ANTILUTEOGENIC EFFECTS OF SERIAL PROSTAGLANDIN F$_{2\alpha}$ ADMINISTRATION IN MARES

Thesis

Presented in partial fulfillment of the requirements for the Master of Science in the Graduate School of The Ohio State University

Elizabeth Ann Coffman

Graduate Program in Comparative and Veterinary Medicine

The Ohio State University

2013

Thesis committee:

Carlos R.F. Pinto PhD, MV, DACT; Dissertation Advisor
Marco A. Coutinho da Silva PhD, MV, DACT; Academic Advisor
Christopher Premanandan PhD, DVM, DACVP
Abstract

For breeding management and estrus synchronization, prostaglandin F$_{2\alpha}$ (PGF) is one of the most commonly utilized hormones to pharmacologically manipulate the equine estrous cycle. There is a general supposition a single dose of PGF does not consistently induce luteolysis in the equine corpus luteum (CL) until at least five to six days after ovulation. This leads to the erroneous assumption that the early CL (before day five after ovulation) is refractory to the luteolytic effects of PGF.

An experiment was designed to test the hypotheses that serial administration of PGF in early diestrus would induce a return to estrus similar to mares treated with a single injection in mid diestrus, and fertility of the induced estrus for the two treatment groups would not differ. The specific objectives of the study were to evaluate the effects of early diestrus treatment by: 1) assessing the luteal function as reflected by hormone profile for concentration of plasma progesterone; 2) determining the duration of interovulatory and treatment to ovulation intervals; 3) comparing of the number of pregnant mares at 14 days post-ovulation. The study consisted of a balanced crossover design in which reproductively normal Quarter horse mares (n=10) were exposed to two treatments...
on consecutive reproductive cycles. Mares were examined by serial transrectal ultrasonography throughout the study. Once signs of estrus were present in conjunction with a follicle > 35 mm in diameter, ovulation was induced with human chorionic gonadotropin (hCG). When ovulation was detected (day 0), mares were randomly allotted to one of two treatment groups: I, mid diestrus treatment, administration of a single 10 mg dose of dinoprost tromethamine salt (Lutalyse®, Pfizer Animal Health, New York, NY, USA) im on day 10; II, early diestrus treatment, administration of 10 mg PGF im twice daily on days 0, 1, and 2 and then daily on days 3 and 4. Mares in estrus and with a follicle > 35 mm in diameter were artificially inseminated with at least 2 billion motile sperm from a fertile stallion and induced to ovulate with hCG. A viable pregnancy was defined as detection of a growing embryonic vesicle on two consecutive transrectal ultrasound examinations approximately 14 days post ovulation. Serial plasma samples were collected throughout the study period, and RIA was used to determine concentration of plasma progesterone. For all statistical analyses, significance was set at $P < 0.05$ and trend to significance at $0.05 < P < 0.10$. Data were expressed as mean ± SD. A mixed model ANOVA for repeated measures (SAS 9.2, SAS Institute, Inc., Cary, NC, USA) was used to analyze the concentrations of plasma progesterone. Interovulatory and treatment to ovulation intervals were compared by a paired t-test and fertility by a McNemar’s chi-squared analysis. All mares in group I underwent luteolysis after PGF administration, reflected by baseline concentrations (0.25 ± 0.21 ng/mL) of plasma progesterone on day 12, two days after treatment. In group II, all mares
experienced anti-luteogenesis, as mean concentrations of plasma progesterone remained below 1.0 ng/mL and no mare achieved a sustained rise. The mean interovulatory interval in group I was 18.5 ± 2.0 days compared with 13.1 ± 3.7 days in group II (p<0.01). For groups I and II, treatment to ovulation intervals were 8.5 ± 2.0 days and 13.1 ± 3.7 days, respectively (P<0.05). In both groups I and II, nine of ten mares became pregnant (p=1.0).

The protocol developed for the current investigation induced antiluteogenesis in all treated mares without adverse effects on fertility. Scientifically, the results of this study indicate that antiluteogenesis can be reliably accomplished by serial administration of PGF immediately after ovulation. Clinically, this innovative approach can be applied in the early luteal period to prevent luteal function and enhance artificial manipulation of the equine estrous cycle for estrus synchronization and breeding management.
Dedication

This thesis is dedicated to my family, friends, and mentors whom have made these accomplishments possible. I would like to especially acknowledge my parents, John and Cynthia Coffman, for their unconditional love and support- thanks to them I am blessed to live my dream every day; Holly Horn who has demonstrated what true friendship is and been there through all the craziness; and my mentors, Carlos Pinto and Brian Whitlock, who inspire me to be a better student, clinician, teacher, researcher, and friend.
Acknowledgements

I thank all the individuals whose contributions made this work possible: Dr. Kimberly Cole and The OSU Equine Sciences program for animal use, Dan Rhodeback for assistance with farm facilities, Dr. Chris Premanandan for expertise and contribution to my research study, Dr. C. Scott Whisnant and the North Carolina State Animal Science Program for their collaboration in hormonal assays, Tim Vojt for contribution to editing of images for this thesis and dissertation, Jim Chrzan for provision of an ultrasound unit, Chelsey Leisinger and Cydney Hoffman for research assistance, my graduate committee for their contributions to research and the writing of the thesis, and The Ohio State University Veterinary Clinical Sciences for funding. Finally, I must express my appreciation for Holly K. Snyder; without her hard work and dedication, this project would have been more difficult and less fun.
Vita

Oak Ridge High School ................................................................. May 2002

University of Tennessee, BS .......................................................... May 2005
  Majors: Biochemistry, Cellular, and Molecular biology; Psychology

University of Tennessee, DVM ...................................................... May 2009

American College of Theriogenologists, DACT ......................... August 2013
  Residency, The Ohio State University

PUBLICATIONS


Abstracts:


Book Chapter:

Whitlock BK, Coffman EA, Pugh DG. Diseases of the Endocrine System, in Sheep and Goat Medicine, 2nd ed, David G. Pugh, Editor.

FIELDS OF STUDY

Major field: Comparative and Veterinary Medicine
Theriogenology
Table of Contents

Abstract ................................................................................................................................. ii

Dedication ............................................................................................................................. vi

Acknowledgments ................................................................................................................ vii

Vita ........................................................................................................................................ viii

List of Figures ...................................................................................................................... xii

Chapter 1: Introduction .................................................................................................... 1

Chapter 2: Review of the Literature ................................................................................. 3

Chapter 3: Antiluteogenic effects of serial administration of PGF in mares ............... 49

  Introduction ........................................................................................................................ 49

  Materials and Methods ..................................................................................................... 51

  Results ............................................................................................................................... 56

  Discussion ........................................................................................................................ 60

  Figures ............................................................................................................................... 66

Chapter 4: Structural and molecular effects of antiluteogenesis and luteal resurgence in mares following PGF administration in early diestrus ........................................ 80

  Introduction ...................................................................................................................... 80
Materials and Methods .................................................................................................................. 84
Results ........................................................................................................................................ 88
Discussion .................................................................................................................................... 90
Figures .......................................................................................................................................... 94
Chapter 5: Conclusion ................................................................................................................. 102
List of Figures

Figure 3.1. Demographic data on mares used in the study ........................................66

Figure 3.2. Semen evaluation data for the ejaculates used for insemination ........67

Figure 3.3. Interovulatory interval for mares in groups I and II .........................68

Figure 3.4. Treatment to ovulation interval for mares in groups I and II .............69

Figure 3.5. Number of mares pregnant on day 14 following insemination during the induced estrus for mares in groups I and II ..........................................................70

Figure 3.6. Ultrasonographic image of a 10-day (3.5 mm) embryonic vesicle ......71

Figure 3.7. Mean concentrations of plasma progesterone before and after treatment with PGF on day 10 treatment in group I mares ........................................72

Figure 3.8. Mean concentrations of plasma progesterone on days 0 through 4 in group I ........................................................................................................................................73

Figure 3.9. Mean concentrations of plasma progesterone of mares in the diestrus of the induced cycle for groups I and II .................................................................74

Figure 3.10. Selected individual mare profiles for concentrations of plasma progesterone in groups I and II .................................................................75

Figure 3.11. Diameter of the two largest follicles of each mare in group I ......76

Figure 3.12. Diameter of the two largest follicles of each mare in group II ......77

Figure 3.13. Serial ultrasonographic evaluations of a mare in group I who developed a luteinized unruptured follicle .................................................................78

Figure 3.14. Ultrasonographic appearance of the follicle, ovulation, CH, and CL in groups I and II ........................................................................................................79
Figure 4.1. Gross appearance of the CL following ovariectomy in mares from each of the three treatments (control, antiluteogenesis, luteal resurgence)..............................94

Figure 4.2. Ultrasonographic appearance of the CL on the day of ovariectomy in mares following control (no treatment), antiluteogenic, and luteal resurgence protocols of PGF........................................................................................................95

Figure 4.3. Histopathological appearance of corpora lutea removed from mares I, II, and III on day 3 following ovulation and treatment..............................................................96

Figure 4.4. High magnification of the CL from mare II demonstrating the appearance of apoptotic luteal cells and white blood cells.................................................................97

Figure 4.5. Immunostaining of the corpora lutea for StAR in mares I, II, and III. No clear difference was evident........................................................................................................98

Figure 4.6. Immunohistochemistry for factor VIII in mares I, II, and III illustrating decreased vascularity of the CL from mare III as compared to mare I......................99

Figure 4.7. Expression of caspase 3 in mares I, II, and III by immunohistochemistry demonstrating increased apoptosis in mare III.........................................................100

Figure 4.8. Immunostaining for caspase 3 in mare III at higher magnification, illustrating positive-staining apoptotic cells.................................................................101
Chapter 1

Introduction

One of the most common procedures applied in equine reproductive management is administration of a luteolytic dose of prostaglandin F2\textsubscript{2α} (PGF) to ‘short-cycle’ a mare, inducing her return to estrus and shortening the interovulatory interval. Administration of exogenous PGF to effectively shorten diestrus in mares was first described in 1972 (Douglas and Ginther, 1972) and has since been broadly applied clinically in breeding management. Traditional doctrine and standard clinical approaches assert that the equine corpus luteum (CL) is not consistently responsive to the luteolytic effects of a single dose of PGF until at least five days after ovulation (Allen and Rowson, 1973), approximately one third of diestrus in a normal estrous cycle in a nonpregnant mare. Thus, the implications for this postovulatory refractory period significantly impact the equine industry and our ability as veterinarians to manipulate the reproductive cycle. Ability to treat mares early in diestrus (during the ‘refractory period’) could greatly enhance our control of the equine estrous cycle through early luteolysis or prevention of diestrus.

Recent research has begun to challenge the methodology of administering a single dose (10 mg dinoprost tromethamine) of PGF in mid diestrus. Significantly lower doses of PGF (e.g. a single dose of 1.25 mg, two doses of 0.5 mg at a 24-hour
interval) are capable of inducing luteolysis of the mature CL (Irvine et al., 2002, Barker et al., 2006). Furthermore, multiple doses of PGF administered in early diestrus are capable of interrupting CL function. The PGF–induced pharmacologic responses elicited included complete early luteolysis (Holland and Pinto, 2008, Rubio et al., 2008), partial luteolysis followed by luteal resurgence (Bergfelt et al., 2006, Holland and Pinto, 2008, Rubio et al., 2008), and antiluteogenesis (Rubio et al., 2008). These processes are associated with early cessation of luteal function and concomitant decline in concentration of plasma progesterone, decreased concentration of plasma progesterone followed by a return to luteal concentrations, and failure of normal CL formation and rise in concentration of plasma progesterone, respectively.

In an innovative approach, it was demonstrated that administration of 10 mg PGF (dinoprost) twice daily on days 0, 1, and 2 prevented diestrus in four out of six mares, with concentrations of plasma progesterone remaining below 1 ng/mL and resulted in a mean interovulatory interval of 7 ± 1.8 days for those four mares (Rubio et al., 2008). This effect has been christened as ‘antiluteogenesis.’ In the present study, the specific aim was to modify the previously described treatment protocol to elicit complete antiluteogenesis in all mares treated and to assess the effects of such treatment on fertility in mares artificially inseminated in the post-treatment cycles.
Chapter 2

Review of the Literature

The corpus luteum (CL) is a transient endocrine structure formed from the ruptured ovulatory follicle that wields significant reproductive function. The production of progesterone by this gland is responsible for creation of an environment favorable for embryonic and fetal survival (e.g. production of uterine milk by endometrial glands, promotion of uterine quiescence, mediation of cervical closure, prevention of estrual behavior; etc.). In both pregnant and nonpregnant domestic animals, the formation of the CL following ovulation constitutes a period of rapid cellular growth characterized by both hypertrophy and hyperplasia of luteal cells and extensive vascular (capillary) ingrowth to create one of the most highly vascularized tissues in the body (McCracken et al., 1999). In pregnant mares, an embryo-derived signal must prevent cyclical release of the PGF from the endometrium, a process mediated partly via down regulation of expression of cyclooxygenase 2 (Klein and Troedsson, 2011). The signal that provides maternal recognition of pregnancy in mares has been elusive in its identification but mobility of the embryo is essential in the process (Zavy et al., 1984, Mcdowell et al., 1988). In
nonpregnant mares, a series of physiologic events trigger functional and structural regression of the CL, effectively terminating to diestrus. Following luteolysis and the cessation of luteal progesterone steroidogenesis, mares return to estrus. This cyclicity during the breeding season affords multiple opportunities for mares to become pregnant.

Luteolysis in the equine is unique as compared with other species, specifically the bovine (Ginther, 2009). One distinction in mares is the systemic route whereby PGF induces luteolysis in contrast to the local mechanism responsible in ruminants (Ginther, 1974). A number of different experiments provide evidence for this phenomenon. The vascular anatomy of the reproductive tract in mares is different from farm animal species (Ginther et al., 1972); the spatial separation between the uterine vein and ovarian artery rather than an intimate association present in other species precludes countercurrent exchange of the uterine luteolysin for local transport to the ovary. Furthermore, unilateral hysterectomy does not result in differential duration of diestrus regardless of whether the CL is contralateral or ipsilateral to the removed uterine horn (Ginther and First, 1971, Stabenfeldt et al., 1974b). Finally, administration of PGF via a local (intrauterine) versus systemic route does not result in differential dose requirements to induce luteolysis in mares as it does in heifers (Douglas and Ginther, 1975).

There are other unique aspects to the process by which PGF signals the demise of the CL in mares. The equine CL has an 18-fold greater sensitivity to PGF
as compared to the bovine (Douglas and Ginther, 1975, Ginther, 1992). One potential explanation for this difference is increased binding affinity receptors for PGF in the equine CL (Mattioli et al., 1983). In addition, plasma clearance of exogenous PGF is about five times lower in mares as compared to heifers, resulting in a threefold increased in the half-life of the hormone (Shrestha et al., 2012).

**Reproductive Endocrinology of Mares**

Mares are seasonal polyestrous long day breeders. Exposure to prolonged photoperiods with increasing day length results in decreased release of melatonin from the epiphysis. This results in decreased inhibition of the hypothalamic-hypophyseal-gonadal axis, increased secretion of gonadotropin-releasing hormone (GnRH) and resultant increased expression of other reproductive hormones (prolactin, gonadotropins, ovarian steroids, etc.), and cyclicity. The period of vernal transition is characterized by development of steroidogenically incompetent follicles and behavioral estrus without ovulation. Following the first ovulation of the season, mares enter a period of reproductive cyclicity marked by repeated return to estrus and multiple opportunities to reproduce.

