated with 10 mg indomethacin were 19 percent smaller than controls (0 mg indomethacin), CL treated with 0 mg indomethacin + PG were 39 percent smaller than controls (0 mg indomethacin), and 10 mg indomethacin + PG-treated CL were only 22 percent smaller than CL treated with 10

mg indomethacin. There were no differences in weight among any of the PG-treated CL or the indomethacin-treated CL in the bilaterally ovulating ewes.

Messenger RNA in Luteal Tissue.

In CL collected from unilateral ovulations, levels of mRNA encoding StAR were not affected by treatment with indomethacin (0 mg vs. 10 mg indomethacin, $p = 0.87$), and administration of PG caused a decrease in StAR mRNA levels in both the 0 mg indomethacin + PG ($p = 0.05$) and 10 mg indomethacin + PG ($p = 0.07$) treated ewes compared to controls (0 mg indomethacin; Table 4.3). Level of mRNA encoding StAR was positively correlated with serum concentration of progesterone at 24 hours ($r = 0.31$, $p = 0.10$) and concentration of progesterone in the luteal tissue ($r = 0.46$, $p < 0.01$). There were no differences in the current experiment among any of the unilateral treatment groups in 3βHSD expression ($p > 0.83$). Due to the lack of differences in 3β–HSD expression and high standard deviations, there were not any significant correlations between 3β–HSD expression and any steroidogenic indicators.

A similar trend in levels of mRNA encoding StAR and 3βHSD was observed in the bilateral CL (Table 4.3). There were no differences in levels of mRNA for 3β-HSD. All CL exposed to Lutalyse (0 mg indomethacin + PG, $p < 0.01$; 10 mg indomethacin + PG, $p < 0.01$) had less mRNA encoding StAR than control CL (0 mg indomethacin). Corpora lutea treated with 0 mg and 10 mg indomethacin were not different from each other ($p = 0.42$), and the CL exposed to PG were not different from each other ($p = 0.51$).

No differences in levels of mRNA encoding Cox-2 were detected in unilateral CL ($p > 0.23$; Table 4.3). Level of mRNA encoding Cox-2 was negatively correlated with content of progesterone in the luteal tissue ($r = -0.42$, $p = 0.01$), and luteal weight ($r =$ -

0.42, p = 0.01). In the bilaterally ovulating sheep, mRNA for Cox-2 was higher in both the 0 mg indomethacin + PG (p = 0.06) and 10 mg indomethacin + PG (p = 0.03) treated CL compared to controls (0 mg indomethacin). Among the unilateral and bilateral CL, there were no differences between any groups in levels of mRNA encoding caspase-3 (p > 0.16).

	fmol relative to fmol S15			
Treatment	**StAR**	**3β-HSD**	**Cox-2 (x 10^{-4})**	**Caspase-3**
Unilateral CL				
0 mg	2.65±0.778[a]	67.1±42.4	2.88 ± 0.744	1.5±0.599
0 mg + PG	1.03±0.307[b]	54.2±64.8	4.30 ± 1.99	2.07±1.50
10 mg	2.72±0.601[a]	76.9±50.0	4.47 ± 3.90	2.43±1.02
10 mg + PG	1.30±0.429[b]	55.9±36.0	2.57 ± 1.01	1.73±1.02
Bilateral CL				
0 mg	2.38±0.791[c]	40.3±39.2	2.46 ± 0.906[a]	1.26±0.829
0 mg + PG	0.502±0.193[d]	45.1±25.8	7.43 ± 1.82[b,c]	2.19±0.735
10 mg	2.71±0.595[c]	71.2±34.9	3.57 ± 1.20[a,b]	2.14±0.963
10 mg + PG	0.517±0.158[d]	49.3±55.1	6.06 ± 1.13[c]	2.12±1.13

Mean ± SEM

[a,b]Means within a column with different supersripts are different (p < 0.10)
Unilateral data analyzed separately from bilateral data.

Table 4.3. Amount of mRNA corresponding to each gene of interest in luteal samples collected from ewes 24 hours following treatment with PG. Messenger RNA values were obtained by real time RT-PCR and normalized against S15.

Discussion

In a similar experiment, intraluteal indomethacin treatment did not cause the ovine CL to maintain diestrous levels of steroidogenesis at the time of expected luteolysis (Griffeth, 2002), but there was some evidence in the current experiment that indomethacin treatment may have attenuated a PG-induced decline in steroidogenesis. While both groups of ewes with unilateral CL experienced a decline in serum concentrations of progesterone in response to Lutalyse treatment, the serum concentrations of progesterone in the10 mg indomethacin and 10 mg indomethacin + PG

treatment groups were not different from each other at most time points including 24 hours after Lutalyse injection. Additionally, the 10 mg indomethacin + PG-treated ewes had significantly higher serum progesterone than 0 mg indomethacin + PG-treated ewes ($p = 0.04$) 24 hours following PG treatment.

