CONTROL OF LUTEOLYSIS IN THE MARE

Vanda Gonçalves dos Santos

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Ciências Veterinárias

ORIENTADORA: *Elisa Maria Varela Bettencourt*

ÉVORA, MAIO 2015

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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Abstract

Persistent corpus luteum (PCL) is one cause of infertility in mares. However, its nature is poorly understood. Furthermore, role of oxytocin during luteolysis is not clear. A series of experiments were conducted (1) to characterize PCL and to compare PCL with interovulatory-intervals (IOIs) and (2) to evaluate the role of oxytocin during luteolysis. The induction of PCL was also attempted using a PGF2α secretion inhibitor. Oxytocin was used to induce luteolysis. Progesterone (P4) concentration decreased in IOI and PCL until Day 14 postovulation and then diverged, whereas PGFM concentration did not differ between groups. Transient P4 depressions were observed during PCL. Before the end of luteolysis P4 concentration was less in PCL than IOI. Inhibition of PGF2α secretion caused a 1-day increase in the length of the luteal phase. Oxytocin caused a P4 decrease within 8-hours and a PGFM increase within 1-hour after infusion and induced partial luteolysis.

Key-words: Mare, Persistent corpus luteum, PGFM, Progesterone, Oxytocin.

Controlo da luteólise na égua

Resumo

O corpo lúteo persistente (PCL) é uma causa de infertilidade em éguas, no entanto a sua origem não é conhecida. Além disso, o papel da oxitocina na luteólise na égua não está claro. Foram realizados estudos para (1) caracterizar o PCL e comparar o PCL com intervalos-entre-ovulações fisiológicos (IOI) e (2) determinar o papel da oxitocina na luteólise. A indução do PCL foi tentada através da inibição da secreção de PGF2α. A oxitocina foi utilizada para induzir a luteólise. A progesterona (P4) diminuiu em éguas com IOI e PCL até ao dia 14 pós-ovulação divergindo entre grupos posteriormente; a PGFM não diferiu entre grupos. Foram observadas diminuições transitórias de P4 na presença de PCL. Antes da luteólise a P4 era menor em éguas com PCL comparado com IOI. A inibição da secreção de PGF2α aumentou em 1 dia a duração da fase lútea. A oxitocina diminuiu a P4 em 8 horas e aumentou a PGFM em 1 hora após o início da infusão, causando luteólise parcial.

Palavras chave: Égua, Corpo lúteo persistente, PGFM, Progesterona, Oxitocina.

“Nature hides its secrets well.”

O. J. Ginther

Dedication

To Paulo who fought hard to make this possible and makes me a better person.

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Abbreviation	Unabbreviated	Abbreviation	Unabbreviated
CL	corpus luteum	NS	not significant
cm^2	square centimeter	OT	oxytocin
CV	coefficient of variation	P	probability
E2	estradiol	P4	progesterone
FM	flunixin meglumine	PCL	persistent corpus luteum
FSH	follicle stimulating hormone	pg	picogram
h	hour	PGF2α	prostaglandin F2α
HAF	hemorrhagic anovulatory follicle	PGFM	Prostaglandin F2α metabolite
IGF-1	Insulin-like growth factor-1	reFSH	recombinant FSH
IOI	interovulatory interval	reLH	recombinant LH
ir-inhibin	Immunoreactive-inhibin	RIA	Radioimmunoassay
IU	international units	SEM	Standard error of mean
iv	intravenous	y	year
kg	kilogram		
LH	luteinizing hormone		
mg	milligram		
MHz	megahertz		
min	minutes		
mL	milliliter		
mm	millimeter		
mo	month		

Preamble

The estrous cycle of the mare has been subject of study for almost half a century. The fact that mares are a seasonal specie represents a challenge for the study of the physiological estrous cycle because of its short ovulatory season. From mid-summer until fall the characteristics of the estrous cycle begin to change which can lead researchers into erroneous conclusions about the reproductive physiology of the mare. Although numerous reports can be found on different subjects of the estrous cycle of the mare, this specie still holds and insists in keeping its secrets from us.

The objectives of this thesis document were many fold. Firstly, we aimed to determine the role of oxytocin (OT) during luteolysis in mares, testing the hypothesis that luteolysis can be induced through the administration of OT. Of particular importance to this topic our objectives were (1) to determine a dose and method of administration of OT that will stimulate a PGFM pulse similar to a spontaneous pulse and (2) to study the role of OT in luteolysis.

In some reports [1], it has been hypothesized that the failure of secretion of prostaglandin-F2α (PGF2α) at the time of expected luteolysis may be the cause of idiopathic persistent corpus luteum (PCL). Therefore, our second objective was to attempt the induction of the formation of the PCL through the inhibition of PGF2α secretion using a cyclooxygenase inhibitor administered at the time of expected luteolysis. Our third and last objective, was the characterization and description of the hormonal and ovarian changes during the spontaneous development of an idiopathic PCL from Day 12 until Day 22 (during the time of expected luteolysis that failed to occur) and during the maintenance of the idiopathic PCL (normalized to the end of luteolysis and ovulation at the end of an IOI and PCL).

1. Literature review

1.1. Introduction

The estrous cycle, or more precisely the interovulatory interval, begins at ovulation usually associated with estrus and ends at the ensuing ovulation associated with the next estrus. The mean length of an interovulatory interval is typically 22 or 24 days in horses or ponies, respectively. The estrous cycle or interovulatory interval is characterized behaviorally by diestrus (12 to 16 days) and estrus (5 to 9 days) or, physiologically, by luteal phase and follicular phases, respectively [2,3]. In this thesis document, attention will only be given to physiological characteristics of the interovulatory interval and not to the behavioral characteristics, since the latter can be misleading and usually associated with a large variation among individuals. In addition, the literature review presented herein will focus on the physiological events of the estrous cycle of the mare (follicular and luteal phase).

1.2.1. Follicular phase

a) The wave phenomenon

In the domestic species the follicular development occurs in waves [2]. A major follicular wave in mares is defined as a number of several follicles that initially grow in synchrony but eventually dissociate or diverge in diameter [2,4]. Dissociation is characterized by preferential growth of one, occasionally two, members of the wave [2].