The average estrous cycle is approximately 21 to 22 days, with estrus lasting an average of five to seven days and ending approximately 24 to 48 hours after ovulation. The control of reproductive phenomena in mares involves the interaction of many different organs and systems, and many unique aspects of
equine physiology have long been recognized (Stabenfeldt et al., 1975). The basic endocrine mechanisms by which it occurs will be briefly outlined.

**Hypothalamus**

The notion that the brain is a regulator of endocrine function dates back to the 1930s (Marshall, 1936), and the hypothalamus is the neurological center in the brain for integration of body function. Observation and description of the anatomic configuration of the hypothalamic-hypophyseal portal system led to the hypothesis that the hypothalamus plays a role in the regulation of adenohypophyseal function (Harris, 1955). This theory was validated with successful catheterization of the portal vessels and identification of releasing factor hormones (Wilber and Porter, 1970). The hypothalamus is anatomically and physiologically divided into various nuclei, each with specified actions. For example, the medial preoptic nucleus is essential in regulation of the gonadotropins and sexual behavior, and it contains the sexually dimorphic nucleus (Simerly, 2002). Several important releasing hormones from the hypothalamus affect adenohypophyseal function via the hypothalamic-hypophyseal portal system.

The most important hypothalamic factor in reproduction is GnRH, with dopamine and prolactin-releasing hormone also playing noteworthy roles. GnRH is primarily synthesized in the preoptic nuclei of the hypothalamus and is traditionally considered the apex of endocrine control of reproductive function (Melrose et al.,
Only recently have controlling factors affecting the release of GnRH received greater attention [e.g. kisspeptin (Decourt et al., 2008, d'Anglemont de Tassigny and Colledge, 2010)]. GnRH is found in virtually all vertebrate species and is highly conserved. The elucidation of its decapeptide structure in 1971 simultaneously by two different laboratories resulted in the awarding of the Nobel Prize (Burgus et al., 1971, Matsuo et al., 1971, Nair et al., 1971). Release of GnRH from the hypothalamus at the median eminence into the venous portal system and subsequent binding to its receptors in the adenohypophysis stimulates the release of the gonadotropins - follicle-stimulating hormone (FSH) and, especially, luteinizing hormone (LH).

In mares, the release of GnRH is pulsatile, and pulsatility varies with season and hormonal environment. The pulsatility is most evident during anestrus, seasonal transition, and diestrus, yet release is more continuous during estrus (Sharp and Grubaugh, 1987). The GnRH pulses exhibit a high amplitude coupled with low frequency in mid-diestrus (Irvine and Alexander, 1993), and they shift to high frequency and low-amplitude during the preovulatory elevation of LH (Irvine and Alexander, 1994).

Gonadotropin-inhibitory hormone (GnIH) is a recently identified neuropeptide synthesized in the hypothalamus with function opposite of GnRH in that it inhibits the release of LH (Tsutsui et al., 2010). The first vertebrate in which it was isolated was the quail in 2000 (Tsutsui et al., 2000). Since that time, it has
been identified in many other species and its reproductive functions further elucidated. This dodecapeptide is a member of the RF-amide-related peptide family. Its release at the median eminence affects the adenohypophysis in a manner opposite that of GnRH, inhibiting the release of the gonadotropins, LH and FSH. GnIH-releasing neurons have direct anatomic connections to GnRH-releasing neurons in the hypothalamus and decrease secretion of the gonadotropins in the adenohypophysis; it thereby affects gametogenesis and steroidogenesis via modulation of endocrine function and locally in an autocrine/paracrine manner, and GnIH release is modulated by melatonin in birds and mammals (Tsutsui et al., 2010). In mares, findings remain preliminary and functional significance remains hypothetical. Neurons containing this hormone have been identified in the hypothalamus of mares (Velez, 2009), and it has been hypothesized that it may actually contribute to seasonality of mare reproduction more than variation in the frequency or amplitude of GnRH pulsatility does (Cooper et al., 2006).

**Hypophysis**

The hypophysis is an unpaired gland located at base of the brain in the sella turcica of the sphenoid bone. It has dual embryonic origin. The adenohypophysis arises from a dorsal outpouching of the stomodeum (Rathke’s pouch). The adenohypophysis fuses with a ventral outpouching from the diencephalon that comprises the neurohypophysis, and together they make up the hypophysis. This
relatively small gland is one of the most important endocrine centers in vertebrate animals.

**Neurohypophysis- Oxytocin**

In contrast to the adenohypophysis, hormones move from the hypothalamic nuclei to the neurohypophysis via axoplasmic flow. Hormones produced in the hypothalamus travel to the neurohypophysis in long axons to be released from the latter location. Oxytocin is produced in the paraventricular and supraoptic nuclei of the hypothalamus and released from the neurohypophysis; it has integral functions in reproduction. This nonapeptide hormone has multiple and various effects according to its timing and degree of release. In mares, these include but are not limited to milk ejection (Starbuck, 2006), uterine contraction during parturition (Ferguson reflex) (Fowden et al., 2008), and luteolysis (Behrendt-Adam et al., 1999).

Oxytocin has a very short half-life in mares. Following exogenous hormone administration, half-life of oxytocin in mares has been found to be approximately 6.8 minutes (Paccamonti et al., 1999). Its primary effects are mediated by stimulation of smooth muscle contraction. Binding to smooth muscle in the uterus stimulates myometrial contractions of the reproductive tract and in the mammary gland results in milk ejection. Additionally, oxytocin is a critical endocrine component in parturition. Stimulation of the cervix during parturition triggers a neurohormonal reflex (Ferguson reflex) whereby release of oxytocin stimulates further contraction.
(Ferguson, 1941); the result is a positive feedback system that is integral to fetal expulsion. Therefore, increases in concentration of oxytocin are primarily detected during the second stage of parturition in mares (Allen et al., 1973).

The ecbolic effects of oxytocin facilitate its primary pharmacological applications. Exogenous administration of oxytocin induces uterine contraction to facilitate uterine clearance in breeding management and induction of parturition. The use of oxytocin to aid in uterine clearance increases fertility after natural mating or artificial insemination (Pycock and Newcombe, 1996), resulting in widespread clinical utilization in mares with persistent post-breeding endometritis and delayed uterine clearance. It has been suggested that some of the myometrial effects may be propagated and protracted by stimulation of PGF release, as exogenous oxytocin results in increased concentration of PGF as detected by measurement of increased concentration of its metabolite (15-keto-13,14-dihydro-PGF2alpha metabolite, PGFM) (Cadario et al., 1999).

Release of oxytocin by the neurohypophysis concomitantly with an increased number of endometrial oxytocin receptors in late diestrus in the nonpregnant mare is responsible for release of PGF from the endometrium and luteolysis. The neurohypophysis is the sole source of oxytocin triggering luteolysis in mares; by contrast in ruminants, oxytocin is also produced by the CL itself and plays a significant role in the luteolytic process (McCracken et al., 1999). An illustration of the luteolytic role of oxytocin is provided as stimulation of the cervix in mares on
days five and seven of diestrus results in reflex release of oxytocin and premature luteolysis (Handler et al., 2003); however, no increase in PGFM was definitively detected following treatment in that experiment. In contrast, administration of supraphysiologic doses of oxytocin on days seven through fourteen following ovulation can be used to prolong diestrus (Vanderwall et al., 2007). Further elaboration of the mechanisms whereby luteolysis is signaled in the equine is provided in the section detailing luteolysis below.

**Adenohypophysis- Gonadotropins**

The adenohypophysis is responsible for the release of multiple hormones and is a significant center of endocrine function. The adenohypophysis comprises approximately 80% of the hypophysis. It is composed on the pars distalis, pars intermedia, and pars tuberalis. The pars distalis is the primary portion of functional significance will henceforth referred to as the adenohypophysis. Traditionally, the cells of the adenohypophysis have been classified according to their primary hormone product (e.g. gonadotropes secrete gonadotropins). Recent research has suggested the physiology is more complicated with cells capable of secreting more than one hormone (Mignot and Skinner, 2005).

The hypothalamus communicates with the adenohypophysis via a neurohumoral mechanism and the hypophyseal-adenohypophyseal portal system.
Secretion of hypothalamic releasing factors at the median eminence into this specialized vascular system is followed by transport to the adenohypophysis, binding to the hormone receptors on target cells, and release of adenohypophyseal hormones. The primary control for the release of LH and FSH comes from the central nervous system, mainly via GnRH (Pawson and McNeilly, 2005). The steroid hormones, estradiol and progesterone, and the glycoprotein hormones, inhibin and activin, produced by the ovaries provide negative feedback to the hypothalamus and adenohypophysis to down regulate the production of GnRH and the gonadotropins (Fink, 1979, De Jong, 1988, Ying, 1988).

LH and FSH are both glycoprotein heterodimer hormones consisting of two alpha and two beta subunits held together by intermolecular forces (Gharib et al., 1990). The alpha units are identical for FSH, LH, and TSH; it is the beta unit confers the unique tertiary configuration of each hormone and conveys its specificity (Bousfield et al., 1996). Whereas the molecular structure of FSH is highly conserved between species, the glycosylation is variable (Dalpathado et al., 2006). The targets for these hormones are primarily the gonads, where binding to their specific g-protein linked receptors regulates steroidogenesis and gametogenesis.

The secretory patterns of FSH and LH are pulsatile, as with GnRH, and the pulses occur concomitant to increases in plasma GnRH (Irvine and Alexander, 1993). However, immunization of mares against GnRH results in a decrease in concentration of plasma LH but not FSH, suggesting greater specificity for GnRH in
signaling the release of LH as compared to FSH (Garza et al., 1986). The differential expression of FSH and LH is also evident with the observation of a mid diestrus rise in FSH, which occurs at a variable day in the cycle for individual mares and is not coupled with an increase in LH (Ginther et al., 2005). The pattern of LH secretion in the mare is unique among domestic animals. As in other species, a periovulatory surge in LH signals the onset of ovulation, but the elevation of LH in mares is more protracted (beginning with luteolysis and lasting up to 10 days) and peak concentration does not occur until one day after ovulation (Whitmore et al., 1973, Geschwind et al., 1975, Ginther, 1992). Characterization of concentration of plasma LH throughout the cycle described low concentrations from days 5 through 16 of the estrous cycle followed by a slow increase that does not peak until three days after ovulation (Geschwind et al., 1975).

In addition to the gonadotropins, the adenohypophysis secretes prolactin. This 100 amino acid polypeptide hormone (Li and Chung, 1983) is primarily controlled by inhibitory hormones from the hypothalamus (predominantly prolactin-inhibiting hormone, or dopamine). The primary function of prolactin, as the name suggests, is to mediate mammary gland development and milk production and secretion (Worthy et al., 1986). In horses, concentration of prolactin in circulation has also been suggested to play a role in seasonality (Nequin et al., 1993, Hodson et al., 2010) and luteolysis (King et al., 2010).
**Ovaries- Steroids and other hormones**

The primary target organ for the gonadotropins, as their name suggests, is the gonad. The two essential reproductive functions of the gonads are steroidogenesis and gametogenesis, and LH and FSH regulate both processes. Additional hormone products of the ovaries (and testes) also contribute to the regulation of reproductive function.

The primary hormone products of the ovary are the steroids, estrogen and progesterone. These hormones are produced primarily during the follicular and luteal phases of the estrous cycle, respectively. The various effects of estrogen are primarily mediated through binding to its nuclear receptors, estrogen receptors (ER) alpha and beta (Kuiper et al., 1997). As a steroid hormone receptors, ERs are transcription factors containing a ligand-binding domain, and binding of the hormone to the receptor results in alteration in gene expression (Brzozowski et al., 1997). During the follicular phase, estrogen synthesis in the ovary occurs by the two-cell two-gonadotropin model where binding of LH to receptors stimulates androgen production by theca cells which is converted to estradiol by aromatase in the granulosa cells under FSH influence (Short, 1962, Sirois et al., 1991). Consistent with this model, steroid acute regulatory protein (StAR), cytochrome P450 side-chain cleavage family of enzymes (P450_{SCC}, CYP11A1), and cytochrome P450_{c17} (CYP17) are essential enzymes for steroidogenesis found in equine theca cells whereas cytochrome P450aromatase (CYP19) expression is localized to granulosa
cells (Watson et al., 2004). Concentration of estrogens in blood is highest during estrus and reflects follicular growth; peak concentrations occur approximately two days prior to ovulation (Ginther et al., 2008a). It is interesting to note that the equine CL also demonstrates production and secretion of estrogen due to increase expression of cytochrome P450C17 (CYP17) (Daels et al., 1991), but the significance of this change is not clear.

In mares, estrogen has significant reproductive effects [enhancement of reproductive behavior (Andrews and McKenzie, 1941), augmentation of uterine immune defenses and phagocytic activity (Washburn et al., 1982), increased myometrial activity (Taverne et al., 1979) and uterine clearance (Evans et al., 1987), etc.]. Estradiol also apparently increases steroid hormone receptors (for both estrogen and progesterone) in the endometrium in mares as in other species (Spencer and Bazer, 1995) as the highest concentrations of steroid hormone receptors in the endometrium occur during estrus (Hartt et al., 2005).

In addition to its other functions, the feedback effects of estradiol on the expression of other hormones are integral to reproductive cyclicity in mares. Increasing levels of estrogen in mares have been shown to have an inhibitory effect on FSH secretion (Burns and Douglas, 1981). Conversely, the most important effects of estradiol on LH in the horse are primarily stimulatory (Garcia et al., 1979, Burns and Douglas, 1981), ultimately culminating in peak concentrations of estradiol from
the dominant follicle during estrus, followed by a further elevation in LH and ovulation.

In addition to estradiol, follicular production of inhibin (which also occurs primarily in granulosa cells) plays an important role in regulation of reproduction. Inhibin is a glycoprotein hormone and member of the transforming growth factor β superfamily of hormones and consists of alpha and beta subunits connected by disulfide bonds (Ling et al., 1985, Mason et al., 1985). Different forms of inhibin are formed based on post-translational processing of the alpha subunit (Knight and Glister, 2001), and whether the βA or βB subunit is present (designated as inhibin A or B, respectively, and described in mares) (Moore et al., 1994). As follicular divergence occurs, the dominant follicle acquires increased sensitivity to the gonadotropins (particularly LH), and increased production of estrogen and inhibin by the dominant follicle contribute to decreased gonadotropin expression (especially FSH) and atresia of the remaining follicles in the cohort. Inhibin concentration is inversely related to concentration of FSH in mares (Bergfelt et al., 1991), reflecting its role in inhibition in secretion of FSH (Ying, 1988). Additionally, studies in other species and in vitro using equine tissue suggest various paracrine and local effects at the level of the ovary. These include increased LH-induced androgen production by theca cells (Wrathall and Knight, 1995) and inhibition of oocyte maturation (O et al., 1989); the local effects of inhibin on oocytes are theorized but less well-defined.
Activin is a glycoprotein hormone dimer consisting of two β subunits of inhibin joined by disulfide bonds that stimulates, contrarily to inhibin, secretion of FSH from the adenohypophysis (Ling et al., 1986a, b, Doi et al., 1992). However, most circulating activin is bound to the high-affinity binding protein, follistatin (Woodruff, 1998), rendering it biologically inactive. Therefore, its most important functions occur at the ovary (Knight and Glister, 2001). Whereas function of activin in mares is yet to be fully meted out, studies in other species and in vitro using equine-derived cells demonstrate potential effects, including stimulation of granulosa cell proliferation (Li et al., 1995), induction of FSH receptor expression in granulosa cells (Xiao et al., 1992), decrease in theca cell LH- and estradiol-induced androgen production (Wrathall and Knight, 1995), and acceleration of oocyte maturation (Sadatsuki et al., 1993) or developmental competence (Silva and Knight, 1998).