Prostaglandin treatment was sufficient to cause decreased serum concentrations of progesterone in ewes with bilateral CL. Levels of mRNA encoding StAR declined and total content of progesterone was decreased in both groups of CL exposed to PG, so it appears likely that both the indomethacin-treated and 0 mg-treated CL in the bilaterally ovulating ewes had decreased production of progesterone, and so both sets of CL contributed to declining serum concentrations of progesterone. There was no evidence that indomethacin treatement prevented a luteolytic decline in steroidogenesis in ewes with bilateral ovulations.

In the ewes with unilateral CL, there were no differences in luteal concentration of progesterone or size between any of the treatment groups, but when the luteal content of progesterone and CL size in PG-treated ewes were considered together, there was less total progesterone in the PG-treated CL (0 mg indomethacin vs. 0 mg indomethacin + PG), which explains the lower serum concentration of progesterone in the PG-treated ewes. Once again, it appears that indomethacin may have protected production of progesterone somewhat from the effects of Lutalyse. The 10 mg indomethacin and 10 mg indomethacin + PG treatment groups did not differ in total luteal content of progesterone.

Diestrous levels of steroidogenesis were not maintained in bilaterally ovulating ewes exposed to Lutalyse. Both indomethacin-treated and untreated CL exposed to Lutalyse had lower tissue concentrations of progesterone than control CL (0 mg

indomethacin) in the bilaterally ovulating sheep not exposed to PG. Because indomethacin did not provide the same protective effects on steroidogenesis in bilaterally ovulating ewes as was observed in unilaterally ovulating ewes, it seems possible that there was some sort of interaction between the bilateral CL within a single ewe to amplify the luteolytic signal. Interestingly, the indomethacin treated CL (10 mg) not exposed to Lutalyse had higher tissue concentrations of progesterone than controls (0 mg indomethacin). This may be due to suppression of tonic Cox activity. If there is tonic Cox activity, and thus constant PG production, there could be steady inhibition of steroidogenesis through activation of PKC. Corpora lutea treated with 10 mg of indomethacin were lighter in weight than control CL in the same ewes, but with the increased steroidogenesis in the indomethacin-treated CL, the total content of progesterone in these CL was no different than controls.

There were no differences in CL weight among any of the treatment groups in the CL collected from unilaterally ovulating ewes. After a luteolytic dose of PG in ewes, a 20 percent reduction in CL size is expected within 24 hours, which may not reach statistical significance. Braden et al. (1988) treated ewes with two injections of 7.5 mg $PGF_{2\alpha}$ four hours apart, and 24 hours following treatment there were no significant differences in CL weight. For the current experiment, CL were collected at 24 hours post-injection to ensure there would be adequate tissue for biochemical analysis. While not significant, the CL receiving 0 mg indomethacin + PG were, in fact, about 80 percent of the size of the control CL (0 mg indomethacin). The 10 mg indomethacin + PG-treated CL were 90 percent of the size of the 10 mg indomethacin-treated CL. Indomethacin may have

provided some limited protection against structural luteolysis despite PG treatement, but due to the variability among samples and the limited sample size, it was not significant.

All groups of treated CL in the bilateral groups were lighter in weight than control CL (0 mg indomethacin). While indomethacin-treated CL were 19 percent smaller than controls (0 mg indomethacin), treatment with PG caused CL treated with 0 mg indomethacin + PG to be 39 percent smaller than controls (0 mg indomethacin). Again this is a more dramatic effect than was observed in ewes with unilateral CL, suggesting some interaction between the two CL to amplify the luteolytic signal. Indomethacin may have attenuated the Lutalyse effect; CL treated with 10 mg indomethacin + PG were only 22 percent smaller than 10 mg indomethacin-treated CL. While this is the magnitude of decline expected 24 hours after Lutalyse injection, it is not as dramatic a decline in CL weight as was observed in the CL receiving 0 mg indomethacin + PG compared to bilateral controls. Statistically, there were no differences in weight among any of the PG-treated CL or the indomethacin-treated CL.

Messenger RNA in Luteal Tissue.

To gain some insight into the effects of treatment on the steroidogenic pathway, the levels of mRNA encoding StAR and 3β-HSD were quantified. Steroidogenic acute regulatory protein is involved in transporting cholesterol to the inner mitochondrial membrane, which is the rate-limiting step of steroidogenesis (Stocco and Clark, 1996a), and 3β-HSD converts pregnenolone to progesterone in the smooth endoplasmic reticulum (Reviewed in Juengel and Niswender, 1999). Steroidogenic acute regulatory protein mRNA decreases significantly within 4 hours of a luteolytic dose of PG (Juengel and Niswender, 1999), so a decrease in level of mRNA encoding StAR was predicted and

obtained upon luteal exposure to PG. Treatment with indomethacin had no protective effect on levels of mRNA for StAR, so any attenuation by indomethacin of declining serum and tissue concentrations of progesterone occurred through a mechanism other than the retention of mRNA for StAR.