Waves are classified as major (divergence of follicles of a wave into dominant and subordinate follicles) and minor (no divergence) [4]. The largest follicle after divergence is named of dominant follicle, and those that regress after divergence are named of subordinate

follicles. The dominant follicle grows to a large diameter (e.g., > 30 mm), and this characteristic has also been used to define a major wave [2,4]. A minor follicular wave is characterized by the failure of the largest follicle to reach a large size and the apparent absence of dissociation and dominance. The terms secondary and primary waves have also been used for the equine estrous cycle. A secondary wave is a major wave that emerges during late estrus or early diestrus and its dominant follicle becomes anovulatory or terminates in a diestrous or secondary ovulation [4]. The development of a secondary follicular wave occurs in approximately 25% of the estrous cycles [5]. A primary wave is a major wave that originates during diestrus and gives origin to a dominant follicle that terminates in the primary ovulation (ovulation associated with estrus at the end of the interovulatory interval) [4].

b) Emergence

Emergence of follicular waves refers to the earliest ultrasonic detection of follicles compatible with retrospective tracking and is represented in mares by diameters of 6 mm [6]. Emergence of the primary wave occurs in mean on Day 7.4 (Day 0 = ovulation; range from Day 3 to Day 14) [4]. Length of the interovulatory interval is positively correlated with the day of emergence of the primary wave. That is, early or late emergence of a wave is associated with shorter or longer interovulatory intervals, respectively [4]. Emergence of each wave is temporally associated with an FSH surge for both the major and minor waves in mares. The FSH surge reaches a peak or a plateau when the largest follicle reaches about 13 mm [7]. The FSH concentrations then decline [6]. In mares, the future dominant follicle emerges 1 day earlier than the future largest subordinate follicle [7].

c) Acquisition of dominance / Deviation

After emergence, the follicles of a wave enter a common-growth phase involving reported means of 7 to 11 follicles [7]. Usually, in mares, only one of the available follicles develops into the dominant follicle, and this phenomenon is known as folliclular selection. Follicle selection or deviation occurs at the end of the common-growth phase and is characterized by continued growth of the largest follicle to become the dominant follicle and a reduction or cessation of growth by the remaining follicles to become subordinate follicles [6,8]. The beginning of observed deviation in the diameter profiles of individual follicles occurs at the ultrasound examination preceding the first examination with an apparent change in diameter differences between the two largest follicles [9]. Mean diameter observed at the beginning of deviation in mares is 22.5 mm with deviation beginning a mean of 6.2 days after emergence of a 6 mm follicle [6,7]. Between emergence and deviation, the growth rate of the two largest follicles seem parallel in about 50% of individual waves [7].

The follicle that first reached 20 mm (with daily examinations) became the dominant follicle in 93% of 29 waves. This diameter occurred on average at the examination before the beginning of deviation [8]. In adition, on average, the future dominant follicle reached 6 mm before the future largest subordinate follicle and maintained a mean diameter advantage of 3 mm until the beginning of deviation [7]. The difference in diameter between the two largest follicles at beginning of deviation is postulated to allow the largest follicle to establish dominance before the second largest follicle can reach a similar diameter [9]. The difference in diameter between the two largest follicles indicates that the smaller follicle must be inhibited in < 1 day (equivalent to a difference of 3 mm) [6]. The deviation mechanism must prevent the continued growth of the future subordinate follicles because they, as well as the future dominant follicle, are capable of dominance as indicated by studies involving ablation of specific follicles [6,10]. The deviation in diameter is likely preceded by biochemical or

functional deviation. In this regard, echotextural changes were detected in the wall of the largest follicle on the day before the beginning of diameter deviation in mares [11].

The main pituitary hormone in mares involved in the stimulation and regulation of follicle development is FSH [12]. A close two-way functional relationship between FSH and the follicles has been proposed to be an integral component of the deviation mechanism [8]. The depression of FSH concentration could be the critical event in deviation if the FSH is depressed below the quantities required by the smaller follicles, but not the largest follicle. The changes in FSH concentrations and follicle development are closely related [6]. The FSH surge that stimulates emergence of a wave begins to decline when the largest follicle is about 13 mm [6]. The interval from the beginning of the FSH decline to the beginning of deviation is about 3 days. After the beginning of deviation, the concentration of FSH continue to decline reaching a nadir 2 or 3 days after deviation [6,13]. Follicular growth for about 2 days after emergence is independent of FSH. However, during the decline in the wave-stimulating FSH surge and before deviation, growth of follicle is dependent on FSH [13]. During the interval from the beginning of the FSH decline until the beginning of deviation, multiple follicles contribute to the FSH decline [8]. Nevertheless, FSH is needed to maintain follicular viability even after the FSH peak, that is, the declining FSH concentrations is required for continued follicle growth [13]. The FSH:follicle relationship changes from multiple to single-follicle at the beginning of deviation and has been demonstrated by ablating only the largest follicle, only the smaller follicle, or none of the follicles when the largest follicle reached ≥ 20 mm (expected day of deviation) [10]. The decline in FSH concentrations of the wave-stimulating FSH surge continued for several days after ablation in the groups with no ablation or only the smaller follicle ablated. In the group with only the largest follicle ablated, the FSH and diameter of the smaller follicle increased within a day [10]. The continued decrease in FSH at the expected beginning of deviation can be attributed to the largest follicle, with no indication that the smaller follicle is involved [6]. Therefore, by the time of beginning of deviation, the future

dominant follicle develops the ability to suppress circulating FSH to below the concentration required by other follicles and the ability to utilize the low FSH concentrations in its further growth and development [6]. It has been proposed that regression of the smaller follicles involved a direct effect of follicle inhibitors secreted by the larger follicle, but the evidence is not convincing [14].

Inhibin, has been shown to be the FSH inhibitor during the early development of a follicular wave and is produced by the growing follicles of a follicular wave [12,15]. During the estrous cycle, in mares, peripheral concentration of total inhibin increase at the time FSH concentrations are declining, having a known negative feedback effect on FSH secretion [12,16,17]. Inhibin concentrations increase with follicle growth [18], but do not reach circulating concentrations that are sufficient to initiate a decline in the wave-stimulating FSH surge until the largest follicle reaches 12 or 13 mm [19,20]. An FSH-suppressing activity of equine follicular fluid has been demonstrated by an increase in FSH concentrations after ablation of follicles and is attributable to the removal of FSH-suppressing factors of follicle origin [6]. In adition, administration of an inhibin antiserum increased plasma FSH concentrations and increased the number of large follicles and ovulations [6]. The first 2 days of the FSH decrease are caused by inhibin, based on the positive relationship between the number of experimentally retained follicles and the extent of the increase in circulating total inhibin concentrations and the corresponding decrease in FSH. Near the expected day of deviation, total inhibin remained at elevated concentrations and likely contributed to the continuing FSH decline [8]. In this regard, treatment with serum containing inhibin antibodies on the day the largest follicle was 20 mm resulted in an increase in the number of 30 mm follicles [21,22]. After deviation, inhibin continues to be a FSH suppressant in mares [8].