Following the LH elevation, ovulation, and formation of the CL, the predominant steroid product of the ovary is progesterone. During pregnancy in the mare, the fetoplacental unit is an additional source of progesterone in addition to other progestins (the 5-alpha pregnanes) with similar hormonal actions (Allen et al., 2002). As its name suggests, progesterone promotes a reproductive environment supportive of pregnancy. Its effects are so essential to the maintenance of pregnancy that premature luteal regression results in pregnancy loss in mares and administration of exogenous progestins can maintain pregnancy in the absence of
endogenous hormone (Allen, 2001, Canisso et al., 2013). In the absence of pregnancy, release of PGF by the endometrium induces luteolysis, decrease in concentration of plasma progesterone to baseline, and return to estrus.

Progesterone exerts negative feedback on the hypothalamus and adenohypophysis, resulting in decreased concentration of plasma of LH (Gastal et al., 1999, Ginther et al., 2006). In contrast, progesterone does not significantly inhibit FSH expression (Garcia et al., 1979); therefore, follicular growth continues during diestrus. As a result, the most effective method of pharmacologic synchronization of the estrous cycle in mares is through concurrent administration of both steroid hormones (progesterone and estradiol) in conjunction with luteolytic PGF to effectively suppress gonadotropin secretion, follicular growth, and estrus (Lofstedt, 1988).

**Prostaglandins- PGF and PGE**

The primary prostaglandins associated with reproduction are PGF and prostaglandin E2 (PGE). These hormones are eicosanoids- 20-carbon polyunsaturated fatty acids derived from the cleavage of membrane phospholipids. Whereas prostaglandins are primarily known for their inflammatory functions, PGF and PGE are essential reproductive hormones with critical roles in ovulation, maternal recognition of pregnancy, parturition, and luteolysis. In pregnant mares, production of PGE is essential to allow oviductal transport and selective transport of
fertilized embryos at the uterotubal junction (Weber et al., 1991, Freeman et al., 1992). In addition, stimulation of myometrial contraction by embryonic PGF and PGE are essential to promote embryonic motility for maternal recognition of pregnancy in mares (Stout and Allen, 2001) and parturition (Ousey, 2006). The roles of prostaglandins in the dynamic processes of ovulation and luteolysis are perhaps even more critical and will be further elaborated in the following sections.

**Luteogenesis & Luteolysis**

Much of the published information to date regarding the processes of ovulation, luteogenesis or luteinization, and luteolysis has been focused on rodents, primates, and domestic ruminants. The unique aspects of the equine reproduction regulation and function will be highlighted in the following section.

**Ovulation**

Ovulation is the process whereby the follicle ruptures to release the oocyte for capture by the infundibulum, transport in the uterine tubes, and potential fertilization. In fact, it is a much more complicated phenomenon involving oocyte maturation and resumption of meiosis, cumulus expansion and release, and a complex set of cellular changes (Conti et al., 2012); theca and granulosa cell gene expression is altered to mediate these events (e.g. increased granulosa cell
expression of proteases and other genes associated with luteinization coupled with decreased transcription of genes promoting granulosa cell proliferation and steroidogenesis) (Richards et al., 2002). The importance of this process and the mechanism whereby it occurs have long been a target of scientific wonder.

Although historical scientists as early as Aristotle (384-322 B.C.) and Herophilus (335-280 B.C.) demonstrated interest in reproduction and the existence of ovaries as ‘female testes’ (Hunter, 2003), the discovery of the processes integral to female reproductive physiology has been long and ongoing. Regnier de Graaf (1641-1673) is given credit as being the first to recognize the significance of large follicles as the source of the female gamete (Jocelyn and Setchell, 1972). Scientific reviews on ovulation were published as early as the 1920s (Corner, 1923), the processes in the ovary whereby ovulation occurs and its consequence have been further elucidated.

In the 1960s, major advances in determination of the etiological basis of follicular rupture occurred; a 1970 review outlining the hypothesized causes for the physical rupture of the mature follicle at ovulation included increased intrafollicular pressure due to fluid influx or muscle contraction, enzymatic degradation of the follicular wall, or necrotic changes (Rondell, 1970). Research in the 1960s failed to detect an increase in intrafollicular pressure in rats and rabbits prior to ovulation (Blandau and Rumery, 1963, Espey and Lipner, 1963). Instead it seems that changes in follicular wall membrane distensibility result in rupture at normal
pressures, and these changes are mediated by enzymatic collagenolysis (Rodbard, 1968, Rondell, 1970, Espey, 1974).

In what has since become one of the sentinel papers on the subject, the process of ovulation is compared to an inflammatory reaction (Espey, 1980). This parallel is based on similar vascular changes (hyperemia, edema, vascular permeability); white blood cell migration; fibroblast movement; and the roles of similar regulatory molecules (cyclic nucleotides, steroids, histamine, bradykinin, platelet-activating factor, and prostaglandins). The many similarities have perpetuated this analogy between the two events (Espey, 1994, Richards et al., 2002). Whereas steroid hormones, particularly estradiol and progesterone, and gonadotropins, FSH and LH, are the key modulators of ovarian function, a variety of other molecules play integral roles in ovulation, including cytokines (Brannstrom et al., 1994), proteolytic enzymes (Brannstrom et al., 1988), and growth factors (Koos, 1995, Richards et al., 2002). Many of these are also integral to inflammation. Nitric oxide (NO) is one such signaling molecule that is apparently essential to ovulation in mares (Pinto et al., 2002, Pinto et al., 2003). Studies in the horse have also found increased expression of prostaglandin G/H synthase 2 (cyclooxygenase 2) in preovulatory follicles as ovulation approaches (Boerboom and Sirois, 1998). Accordingly, following induction of ovulation by hCG administration in mares, there is an increase in PGF, PGE₂, 6-keto-PGF₁α, and thromboxane B₂ in follicular fluid (Watson and Sertich, 1991). Furthermore, administration of indomethacin (a non-
steroidal anti-inflammatory drug) resulted in prolonged interval to ovulation following hCG administration and lower production of progesterone for the first five days of the diestrus (Watson and Sertich, 1991).

There are many changes within the mature preovulatory follicle that occur to afford rupture and formation of the corpus hemorrhagicum. In most animal species, follicular rupture and oocyte release occur at the stigma located at the apex of the ovulating follicle; this portion consists of five cell layers that undergo dramatic changes, including marked thinning and dissociation of the fibroblasts (Espey, 1967). Mares are unique in that the ovarian cortex and medulla are inverted, and ovulation must occur through a depression in the ventral border known as the ovulation or ovarian fossa (Witherspoon and Talbot, 1970, Witherspoon, 1975). A similar apex of the preovulatory follicle has been observed via transrectal ultrasonography in the equine at the ovarian fossa (Gastal et al., 2007).

Following rupture, collapse of the follicular wall and interdigitation of the cells and blood vessels creates a network for cellular migration and restructuring to form the CH, then the CL. Structural changes in the preovulatory follicle prepare it for CH formation. Increased blood flow to the theca cells in the equine preovulatory follicle is reflected by increased immunostaining for VEGF, foreshadowing the formation of the highly vascular corpus luteum (Watson and Al-Zi’abi, 2002). Furthermore, immunohistochemical staining illustrates strong expression of angiopoietin 2, VEGF A, Tie2 (angiopoietin receptor), and VEG-receptor 2 in the
theca, granulosa, luteal, and endothelial cells of the periovulatory follicle of mares (Muller et al., 2009).

Changes within the follicle itself begin the functional as well as structural transition from follicle to CL. As LH levels increase, the mature follicle responds by decreasing estradiol and increasing progesterone secretion (Richards and Hedin, 1988). Based on follicular response to human chorionic gonadotropin (hCG) administration in the mare, it is hypothesized that as concentration of plasma LH reaches a threshold level, the follicle attains a static state of growth and there is a negative impact on estradiol production (Gastal et al., 2006). This is supported as administration of hCG results in immediate decrease in concentration of plasma estradiol (Gastal et al., 2006). After gonadotropin injection, equine follicular fluid is characterized by increased concentration of progesterone in addition to the decrease in concentration of estradiol (Belin et al., 2000), and in vitro equine granulosa cells secrete progesterone in response to recombinant equine LH (Bott et al., 2011). The significant hormone components of the large volume of follicular fluid are absorbed from the abdomen, causing a transient suppression of LH and FSH (Nambo et al., 2006). A recent review highlights our understanding of the hormonal interrelationships among steroids, gonadotropins, and inhibin in the periovulatory period in mares (Ginther et al., 2008a).

The hormonal changes can be evaluated according to the key regulators responsible for steroidogenesis (Smith et al., 1994). Decrease in estradiol
production and increased progesterone production in mares may be mediated by decreased androgen production secondary to decreased cytochrome P450 17alpha-hydroxylase (CYP17) in theca cells (Boerboom et al., 1999), decreased aromatase (CYP19) expression by granulosa cells (Schauer et al., 2013), and increased expression of steroid-acute regulatory protein (StAR) (Kerban et al., 1999). These distinct changes in the periovulatory period have been documented in mares and are suggested to serve as molecular markers of luteinization (Murphy, 2000). In addition, an increase in expression of enzymes responsible for the sulfoconjugation of estrogens render the synthesized estradiol biologically inactive (Brown et al., 2006). In contrast to other species (Couet et al., 1990), expression of 3ß hydroxysteroid dehydrogenase (3ß-HSD) was not found to change following hCG induction of ovulation in equine follicles (Boerboom and Sirois, 2001). The biological significance of microRNAs in regulation of gene expression is only recently being recognized, and changes in miRNA expression are implicated in the regulation of steroidogenic enzymes (Schauer et al., 2013).

Ovulation must occur appropriately and precede luteinization. Mice null for cAMP-specific phosphodiesterase 4 (Jin et al., 1999) and progesterone receptor (Lydon et al., 1995) have ovulation delayed and luteinization accelerated, respectively. The oocyte then becomes trapped in the forming CL. This highlights the importance of temporal regulation of these complex events. Therefore, alterations within the ovary begin at ovulation to prepare for luteal formation.
Formation of the Corpus Luteum: Luteogenesis

Following ovulation and collapse of the mature follicle, the ovarian follicular tissue must undergo significant reorganization (luteinization) to form the structure of the CL- a gland with great reproductive function during its relatively limited lifespan in nonpregnant and pregnant mares. Just as an analogy can be drawn between ovulation and inflammation, luteinization has been compared to the process of wound repair (Smith et al., 1994). Marcello Malpighi (1628-1694) is credited as being the first to describe corpora lutea and coining the term ‘corpus luteum’ based on observation of the yellow structures on cow ovaries (Motta, 1989). Interestingly, he even recognized the glandular nature of the CL, but he erroneously believed the CL to be the source of the oocyte (Hunter, 2003). The functional importance of the CL was not recognized until 1901, when ovariectomy and enucleation of corpora lutea from pregnant rabbits resulted in loss of pregnancy (Fraenkel, 1910, Diaz et al., 2002). It was not until the 1930s that the steroid hormone produced by the CL was purified (Allen and Wintersteiner, 1934, Butenandt et al., 1934, Hartmann and Wettstein, 1934, Slotta et al., 1934) and named progesterone (Slotta et al., 1934).

Following ovulation, the collapsed follicular cells terminally differentiate to luteal cells (a process initiated prior to ovulation) and significant neovascularization occurs (Smith et al., 1994). Breakdown of the basement membrane and in folding of
the follicular wall enable migration of endothelial cells, fibroblasts, theca cells, and macrophages (Smith et al., 1994). In mares, only the granulosa cells contribute to the forming CL and develop into luteal cells (Broadley et al., 1994). Nonetheless, small and large luteal cells have been described in the equine CL. Large luteal cells increase in number as the CL develops parallel with progesterone production and are the only type with progesterone receptors (Ferreira-Dias et al., 2002). Both cell types are capable of progesterone production (Broadley et al., 1994). The fate of theca cells has yet to be fully elucidated. However, it is possible that programmed cell death plays a critical role as in other species (Tilly et al., 1991), as treatment of rat luteal cells in vitro with gonadotropins results in increased expression of caspases 3 and 7 reflecting increased apoptosis (Yacobi et al., 2004).

The forming CL grows very rapidly, doubling in area every 60 to 70 hours during its rapid growth phase in the bovine, a rate of cellular growth equivalent to the most aggressive forms of neoplasia (Ricke et al., 1995, Reynolds et al., 2000). In addition, the CL achieves one of the highest levels of blood flow of any tissue in the body (Abdul-Karim and Bruce, 1973, Ford and Chenault, 1981, Wiltbank et al., 1988). In order to accomplish this high rate of growth and progesterone production, rapid and controlled vascular ingrowth must occur (Reynolds et al., 2000, Shirasuna et al., 2012). Endothelial cell proliferation in the early luteal phase paves the way for increased blood flow for provision of metabolic support of the division and metabolism of other cell types. The rate of angiogenesis in some species has been documented as up to four to 20 times greater than the most
aggressive forms of cancer (Tamanini and De Ambrogi, 2004). Furthermore, the CL has the highest rate of blood flow of any organ in the body (Reynolds, 1986, Zheng et al., 1993, Redmer and Reynolds, 1996), and vascular cells comprise a greater proportion of cells in the CL (~80% in the bovine) than steroidogenic cells (~30%) (O’Shea et al., 1989). In mares, the angiogenic factors of the CL have come under investigation predominantly in the last decade. One of the seemingly most important, and most studied, factors controlling endothelial cell proliferation within the developing equine CL is VEGF (Al-zi’abi et al., 2003, Tamanini and De Ambrogi, 2004, Muller et al., 2009). The strong expression of angiopoietin 2, VEGF A, Tie2, and VEG-R2 in the theca, granulosa, luteal, and endothelial cells of the periovulatory follicle that begins at ovulation in mares continues into the early luteal period (Muller et al., 2009). In the equine CL, tumor necrosis factor alpha and nitric oxide may regulate the expression of angiogenic factors and PGE (Ferreira-Dias and Skarzynski, 2008). It is suggested in mares that the synchronized proliferation of hormone-producing luteal cells in conjunction with vascular cells allows for the early synthesis of progesterone by the equine CL (Ferreira-Dias and Mateus, 2003). The cytokines, tumor necrosis factor alpha, interferon gamma, and Fas ligand, play a role in equine luteal angiogenesis via modulation of VEGF (Galvao et al., 2012).