There were no differences in 3β–HSD expression among any of the treatment groups in either unilateral or bilateral CL. It is known that mRNA for 3β–HSD decreases within 4 hours in ovine CL exposed to a luteolytic dosage of PG (Juengel and Niswender, 1999) and is decreased 80 percent within five hours (Hawkins et al., 1993). Within 4 hours of PG injection, a decrease in mRNA encoding 3β–HSD is also detectable in bovine luteal tissue (Tsai and Wiltbank, 1998). Declining mRNA for 3β–HSD is a clearly defined aspect of luteolysis, and so a lack of a similar trend in this experiment is concerning. The lack of differences in this experiment is likely due to the large variability in levels of mRNA encoding 3β–HSD from ewe to ewe. The standard errors were large and the effect of PG appears to have been inconsistent. The efficacy of the single, 10 mg dosage of Lutalyse administered i.m. comes into question.

Cyclooxygenase-2 is responsible for the rate-limiting step in the production of PG, and so levels of mRNA were quantified to indicate whether the CL were producing intraluteal PG in response to Lutalyse. Cyclooxygenase-2 is normally present in tissues, but in very small quantities that are nearly undetectable (O'Neill and Ford, 1993). Others have reported undetectable levels of mRNA for Cox-2 in day 13 ovine CL (Silva, 2002), so the very low levels detected in this experiment do not contradict what has been reported. It is known that bovine luteal tissue exposed to PG will respond by producing more PG (Tsai and Wiltbank, 1998), and so Cox-2 is expected to increase in response to

Lutalyse injection. The timing of the induction of mRNA encoding Cox-2 is variable in the literature, with some reporting an increase within 30 minutes of treatment, and a return to baseline values by 4 hours post-injection (Tsai et al., 2001). Other reports indicate a 3-fold increase in mRNA for Cox-2 at 4 hours post-injection (Tsai and Wiltbank, 1998). An increase in mRNA encoding Cox-2 was not observed in the unilateral CL but was significant in both groups of bilateral CL collected from ewes treated with Lutalyse (0 mg indomethacin + PG; 10 mg indomethacin + PG). It is possible that Cox-2 induction had occurred in the unilateral CL soon after treatment with PG and had returned to baseline values by 24 hours, as has been observed in rats (Narayansingh et al., 2002). If this is the case and luteal Cox-2 induction occurs quickly in response to uterine or exogenous PG, an increase in mRNA may have simply been missed due to the timing of sampling. Again, it is possible that the Lutalyse dosage may not have been completely effective in inducing luteolysis and that there is some interaction between the two CL in bilaterally ovulating ewes to cause more dramatic effects than are observed in unilateral CL. It is interesting to note that the higher levels of Cox-2 mRNA in the bilateral CL were associated with much more dramatic signs of luteolysis, indicating there could indeed be a relationship between intraluteal PG production and luteolysis.

Levels of mRNA encoding caspase-3 were also quantified. Activation of caspase-3 is one of the final stages of apoptosis, leading to cleavage of key structural and functional cellular proteins (Rueda et al., 1999). Caspase-3 is a critical component of luteolysis in mice (Carambula et al., 2002) and mRNA for caspase-3 is increased in ovine CL 24 hours following PG administration (Rueda et al., 1999). Among the unilateral and

bilateral CL, there were no differences between any groups in caspase-3 expression. While caspase-3 is stored in an inactive form and it becomes activated during apoptosis, this is usually accompanied by an increase in mRNA (Rueda et al., 1999). The lack of differences between treatment groups again brings into question the efficacy of the Lutalyse dose and/or the timing of sampling.

All of these data taken together raises two important questions: 1. Was the Lutalyse dose ineffective and if so, why was it ineffective? 2. What interaction might be occurring between the bilateral CL to enhance the luteolytic signal? The lack of significant differences in the unilateral CL in terms of tissue concentration of progesterone, CL weights, and levels of mRNA encoding 3β-HSD, Cox-2, and caspase-3 are cause for concern. It is not clear from these data that luteolysis had been initiated. While activation of PKC is responsible for inhibiting production of progesterone, it does not appear that the cytotoxic effects of PG are mediated through PKC in luteal cells (Wiltbank et al., 1991). Pharmacologically activating PKC in large luteal cells has been shown to decrease production of progesterone in ovine CL without initiating luteolysis (McGuire et al., 1994). It is believed that an influx of calcium is necessary to stimulate apoptosis (Wiltbank et al., 1991). Luteolytic doses of exogenous PG activate PKC and cause a calcium influx, possibly through a PG-gated Ca^{++} channel. It is possible that the single 10 mg dose of Lutalyse administered i.m. was sufficient to activate PKC, but not enough to cause a sustained Ca^{++} influx. Others have shown that low doses of $PGF_{2\alpha}$ stimulate intracellular calcium influxes into only a small percentage of LLC (Wiltbank et al., 1991) and that low doses of $PGF_{2\alpha}$ cause only transient decreases in serum progesterone and do not cause luteolysis (Juengel and Niswender, 1999).