In mares, an increase in estradiol (E2) occurred in the follicular fluid [23], and echogenic changes characteristic of estrogen stimulation occurred in the wall of the future dominant follicle the day before the beginning of deviation [11]. The beginning of increased

local availability of E2 approximately coincides with the beginning of deviation. Reported autocrine and paracrine activities of E2 in the granulosa cells include enhancing aromatase enzyme activity, promoting expression of LH receptors, and enhancing the sensitivity to FSH and LH [8]. The production of E2 by the follicle at deviation has been shown to be dependent on gonadotropins [24]. The increased E2 produced by the developing dominant follicle near the beginning of deviation may be attributed to the transient elevation in LH concentrations and the increased expression of LH receptors by the granulosa cells [8]. Direct LH-induced secretion of E2 within the granulosa apparently has not been demonstrated in horses [25]. However, E2 does not begin to increase in the circulation until 1 or 2 days before the beginning of deviation in mares [19,23]. Thus, on a temporal basis, increased E2 apparently does not contribute to the FSH decline until the day before deviation. Ablation of the largest follicle at the expected beginning of deviation prevented the continuing E2 increase and resulted in associated FSH increase [10]. However, an FSH increase does not occur when only the second largest follicle is ablated [8]. Both E2 and inhibins are lost upon ablation of the largest follicle and a decrease in either or both could account for the resulting increase in FSH [8]. Inhibin from the wave of growing follicles apparently is the principal, if not the only, systemic FSH supressant during most of the common-growth phase. Circulating E2 concentrations increase near the beginning of deviation, and it appears that both inhibin and E2 contribute to the subsequent continuation of the FSH decline [8].

Elevated LH concentrations were temporally associated with deviation and often were represented by a distinct part of the prolonged ovulatory LH surge in this species [7]. The granulosa cells of the future dominant follicle acquire LH receptors just before the beginning of deviation, providing a pathway for a functional LH effect of the early portion of the transient LH elevation [8]. Detectable amounts of LH receptors are present in granulosa cells of all follicles more than 5 mm in diameter in the mare and LH receptor content in granulosa cells increases with follicular diameter [18,26] being greater when the follicles are 20-24 mm than

15-19 mm [18], but a differential change in LH receptor content in granulosa cells of the future dominant versus subordinate follicles at the beginning of deviation has not been reported. Experimental reduction of LH concentrations decreased the diameter of the dominant follicle beginning 1 or 2 days after the beginning of deviation in mares [27,28]. A study with acyline induced gonadotropins supression and with administration of reFSH, reLH, or both showed that follicle diameters are dependent only on FSH during the first 48 hours after the beginning of deviation, with no aditional dependence on LH [24]. In adition, the reduced LH did not affect the second-largest follicle. Nevertheless, the onset of deviation was not delayed by the reduction of LH, but the post-deviation growth of the largest follicle was reduced [27]. The transient elevation in LH at deviation stimulates the IGF system, as well as the steroid system. At the beginning of deviation, the largest follicle is able to utilize the low concentrations of FSH, presumably enhanced by local effects of E2 and IGF-1. The second largest follicle has not developed to a similar extent and therefore cannot withstand the low FSH concentrations. Thereby, deviation is established before the second-largest follicle can develop to a stage that would allow it to assume the biochemical characteristics of a future dominant follicle [8].

Before the beginning of deviation, differential concentration changes occur between the future dominant and subordinate follicle in E2, free insulin-like growth factor (IGF)-1, activin-A, and inhibin-A [29,30]. The IGF-1 system seems to be the most critical among these factors for the initiation of deviation in mares. In experimental deviation, an increase in IGF-1 occurs before deviation, whereas E2, activin-A, and inhibin-A concentrations increase after deviation [12,30]. Acyline-treated mares were given reFSH, reLH, or both, and in the groups that were not given FSH replacement, the IGF-1 concentrations were lower in both the dominant and second largest follicles and the lower IGF-1 was assotiated with retardation of follicular growth. The temporal availability of IGF-1 as an enabling factor in deviation in mares was also supported by the greater concentration of free IGF-1 in the dominant follicle than in the second largest follicle at 48 hours after the expected beginning of deviation [24]. In adition, injection

of recombinant human IGF-1 into the second largest follicle at the expected beginning of deviation induced an increased incidence of dominance in the treated follicle [31,32] and injection of IGF binding proteins-3 (IGF antagonist) into the dominant follicle caused the regression of the dominant follicle and the subordinate follicle to become dominant [33]. Furthermore, the injection of recombinant human IGF-1 into the dominant follicle at the expected beginning of deviation maintained follicular growth in mares treated with acyline [34]. These findings lead to the conclusion that gonadotropins are needed for increased follicular fluid concentrations of free IGF-1 at the beginning of deviation in the mare, and the increase in intrafollicular free IGF-1 in the future dominant follicle is FSH dependent [24,34].

d) Follicular growth, periovulatory period and ovulation

After deviation, the ovulatory follicle grows at a rate of 3 mm/day, reaching 35 mm 4 days before ovulation or at the beginning of the preovulatory period. The growth rate continues until 2 days before ovulation when it reaches a mean preovulatory plateau of 41 mm [35].