Progesterone production by the CL is arguably its most important function (Niswender and Nett, 1988). In mares, CYP11A1 is primarily expressed in theca cells prior to ovulation, but expression increased significantly in granulosa cells
throughout the ovulatory process (Boerboom and Sirois, 2001). This is coupled with decreased CYP17 beginning at ovulation (Boerboom et al., 1999). Continued expression of these steroidogenic enzymes by luteal cells affords expression and secretion of luteal concentrations of progesterone. Serial biopsies of the CL and RT-PCR for gene expression show an increase in expression of 3ß–HSD and a positive correlation between the two (Slough et al., 2011). Mature corpora lutea in nonpregnant mares are negative by immunostaining for CYP17, and the CL in the pregnant mare does not stain positively for this enzyme until the time of onset of equine chorionic gonadotropin secretion around day 35 (Rodgers et al., 1998). There is an increased expression of CYP17 by the CL of pregnancy that is associated with an increase in concentration of plasma estradiol at the time, but the significance is unknown (Daels et al., 1998, Watson, 2000). To complement the down-regulation of estradiol expression, there is increased production of 17ß hydroxysteroid dehydrogenase type 4 (an enzyme capable of inactivating 17ß estradiol) during luteinization (Brown et al., 2004).

**Luteolysis**

Luteolysis at the end of diestrus in nonpregnant mares affords the return to estrus and multiple opportunities to reproduce. The first evidence for production of a uterine luteolysin in mares, as in other species, came from experiments demonstrating prolongation of CL function following hysterectomy (Ginther and
First, 1971). It was also demonstrated the administration of exogenous PGF results in shortening of diestrus and return to estrus, first in rats (Pharriss and Wyngarde, 1969) and soon thereafter in mares (Douglas and Ginther, 1972). However, it was not until over half a decade later that the endogenous PGF metabolite (PGFM) was first identified and measured in the uterine vein of anesthetized mares (Douglas and Ginther, 1976) and increased PGFM concentrations were detected concomitant with occurrence of luteolysis during the estrous cycle (Neely et al., 1979a). These experiments provided compelling evidence PGF is the luteolytic signal in mares. Up regulation of prostaglandin H synthase-I, prostaglandin F synthase, and prostaglandin E synthase in the equine endometrium at the time of luteolysis allow the endometrium to express high levels of prostaglandin (Atli et al., 2010).

Inflammation of the endometrium as with endometritis can also cause premature prostaglandin release and premature luteolysis resulting in a shortened interovulatory interval (Adams et al., 1987). Luteolysis requires pulsatile release of PGF from the endometrium (Kindahl et al., 2000, Shand et al., 2000). Studies vary somewhat, but hourly sampling suggests two to three pulses of PGF(M) occur within a 24-hour period (Ginther et al., 2008b).

The stimulus for PGF secretion by the endometrium is oxytocin. Prolonged progesterone exposure results in down regulation of progesterone receptors and decreased inhibition of estradiol, and estrogenic up regulation of oxytocin receptors follows (Leavitt et al., 1985, Sharp et al., 1997). The endometrium responds to pulsatile release of oxytocin from the neurohypophysis with pulsatile release of PGF.
Increases in circulating concentrations of oxytocin are temporally related to those of PGFM in mares (Ginther and Beg, 2011, Ginther et al., 2011b). Additionally, administration of exogenous oxytocin to mares has been observed to increase PGFM and terminate luteal function (Betteridge et al., 1985). The corpus luteum does not appear to be a significant source of oxytocin in mares (Stevenson et al., 1991) as it is in domestic ruminants (McCracken et al., 1999). The most likely source of luteolytic oxytocin in mares is therefore the neurohypophysis. In addition, oxytocin-neurophysin-I mRNA levels in the endometrium were negatively correlated with concentration of plasma progesterone and uterine oxytocin has been proposed to play a role in cyclicity and luteolysis (Behrendt-Adam et al., 1999), but the significance of uterine oxytocin remains to be clarified.

In many species, prostaglandins are also produced by the corpus luteum itself. This process is primarily regulated at the levels of phospholipase A2 and cyclooxygenase (Wiltbank and Ottobre, 2003). PGF, PGE, and a prostacyclin metabolite have been isolated from the equine CL (Watson and Sertich, 1990). Prostaglandin production by the CL provides a mechanism whereby the luteolytic signal may be amplified in a positive feedback loop during luteolysis.

PGF has a direct negative effect on progesterone synthesis (McCracken et al., 1999), and concentration of plasma progesterone in mares shows a linear decline with exposure to physiologic levels of PGF (Ginther et al., 2009a, Ginther and Beg, 2011, Ginther et al., 2011a). Most of the functional effects of PGF are mediated by
the protein kinase C (PKC) second messenger system (Pate, 1994). In short, binding of PGF to its receptor activates phospholipase C. This results in phospholipid cleavage to inositol triphosphate to diacylglycerol. These then mediate the activation of protein kinase C and influx calcium in luteal cells. Studies in ewes in vitro (Wiltbank et al., 1990) and in vivo (Mcguire et al., 1994) demonstrate that PKC has negative effects on steroidogenesis and is a mediator of the effects of PGF.

Many of the functional antisteroidogenic effects of PGF are due to decreased cholesterol availability. Steroidogenic acute regulatory protein (StAR) is responsible for transport of cholesterol from the outer to the inner mitochondrial membrane- the rate-limiting step in steroidogenesis. Its expression has been described in the equine CL (Watson et al., 2000). A decrease in StAR mRNA transcription has been detected by RT-PCR during natural luteolysis between days 12 and 14 of diestrus (Slough et al., 2011). Equine corpora lutea also show a decrease in 3ß-HSD expressions between days 12 and 14 (Slough et al., 2011).

Structural regression and involution of the corpus luteum must follow cessation of function. A descriptive study of the ultrastructural characteristics of the CL at different stages based on electron microscopy highlights some of the structural changes apparent in the equine CL. Those observed include mitochondrial rarefaction, vesiculation and whorling of the smooth endoplasmic reticulum, decreased multivesicular bodies, collagen accumulation, and pyknosis and irregularity of cell nuclei (Levine et al., 1979). Immunohistochemistry of the
apoptotic markers, caspase 3 and histone 3, in the CL of mares suggests vascular and hormone-producing cells may experience a mostly simultaneous demise (Aguilar et al., 2006). Maximal vascular development of the equine CL occurs approximately midway through the luteal phase followed by a decrease in endothelial proliferation. This occurs parallel to decreasing concentration of plasma progesterone (Al-zi’abi et al., 2003). Consistent with these changes, expression of angiogenic factors and receptors (particularly VEGF) decreases as luteolysis progresses (Muller et al., 2009).

Early reports hypothesized that decreased progesterone secretion could be attributed to decreased luteal blood flow due to the vasoconstrictive properties of PGF (Pharriss et al., 1970), but there is no change in blood flow during luteolytic pulses of PGF in mares (Ginther et al., 2008b) in contrast to cows, where changes in concentration of plasma progesterone reflect changes in blood flow (Acosta et al., 2002, Shrestha et al., 2010). Nonetheless, the extensive vasculature must regress. Nitric oxide may be involved in this process of luteolysis, as eNOS expression is increased in the late luteal phase (Ferreira-Dias et al., 2011). Also, increased expression of the cytokines, tumor necrosis factor, interferon gamma, and Fas ligand is associated with increased PGF and reduced VEGF are implicated for controlling regression of the equine luteal vasculature in late diestrus (Galvao et al., 2013).

Both apoptotic and non apoptotic factors appear to be involved in luteal regression in mares (Al-zi’abi et al., 2002). Apoptosis is triggered by intrinsic or
extrinsic pathways, depending on whether the initial signal is intracellular or extracellular (Nagata, 1997, Adams and Cory, 1998). In a multispecies review of the literature, both pathways appear to play important roles in luteal regression, with perhaps a greater role for the extrinsic pathway (Yadav et al., 2005). Thus, many scientific investigations employ indicators that are common to both pathways (e.g. caspase, DNA fragmentation, etc.). Bovine oligosome formation increases after the decrease in concentration of plasma progesterone during natural and PGF induced luteolysis (Juengel et al., 1993), reflecting a potential role of apoptosis in structural luteal regression. Caspases are protein enzyme mediators of apoptosis that cleave essential cellular components and precipitate the morphologic changes that occur (Cohen, 1997). Mice deficient in caspase 3 demonstrate functional luteal regression (decrease in concentration of plasma progesterone) but not structural regression by day 6 (Carambula et al., 2002). In mares, caspase 3 expression, as assessed by Western blotting and immunocytochemistry, increases in the CL as compared to the corpus hemorrhagicum and corpus albicans, suggesting that it may be an apoptotic mediator in luteal regression (Ferreira-Dias et al., 2007). Histone 3 has also been used as an apoptotic marker and increases during luteolysis in mares (Aguilar et al., 2006).

Finally, the function of immune cells in luteal function and luteolysis has been recently investigated (Pate et al., 2010). White blood cells and other immune cells are recruited to the CL throughout its lifespan, and macrophages and
lymphocytes serve as essential producers of cytokines that are critical signaling molecules. In the guinea pig, ultrastructural studies have illustrated the role of leukocytes in removing dead cells and debris (Paavola, 1979). In mares, there is an increase in T-lymphocytes in the late luteal phase, with CD8+ cells increasing before functional luteolysis, illustrating a potential role for immune cells in the luteolytic pathway (Lawler et al., 1999).

The regression of the CL occurs every 21 to 22 days in a cycling mare. Ovulation, luteinization, and luteolysis are complex physiological events that occur in a cyclical manner and are integral to female reproductive function. Due to its critical role, artificial manipulation of luteal function is an invaluable tool for equine practitioners in breeding management.

**Artificial Modulation of Luteal Function in Mares**

**Prolongation of luteal function**

Artificial prolongation of luteal function is desirable insofar as it prevents expression of estrus behavior (Hedberg et al., 2005). It has long been recognized that periods of prolonged diestrus occasionally occur naturally in mares (Stabenfeldt et al., 1974a), and diestrus ovulations occur sporadically. Additional causes of prolonged diestrus include failure to release PGF from a damaged (fibrotic) endometrium secondary to underlying uterine pathology (Hughes et al.,
1979) and early embryonic death. However, ability to manipulate luteal function in individual mares can be clinically useful.

Daily treatment with exogenous progesterone or progestagens is a common and well-established method to prevent estrus by mimicking luteal function (Loy and Swan, 1966). Additionally, mares occasionally ovulate during treatment with the synthetic progestagen, altrenogest, resulting in an increased interval to estrus even after discontinuation of therapy (Daels et al., 1996). Nonetheless, prolongation of the CL lifespan and endogenous progesterone production is desirable to decrease hormone handling and frequency of treatment. Induction of diestrus ovulation in mares who develop an at least 30 mm follicle during diestrus has been employed effectively (Hedberg et al., 2006). However, not all mares develop large follicles and frequent ultrasound monitoring is required. It would be preferable to develop a protocol with a greater success rate and fewer technical requirements. Towards that end, research efforts have aimed to develop artificial mechanical and pharmacologic methods to prolong luteal function.

Pregnancy is one of the most common reasons for observed failure to cycle. Accordingly, simulation of pregnancy can be employed to prolong luteal function. One approach to increasing diestrus is to breed females and terminate pregnancy after the critical point of maternal recognition of pregnancy. This tactic was effective in one hundred percent of 11 pony mares whose pregnancies were manually crushed on day between days 16 and 22, resulting in diestrus signs for a mean of 82 days (Lefranc and Allen, 2004) Breeding and pregnancy termination
require technical skill, and it is not commonly employed. A similar approach employs placement of a 35 mm glass marble into the uterus within 24 hours of ovulation to imitate embryonic movement in the reproductive tract, resulting in a prolonged luteal phase (88.8 days) in 40% of mares (Nie et al., 2001).

Oxytocin is one of the most widely used drugs in mare reproductive management, mainly due to its ecbolic effects, but it can also be used to prolong luteal function. It has previously been recognized that continuous infusion of oxytocin can result in a protracted diestrus (Stout et al., 1999). However, continuous infusion does not provide a very practical approach for clinical application. A more recently described method to pharmacologically prolong the luteal phase employs twice daily administration of supraphysiologic (60 IU) doses of exogenous oxytocin on days 7 to 14 of diestrus (Vanderwall et al., 2007).

Interestingly, this process was first described in the 1970s when a single mare developed prolonged luteal function following attempted pharmacological induction of luteolysis via oxytocin administration (Neely et al., 1979b). A subsequent study did not find a difference in the proportion of mares with prolonged diestrus when treated once or twice daily by this regimen (five of eight versus five of seven, respectively) (Vanderwall et al., 2012). It was theorized that the efficacy of this treatment might be mediated via down regulation of oxytocin receptors in the endometrium, thereby disrupting the luteolytic pathway. However, there was not a measurable difference in the expression of oxytocin receptors in the endometrium on day 15 in control versus oxytocin treated mares (Vanderwall et al., 2012). Twice
daily administration of 60 IU of oxytocin on days 8 through 14 decreased
cyclooxygenase-2 expression and plasma concentration of PGFM (Keith et al., 2013),
and increasing the number of days of treatment increased the proportion of mares
responding.

Recently, it was demonstrated that intrauterine infusion of plant oils may
result in prolonged luteal function (Wilsher and Allen, 2011). In a serendipitous
experiment, it was discovered that infusion of coconut and peanut oils (originally
designed as control treatments) on day 10 extended the luteal period of at least 30
days (mean not reported) in 92% of mares treated without adverse reproductive
effects (Wilsher and Allen, 2011). The hypothesized mechanism of action is via
decreased endometrial prostaglandin production due to the polyunsaturated fatty
acids in the plant oils.

From the numerous approaches employed with varying degrees of success,
the desirability of controlling luteal function is clearly evident. Depending on the
goals and constraints of the artificial manipulation, a variety of methods may be
attempted.

**Pharmacologic luteolysis**

One of the most commonly utilized methods for manipulation of the estrous
cycle in mares is induction of luteolysis and return to estrus by administration of
exogenous PGF (dinoprost or synthetic analogues). However, other methods are available, and PGF itself may be utilized by various approaches depending on the desired outcome. The principal pharmacologic agent discussed will be dinoprost tromethamine, as it is the only PGF product labeled for this application in the equine in the United States of America.

Even before PGF was identified as the luteolysin, methods for termination of luteal function were developed that relied on stimulation of the release of endogenous PGF from the endometrium. As early as the 1950s, it was recognized that uterine lavage with saline solution can induce a return to estrus in mares exhibiting a prolonged luteal phase in the absence of pregnancy (Burkhardt, 1954, Zafrakas, 1964, Arthur, 1970), and this became a treatment for persistent CL function. In a controlled experiment, mares were infused with 1.65 mL/kg of warm saline solution on day 1, 6, or 11, and treatment on day 6 resulted in a decreased duration of diestrus compared to the other two treatments (Ginther and Meckley, 1972).

The administration of exogenous PGF to pharmacologically induce luteolysis, resulting in a shorter diestrus period was first described by Douglas and Ginther in 1972 (Douglas and Ginther, 1972). In this study, based on transrectal palpation, intramuscular administration of 1.25, 2.5, 5.0, or 10.0 mg of PGF on day 6 of diestrus resulted in a mean interovulatory interval of 17.3-21.3 days in treated mares compared to 30.0 days in the control group (n=3 mares/group). The data acquired
provided the first evidence that exogenous PGF may be used in mares to induce luteolysis and hasten onset of the subsequent estrus, effectively ‘short-cycling’ mares. Subsequently, many studies- including those incorporating the use of transrectal ultrasonography- have validated the use of PGF and its analogues to short-cycle mares by inducing luteolysis to shorten diestrus and induce return to estrus (Douglas and Ginther, 1972, 1975, Kiefer et al., 1979, Gastal et al., 2005, Barker et al., 2006, Ginther et al., 2007, Utt et al., 2007, Holland and Pinto, 2008, Rubio et al., 2008, Samper, 2008, Ginther et al., 2009a, Cuervo-Arango and Newcombe, 2012).