Other similarities between reports by McGuire et al. (1994) and the current experiment provide further evidence that the Lutalyse injection may have activated PKC without initiating complete luteolysis. In McGuire et al. (1994), activation of PKC caused a reduction in 3β–HSD within three hours of treatment, but by 24 hours levels of mRNA encoding 3β–HSD had recovered so they were no longer different than controls. Since quantification of mRNA for 3β–HSD was performed in CL collected 24 hours after PG injection in the current experiment, it is possible that mRNA encoding 3β–HSD had declined and recovered by the time samples were collected. Another observation from McGuire et al. (1994) is that activation of PKC caused a decrease in serum concentrations of progesterone from 3-12 hours, but serum progesterone increased from 24-48 hours and were not different than controls. Serum concentrations of progesterone in 0 mg indomethacin + PG-treated ewes increased from 20-24 hours post PG injection. These observations all agree with prior observations associated with PKC activation, but not necessarily a sufficient Ca^{++} influx leading to apoptosis.

The 10 mg dosage of Lutalyse used in this study was chosen based on earlier work in our laboratory where W. Silvia had demonstrated that 7.5 mg was the minimal dosage required to cause decreased serum concentrations of progesterone; apoptotic processes were not evaluated in this research. Another consideration is that Lutalyse is generally given to ewes in a series of two injections, and it is possible that a pulsatile PG exposure is necessary for the ovine CL to complete luteolysis. Speculation about why pulses of PG might have been more effective at inducing luteolysis was the inspiration for another experiment. (See Slough, Chapter 5.)

The ewes with bilateral CL responded to Lutalyse in virtually every aspect evaluated with a much more dramatic response than unilateral CL, which indicates there was likely an interaction between the two CL within a single ewe that amplified the exogenous PG signal to initiate luteolysis. A recent paper suggests that the presence of $PGF_{2\alpha}$ in the CL causes a massive release of oxytocin, which leads to an increase in endothelin-1, and production of progesterone decreases. The authors propose that the endothelin-1 interacts with tumor necrosis factor-α to cause structural luteolysis in the ewe (Ohtani et al., 2004). It is possible that both CL responded to Lutalyse by producing PG, oxytocin, endothelin-1, or another mediator that traveled through systemic circulation to the contralateral CL and served to amplify the luteolytic signal. Bennegard-Eden et al. (1995) found that oxytocin injected into human ovaries contralateral to a CL caused reduced secretion of progesterone.

Indomethacin treatment may have offered some protection for the CL against luteolysis in unilateral CL, but there is no conclusive evidence from this study. There are some trends that are encouraging and some data that support the hypothesis that intraluteal PG production is a required component of luteolysis, but further studies will be necessary to either prove or disprove the theory that intraluteal PG production is required for luteolysis to occur in the ewe.

Chapter 5 A Proposed Model of Luteolysis, Focusing on Intraluteal Factors

Luteolysis is a critical component of the estrous cycle in domestic animals, and so it is important to understand this dynamic process. A decline in progesterone production creates the environment required to allow another estrous cycle and another opportunity for pregnancy. Many models describing luteolysis have been proposed, but as more data are collected, the model must be modified to become more detailed and complete.

Currently Accepted Model

Luteolysis is temporally associated with pulses of prostaglandin (PG) $F_{2\alpha}$ released from the uterus (Kindahl et al., 1976; Thornburn et al., 1973). Exogenous administration of $PGF_{2\alpha}$ will cause luteolysis (Hansel et al., 1973; McCracken et al., 1970; Schramm et al., 1983) and removal of the uterus will inhibit luteolysis (Anderson et al., 1961; Anderson et al., 1966; Ginther and First, 1971; Malven and Hansel, 1964; Wiltbank and Casida, 1956). Therefore, there is little question that the uterus and $PGF_{2\alpha}$ both play a critical role in the luteolytic process.

Prior to luteolysis, a large follicle is usually present on the ovary. This follicle secretes estrogen, and it is believed that high levels of estrogen cause the hypothalamus to secrete oxytocin (OT) (McCracken et al., 1996). Oxytocin, in turn, stimulates the uterus to release $PGF_{2\alpha}$ (Al-Matubsi et al., 1997; Fairclough et al., 1980; King and Evans, 1984; Lafrance and Goff, 1990; Roberts et al., 1976). A countercurrent venous exchange

between the uterus and ovary delivers $PGF_{2\alpha}$ to the corpus luteum (CL) in ruminants. The CL responds to $PGF_{2\alpha}$ exposure by releasing OT (Al-Matubsi et al., 1997; Flint and Sheldrick, 1983b; Moore et al., 1986; Tsai and Wiltbank, 1997). This sets up a positive feedback loop whereby $PGF_{2\alpha}$ from the uterus stimulates the release of OT from the CL, which stimulates further release of uterine $PGF_{2\alpha}$. The net result is luteolysis (Reviewed in Niswender et al., 2000).