The E2 and LH periovulatory surges begin approximately 10 days before the periovulatory period or near the end of luteolysis and a day or two before follicle deviation as mentioned previously [35,36]. Estradiol from the dominant follicle forms a surge in the plasma that reaches a peak two days before ovulation and then recedes. The LH concentrations in the ovulatory surge increase slowly and then more rapidly, with the transition between the slow and rapid increases occurring at the peak of the E2 surge. The enhanced output of LH reaches maximum 1 day after ovulation [35,36]. The change in rate of LH output during the surge in mares is attributable to a negative effect of E2 on LH throughout the LH surge [37]. A negative effect of the follicles [15] and specifically E2 [37] on LH has been demonstrated by follicle ablations and treatments with E2. The decrease in E2 and the reduction or cessation in growth of the preovulatory follicle beginning 2 days before ovulation are attributable to the

development of a reciprocal negative effect of LH on follicular E2 production when LH reaches a critical concentration. This conclusion is based on temporal relationships and on the E2 decrease and reduction in follicle expansion beginning immediately after human chorionic gonadotropin treatment [38]. The initial rapid decrease in E2 from 2 days before to 1 day after ovulation is attributable to the negative effect of the rapidly increasing LH on E2, and the slower decrease after 1 day postovulation is attributable to the diminishing negative effect of the decreasing LH [39].

One to three surges in FSH concentrations occur during the luteal phase. The first surge begins just before ovulation when E2 is decreasing, and the peak of the last surge occurs when the largest follicle of the resulting ovulatory follicular wave is approximately 13 mm. Concentrations of FSH reach a nadir between the last surge of the estrous cycle and the first surge that will peak during the next cycle. The nadir occurs concomitantly with the preovulatory E2 peak. The occurrences of surges at different times and variation in the number of surges among mares account for the plateau in the mean FSH profile [35]. A negative effect of E2 on FSH accounts for the periovulatory E2/FSH temporal relationships; the negative effect has been demonstrated by the administration of E2 [20].

A slight but significant increase in circulating progesterone (P4) occurs with consistency among mares on the day of detection of ovulation or a collapsed follicle. The LH decrease after the peak of the LH surge on day 1 is related to a negative effect of the postovulatory increase in P4. The negative effect of P4 on LH is indicated by the temporal relationships at the beginning and end of the luteal phase [36] and by a demonstrated negative effect of exogenous P4 on LH [27].

There is indication that considerable amounts of follicular fluid with high concentrations of hormones passes through the fimbrae of the oviduct into the peritoneal cavity at ovulation [5]. The hormones are absorbed from the peritoneal cavity and alter circulating concentrations of E2 and inhibin, which in turn alter concentrations of LH and FSH. A

preovulatory collection of fluid external to the ovary in the infundibular area has been detected by transrectal ultrasonic imaging [40,41]. As ovulation approaches, a bulge at the apex of the follicle can be detected at the ovulation fossa by ultrasonic imaging [41]. The follicular fluid enters the infundibular fluid during follicle evacuation at ovulation. Most of the follicular fluid passes into the abdomen, based on continuous ultrasonic imaging in mares [40].

The gradual preovulatory mean increase in each gonadotropin is temporarily disrupted at ovulation. Concentrations of LH and FSH increased significantly between 1 and 0.5 days before ovulation and 0.5 and 1 days after ovulation but not between 0.5 days before and 0.5 days after ovulation. Concentrations of E2 decreased significantly between 1 and 0.5 days before ovulation and between the day of ovulation and 0.5 days after, but the decrease between 0.5 days before ovulation and the day of ovulation was not significant [5,42]. The transient disruption in LH increase can be attributed to release of the E2 content of the follicular fluid into the peritoneal cavity, followed by absorption into the circulatory system [5,42]. A distinct spike in circulating immunoreactive (ir)-inhibin also occurs on the day of ovulation in mares [17]. It has been shown that inhibins increased immediately after ovulation and returned to basal concentrations in 12 hours. Sampling of peritoneal fluid showed inhibin-A concentrations that were 300 times greater immediately after ovulation than at other stages of estrus [43]. The spike in (ir)-inhibin concentrations in association with evacuation of the follicle at ovulation only partly accounts for the depicted transient suspension in the FSH increase from 0.5 days before to 0.5 days after ovulation [17]. In addition, a negative effect of E2 on FSH has been demonstrated by treatment with E2 [20]. The synergistic effect of E2 and inhibin from the follicular fluid that entered the peritoneal cavity more completely accounts for the transient suppression of the incline in FSH. The E2 content of the discharged follicular fluid on the day of ovulation [5] is adequate for disrupting the LH surge [37] and contributing to disruption of the FSH increase.

Ovulation is readily detected ultrasonically by the disappearance of a large follicle that was present at a recent previous examination [2].

1.2.2. Luteal phase

a) Corpus luteum

The corpus luteum (CL) is a transient endocrine gland involved in establishment and maintenance of pregnancy due to production of P4 and has a critical role in the estrous cycle periodicity [44]. The CL goes through a developmental, maintenance and regressive stages [2], and forms at the site of ovulation, developing from cells that remain in the follicle following ovulation but is eventually composed of multiple, distinctive cell types including steroidogenic cells (small and large luteal cells) and non-steroidogenic cells (endothelial cells, pericytes, fibrocytes, and immune cells) [45]. The luteal phase has a mean length of 14 days.

After ovulation there is an immediate increase in P4 concentrations, starting on the ovulation day (Day 0) and reaches a maximum on Day 8. After Day 8, concentrations of P4 decrease gradually until the beginning of luteolysis. The period between Day 8 and 14 is designated as preluteolytic period. The area of the corpus luteum reaches a maximum on Day 4 and progressively decreases thereafter until Day 19. During preluteolysis the rate of decline in plasma P4 and area of CL are not different. The gradual decline in P4 concentrations during the preluteolytic period is temporally related to a gradual decline in LH concentrations from the ovulatory LH surge. The concomitant gradual decline in both P4 and luteal area seems attributable to a decreasing positive effect of LH on the CL [46].

b) Corpus hemorragicum

After ovulation, 68% of the luteal glands develop a large, fluid-filled, central area. The central area was attributable to blood [47], and the structure was designated by corpus hemorragicum. The central area develops gradually. It increases in area for the first 52 hours reaching a maximum at 72 hours. Echogenic lines within the central area were first detected, on average, 44 hours after follicle evacuation. The echogenic lines within the central area are attributable to clotting and fibrinization of the contents. Fibrin-like material within the blood clot is echogenic, so images of corpora lutea with this morphology had an echogenic network interspersed within the anechoic area [48]. The development of a central fluid area did not alter circulating P4 concentrations or the length of the interovulatory interval [2].