PGF is rapidly metabolized in mares [following intravenous administration, the distribution half-life of the native dinoprost salt is 94.2 seconds and the elimination half-life of 25.9 minutes (Shrestha et al., 2012)]. Therefore, most studies to determine plasma concentration and secretory patterns of PGF instead measure PGFM (15-keto-13,14-dihydro-PGF2alpha metabolite) (Goff et al., 1984). It has been confirmed that PGF is responsible for triggering luteolysis rather than its metabolite, PGFM. In an experiment where equivalent doses (10 mg) of PGF and PGFM were administered to mares on day 9 of diestrus, all mares treated with PGF underwent luteolysis as evidenced by a drop in concentration of plasma progesterone whereas those treated with PGFM did not undergo luteolysis (Vanderwall et al., 2000).

In light of the plenitude of experimental evidence that PGF administration effectively shortens diestrus and the interovulatory interval in mares, it is
interesting that some authors have recently questioned the ability to short-cycle mares at all (Lofstedt, 2011). This argument is based on the premise that the majority of mares have only one follicular wave (Ginther, 2000), and that follicular growth would not be accelerated by PGF administration. Nonetheless, there is ample experimental evidence that administration of exogenous PGF does induce luteolysis, hasten return to estrus, and shorten the interovulatory interval (Douglas and Ginther, 1972, 1975, Kiefer et al., 1979, Gastal et al., 2005, Barker et al., 2006, Ginther et al., 2007, Utt et al., 2007, Holland and Pinto, 2008, Rubio et al., 2008, Samper, 2008, Ginther et al., 2009a, Cuervo-Arango and Newcombe, 2012).

Administration of exogenous PGF is different from physiological luteolysis in that it generally employs a single bolus treatment of supraphysiologic hormone concentrations. This is in contrast to the physiological process and pulsatile PGF release previously reviewed. It is therefore inherently flawed to draw conclusions about the natural luteolytic process based on these pharmacologic manipulations. A single low-dose (physiologic) of dinoprost (0.05 or 0.1 mg iv) is inadequate to achieve a sustained decrease in concentration of plasma progesterone (Ginther et al., 2009b). Some research has highlighted the differences between pharmacologically induced and naturally occurring luteolysis in mares (Ginther et al., 2009a). For example, concentration of plasma progesterone initially increases following bolus administration of PGF; this initial increase reaches a peak by 10-15 minutes and is followed by a decline and sub-luteolytic concentrations are achieved by approximately 24 hours post-treatment (Noden et al., 1978, Ginther et al., 2007,
Ginther et al., 2009a). By contrast, such a rise in progesterone is not observed during natural luteolysis nor during administration of more physiologic doses of exogenous PGF; rather there appears to be a more linear decline in concentration of plasma progesterone (Ginther and Beg, 2009a, Ginther et al., 2009a, Ginther et al., 2011b). The concentration of plasma estradiol has been reported to reflect various responses to bolus PGF administration (Noden et al., 1978, Ginther et al., 2007). Recent literature suggests that concentrations of plasma estradiol increases following both bolus and low-dose infusion (physiologic) PGF administration (Ginther et al., 2009a), and the concentration of plasma estradiol rises in parallel with the ascending portion of the PGF pulse during natural luteolysis (Ginther and Beg, 2009b). Whether this increase is related to the effects of PGF itself or is a consequence of the timing wherein follicular growth and beginning of estrus occurs contemporaneously with luteolysis has not been determined. Administration of exogenous PGF also results in increased concentration of plasma oxytocin (Utt et al., 2007). Additional endocrine changes observed only with bolus doses of PGF are increases in LH, FSH, and cortisol (Ginther et al., 2009a).

Besides the differential endocrinologic effects, bolus PGF-induced luteolysis exhibits additional deviation as compared to the physiologic luteolytic event. According to RT-PCR of luteal biopsies, treatment with exogenous PGF resulted in decreased production of LH receptor, StAR, and aromatase but an increase in luteal cyclooxygenase 2 (Cox-2) expression within 12 hours (Beg et al., 2005), whereas biopsy of the CL at different time points in a natural cycle did not detect a change in
Cox-2 expression (Slough et al., 2011). Furthermore, luteolysis induced by exogenous PGF resulted in observation of a higher number of eosinophils and fewer CD4+ and non-specific esterase staining cells (Lawler et al., 1999). An influx of a large number of neutrophils into the CL has also been observed at least 12 hours following PGF administration (Al-zi’abi et al., 2002).

The methodology of administering a single dose (10 mg dinoprost) of PGF in mid-diestrus has been challenged. Significantly lower doses of PGF are capable of inducing luteolysis of the mature CL. Irvine et al. demonstrated that administration of two injections of 0.5 mg PGF administered at a 24 hour interval was sufficient to induce luteolysis in mares. All mares treated in this manner were at least seven days into diestrus and displayed signs of estrus or a decrease in concentration of plasma progesterone consistent with luteolysis (Irvine et al., 2002). It has also been demonstrated that a single injection as low as 1.25 mg is sufficient to induce luteolysis of the mature CL (6 to 12 days post-ovulation), which may be a more convenient regimen in terms of dosing frequency and volume of drug administered (no dilution of the commercial product is required) (Barker et al., 2006). Studies suggested a dosage of 9 ug/kg of the free acid or 1.2 ug/kg of tromethamine salt were adequate to cause luteolysis with a single dose (Oxender et al., 1975). Similarly, treatment of pony mares with 0.2, 0.4, or 0.8 mg/kg of PGF all resulted in significant shortening of the interval from treatment to ovulation and decreased AUC for progesterone compared to saline- treated mares, and there was not a difference between the different doses administered (Handler et al., 2004).
In addition, the idea of a refractory period has been questioned to a greater degree. Even early on, it was recognized that some mares undergo luteolysis following administration of PGF or its analogues in early diestrus (Oxender et al., 1975). The proportion of mares who respond to a single treatment with PGF in early diestrus increases according to the day of treatment (Gastal et al., 2005). It is now recognized that even administration of low doses (especially multiple doses) in early diestrus (sometimes applied clinically for uterine clearance) can impair luteal function and shorten the interovulatory interval (Troedsson et al., 2001, Nie et al., 2003b).

In addition to low dose protocols, recent research has shown that the administration of not one but multiple doses of PGF during early diestrus (the period during which the CL was previously considered ‘refractory’ to the effects of exogenous PGF administration) may induce luteolysis (Holland and Pinto, 2008, Rubio et al., 2008). In the aforementioned studies, 6 of 10 mares treated with 2.5 mg of PGF intramuscularly on days 2, 3, and 4 following ovulation underwent luteolysis 3.3 ± 0.2 days after the beginning of treatment on day 2 based on concentration of plasma progesterone. However, individual mare variation exists, and response rate with early diestrus administration is well below the nearly 100 percent response rate observed after day 5.

**Luteal resurgence**
Another described response of the early diestrus CL to exogenous PGF administration is incomplete luteolysis followed by a partial recovery and resurgence in progesterone production, otherwise known as luteal resurgence. One of the first references to a ‘transitory luteolysis’ in the literature came in the 1970s in two mares treated with the PGF analogue, RS 9390 (Thompson and Witherspoon, 1974). This incomplete luteolysis followed by recovery in function was also described in mares following administration of 2.0 mg of the PGF analogue prostalene on day 6 of diestrus (Kiefer et al., 1979). This response was observed in 4 (6.2%) of the treatment cycles with complete luteolysis occurring 9.5 ± 1.7 days post-injection. Interestingly, this phenomenon in mares was initially used as evidence for the apparent CL ‘refractory period’ in mares and did not actually get defined until thirty years later, when early diestrus PGF administration in mares and the idea of a refractory period came under further consideration.

Clinically, administration of PGF in the periovulatory period is applied for its ecbolic effects to aid in treating delayed uterine clearance and endometritis. However, critical consideration of the effects of this treatment on luteal function provided some of the first evidence of luteal resurgence. In evaluating the effects of this periovulatory PGF treatment, the effects of administration of 500 ug of the PGF analog, cloprostenol, on days 0, 1, and 2 or on day 2 affected luteal function and pregnancy outcome (Troedsson et al., 2001). Both treatments had a transient negative impact on luteal steroidogenesis as determined by plasma concentration of progesterone, and pregnancy rate was significantly lower following PGF treatment.
as compared to no PGF treatment. In another report, it was found that administration of two standard (250 ug) or micro (25 ug) doses of cloprostenol on day 0, 1, or 2 resulted in decreased concentrations of plasma progesterone, slower rise in progesterone, and a shortened interovulatory interval (Nie et al., 2003b). However, studies from this group did not show a negative impact on pregnancy rate (Nie et al., 2003a). Nonetheless, the inhibitory effects of serial dosing of PGF in the early luteal period was put forth; whereas the focus of these studies was to focus on potential negative effects of this therapeutic approach on fertility, the potential application in non pregnant animals was not overlooked. Luteal resurgence was also again evident although it was the primary intent of these studies.

It was only recently that the idea of a refractory period began to be challenged as scientists begin to investigate early diestrus PGF administration with renewed vigor, and the idea of CL resurgence was again described and considered. Investigation of the effects of administration of a single dose or multiple administrations of 5 mg of dinoprost in early diestrus described potential responses as none, luteolysis, and luteal resurgence (Gastal et al., 2005).

In 2006, two different published studies showed additional evidence for this phenomenon. In the first, a transient decrease in plasma concentration of plasma progesterone was noted in mares treated with 250 ug cloprostenol or 5 mg dinoprost im once daily on days zero through two which was followed by a return to control levels by days 12 to 14 of diestrus (Mocklin et al., 2006). Administration of a single injection of 10 mg dinoprost on day 3 of diestrus resulted luteal resurgence in
37.5% of mares (Bergfelt et al., 2006). In this study, the observed rebound in luteal function (as reflected by concentration of plasma progesterone) was classified as ‘minor’ or ‘major’ resurgence according to the amount and duration of increase in concentration of plasma progesterone following treatment (<1.5 ng/mL for approximately 2 days versus >3 ng/mL for approximately six days, respectively). Interovulatory interval for mares treated on day 3 was shorter than mares treated on day 10 regardless of presence or degree of luteal resurgence although mares with a major luteal resurgence had a longer interovulatory interval than mares with minor or no resurgence in luteal function. Thus, this study effectively demonstrated that the CL is not refractory to PGF administered before day 5 and that such treatment results in a shorter period of diestrus.

Further studies on the phenomenon of luteal resurgence documented its occurrence but did not classify the luteal response according to degree (i.e. ‘major’ versus ‘minor’). In one study, complete luteolysis occurred in six out of ten mares treated with 2.5 mg of dinoprost intramuscularly on days two through four of diestrus (Holland and Pinto, 2008). In the remaining four mares, CL resurgence was observed. Interestingly, 75% (three out of four) of mares that underwent resurgence ovulated with relatively elevated concentration of plasma progesterone (>1 ng/mL). Hence, it became apparent that the early CL does respond to PGF with variable responses according to the dosage, frequency, and timing of administrations.
Further investigation of different dosage regimens was undertaken to determine what responses would be elicited according to the timing and dosage of administration (Rubio et al., 2008). All mares treated with 2.5 mg of PGF in the muscle twice daily on days zero through two of diestrus, beginning within 24 hours of ovulation, demonstrated luteal resurgence with a rebound in plasma concentration of plasma progesterone. Whereas only seven mares were treated in this manner, the universal CL resurgence observed in those mares may suggest that this regimen could be used as a model in the investigation of resurgence.

Furthermore, diestrus was variable with interovulatory interval ranging from 14 to 26 days after treatment, and one mare remained anovulatory.

Administration of 5 mg of dinoprost once daily on days 0, 1, and 2 resulted in concentration of plasma progesterone remaining below 2 ng/mL until after the cessation of treatment on day 4 (Gastal et al., 2005). The subsequent increase in concentration of plasma progesterone to luteal values was consistent with luteal resurgence. However, the authors commented on the progesterone-retarding effects of this treatment, which became further evident in antiluteogenesis.

**Anti-luteogenisis**

In a novel reported approach, serial administration of multiple doses of PGF in early diestrus (beginning at day 0) to prevent and interrupt luteal function of the
developing CL was defined as anti-luteogenesis (Rubio et al., 2008). Administration of 10 mg PGF twice daily on days 0, 1, and 2 prevented diestrus in four out of six mares, with concentration of plasma progesterone remaining below 1 ng/mL, and resulted in a mean treatment to ovulation interval of 7 ± 1.8 days. Refinement of PGF dosing regimen and further characterization of the endocrine mechanisms behind this phenomenon are necessary to justify clinical application of PGF treatment at any stage of diestrus.

**Rationale for Study**

Antiluteogenesis (serial administration of PGF in early diestrus to prevent normal CL development and luteal function) is a novel approach in pharmacological manipulation of the estrous cycle in mares. It was surmised that modification of previously described treatment protocols could elicit antiluteogenesis in one hundred percent of treated mares and induce a subsequent normal estrus not preceded by diestrus. The effects of this treatment on fertility of the induced estrus have not been empirically assessed.
Chapter 3

Antiluteogenic effects of serial PGF administration in mares

Introduction

Administration of exogenous PGF is one of the most commonly utilized methods of pharmacological manipulation of the equine estrous cycle. It is commonly accepted that luteolysis can only be consistently induced with a single injection of PGF at least five or six days ovulation. Based on this practice, it is erroneously assumed that the early CL is refractory to luteolytic actions of PGF. Recently, it has been reported that serial treatment with PGF in early diestrus can interrupt luteal function. The effects of PGF on the early CL may depend on the dosage employed, frequency of dosing, and timing of administration. Administration of not one but multiple doses of PGF early in diestrus can interrupt luteal function (as evidenced by changes in concentration of plasma progesterone) in one of three ways: early induction of luteolysis (Holland and Pinto, 2008, Rubio et al., 2008), luteal regression and resurgence (Bergfelt et al., 2006, Holland and Pinto, 2008, Rubio et al., 2008), and anti-luteogenesis (Rubio et al., 2008). These
processes are associated with early cessation of function and concomitant decline in concentration of plasma progesterone, decreased concentration of plasma progesterone followed by a resurgence, and failure of normal CL formation and rise in concentration of plasma progesterone, respectively.

In a novel approach, administration of multiple doses of PGF beginning at ovulation resulted in prevention of normal luteal function: antiluteogenesis (Rubio et al., 2008). Four of six mares treated with twice daily injections of 10 mg of dinoprost on days zero through two, beginning within 24 hours of ovulation, resulted in failure to achieve a sustained rise in plasma concentration of plasma progesterone in four of six mares and an interovulatory interval of 9 ± 1.8 days in those mares. Previous reports have not evaluated the effects on fertility.