If a female becomes pregnant, then luteolysis must be prevented. The CL must remain functional and continue to produce progesterone. Uterine-ovarian venous concentrations of $PGF_{2\alpha}$ are no different in pregnant ewes compared to cyclic ewes, although uterine production of PGE_2 is elevated (Nett and Niswender, 1981; Silvia et al., 1984). Maternal recognition of pregnancy in ruminants is now known to occur in response to interferon tau (IFN-τ) secreted by the embryo. The embryo trophectoderm produces IFN-τ for a limited time in early gestation; in sheep this protein is produced by day 13-21 embryos (Flint and Sheldrick, 1986; Martal et al., 1979; Moor and Rowson, 1966). Days 12-13 following ovulation in the ewe seem to be critical for the determination of whether to maintain or abolish the CL (Moor and Rowson, 1966; Silvia and Niswender, 1986). During this period, the CL of pregnant ewes are resistant to exogenous treatment with $PGF_{2\alpha}$, with progesterone declining and then returning to pretreatment values in response to $PGF_{2\alpha}$ (Silvia and Niswender, 1986).

The Role of Oxytocin

Whether or not OT is an essential component of the luteolytic cascade remains a topic of debate. There is evidence supporting the hypothesis that OT is an important factor in luteolysis: administration of an OT antagonist in cyclic ewes delays luteolysis

(Mann et al., 2003), as does immunization against OT (Milvae and Hansel, 1980b). Other evidence indicates that OT may not be required for luteolysis: depletion of luteal OT stores in ewes does not diminish the luteolytic response to exogenous $PGF_{2\alpha}$ (Sheldrick and Flint, 1983) and neither OT depletion (Kotwica and Skarzynski, 1993) nor the administration of OT antagonists (Kotwica et al., 1997) are effective in prolonging the luteal phase in cows.

While traditional models of luteolysis state that OT is secreted from the hypothalamus during luteolysis (Hooper et al., 1986), more recently authors suggest that up to 90 percent of the OT originates from intraluteal sources (McCracken et al., 1996). It has long been accepted that the CL produces OT (Flint and Sheldrick, 1983b), but whether OT plays a local role within the luteal tissue itself has not been closely investigated.

Large luteal cells (LLC) contain receptors for $PGF_{2\alpha}$ and small luteal cells (SLC) have very few, if any, receptors for $PGF_{2\alpha}$ (Fitz et al., 1982; Hoyer, 1998; Juengel et al., 1996); therefore, the luteolytic effects of $PGF_{2\alpha}$ must be mediated through LLC. Upon exposure to $PGF_{2\alpha}$, there is a dramatic release of oxytocin (OT) from the CL (Ohtani et al., 1998; Ohtani et al., 2004; Shaw and Britt, 2000). Oxytocin granules are localized to LLC (Fields et al., 1992; Sawyer et al., 1986; Schams, 1987), so authors have concluded these cells are the source of luteal OT. While bovine luteal cells contain receptors for OT (Okuda et al., 1992), there is significantly more OT binding in membrane fractions isolated from porcine SLC than from LLC (Pitzel et al., 1993a). If this is also the case in ruminants and SLC have significant numbers of OT receptors, it seems possible, and even likely, that the luteolytic effects of $PGF_{2\alpha}$ on SLC in ruminants could be mediated

by the release of OT from LLC, resulting in decreased steroidogenesis and/or apoptosis in SLC.

The Role of Intraluteal Prostaglandin Production

There is a growing body of evidence that supports the hypothesis that intraluteal $PGF_{2\alpha}$ production is a critical component of luteolysis. There appears to be an "autoamplification loop" in place that results in increased luteal production of $PGF_{2\alpha}$ as a result of treatment with exogenous $PGF_{2\alpha}$ in ewes (Rexroad and Guthrie, 1979) and sows (Guthrie et al., 1979). Increased production of $PGF_{2\alpha}$ could be due to increased phospholipase A_2 (PLA_2) activity, increased mitogen activated protein (MAP) kinase activity, activation of protein kinase C (PKC), or increased mRNA and protein for cyclooxygenase-2 (Cox-2) (Reviewed in Wiltbank and Ottobre, 2003). This autoamplification loop seems to be functioning only during phases of the estrous cycle when the CL is susceptible to luteolysis (Tsai and Wiltbank, 1998), indicating that intraluteal production of $PGF_{2\alpha}$ may indeed be essential to the luteolytic process.

Griffeth (2002) also suggests that intraluteal $PGF_{2\alpha}$ production is a necessary component of luteolysis. Indomethacin inhibits the activity of cyclooxygenase-2 (Cox-2), a critical enzyme involved in the rate-limiting step of $PGF_{2\alpha}$ production. Griffeth demonstrated that treatment with indomethacin in ovine CL, and thus inhibition of luteal $PGF_{2\alpha}$ production, inhibits structural regression of the CL, although a decline in progesterone production was still observed. Griffeth (2002) also detected peaks of 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM), the primary metabolite of $PGF_{2\alpha}$, in hysterectomized ewes. Because this PGFM could not have originated in the uterus, it provides further evidence for a luteal source of luteolytic pulses of $PGF_{2\alpha}$.