c) Luteolysis

In the absence of a viable embryo, functional and structural regression of the CL occurs, event named luteolysis. A chronology of research findings in luteolysis in the mare for the past 40 years has been reported [49]. Luteolysis is divided in three stages: preluteolysis, luteolysis, and postluteolysis. Preluteolysis occurs before the beginning of regression of the corpus luteum (defined as Days 8–14). Luteolysis is the period during regression of the corpus luteum until P4 has decreased to < 1 ng/mL. The period after luteolysis when concentrations are < 1 ng/mL is termed postluteolysis. Luteal transition refers to the hour at the end of preluteolysis and beginning of luteolysis, and is based on P4 determinations every hour [50]. The transitional hour is selected subjectively by inspection of the P4 profile for each animal and is based on the beginning of a progressive decrease in P4 in the hourly blood samples [51]. In the mare, luteolysis begins on average 14 days after ovulation or 9 days before the next ovulation, and it lasts approximately 23 hours, based in hourly blood sampling [50,52]. In many species,

including mares, CL regression is due to multiple pulses of prostaglandin F2α (PGF2α) secreted in a pulsatile way by the nonpregnant uterus [53]. Concentrations of PGF2α in the systemic circulation are usually assessed by measuring its metabolite, 15-keto-13,14-dihydro-prostaglandin F2α (PGFM), owing to the short half-life of PGF2α [54,55]. Luteal regression involves secretion of PGF2α in several sequential pulses during the length of the luteolytic period (two to four pulses) [52,56]. The peak concentration does not differ between a preluteolytic and the transitional pulse (~ 45 pg/mL), but the peak concentrations increase progressively between the first luteolytic pulse (~ 78 pg/mL) and the second luteolystic pulse (~ 193 pg/mL) to the postluteolytic pulse (~ 305 pg/mL) [52]. The base of the PGFM concentrations is maintained throughout the three periods, but mechanisms exist for increasing the prominence of the pulses during luteolysis and postluteolysis. Contributing factors in this regard may be the increase in circulating E2 concentrations which begin to increase a few hours after transition and the decreasing P4 beginning at transition [52].

In mares the PGF2α pulse that initiates luteolysis (transitional pulse) is relatively small (approximately 50 pg/ml). Approximately eight hours before the transitional pulse, the CL is exposed in the majority of the mares (67%) to two or three preluteolytic PGFM pulses similar in magnitude to the transitional pulse at a mean of peak to peak interval of 8 hours, but luteolysis is not initiated at this time [52]. The absence of PGFM pulses during preluteolysis in 33% of the mares and the small pulses in the other 67% indicate that the PGFM pulses during preluteolysis are not physiologically essential to subsequent luteolysis in mares [52]. In addition, rhythmicity of PGFM pulses was not detected during preluteolysis, but was detected in seven of nine mares during luteolysis and postluteolysis [52]. Other temporally related hormones or factors seem to be involved in the initiation of luteolysis in mares by increasing the response of the CL to the small PGFM pulse at the hour of transition in mares [50,52]. Cortisol and E2 were associated temporally with a PGFM pulse during luteolysis but not with a pulse during preluteolysis or with the transitional pulse [57]. Estradiol does not begin to

increase until 5 hours after the transitional pulse [52]. Oxytocin concentrations don't increase during the last PGFM preluteolytic pulse, but they increase and decrease simultaneously with the ascending and descending portion of the PGFM transitional pulse and with the first PGFM luteolytic pulse. The oxytocin increase seems to account at least partly for the decrease in P4 concentrations at the moment of transition from preluteolysis to luteolysis [50,57]. Oxytocin is synthesized in the hypothalamus and stored in the posterior pituitary. It is secreted in a pulsatile way, but its regulation during luteolysis is still unknown [58,59]. In mares the ovary is not a source of oxytocin contrarily to what is seen in domestic ruminants, where the CL synthesizes and secretes oxytocin during the estrous cycle [60,61]. The studies made to clarify oxytocin's role during the estrous cycle showed that it appears to have a role in mares' luteolysis as the intermediary that stimulates PGF secretion from the uterus [58,59]. However, it is not clear if oxytocin has a crucial role in the beginning of luteolysis.

In mares, P4 concentrations during luteolysis decrease linearly during the hours of a PGFM pulse and between the ending nadir of a pulse and the beginning nadir of the next pulse [52,62,63]. Substantial pulses of LH are detected during preluteolysis and luteolysis. During luteolysis, LH concentration approximately doubles relatively to preluteolysis. However, concentrations of P4 don't increase within the 3 to 5 hours of an LH pulse, and a P4 fluctuation does not occur in synchrony with the LH pulse [52].

Concentrations of P4 $\leq$ 0.9 ng/mL are used to represent postluteolysis. Progesterone continues to slowly decline after the defined end of luteolysis. The decline in P4 from 0.9 to 0.1 ng/mL requires 22 to 32 hours [52]. It is unknown if the prominent PGFM pulses after the defined end of luteolysis are needed for the slow continuation of the decrease in P4 output by the corpus luteum or are used for another purpose [52].

1.2.3. Abnormalities of the estrous cycle

a) Hemorrhagic anovulatory follicles

A hematoma that forms in the antrum of a follicle instead of ovulation has been termed a hemorrhagic anovulatory follicle (HAF). Most of HAFs have a peripheral wall of luteal tissue, as indicated by echotexture [48]. A distinguishing feature between a corpus hemorrhagicum and an HAF is the clotting of blood immediately during extravasation into the evacuated antrum or developing corpus luteum versus delayed clotting when blood enters follicular fluid [64]. Also, anovulation is a requisite component of the HAF syndrome. In this regard, no pregnancies occurred in 71 inseminated mares that developed an HAF [65]. The oocyte is not discharged into the oviduct before or during HAF formation, but whether oocytes are trapped or detrimentally affected in septated evacuations is not known. The incidence of hemorrhagic anovulatory follicles is approximately 5% and 20% of estrous cycles during the early and late ovulatory season, respectively and are especially common (36%) in mares 20 or more years old [64]. In addition, 44% of the mares that formed an HAF repeated the formation during a subsequent estrous cycle [64]. An HAF can occur without a simultaneous ovulation or a second HAF during the late follicular phase, in conjunction with ovulation of another follicle, or during the luteal phase [64].