In the present study, it was hypothesized that administration of multiple doses of PGF in early diestrus can interrupt luteal function, inducing a return to estrus similar to mares treated with a single dose during mid diestrus. It was further theorized that the fertility of the induced estrus between the two groups would not differ. The specific objectives of the study were to evaluate the effects of early diestrus treatment by: 1) assessment of luteal function as reflected by hormone profile for concentration of plasma progesterone; 2) determination of the duration of interovulatory and treatment to ovulation intervals; 3) comparison of the number of pregnant mares at 14 days post-ovulation.
Materials and Methods

Ten reproductively normal, cycling Quarter horse mares were evaluated daily by transrectal ultrasonography and teasing. Estrus was defined as presence of uterine edema detected by transrectal ultrasonography, relaxation of the cervix and reproductive tract detected by transrectal palpation, and display of sexually receptive behavior (urinating, squatting, eversion of the clitoris) when teased with a breeding stallion. Mares in estrus with a follicle measuring at least 35 mm in diameter were induced to ovulate by administration of 2500 IU of human chorionic gonadotropin (hCG; Chorulon®, Intervet, Millsboro, USA) intravenously. Reproductive examinations by ultrasonography were performed twice daily (~q12hrs) beginning 24 hours after hCG administration until detection of ovulation.

Following ultrasonographic detection of ovulation (day 0), mares were randomly assigned to one of two treatment groups. In group I (mid diestrus group, n=10), mares were administered a single 10 mg dose of the native dinoprost tromethamine salt (Lutalyse®, Pfizer, New York, New York, USA) on day 10. In group II (early diestrus group, n=10), mares were treated with 10 mg PGF twice daily on days 0, 1, and 2 and then daily on days 3 and 4. Following treatment, once in estrus with a follicle at least 35 mm in diameter, mares were artificially inseminated.
and induced to ovulate with 2500 IU hCG intravenously. Pregnancy was defined as detection of a growing embryonic vesicle on two consecutive examinations at least 24 hours apart by transrectal ultrasonography at approximately 14 days post ovulation and terminated by administration of 10 mg PGF intramuscularly. Following completion of one cycle, the mare was allotted to the opposite group on subsequent ovulation such that all mares received both treatments in a balanced crossover design.

**PGF Treatment**

Two milliliters (10 mg) of dinoprost tromethamine were delivered into the muscles of the neck using a 3 mL syringe and a one-inch, 22-gauge hypodermic needle.

**Transrectal ultrasound examinations**

Serial transrectal ultrasonography was performed throughout the study period with a portable ultrasound unit equipped with a 5.0-12.0 MHz multifrequency linear transducer (MyLab OneVET™, Esaote). Ultrasound examinations were performed to monitor follicular development (including presence of an inducible follicle), appearance of the reproductive tract, appearance of the corpora lutea, and ovulation. The initial ovulation was detected within 12 hours of its occurrence by twice daily monitoring. Mares in group I were monitored through day four following ovulation, and then on Monday, Wednesday, and Friday until they
again displayed signs of estrus. Mares in group II were monitored daily until
subsequent ovulation occurred. One mare in group II was noted to have a
protracted interovulatory interval characterized by ovarian quiescence; therefore,
monitoring on this mare decreased to thrice weekly (Monday, Wednesday, and
Friday) as with group I mares until at least one follicle ≥ 20 mm was detected.

**Semen collection and artificial insemination**

For artificial inseminations, semen was collected from a known fertile
stallion using a Missouri-model artificial vagina (AV) equipped with a latex liner and
filled to 12 pounds with warm water. The AV was lubricated with a non-spermicidal
gel (Priority Care™, First Priority Inc., Elgin, IL USA) immediately prior to collection.
The ejaculate was immediately assessed for concentration with a commercial
spectrophotometer (591B Densimeter™, Animal Reproduction Systems, Chino, CA,
USA) according to manufacturer’s instructions. A breeding dose was calculated
consisting of between three and four billion total sperm so that all mares would be
inseminated with at least 2 billion motile sperm and 1 billion progressively motile
sperm extended to 45 mL total volume in milk-based, Kenney extender with
amikacin (Next Generation™, Partnar Animal Health, Iderton, ON, Canada.). Prior to
artificial insemination procedure, the mare perineum was cleanly prepared with
water and betadine scrub. Mares were artificially inseminated with standard clean
technique. Ovulation was induced with 2500 IU hCG intravenously immediately
following insemination. Any mare not ovulating within 48 hours of artificial
insemination and hCG administration was artificially inseminated a second time. Mares with fluid in their uteri 24 hour post-insemination were treated with 20 IU oxytocin intramuscularly twice daily for up to four treatments or until the uterus was free of intraluminal fluid as determined by transrectal ultrasonography.

Pregnancy was defined as detection of a growing embryonic vesicle on at least two serial transrectal ultrasound examinations at least 24 hours apart. All pregnant mares were treated with 10 mg of PGF on day 14.

**Plasma sampling**

Serial plasma samples for hormone analyses were collected in all mares throughout the experimental period. Sampling for all mares commenced at induction of ovulation and continued twice daily on days 0 to 4. Mares treated in mid diestrus (day 10) were sampled daily on days 4 to 9, twice daily on days 10 to 14 (five days following PGF administration), daily until day 4 following subsequent ovulation, and then thrice weekly until subsequent estrus. For mares in group II, sample collection continued daily until four days following subsequent ovulation, then thrice weekly until subsequent estrus. Ten milliliters of whole blood was collected in vacutainer tubes (BD Vacutainer®, Bectin, Dickinson and Company, Franklin Lakes, New Jersey, USA) containing lithium heparin with 20-gauge, 1-inch vacutainer needles. Blood samples were placed on ice until processing. Plasma was harvested following centrifugation of the blood samples at 600 g for 10 minutes.
within two hours of sampling. Plasma samples were then transferred to two labeled 1.5 or 1.8 mL cryovials with 3 mL transfer pipettes and stored at -80°C.

**Progesterone radioimmunoassay**

Progesterone radioimmunoassay was performed at North Carolina State University by a technician blinded to treatment protocols and groups. Radioimmunoassay technique employed has been previously validated for use in mares (Whisnant and Burns, 2002, Barker et al., 2006). A commercially available radioimmunoassay kit (Coat-A-Count™, Diagnostic Products Corporation, Los Angeles, CA) was used according to the manufacturer’s instructions. All samples were run in duplicate.

**Data and statistical analysis**

For all data analysis, statistical significant was set at $p < 0.05$ and trend to significance at $p < 0.1$. Data for inter ovulatory interval and treatment to ovulation interval were compared by a paired t-test. Concentrations of plasma progesterone were analyzed by a mixed model ANOVA for repeated measures with day and treatment specified as fixed factors along with an interaction between the two (SAS 9.2, SAS Institute, Inc., Cary, NC, USA). Pregnancy outcome for the two groups were compared using a McNemar’s chi-squared analysis. Data are reported as mean ± standard deviation.
Results

The demographic data for the mares used for the present study are presented in Figure 3.1. Following induction of ovulation with hCG at initiation of the experiment, time to ovulation was 52.8 ± 31.0 and 58.7 ± 23.6 hours for groups I and II, respectively (p > 0.05). The follicular diameter at the time of hCG administration was 37.8 ± 2.3 mm for group I and 38.9 ± 3.1 for group II (p > 0.05).

The data regarding the ejaculates and insemination doses is provided in Figure 3.2. Mean interval between hCG administration and ovulation for groups I and II were 2.3 ± 0.8 and 1.9 ± 0.7 days, respectively (p>0.05) (monitoring was performed once daily). The follicular diameter at the time of insemination and induction of ovulation was 37.1 ± 2.0 for group I and 37.0 ± 2.4 for group II (p > 0.05). All mares ovulated by one to four days after hCG administration. Two mares in group I had to be inseminated twice, and both became pregnant.

When compared to the group I treatment of single injection in mid diestrus, serial PGF treatment in group II resulted in a shorter mean interovulatory interval (p<0.05; 18.5 ± 2.0 vs. 13.1 ± 3.7 days, respectively; Figure 3.3ab). Only one mare had a longer interovulatory interval with this treatment. By comparison, treatment
to ovulation interval (as calculated from the first day of treatment) was shorter in mares treated on day 10 compared to those treated serially in early diestrus (p<0.05; 8.5 vs. 13.1 days, respectively; Figure 3.4ab).

Pregnancy outcome between the two groups was not different (Figure 3.5). For both early diestrus treatment and control groups, 9 of 10 mares were pregnant on ultrasonographic examination following insemination (p=1.0).

For the progesterone radioimmunoassay, intraassay and interassay coefficients of variation were 4.2 ± 0.6% and 5.3%, respectively. Concentration of plasma progesterone reflected the effect of PGF on luteal function. Functional luteolysis was achieved by administration of 10 mg of dinoprost on day 10, as concentration of plasma progesterone dropped to < 1 ng/mL in all mares within 48 hours (P<0.05, Figure 3.7), and day 12 values were below those observed in a normal diestrus.

For group II, mean plasma concentration of plasma progesterone during the treatment period (days 0-4) remained beneath luteal values of 1.0 ng/mL (Figure 3.8a), and no mare achieved a sustained rise despite a brief elevation in some mares. There was an effect of treatment and day and an interaction of treatment*time (p<0.001). Five of 10 mares had a transient rise in concentration of plasma progesterone > 1 ng/mL; three of those five had a concentration of plasma progesterone that remained above 1 ng/mL for two data points (i.e. more than 24 hours). No resurgence in concentration of plasma progesterone (luteal resurgence)
was observed after the treatment period (data not shown). Compared to the same period during a normal diestrous in the same group of mares, there is a difference in the progesterone profile on days 1-4 (Figure 3.8b).

The post-treatment diestrus was also considered to determine whether prevention of the previous diestrous by early dinoprost treatment would alter subsequent luteal function. A single mare (E) in group II had a quintuple ovulation on the diestrous following treatment, resulting in a concentration of plasma progesterone of 32.17 ng/mL on day 4. As this represents an outlier, the data was analyzed without this observation. There was no difference in concentration of plasma progesterone following treatment between the two groups (p > 0.05, Figure 3.9).

Figure 3.10 illustrates typical profiles from two randomly chosen mares in group I and II. The mares in group II do not achieve a sustained rise in plasma concentration of plasma progesterone until after the ovulation subsequent to treatment. The mares in the group I exhibit normal luteal levels of progesterone followed by a sharp decline in progesterone after administration of single injection of dinoprost on day 10.

Diameters of the largest and second largest follicle for the mares throughout the study are presented in Figure 3.11 and 3.12. All mares exhibited a single follicular wave in both treatment and control cycles. One mare (X) experienced double ovulation on every cycle including induction of ovulation at the
commencement of treatment. That mare was pregnant with twins when in group I but was not pregnant when in group II. One mare (E) in group II had a quintuple ovulation following treatment; however, one embryonic vesicle was detected on pregnancy evaluation. Based on this data, the mean time of follicular deviation was on day 11.9 ± 3.8 for group I compared to day 8.1 ± 3.9 for group II (p<0.05).

One mare in group I (S) had a 40 mm follicle on the day of PGF administration and concentration of plasma progesterone decreased from 7.3 ng/mL at the time of PGF administration on day 10 to 0.9 ng/mL on day 12. Subsequently, the large follicle grew to 56 mm diameter and began to show signs of luteinization on ultrasonography (thickening of the follicular walls) (Figure 3.13). A second 47 mm follicle developed on the opposite ovary, so it was elected to proceed with artificial insemination. Concentration of plasma progesterone at the time of insemination was determined to be 2.2 ng/mL. The second ovulation occurred on day 20 (ten days following injection of PGF). This mare was pregnant on examination at day 14. The data for follicular diameter from mare S are therefore not included in figure 3.11.

Corpora lutea began to show ultrasonographic signs of regression by day 4 of treatment in all mares in group II (Figure 3.14). Signs of structural regression as evidenced by decrease in the imaged area coupled with increased echogenicity of the core surrounded by a relatively hypoechoic margin were evident by day 4.
Discussion

To our knowledge, this is the first study to reliably induce antiluteogenesis in mares following ovulation. It is also the first to investigate the effects of such treatment on fertility. All mares in group I underwent luteolysis (reflected by concentration of plasma progesterone < 1 ng/mL) by 48 hours following administration of a single injection of PGF in mid diestrus on day 10. Mares in group II treated with serial administration of PGF beginning at ovulation all underwent antiluteogenesis, as concentration of plasma progesterone did not achieve a sustained rise (>1 ng/mL) in any mare. Fertility of the induced estrus was apparently normal and not different between the two groups.

Profiles for plasma concentration of plasma progesterone during serial early diestrus treatment in group II resulted in prevention of normal luteal function as suggested by a failure of any mare to achieve a sustained rise in concentration of plasma progesterone to luteal concentrations of > 1 ng/mL. Five of the ten treated mares had at least one data point > 1 ng/mL (range 1.08-2.2 ng/mL). Only one mare had a single time point of > 2 ng/mL. Three of the five mares maintained the elevation in concentration of plasma progesterone for at least two data points, or more than 24 hours. The elevations in concentration of plasma progesterone
occurred between 1 and 3 days post ovulation, and all mares had sub-luteal concentrations by day 4. Mares have been treated with multiple doses of PGF in early diestrus, resulting in a transient decrease in progesterone (Mocklin et al., 2006, Holland and Pinto, 2008, Rubio et al., 2008), but the antiluteogenic effects of serial prostaglandin administration of relatively higher doses in early diestrus represent a novel approach to pharmacological manipulation of luteal function (Rubio et al., 2008). The addition of two additional doses at 24 hour intervals on days 3 and 4 in the current study mitigated luteal resurgence and prevented luteal formation in all treated mares, increasing the response rate from two thirds (four out of six mares) (Rubio et al., 2008) to all mares (ten out of ten).

Recently, the efficacy of different doses of the PGF analogue cloprostenol in inducing luteolysis in early diestrus (80 to 112 hours) has been investigated (Cuervo-Arango and Newcombe, 2012). There was an effect of dosage on proportion of mares responding, but only after 96 to 104 hours, and significantly more mares underwent luteolysis at 104 compared with 96 hours. In that study, luteolysis was defined as presence of clinical signs of estrus, and no determination of concentration of plasma progesterone was performed.

It is relevant to note that return to estrus was uneventful following treatment, and ovulation occurred in all post-treatment cycles. For breeding management, only two mares had to be inseminated twice according to the stallion schedule used, which was chosen as a clinical example such as those used in
practice. Furthermore, both mares that required two inseminations were in the group I. Thus, antiluteogenic treatment in early diestrus induced a return to estrus in such a way that ovulation was predictable and standard breeding management practices were effective to successfully impregnate mares.

Administration of dinoprost to mares in both early and mid diestrus resulted in a shortened diestrus. Interovulatory interval following mid and early diestrus treatment was decreased as compared with the universally accepted normal of 21 to 22 days (Ginther and Pierson, 1984, Ginther et al., 2004, Aurich, 2011). This is contrary to the argument that mares can not be effectively ‘short-cycled’ due to the existence of only one follicular wave (Lofstedt, 2011). However, in the present study, administration of serial doses of PGF in early diestrus resulted in an earlier day of follicular deviation compared to administration of a single dose on day 10 (8.1 ± 3.9 days compared to 11.9 ± 3.8 days, respectively; p < 0.05). This is consistent with data demonstrating that administration of exogenous progesterone can alter follicular dynamics and day of follicle deviation, and serial administration in early diestrus resulted in an earlier time of follicle deviation. Administration in early diestrus resulted in a shorter interovulatory interval as compared to mid diestrus treatment, suggesting that implementation of treatment earlier may afford greater control over manipulation of the estrous cycle.