The Role of Progesterone

There are two well-defined stages of luteal regression: 1. a reduction in serum concentration of progesterone and 2. structural regression of the luteal tissue (Hoyer, 1998). Reduced serum concentrations of progesterone typically precede a reduction in luteal weight (Juengel et al., 2000; Juengel et al., 1998; Juengel et al., 1995). Treatment with $PGF_{2\alpha}$ leads to activation of PKC and an influx of Ca^{++}. The activation of PKC leads to decreased steroidogenesis in ovine LLC (Wiltbank et al., 1991). The influx of Ca^{++} into LLC leads to apoptosis (Wiltbank et al., 1991; Wiltbank et al., 1989).

Progesterone can inhibit ligand-induced increases in intracellular calcium (Barbagallo et al., 2001; Crews and Khalil, 1999; Luciano et al., 1994), and progesterone is known to have anti-apoptitic effects in some cell types (Luciano et al., 1994; Peluso and Pappalardo, 1994; Rotello et al., 1992), including luteal tissue (Duffy et al., 1994; Goyeneche et al., 2003; Kuranaga et al., 2000; Rueda et al., 2000; Telleria et al., 2001). Thus, it seems possible that high intraluteal concentrations of progesterone may inhibit $PGF_{2\alpha}$ -induced increases in intracellular calcium in LLC and thus effectively block apoptosis and structural luteolysis.

It is also well-accepted that two injections of $PGF_{2\alpha}$ are more effective in inducing luteolysis in domestic livestock than one injection (Irvine et al., 2002; Wade and Lewis, 1996). It seems possible that a first injection of $PGF_{2\alpha}$ might activate PKC and cause decreased steroidogenesis. A decline in concentration of progesterone could then remove a block to apoptosis and allow a second injection to be more effective in initiating the structural demise of luteal tissue.

Progesterone could also inhibit luteolysis in a similar manner at the level of the SLC if OT does indeed trigger a calcium influx into SLC and this influx is associated with apoptosis. Progesterone has been reported to inhibit binding of OT to its receptor in murine endometrial membranes (Dunlap and Stormshak, 2004) and to have a direct effect on the OT receptor that results in decreased calcium mobilization (Grazzini et al., 1998).

Preliminary experiments were conducted to test three separate hypotheses: treatment with OT of ovine SLC will cause an increase in free intracellular Ca^{++}, high concentrations of progesterone will inhibit $PGF_{2\alpha}$ -triggered increases in free intracellular Ca^{++} in LLC, and high concentrations of progesterone will inhibit OT-triggered increases in free intracellular Ca^{++} in SLC.

Materials and Methods

Western range ewes were superovulated (Hild-Petito et al., 1987), and on day 9 following estrus CL were collected via midventral laparotomy (Fitz et al., 1982). Following pooling and dissociation, the cells were separated into small and large luteal cells via centrifugal elutriation (Fitz et al., 1982). Large (2×10^4) and small (1×10^5) luteal cells were then plated on Matrigel-coated 35 mm glass bottom culture dishes and cultured overnight in Medium 199 (M199) with charcoal-stripped fetal calf serum.

The following morning, cells were rinsed with M199 and washed twice with fluorescence buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2PO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose; pH 7.4). Cells were incubated at room temperature for 30 minutes with 3 μM Fura-2 acetoxymethyl ester (Frua-2AM) and

0.06% Pluronic F-127. Following Fura-2 loading, the cells were washed again with fluorescence buffer and incubated for an additional 30 minutes in the fluorescence buffer.

Response to oxytocin. The intracellular calcium response to oxytocin treatment in SLC was then monitored using an InCyt2 Im2 imaging system (Intracellular Imaging Inc.). After determining baseline Ca^{++} concentrations, cells were treated with 10 μM oxytocin (OT; 10 ng/ml) (Mayerhoffer et al., 1993). Free intracellular Ca^{++} changes were monitored by Fura 2 fluorescence in individual cells. The 340 and 380 nm excitation and 510 nm emission wavelengths were measured and a calcium response was defined as a simultaneous increase in 340 nm and decrease in 380 nm excitation wavelengths at the time of treatment.