The morphology of ovulatory-sized follicles that formed HAFs during the late follicular phase has been compared with those of a solitary follicle that ovulated [66]. The diameter of a preovulatory follicle or future HAF one day before ovulation or formation of HAF, respectively, was similar between the two groups. The HAF formation involves entry of blood into a follicle that does not have altered structure. In addition, the follicle cells are viable, as indicated by luteinization of the wall of the HAF (luteinization is based on echotexture and approximately normal P4 production). However, the percentage of follicle wall with color

Doppler signals one day before ovulation or HAF formation was greater for the HAF group. The difference between the two groups in percentage of wall with color signals was attributable to vascularization of the follicle wall in the area of expected ovulation (apical area) in mares that formed an HAF but not in mares that ovulated. A difference in vascularity at the apex is compatible with null or minimal hemorrhage during ovulation and massive hemorrhage during HAF formation [64]. Concentrations of FSH, LH, and P4 were not different before ovulation between mares that developed HAF and mares that ovulated [66]. In both groups, the preovulatory E2 surge reached a peak two days before ovulation or anovulation or 3 days before the peak of the LH surge, as expected for ovulating mares [39]. Endometrial echotexture on the day of HAF formation is not different for mares that do not develop HAF. However, E2 was higher for mares that developed an HAF 3 days before HAF formation [66]. The role of elevated E2 in the formation of the HAF is not known.

Increased thickness of the wall of the expanding HAF presumably represents luteal tissue and is associated with P4 production during HAF development. The luteinized wall is well vascularized, and the vascularization remains extensive as the structure regresses. Circulating P4 concentrations increase from 1 day before to the day of formation of the HAF, similarly to what is observed for an ovulation. In addition, the length of the luteal phase and interovulatory intervals are similar between mares that develop an HAF and mares that ovulate [64].

b) Idiopathic persistence of the corpus luteum

As referred previously, luteolysis in mares begins in average 14 days after ovulation (range Day 14 to 16). A CL that fails to regress during the expected time of luteolysis in a non-pregnant mare is considered to be pathologically persistent [67]. This condition can affect 25% of estrous cycles [2]and can be related to ovulations late in diestrous, embryonic loss after the

time of maternal recognition of pregnancy and chronic uterine infections. However, this condition can be observed without any of these conditionants [67], and this is named as idiopathic persistent CL. A wide spectrum of terminology has been used throughout the years to name this syndrome. Terms as persistent corpus luteum, prolonged diestrus, prolonged luteal activity or pseudopregnancy were used for similar conditions that if analyzed carefully may differ from each other [68]. Animals presenting persistent CL have P4 concentrations >1 ng/ml for about 63 days (range: 35 to 95 days) [2,68-70].

The idiopathic persistent CL has been suggested to be associated with low concentrations of PGF2α secreted from the uterus in the days that luteolysis is expected to occur, but the mechanism behind this condition is still unknown [2,68,71]. Most of the studies done in this area were made before the availability of ultrasound technology, so it was difficult to know if the cause of the prolonged diestrus was a prolonged primary CL (CL formed from a follicle that ovulated at the last estrus), a CL derived from an ovulation late in diestrus (CL is immature and subsequently non-responsive to PGF2α secreted at the normal time of luteolysis) or from an hemorrhagic anovulatory follicle with luteinized tissue [68]. Further studies with maintenance of the identity of the primary CL will be needed to characterize the mechanism that leads to idiopathic persistent CL.

List of publications

Published papers

Oxytocin induction of pulses of a prostaglandin metabolite and luteolysis in mares.

Santos, V.G., Castro, T., Bettencourt, E.M., Ginther, O.J.. Oxytocin induction of pulses of a prostaglandin metabolite and luteolysis in mares. Theriogenology 2015; 83:730–738.

Role of PGF2α in luteolysis based on inhibition of PGF2α synthesis in the mare.

Santos VG, Beg MA, Bettencourt EM, Ginther OJ. Role of PGF2α in luteolysis based on inhibition of PGF2α synthesis in the mare. Theriogenology 2013; 80:812–20.

Hormonal, luteal, and follicular changes during initiation of persistent corpus luteum in mares.

Santos, V.G., Bettencourt, E.M., Ginther, O.J.. Hormonal, luteal, and follicular changes during initiation of persistent corpus luteum in mares. Theriogenology 2015; 83:757–765.

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Papers submitted to publication

Long-term characteristics of idiopathic persistent corpus luteum in the mare.

Santos, V.G., Bettencourt, E.M., Ginther, O.J.. Long-term characteristics of idiopathic persistent corpus luteum in the mare. Theriogenology (Submitted).

2. Oxytocin induction of pulses of a prostaglandin metabolite and luteolysis in mares.

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Abstract

A procedure for oxytocin (OT) administration on Day 13 postovulation was developed in mares for stimulation of a pulse of PGFM (a PGF2α metabolite) that mimics a natural PGFM pulse during luteolysis. Bolus treatment with each of five OT doses (1 to 10 IU/mare, n = 3) stimulated a burst of PGFM that was maximum in 4 min and was unlike a natural pulse. A 2-h OT infusion of 1.25, 2.5 or 5 IU/100 kg (n = 4) induced a PGFM pulse similar to reported pulses; lower doses did not. The peak of an induced pulse (~260 to 380 pg/mL) seemed similar to reported natural peaks (~200 to 300 pg/mL), using the same assay system. The interval from nadir to nadir was 6.6 ± 0.2 h. Percentage decrease in progesterone (P4) within 8 h was greater ($P < 0.05$) for doses of 1.25, 2.5 or 5 IU/100 kg (43 to 50%) than for a vehicle group (11%). Treatment with flunixin meglumine (1.0 mg/kg), a cyclooxygenase inhibitor, decreased ($P < 0.008$) PGFM concentration but treatment 2 h before the beginning of OT infusion (2.5 IU/100 kg) did not prevent the OT-induced PGFM pulses and the decrease in P4. In conclusion, a PGFM pulse was simulated by infusion of OT during 2 h but not by a single OT bolus, and an OT-simulated PGFM pulse stimulated a decrease in P4 that was not prevented by a cyclooxygenase inhibitor. These are the first firm demonstrations that OT in mares as in other species has a role in luteolysis.

Keywords: Luteolysis; Mares; Oxytocin; PGFM; Progesterone.