In a previous study, four of six mares treated twice daily on days 0 to 2 returned to estrus and ovulated 7 ± 1.8 days from treatment, or 9 ± 1.8 days from
previous ovulation (Rubio et al., 2008). The observed interovulatory interval in this study of 13.1 ± 3.7 days was longer. One proposed theory to explain this apparent discrepancy is based on the follicular dynamics of the mare population used in this study. Complex follicular mapping for study of deviation and follicular wave emergence was beyond the scope of the current study design. However, mapping of the largest and second-largest follicles was supportive of the existence of one follicular wave (Figures 3.11 and 3.12). Furthermore, previous studies of Quarter Horse and Appaloosa type mares suggests that as few as 25% of cycles have a secondary or minor wave (Ginther, 1993, Ginther et al., 2004). There may be a greater number of mares with two waves of follicular growth among Thoroughbreds, Warmbloods, and other non-Quarter Horse breeds (based on occurrence of diestrus ovulation) (Stabenfeldt et al., 1972, Vandeplassche et al., 1979, Ginther and Pierson, 1989). Therefore, a difference in study population could account for the observed difference between the two studies. It is interesting to note that even the mares in the current study, all of which apparently had a single follicular wave, demonstrated a shortened interovulatory interval. This further supports the notion that PGF can be used to ‘short-cycle’ mares. Furthermore, anecdotally, we have observed mares to ovulate spontaneously as few as five days after previous ovulation with the application of this antiluteogenic treatment protocol.
Calculation of the treatment to ovulation interval could be taken from the first or the last day of treatment in the early diestrus treatment group. There is no difference between early and mid diestrus treatment groups (9 + 3.7 versus 8.5 + 2.0 days, respectively) by the former method, as compared with the latter. Furthermore, there is no difference between the aforementioned intervals, the previously described study on antiluteogenesis (7.0 + 1.8), and the accepted treatment to ovulation interval for PGF treatment to ovulation in the literature of seven to ten days (Samper, 2008). The fact that one half of treated mares displayed a transient rise in concentration of plasma progesterone may contribute to the difference in the calculation when taken from the last versus the first day of treatment; however, there was no difference between the treatment to ovulation interval in mares who did not demonstrate a transient rise versus those who did. Nonetheless, the shortest interovulatory interval (9 days) was observed in two mares who did not achieve a concentration of plasma progesterone > 1 ng/mL.

Consistent with our own clinical experiences and that of others, antiluteogenic treatment with PGF did not have a significant negative effect on fertility of the induced estrus based on the same pregnancy outcome between the two treatment groups. Studying the effects of treatment on fertility in large animals is complicated by the inherent limitations in power of statistical tests applied in studies with a relatively small number of experimental units. Nonetheless, the absence of a large negative impact certainly aids in the argument that this treatment
could be beneficial for specific, selected cases without concern about negatively impacting fertility.
Figures

<table>
<thead>
<tr>
<th>Mare</th>
<th>Age</th>
<th>Wet (W)/Dry (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mare A</td>
<td>12</td>
<td>D</td>
</tr>
<tr>
<td>Mare B</td>
<td>7</td>
<td>W</td>
</tr>
<tr>
<td>Mare C</td>
<td>10</td>
<td>W</td>
</tr>
<tr>
<td>Mare E</td>
<td>7</td>
<td>D</td>
</tr>
<tr>
<td>Mare H</td>
<td>7</td>
<td>W</td>
</tr>
<tr>
<td>Mare L</td>
<td>15</td>
<td>W</td>
</tr>
<tr>
<td>Mare R</td>
<td>8</td>
<td>D</td>
</tr>
<tr>
<td>Mare S</td>
<td>7</td>
<td>W</td>
</tr>
<tr>
<td>Mare Y</td>
<td>18</td>
<td>W</td>
</tr>
<tr>
<td>Mare X</td>
<td>15</td>
<td>W</td>
</tr>
<tr>
<td>Mean</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Std Dev</td>
<td>4.1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.1. Demographic data on the mares used in the present study.
<table>
<thead>
<tr>
<th>Date</th>
<th>Volume</th>
<th>Concentration (densimeter) (x10^6)</th>
<th>Total Sperm/ Ejaculate (x10^6)</th>
<th>Motility</th>
<th>Morphology</th>
<th>Volume Raw for AI (mL)</th>
<th>Sperm AI (x10^9)</th>
<th>PMS/AI (x10^9)</th>
<th>PM, normal (x10^9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/6/12</td>
<td>50</td>
<td>220</td>
<td>11</td>
<td>97</td>
<td>51</td>
<td>15</td>
<td>3.30</td>
<td>1.68</td>
<td>1.09</td>
</tr>
<tr>
<td>7/9/12</td>
<td>??</td>
<td>509</td>
<td>220</td>
<td>90</td>
<td>65</td>
<td>7</td>
<td>3.56</td>
<td>2.31</td>
<td>1.41</td>
</tr>
<tr>
<td>7/13/12</td>
<td>44</td>
<td>215</td>
<td>9.46</td>
<td>81</td>
<td>50</td>
<td>15</td>
<td>3.22</td>
<td>1.61</td>
<td>1.22</td>
</tr>
<tr>
<td>7/16/12</td>
<td>54</td>
<td>141</td>
<td>7.61</td>
<td>85</td>
<td>44</td>
<td>??</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/18/12</td>
<td>37</td>
<td>199</td>
<td>7.36</td>
<td>87</td>
<td>46</td>
<td>15</td>
<td>2.99</td>
<td>1.38</td>
<td>0.85</td>
</tr>
<tr>
<td>7/20/12</td>
<td>43</td>
<td>263</td>
<td>11.3</td>
<td>93</td>
<td>27</td>
<td>15</td>
<td>3.95</td>
<td>1.06</td>
<td>0.67</td>
</tr>
<tr>
<td>7/28/12</td>
<td>58</td>
<td>215</td>
<td>12.5</td>
<td>84</td>
<td>46</td>
<td>15</td>
<td>3.22</td>
<td>1.48</td>
<td>1.20</td>
</tr>
<tr>
<td>7/30/12</td>
<td>110</td>
<td>120</td>
<td>13.2</td>
<td>79</td>
<td>47</td>
<td>25</td>
<td>3.00</td>
<td>1.41</td>
<td>1.03</td>
</tr>
<tr>
<td>8/10/12</td>
<td>40</td>
<td>281</td>
<td>11.2</td>
<td>86</td>
<td>51</td>
<td>13</td>
<td>3.65</td>
<td>1.86</td>
<td>1.21</td>
</tr>
<tr>
<td>8/13/12</td>
<td>46</td>
<td>277</td>
<td>12.7</td>
<td>88</td>
<td>50</td>
<td>14</td>
<td>3.88</td>
<td>1.94</td>
<td>1.38</td>
</tr>
<tr>
<td>8/17/12</td>
<td>39</td>
<td>597</td>
<td>23.3</td>
<td>87</td>
<td>58</td>
<td>6</td>
<td>3.58</td>
<td>2.07</td>
<td>1.24</td>
</tr>
<tr>
<td>8/24/12</td>
<td>39</td>
<td>189</td>
<td>7.37</td>
<td>91</td>
<td>62</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/27/12</td>
<td>46</td>
<td>199</td>
<td>9.1</td>
<td>80</td>
<td>53</td>
<td>18</td>
<td>3.58</td>
<td>1.90</td>
<td>1.12</td>
</tr>
<tr>
<td>8/31/12</td>
<td>50</td>
<td>491</td>
<td>24.5</td>
<td>85</td>
<td>55</td>
<td>8</td>
<td>3.93</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>9/3/12</td>
<td>56</td>
<td>262</td>
<td>14.7</td>
<td>92</td>
<td>53</td>
<td>13</td>
<td>3.41</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>9/10/12</td>
<td>46</td>
<td>256</td>
<td>11.7</td>
<td>74</td>
<td>48</td>
<td>15</td>
<td>3.84</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>50.53</td>
<td>277.13</td>
<td>12.48</td>
<td>86.19</td>
<td>50.38</td>
<td>66.42</td>
<td>26.92</td>
<td>14.27</td>
<td>3.51</td>
</tr>
<tr>
<td>Std Dev</td>
<td>17.64</td>
<td>135.87</td>
<td>5.14</td>
<td>5.84</td>
<td>8.51</td>
<td>7.28</td>
<td>7.62</td>
<td>4.85</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Figure 3.2. Semen evaluation data for ejaculates used for insemination.
Figure 3.3. Mean (a) and individual (b) interovulatory interval for mares when in groups I and II.
a. Figure 3.4. Mean (a) and individual (b) treatment to ovulation interval for mares when in Groups I and II.

b. Mean Treatment to Ovulation Interval

![Bar chart showing mean treatment to ovulation interval for mares in Groups I and II.](chart)

- **Group I (n=10)**
- **Group II (n=10)**

*p < 0.05

- Treatment to Ovulation Interval for Individual Mares

![Bar chart showing treatment to ovulation interval for individual mares in Groups I and II.](chart)

- **Mare A**
- **Mare B**
- **Mare C**
- **Mare E**
- **Mare H**
- **Mare L**
- **Mare R**
- **Mare S**
- **Mare Y**
- **Mare X**
Figure 3.5. Number of pregnant mares following post-treatment insemination as detected by serial transrectal ultrasonography up to 14 days post-ovulation.
Figure 3.6. A 10-day embryonic vesicle in Mare A. The embryonic vesicle (arrow) measured 3.5 mm in diameter. Pregnancy was confirmed as a growing embryonic vesicle detected on at least two consecutive ultrasound examinations.
Figure 3.7. Mean concentration of plasma progesterone on the day of treatment and 48 hours after PGF administration in group I (p<0.05).
Figure 3.8. Mean concentration of plasma progesterone during the treatment period with in Group II. No mare achieved a sustained rise in progesterone. When compared to the diestrous following treatment (post-treatment cycle), the early diestrous period was different from the normal cycle, which were not different from one another.
Figure 3.9. Mean progesterone profile for mares during the diestrus period following treatment and insemination. There was no difference between experimental groups (p>0.05)
Figure 3.10. Selected progesterone profiles (n=2) from group I (a) and II (b) mares. Arrows on the x-axis represent ovulation; blue arrow denotes PGF administration for the group I mares.
Figure 3.11. Diameter of the two largest follicles throughout the treatment period for mares group I. Mare X had a double ovulation following treatment.
Figure 3.12. Diameter of the two largest follicles throughout the treatment period for mares in group II. Mares E and X had multiple ovulations following treatment.
Figure 3.13. Serial ultrasonographic images of the ovaries from mare S when in group I illustrating the formation of a luteinized unruptured follicle (LUF). On the day of PGF administration, she had a 40 mm follicle (yellow arrows) next to the existing functional CL (blue arrows). Thickening and irregularity of the follicle were evident and it had grown to 56 mm diameter by day 14, four days after PGF administration (B). At the time of insemination (C) on day 19, increased echogenicity of the follicular contents was apparent (yellow arrows) and a new 47 mm follicle had developed on the contralateral ovary (orange arrows). The follicle ovulated normally and formed a CH (D) following insemination and induction of ovulation with hCG while luteinization continued.
Figure 3.14. Ultrasonographic images of a CL from Mare A on day -1 (preovulatory follicle), day 0 (ovulation), day 1 (corpus hemorrhagicum), and day 4 (corpus luteum) when in group I (A to D) and group II (E to H) treatment cycles. The CL during early diestrus treatment demonstrated signs of structural regression (decreased area, hyperechoic center surrounded by a relatively hypoechoic margin) consistent with the functional changes in progesterone secretion. Concentration of plasma progesterone for each of the images was 0.5, 0.1, 0.7, and 6.5 ng/mL for A to D (representing group I), respectively. Concentration of plasma progesterone was 0, 0.1, 0.32, and 0.13 ng/mL for E-H (group II), respectively.
Chapter 4

Structural and molecular effects of antiluteogenesis and luteal resurgence in mares following PGF administration in early diestrus

Introduction

Recent research, from our laboratory and others, has challenged the traditional dogma and approach of giving relatively high doses (10mg dinoprost tromethamine) of PGF during mid diestrus to induce luteolysis in the mare. Rather, the CL is responsive to significantly lower doses of PGF [as low as a single dose of 1.25mg dinoprost (Barker et al., 2006) or two doses of 0.5mg (Irvine et al., 2002) at a 24 hour interval]. Additionally, the early diestrus CL (considered immature if <5 days old) is capable of responding to PGF, the PGF–induced pharmacologic responses elicited include complete luteolysis (Holland and Pinto, 2008, Rubio et al., 2008), partial luteolysis followed by luteal resurgence (Bergfelt et al., 2006, Holland and Pinto, 2008, Rubio et al., 2008), and anti-luteogenesis (Rubio et al., 2008) (failure of the CL to function). These processes are associated with early cessation of function and progesterone production, decreased progesterone production followed by recovery and increased levels, and failure of normal formation and
initiation of progesterone production, respectively. The response elicited depends on the dosage, timing, and protocol of administration. To date, the structural and molecular characteristics of these phenomena have not been investigated.

Assessment of natural and induced luteolysis has previously been reported based on microstructural characteristics, immunohistochemistry, and RT-PCR. A procedure has been described for conducting serial biopsies of the CL (Kot et al., 1999) and has been successfully used to characterize gene expression in the CL during PGF-induced luteolysis in the bovine (Tsai et al., 2001) and the equine (Beg et al., 2005, Slough et al., 2011). Similarly, microscopy and immunohistochemistry at different time points during diestrus or following PGF administration have been employed for descriptive observations of the changes occurring (Levine et al., 1979, Al-zi’abi et al., 2002, Al-zi’abi et al., 2003, Ferreira-Dias and Mateus, 2003, Ferreira-Dias et al., 2006, Muller et al., 2009). In the current study, immunohistochemistry for steroidogenic acute regulatory protein (StAR), factor VII, and caspase 3 to assess steroidogenic function, vascularization, and apoptosis, respectively.

StAR is a protein responsible for the transport of cholesterol from the outer to the inner mitochondrial membrane; this is a rate-limiting step in steroidogenesis. Its expression has been previously described in the equine CL (Watson et al., 2000). Because of its association with steroidogenesis, gene expression is correlated with changing progesterone synthesis (reflected by concentration of plasma
progesterone) during physiologic (Slough et al., 2011) and PGF-induced (Beg et al., 2005) luteolysis.

The CL achieves one of the highest levels of blood flow of any tissue in the body (Abdul-Karim and Bruce, 1973, Ford and Chenault, 1981, Wiltbank et al., 1988, Reynolds et al., 2000, Shirasuna et al., 2012). Therefore, luteogenesis involves rapid angiogenesis at a rate up to four to twenty times that of the most aggressive forms of cancer (Tamanini and De Ambrogi, 2004). Ultimately, vascular cells comprise a greater proportion of cells in the steroidogenic cells (representing 80% of the cell population in the bovine) (O'Shea et al., 1989). Factor VIII is a procoagulant factor that forms a complex by non-covalent bonding with von Willebrand factor and plays an integral role in the coagulation cascade. Because it is produced in endothelial cells, this is one of the most commonly used markers to assess angiogenesis and microvascular density (Meert et al., 2002, Ordonez, 2012).