Role of progesterone. Ovine LLC and SLC were cultured in Medium 199 with charcoal-stripped serum and incubated in either their own endogenous progesterone (control, C), in the presence of 215 μM (50 μg/ml) aminoglutethimide (AG) to inhibit synthesis of progesterone (Cannon et al., 2003), in the presence of 64 μM (27.5 μg/ml) RU-486 (RU) (Peluso and Pappalardo, 1994), or in 100 μM (31.45 μg/ml) of additional progesterone (P4) for four hours. Cells were then incubated in fluorescence buffer and loaded with Fura-2AM as described above, with the addition of the applicable treatments (C, AG, RU, P4) to the fluorescence buffer. After obtaining baseline intracellular Ca^{++} concentrations, SLC were treated with 10 μM oxytocin (Mayerhoffer et al., 1993) and LLC were treated with 0.5 μM $PGF_{2\alpha}$ (Wiltbank et al., 1989). The intracellular Ca^{++} response was then monitored as described above.

Oligionucleosome formation. Following the intracellular Ca^{++} measurements, the luteal cells were then rinsed and frozen at –20°C. TriReagent (Sigma Aldrich, St. Louis,

MO) was later used to isolate DNA from the cells. The DNA was run on a 1.5% agarose gel to determine whether treatment with oxytocin or $PGF_{2\alpha}$ caused oligionucleosome formation, a hallmark of apoptosis.

Hormone Quantification. The concentrations of progesterone (Niswender, 1973) in the media and buffer removed from the cells were determined using radioimmunoassay (RIA).

Statistical analysis. The number of cells responding to treatment and the magnitude of Ca^{++} response were analyzed in all treatment groups. Each plate of cells was treated as a split plot, and selected pairwise comparisons were made using a Fisher's protected LSD in proc mixed of SAS.

Results

Response to oxytocin. Following OT treatment, 24.9 percent of SLC responded with an increase in free intracellular Ca^{++}. On average, the cells that responded demonstrated a 0.50 increase in the 340/380 ratio. There was enough variability among the 23 plates of cells that the results were not significant.

Response to progesterone. There was no effect of treatment on intracellular Ca^{++} response of SLC to OT or the response of LLC to $PGF_{2\alpha}$. Following this, the culture media was analyzed for content of progesterone using radioimmunoassay (RIA) (Niswender, 1973). While the dosage of AG utilized was based on previous reports in the literature (Cannon et al., 2003), it was ineffective in reducing secretion of progesterone into the media of these luteal cells (Table 5.1).

		Mean Hormone Concentrations in Media Collected from Cultured Cells
Cell Type	Treatment	Progesterone in ng/ml
LLC	C	83.3
LLC	AG	113.6
LLC	RU	192.66
LLC	P	8737.2
SLC	C	5.9
SLC	AG	6
SLC	RU	83.2
SLC	P	4896.6

Table 5.1. Mean concentrations of progesterone in media collected from cultured ovine large luteal cells (LLC) and small luteal cells (SLC). Cells were treated with aminogluthemide (AG), RU-486 (RU), progesterone (P4), or left as untreated controls (C). Concentrations of progesterone were measured in the media using radioimmunoassay (RIA).

	Average Calcium Influx Response in Large Luteal Cells Treated with PG	
Treatment	% Cells Responding	Change in 340/380 Ratio
C	45.5	0.72
AG	65.6	0.71
RU	48.2	0.51
P	79.1	0.61

	Average Calcium Influx Response in Small Luteal Cells Treated with OT	
Treatment	% Cells Responding	Change in 340/380 Ratio
C	8.9	0.43
AG	44.5	0.7
RU	32.1	0.68
P	5.5	0.84

Table 5.2. Summary of data obtained from cultured LLC treated with PG and SLC treated with OT. Control (C), aminoglutethemide (AG), RU486 (RU), and progesterone (P4) treatments were administered to cultured cells. Cells were then treated with PG (LLC) or OT (SLC) and the concentration of free intracellular calcium was monitored. The 340/380 ratio is used to indicate the magnitude of calcium response.

Oligionucleosome formation in the cultured luteal cells was also monitored as an indicator of apoptosis (Figure 5.1). The DNA from all groups of cells showed DNA laddering when run on a 1.5% agarose gel. Thus there was no observable treatment effect and all groups of cells were experiencing some level of cellular death.

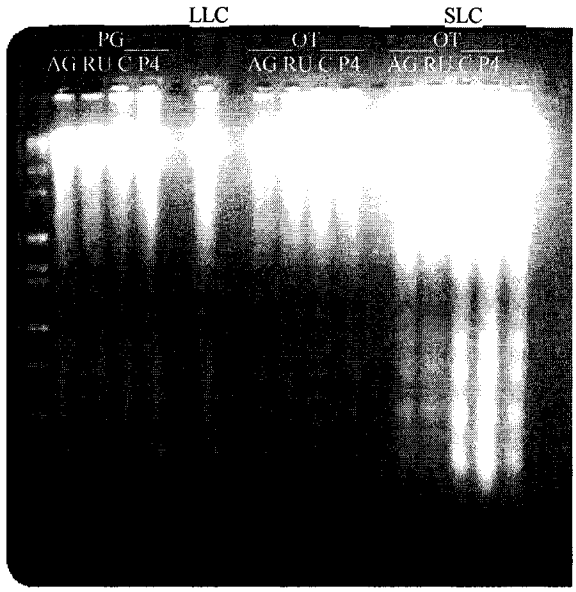

Figure 5.1. Sample agarose gel containing DNA from control LLC receiving no treatment; LLC treated with AG, RU, C, or P4 and treated with PG; LLC treated with AG, RU, C, or P4 and treated with OT; and SLC treated with AG, RU, C, or P4 and treated with OT; and SLC receiving no treatment. DNA laddering was observed in all lanes.