2.1. Introduction

The regression of the corpus luteum (CL) or luteolysis is a pivotal reproductive event during the estrous cycle in farm species, including mares. Luteolysis represents a decrease in progesterone (P4) from the response of the CL to the secretion of prostaglandin F2α (PGF2α) by the endometrium (review [1]). In the mare, luteolysis begins on average on Day 14 (Day 0 = ovulation) and lasts 23 h based on hourly blood sampling [2]. The end of luteolysis is defined as a P4 decrease to < 1 ng/mL [3]. The main plasma PGF2α metabolite is 15-keto-13,14-dihydro-prostaglandin F2α (PGFM) [4]. The metabolite has a longer half-life and is often used to represent circulating concentrations of PGF2α [5]. In many species, including mares, complete luteolysis requires secretion of multiple pulses of PGF2α by the nongravid uterus [6]. The interval between the peaks of sequential PGFM pulses in mares is 9 h and the interval from nadir to nadir at the PGFM base is 5 h [6]. Inhibition of PGF2α secretion at the expected time of luteolysis with a cyclooxygenase inhibitor (flunixin meglumine) induces a delay in the beginning of luteolysis [7].

Based on hourly blood sampling, the transition into the luteolytic period in mares is manifested within 1 h [8]. Pulses of PGFM are relatively small before luteolysis (e.g., peak 30 ng/mL) compared to during luteolysis (e.g., 190 ng/mL) [8]. Each PGFM pulse that occurs during luteolysis is temporally associated with a pulse of oxytocin (OT) [9]. The small transitional pulse at the hour of the initiation of luteolysis is also associated with a pulse of OT, whereas previous small PGFM pulses are not [10]. Oxytocin therefore may play a role in the effectiveness of a PGF2α pulse during luteolysis as well as the effectiveness of the small transitional pulse at the initiation of luteolysis in mares (review [2]).

Oxytocin is synthesized in the hypothalamus, stored in the posterior pituitary, and secreted in pulses [11,12]. The CL also synthesizes and secretes OT during the estrous cycle in domestic ruminants [13,14], but the CL apparently does not secrete OT in mares [15].

Circulating concentration of OT in mares is greater at the expected time of luteolysis (Day 15) than before luteolysis (Days 0, 3, or 7) [12]. The role of OT during spontaneous luteolysis has not been clarified in mares, but in other farm species OT is an intermediary in the stimulation of PGF2α secretion from the uterus (review, [16]). In previous studies on the effect of exogenous OT on PGFM in mares, the doses were apparently based on clinical recommendations (e.g., 20 IU to evacuate the uterus [17]). Treatment of nonpregnant mares with OT doses of 10 to 25 IU [18-21] or the OT response to uterine biopsy [22,23] stimulates an increase in PGFM. The OT treatments are especially effective near the expected day of luteolysis when the number of uterine OT receptors is maximum (Days 14 to 17) [22–24]. Despite the induced increase in PGFM, a negative effect of OT treatment on circulating P4 concentration has not been documented in mares.

Treatment with OT early in the estrous cycle in cattle is luteolytic [25], but in mares treatment on Days 1 through 7 with a high dose (200 IU [26]) or on Days 4 to 8 (150 IU [27]) did not induce luteolysis or shorten the interovulatory interval (IOI). In this regard, the number of OT receptors in the uterus is minimal on Days 4 to 8 in mares [27]. Despite the stimulation of PGFM by OT doses of 10 to 25 IU/mare [18,20], chronic administration of OT in high doses (e.g., 60 IU/day on Days 7 to 14) prolongs the luteal phase at least until Day 30 [28–31]. Furthermore, 60 IU of OT on Days 8 to 14 prolongs the luteal phase (P4 > 1.0 ng/mL through Day 30), reduces endometrial cyclooxygenase-2 expression, and lowers plasma concentrations of PGFM [31].

The dose and method of OT administration that could be considered physiologic in mares are unknown. That is, the effect of OT treatment on the CL has been examined only on a pharmacologic basis. The objectives for the current studies in mares were (1) to determine a dose and method of administration of OT that will stimulate a PGFM pulse similar to a spontaneous pulse and (2) to study the role of OT in luteolysis. The hypotheses were (1) simulation of a PGFM pulse can be done by infusion of a specific dose of OT during 2 h but

not by a single OT bolus dose, (2) OT induction of a simulated PGFM pulse stimulates a decrease in P4, and (3) OT exerts a luteolytic effect only through stimulation of PGF2α secretion.

2.2. Materials and methods

2.2.1. Mares and treatments

Mixed breeds (light, riding-type horses and apparent pony-horse crosses) of 21 nonlactating mares aged 4 to 18 y and weighing 300 to 600 kg were used in the northern temperate zone. Abnormalities of the reproductive tract were not detected by transrectal ultrasonic scanning [32]. The mares had not been bred for at least 3 y. The mares were housed under natural light in an open shelter and outdoor paddock and were maintained by free access to primarily grass hay, trace-mineralized salt, and water. All mares remained healthy and in good body condition throughout the studies. Animals were handled according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching. Mares were examined daily throughout each of four experiments by transrectal ultrasonic imaging as described [32]. A duplex B-mode (gray scale) and color-Doppler instrument equipped with a linear-array 7.5-MHz transducer was used. The day of ovulation was designated Day 0.

Mares were assigned by randomization to experimental groups, including a vehicle or control group (0 dose). Each designated treatment in each experiment was given into a jugular vein. Oxytocin (Oxytocin injection®, Bimeda, Inc., Le Sueur, MN, USA) was used in each experiment. Flunixin meglumine (FM; FluMeglumine, Phoenix, St Joseph, MO, USA) was also used in experiments 3 and 4. The FM acts on cyclooxygenase enzymes in the endometrium

[33] and thereby blocks PGF2α synthesis [34]. A single systemic treatment with FM inhibits PGF2α secretion in the mare, but the effect wanes by 8 h [7].