Apoptosis is one important component of cellular and structural regression in the corpus luteum (Al-zi‘abi et al., 2002). Activated caspase-3 is a heterotetramer and one of a family of enzymes critical to apoptosis (Cohen, 1997), and it serves as a marker of programmed cell death. In the equine CL, immunostaining for caspase-3 increases significantly in late diestrus (days 14 and 17) and also 36 hours after cloprostenol injection (Aguilar et al., 2006). Similarly, by Western blot, there was a twofold increase in caspase 3 in the mid-CL, late-CL, and corpus albicans as
compared to the corpus hemorrhagicum, and these results were corroborated by immunocytochemistry (Ferreira-Dias et al., 2007).

The ability to observe protein expression by immunohistochemistry provides an opportunity to gain insight and understanding into various physiological processes. Responses of the CL to PGF during early diestrus (i.e. the ‘refractory period’) include early luteolysis, regression and resurgence of the early CL, and antiluteogenesis. These recently described phenomena in the equine CL have not been fully elucidated at the molecular level. Therefore, the objectives of the current study were to assess the changes observed by histology and immunohistochemistry for StAR, caspase-3, and factor VIII. It was hypothesized that structural and molecular changes observed in the CL would reflect functional changes in steroidogenesis (concentration of plasma progesterone) during the processes of luteal resurgence and antiluteogenesis in early diestrus.
Materials and Methods

Three reproductively and physically normal Standardbred mares were monitored by transrectal palpation and ultrasonography. Once signs of estrus (endometrial edema, sexual receptivity) were present in conjunction with a follicle at least 35 mm in diameter, ovulation was induced with 2500 IU of human chorionic gonadotropin (hCG; Chorulon®, Intervet, Millsboro, USA) intravenously. At detected ovulation (day 0), each mare was randomly assigned to receive one treatment. Mare I (the control mare) was not administered any further medications. Mare II (the antiluteogenesis mare) was administered twice-daily injections of 10 mg of PGF (Lutalyse®, Pfizer, New York, New York, USA) intramuscularly on days zero through two (total of six injections). Mare III (the CL resurgence mare) was injected twice daily with 2.5 mg PGF im on days zero through two. All injections were given in the neck with 22-gauge, 1-inch needles.

All mares were ovariectomized via colpotomy on day 3 post ovulation. For surgery, mares were restrained in stocks and administered 5-10 mg of detomidine intravenously (Dormosedan™, Pfizer, New York, New York, USA). The perineum was aseptically prepared for obstetrical procedures with betadine scrub. After
preparation, using clean technique, a douche of the vagina was performed with 1 L of 0.5% betadine solution in LRS.

Standard surgical technique was employed for ovariectomy. Briefly, a stab incision was made at the 2 o’clock position relative to the cervix in the cranial vagina using a pair of standard surgical scissors. Following penetration of the cranial vaginal wall, the incision was expanded and the peritoneum penetrated by blunt dissection. Once the reproductive tract was located, a chain ecraseur was introduced through the surgical site and the chain placed around the ovarian pedicle. The surgeon maintained the position of the ecraseur while another operator tightened the chain slowly for proper ligation and hemostasis. The procedure was then repeated on the contralateral ovary. Mares were administered 500 mg of flunixin meglumine (Banamine™, Merck Animal Health, Summit, NJ USA) intravenously and kept in a box stall for one to three days post-surgery.

Immediately following its removal via colpotomy, the ovary containing the corpus luteum was photographed and then sectioned for later analysis. At least two sections were flash frozen in liquid nitrogen inside labeled cryocassettes, two were placed into formalin tubes, and two frozen in optimal cutting temperature embedding medium (Tissue Tek® O.C.T. embedding media, Sakura Finetek, Torrence, CA, USA) in wells floating on liquid nitrogen.

Plasma samples were collected from mares at the time of induction of ovulation and at the time of surgery. Blood was collected via a 20-gauge, 1-inch
needle into a 10 mL Vacutainer (BD Vacutainer®, Bectin, Dickinson and Company, Franklin Lakes, New Jersey, USA) containing lithium heparin. Samples were kept on ice and centrifuged at 600 g for 10 minutes. Plasma was harvested, transferred to two cryovials, and stored at -80 C.

Tissues for histopathology were fixed in 10% neutral buffered formalin for a minimum of 24 hours. Hematoxylin and eosin stained sections were processed by routine methods. Immunohistochemistry was performed by standard methods utilizing primary antibodies for StAR (Biorbyt™, Orb7014, San Francisco, CA, USA), factor VIII (Dako, A0082, Campinteria, CA, USA), and Caspase-3 (Cell Signaling Technology, 9661, Boston, MA, USA) and secondary antibody conjugated to horseradish peroxidase. A peroxidase avidin-biotin complex method was used. Briefly, the sections were deparaffinized and then hydrated in xylene and distilled water, respectively. They were then pre-treated by heating to 125° C in citrate buffer (pH 6.1) (Dako Target Retrieval Solution™, Dako North America Inc, Campinteria, CA, USA) for epitope demasking followed by a 10 minute incubation in 3% hydrogen peroxide. A protein block was achieved by 10 minute exposure to a serum-free protein block. The antibodies were applied at the appropriate dilution in antibody diluent containing background reducing agents for 30 minutes. The dilution factors were 1:100, 1:200, and 1:180 for the StAR, factor VIII, and caspase 3, respectively. The slides were then incubated in a dilute biotinylated secondary antibody [anti-species antibody (Vector Laboratories, Burlingame, CA, USA)] diluted
to 1:500 in protein block followed by Vectastain RTU ABC Elite reagent™ (Vector laboratories) for 30 minutes. They were stained by a 5 minute incubation in 3,3’-diaminobenzidine for signal detection and counterstained in hematoxylin for 45 to 60 seconds. The sections were dehydrated in ethanol and cleared in xylene. A veterinary pathologist familiar with reproductive histopathology examined all slides.
Results

The gross and ultrasonographic appearances of the corpora lutea at the time of ovariectomy on day 3 are shown in Figures 4.1 and 4.2. Mare I (the untreated mare) had a synchronous double ovulation (both occurred within 24 hours apart from each other). Although no formal imaging analysis was performed, the corpora lutea exhibited different echotextures from one another. The corpus luteum from mare I was normal for a day 3 CL in the equine (large, uniform echogenicity) whereas it was hard to distinguish the CL in mare II from surrounding ovarian tissue and the CL from mare III demonstrated signs of luteal regression (increased echogenicity of the center surrounded by relatively hypoechoic tissue, decreased area of the CL).

All mares had a concentration of plasma progesterone less than 1 ng/mL, consistent with estrus, at the time that ovulation was induced at the commencement of the experimental period. On the day of surgery (day 3), concentrations of progesterone were 16.37 ng/mL, 0.1 ng/mL, and 0.15 ng/mL for mares I, II, and III, respectively. Histopathology of sections stained with H and E from each mare are shown in Figure 4.3. In mare I, the corpus luteum exhibited normal luteal cells as well as signs of hemorrhage and fibrin deposition within the interstitium of the
corpus luteum. Mare II (that received the antiluteogenesis treatment) had decreased cellularity and organization of the CL, resulting in appearance of interstitial hemorrhage and moderate to severe parenchymal hemorrhage within the CL. The tissue from mare III (that was treated to elicit luteal resurgence) had less intraparenchymal hemorrhage than mare II and was more comparable to mare I, with interstitial hemorrhage rather than parenchymal. In contrast with the CLs from mares I and II, a significantly greater number of apoptotic luteal cells were present in mare III (figure 4.4). There was a greater degree of neutrophilic and eosinophilic infiltration into the parenchyma of the CL from mare II as compared to mares I and III.

Immunohistochemistry results are presented in Figures 4.5 to 4.8. All three mares had positive cytoplasmic staining for StAR by immunostaining (Figure 4.5). In mare II, factor VIII staining was evident throughout the interstitium and parenchyma in the areas of fibrin and hemorrhage accumulation, demonstrating the same changes evident with H and E staining (Figure 4.6b). Mare III subjectively had decreased vascular profiles (decreased endothelial factor VIII staining) as compared to mare I. A greater number of cells in the CL from mare II stained positive for caspase 3, denoting greater apoptosis as compared to mares I and II.
Discussion

Serial treatment with dinoprost in early diestrus at 10 or 2.5 mg resulted in subluteal concentration of plasma progesterone in mares and II and III, respectively, as compared to the untreated mare I. The baseline value of concentration of plasma progesterone for mare III (model for luteal resurgence) is consistent with previous experiments, where increases to luteal concentrations were not observed until approximately 24 hours after the cessation of treatment. The treatment regimen employed was chosen because it was a reliable model for luteal resurgence in previous experiments (Rubio et al., 2008), successfully inducing in luteal resurgence in all mares treated with sub-luteolytic doses of PGF.

Histologic examination of the corpora lutea in experimental mares in the present study was consistent with their ultrasonographic appearance and the functional changes occurring following ovulation and PGF treatment. Mare I demonstrated normal formation of a corpus hemorrhagicum or corpus luteum with hemorrhage and fibrin deposition within the luteal gland interstitium. The sections from mare II (treated with higher, antiluteogenic doses of PGF) had decreased cellularity and organization of the early CL characterized by apparent marked parenchymal hemorrhage as compared with the other two mares. These findings
are more suggestive of a delayed or impaired formation of the luteal gland as compared with the control mare (Mare I). The CL from mare III demonstrated increased white blood cell (neutrophil and eosinophil) infiltration as compared with mares I and II. This is consistent with the description of increased neutrophil infiltration with induction of luteolysis by administration of supraphysiologic doses of exogenous PGF but not during natural luteolysis (Lawler et al., 1999, Al-zi’abi et al., 2002). There was also evidence of apoptosis on histopathology in mare III, consistent with previously reported changes observed in natural and PGF-induced luteal regression in late diestrus (Al-zi’abi et al., 2002). Thus, mare III showed signs that were consistent with partial luteal regression whereas those observed in mare II were more suggestive of prevention or impairment of normal luteal formation (antiluteogenesis).

Previous studies have focused on VEGF as an essential modulator of angiogenesis in the CL (Reynolds and Redmer, 1998). Alterations in the expression of VEGF have been temporally associated with changes in CL angiogenesis in the horse (Al-zi’abi et al., 2003). A decrease in VEGF expression and vascularization is associated with natural and induced luteolysis in the bovine (Niswender et al., 1976, Reynolds and Redmer, 1998), ovine (Nett et al., 1976), and equine (Al-zi’abi et al., 2003). For the present study, factor VIII was chosen as alternate marker of angiogenesis and vascular profile. The decreased vascular profile in the CL from mare II as compared to the other mares was consistent with those described for
VEGF in the equine CL during luteolysis in mares. Once again, these findings are consistent with partial demise of the CL in mare III but prevention of its formation altogether in mare II.

Future studies will expand on the findings in the current study. A greater number of study subjects are necessary for broader conclusions. Additionally, serial biopsies and RT-PCR may be employed as previously described to document temporal changes and potential alterations in gene expression (in addition to protein expression) in response to serial administration in the early post-ovulatory period.

In the present investigation, findings were consistent with our hypothesis that changes in histologic appearance and protein expression reflected functional changes in steroidogenesis. During luteal resurgence despite the evidence of luteal regression (apoptosis, white blood cell infiltration, decreased vascular profile), the histologic appearance of luteal cells appears normal, possibly indicating their ability to maintain steroidogenic potential. Accordingly, there was no difference in StAR expression evident on IHC among the treated mares in the present study. Taken together, it seems partial structural luteal regression observed in mare III with preservation of steroidogenic potential may contribute to the return (resurgence) to luteal concentrations of plasma progesterone following with sub-luteolytic doses of PGF. Prevention of functional luteal formation by administration of serial PGF at higher doses in early diestrus has previously been coined as, ‘antiluteogenesis,’ and
the present study demonstrates that structural luteal formation is at least partially interrupted, as there were marked changes in cellularity and structure without the signs typically associated with luteal regression. Therefore, structural luteal formation appears to be interrupted in addition to steroidogenesis.
Figure 4.1. Gross appearance of the corpora lutea immediately following colpotomy on day 3 in mare I (A&C), II (B), and III (C). Mare I had a double ovulation.
Figure 4.2. Ultrasonographic appearance of the corpora lutea of mares just prior to ovariecomy on day 3 in mare I (A), II (B), and III (C).
Figure 4.3. CL from mare I (a, b), II (c, d), and III (e, f) stained with H and E. Mare I showed normal luteal formation, with evident interstitial hemorrhage and fibrin deposition (black arrows). Mare III had similar interstitial hemorrhage in addition to apoptotic cells and white blood cells. Mare II had marked parenchymal hemorrhage (white arrows).
Figure 4.4. H and E stained section from the CL of mare III demonstrating apoptotic luteal cells (black arrows) and white blood cell infiltration (white arrows) within the luteal gland.
Figure 4.5. Immunostaining for StAR was evident in the cytoplasm of the luteal cells in mare I (a), II (b), and III (c). No clear difference was evident in staining for StAR among the three mares.
Figure 4.6. Immunostaining for factor VIII in the CLs from mare I (a), II (b), and III (c), demonstrating decreased cellularity and intraparenchymal hemorrhage in mare II (as with H and E staining) and decreased vascular profile (decreased positive staining) in mare III compared to mare I.
Figure 4.7. Immunohistochemistry for Caspase 3 in the corpora lutea of mares I (a), II (b) and III (c). There was an increased amount of positive staining, indicating increased apoptosis, in mare III.
Figure 4.8 Higher magnification of immunohistochemistry for Caspase 3 in the CL section from mare III, demonstrating the positive-staining apoptotic cells.
Chapter 5

Conclusion

Recent research, including that investigated herein, has offered considerable challenge to the dogma that the early CL is refractory to PGF. Most significantly, we have demonstrated that serial administration of PGF (dinoprost) in early diestrus can reliably prevent function of the CL and steroidogenesis, thereby inducing a return to estrus similar to mares treated with a single dose later in diestrus. Moreover, there are no inherent negative effects on the fertility of the induced estrus following antiluteogenic treatment.

For the first time, structural and cellular effects of antiluteogenesis and luteal resurgence were examined. Luteal resurgence showed changes similar to those seen in luteal regression (apoptosis, white blood cell infiltration) and some decrease in cell organization. Antiluteogenic treatment resulted in at least some interruption in structural luteal formation characterized by decreased cellularity and organization, consistent with the term ‘antiluteogenesis.’

The protocol utilized in the present study is not being proposed as a replacement for the existing practice of administering one single injection of PGF at
least five days following ovulation. However, this approach could be used clinically for select cases where mares may benefit from immediate prevention of luteal formation in an attempt to induce estrus and ovulation. The consistency of the evoked response to the protocol described in the present study and its lack of negative effects on fertility make this a potentially valuable clinical tool for breeding management in the mare. Manipulation of luteal function is one of the most common methods of manipulating the equine estrous cycle for timed breeding and estrus synchronization, and administration of serial doses of PGF beginning at immediately after ovulation to induce antiluteogenesis represents a novel clinical approach.
References:


Handler, J., A. Wustenhagen, D. Schams, H. Kindahl, and C. Aurich. 2004. Estrous cycle characteristics, luteal function, secretion of oxytocin (OT) and plasma concentrations of 15-


Nie, G. J., K. E. Johnson, J. G. Wenzel, and T. D. Braden. 2003b. Luteal function in mares following administration of oxytocin, cloprostenol or saline on day 0, 1 or 2 post-ovulation. Theriogenology 60(6):1119-1125.