Discussion

While 24.9% of the SLC demonstrated an increase in intracellular Ca^{++} in response to OT treatment, these results are preliminary and should be followed up with further experiments utilizing more cells, different concentrations of OT and varying culture times to determine whether this is a real effect. If SLC do respond to OT with an increase in intracellular Ca^{++}, this response could initiate important intracellular signaling events critical to the luteolytic cascade.

Because none of the cells in the progesterone experiments were exposed to sub-normal progesterone concentrations, it is impossible to determine from these experiments whether reduced progesterone would have any effect on intracellular Ca^{++} response to $PGF_{2\alpha}$ or OT exposure (Table 5.2). The RU486 treatment did not appear to have an effect on intracellular Ca^{++} response to treatment, but because this antagonist acts only on the nuclear receptor, it is possible that progesterone could be mediating an effect at the level of the membrane.

Many other factors are involved in the production of intraluteal PG, which, in many cases, can be inhibited by high concentrations of progesterone. Cytokines (both interleukin-1β and tumor necrosis factor-α), which are detected in luteal tissue during luteolysis, stimulate production of luteal PG (Benyo and Pate, 1992; Nothnick and Pate, 1990; Townson, 1994). Interestingly, treatment of cells with progesterone inhibits the increased production of $PGF_{2\alpha}$ stimulated by cytokines (Nothnick and Pate, 1990; Townson, 1994). Another peptide found in luteolytic CL is endothelin-1 (ET-1), and ET-1 has been shown to decrease production of progesterone (Girsh et al., 1996; Hinckley and Milvae, 2001) and increase production of $PGF_{2\alpha}$ within human CL (Miceli et al., 2001). Miyamoto et al. (1997) found that ET-1 could only decrease production of progesterone after luteal exposure to $PGF_{2\alpha}$, thus ET-1 may be important only after the progesterone decline initiated by the $PGF_{2\alpha}$ released from the uterus. Other factors previously reported to be involved in luteolysis have been shown to increase luteal production of $PGF_{2\alpha}$: OT, noradrenaline, and nitric oxide (Reviewed in Wiltbank and Ottobre, 2003). While the exact interaction among all of these various signaling mechanisms has yet to be fully understood, there is redundancy in the effect on the CL:

these factors associated with luteolysis stimulate intraluteal production of $PGF_{2\alpha}$ and can often be inhibited by high concentrations of progesterone.

An Updated Model of Luteolysis

The basic theory of luteolysis is generally correct, but there are modifications that would make this model more complete. Prostaglandin released from the uterus probably initiates the luteolytic cascade (Kindahl et al., 1976; Thornburn et al., 1973). When all the evidence is considered, it appears that intraluteal production of $PGF_{2\alpha}$ is an important component of luteolysis (Griffeth, 2002; Tsai and Wiltbank, 1998). The uterus likely initiates luteolysis with pulses of $PGF_{2\alpha}$, but intraluteal production of $PGF_{2\alpha}$ may be required to complete the luteolytic process.

Whether the SLC simply undergo necrosis during luteolysis due to a decreased blood supply or whether they undergo apoptosis is another question to consider. It seems possible that OT could be a signaling molecule between LLC and SLC, but further research in this area must be conducted before any conclusions can be drawn. There is no question that OT is detected in the blood of animals undergoing luteolysis (Flint and Sheldrick, 1983b; Tetzke et al., 1987; Vanderwall et al., 1998; Wathes et al., 1984), but there remains some doubt as to the necessity of OT in luteolysis. It is possible that OT plays an intraluteal role and if OT is serving as a LLC-to-SLC communication aid, then the required levels of OT may be so minute that it is virtually impossible to deplete luteal stores of OT sufficiently to eliminate the effect of OT.

Finally, progesterone is also important in the luteolytic cascade. The first pulses of $PGF_{2\alpha}$ from the uterus activate PKC in the LLC. Protein kinase C activation leads to decreased steroidogenesis and decreased concentrations of progesterone. Activation of

PKC also leads to the intraluteal production of $PGF_{2\alpha}$, which can be prevented by high concentrations of progesterone (Pate, 1988). As progesterone is decreasing and intraluteal PG is being produced, a potential progesterone block to calcium influx may be removed and thus PG can trigger an influx of calcium into LLC. This initiates apoptosis in LLC and could trigger the release of OT, which in turn may initiate apoptosis in SLC. The net result is luteolysis, followed by another ovulation.

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