2.2.2. Experiment 1. Effect of bolus treatment with OT on PGFM

This experiment was done to determine if a single injection of OT would induce a pulse of PGFM that would be similar to an endogenous pulse. On Day 13, mares in a vehicle group were given 2 mL of saline, and mares in five OT-treated groups were given a single bolus treatment of OT as follows: OT-1.0 (1.0 IU of OT/mare), OT-2.5, OT-5, OT-7.5 and OT-10.0 (n = 3/group). The doses of OT were diluted in saline to a final volume of 2 mL for each dose. The minute of the treatment was designated Minute 0. Blood samples were collected into heparinized tubes by venipuncture of the jugular vein. The mares were docile and did not object to venipuncture with a 20-gauge needle, as indicated by collection without head restraint. Samples were collected at Minutes 0, 1, 2, 3, 4, 5, 10, 15, 30, 45, and 60 and hourly thereafter until Hour 4. The Minute 0 sample was collected immediately before treatment. Plasma samples were assayed for PGFM.

2.2.3. Experiment 2. Effect of 2-h infusion of OT on PGFM pulses

This experiment was done to determine a dose of OT that would simulate an endogenous pulse of PGFM when infused for 2 h. An infusion period of 2 h was selected because a 2-h infusion of PGF2α approximately simulates a PGFM pulse in mares [35]. On Day 12, an indwelling catheter was inserted into a jugular vein as described [8]. On Day 13, vehicle (n = 8) or a specified dose of OT (n = 4/group) was infused into the vein during 2 h. The doses for the OT-treated groups were OT-0.05 (0.05 IU of OT/100kg), OT-0.5, OT-1.25, OT-2.5, and OT-5.

The beginning of the infusion was designated Hour 0. Infusion of OT was done at a constant rate using a variable-flow peristaltic minipump (Number 18-876-4; Fisher Scientific, Pittsburgh, PA, USA). The pump was calibrated to deliver the specified dose of OT in 8 mL of saline vehicle during 2 h. During the infusion, the mare was confined in a chute but the head was not restrained. Ten hourly blood samples were collected from Hours –1 through 8, and a last sample was collected at Hour 48. Determination was made of the lowest dose of OT that simulates a PGFM pulse that is similar to a reported [6,8] spontaneous PGFM pulse during luteolysis. Blood samples were assayed for PGFM and P4.

Pulses of PGFM were identified in each set of hourly blood samples. To distinguish between fluctuations and pulses, the intra-assay coefficient of variation (CV) was used as described [36]. Each PGFM fluctuation with an increase and decrease encompassing at least four hours, including nadirs, was evaluated. A fluctuation was defined as a pulse when the CV was at least three times greater than the mean intra-assay CV. The first defined pulse with a peak after Hour 0 was used in the analyses. The peak of an identified PGFM pulse was designated 0 hour.

2.2.4. Experiment 3. Effect of FM bolus treatment and OT infusion on P4.

This experiment was done to determine if FM treatment before and after OT infusion would have an effect on P4 concentration. Four groups (n = 3 or 4/group) were used: control (no vehicle), FM (1.0 mg/kg), OT (2.5 IU of OT/100kg), and FM/OT (1.0 mg/kg FM, and 2.5 IU of OT/100kg). On Day 12, about 16 h before treatment on Day 13, an indwelling catheter was inserted into a jugular vein as for experiment 2. The FM was given on Days 13.0 and 13.3 (8 hours after Day 13.0), and a 2-h infusion of the dose of OT was begun 2 h after the first FM treatment. Blood samples were collected on Days 13.0, 13.3, 14 and 15 and were assayed for P4.

2.2.5. Experiment 4. Effect of FM bolus treatment and OT infusion on PGFM and P4.

This experiment was done as a follow-up to experiment 3 to determine if blood sampling at hourly intervals would better determine the effect of OT and FM on P4 and to detect and characterize PGFM pulses. The beginning of the 2-h OT infusion was designated Hour 0. The groups (n = 5 mares/group) and protocol were the same as for experiment 3, except that 11 hourly blood samples were collected from Hours –2 to 8 and at Hour 24. The FM was administered at Hours –2 and 4. Plasma samples were assayed for PGFM, and PGFM pulses were identified as described for experiment 2. Samples were also assayed for P4 to determine if OT alone had at least a partial luteolytic effect.

2.2.6. Hormone assays

Blood samples were immediately placed in ice water for 10 min after collection followed by centrifugation (2,000 X *g* for 10 min). The plasma was decanted and stored (–20º C) until assayed. Plasma samples were assayed for P4 by a solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure has been validated and described for mare plasma in our laboratory [37]. Plasma concentrations of PGFM were determined by an ELISA that was developed and validated in our laboratory for use in mare plasma [38]. For PGFM in experiment 1, the intra- and interassay CV and sensitivity, respectively, were 5.2%, 17.1%, and 4.6 pg/mL. For PGFM in experiment 2, the intra- and interassay CV and sensitivity, respectively, were 14.5%, 12.1%, and 5.8 pg/mL, and for P4 the intra-assay and sensitivity, respectively, were 5.5% and 0.03 ng/mL. For P4 in experiment 3, the intra- and interassay CV and sensitivity, respectively, were 7.9%, 4.3%, and 0.02 ng/mL. For PGFM in experiment 4,

the intra- and interassay CV and sensitivity, respectively, were 18.0%, 18.9%, and 6.6 pg/mL, and for P4 the intra-assay CV and sensitivity, respectively, were 7.2% and 0.03 ng/mL.

2.2.7. Statistical analyses

Data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were resolved by transformation to natural logarithms or ranks. The rank transformation was used when logarithms did not resolve a lack of normality. A value defined as an extreme outlier by the interquartile range analysis was removed from the analyses and figures. Determination was made of the main effects of group and time (day, hour, or minute) and the interaction of group and time. The statistical analyses were done by using SAS PROC MIXED (Version 9.3; SAS Institute Inc., Cary, NC, USA) with REPEATED statement, owing to repeated measures in each mare. Autocorrelation was accounted for by modeling the first-order autoregressive or spatial power between sequential measurements. When a factor or interaction was significant or approached significance, least significant difference from the SAS procedure was used for comparisons among patterns within a time and to compare times within a group. Student's paired and unpaired t-tests were also used but on a preliminary basis. Either chi-square or Fisher's exact test was used at the discretion of the computer program to determine differences in frequency among groups in the proportion of PGFM pulses and for the proportion of mares that ended luteolysis on a given day. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to ≤ 0.10 indicated that significance was approached. Differences that approached significance were included on a preliminary basis for potential consideration in further studies. Data are presented as the mean ± SEM, unless otherwise indicated.

2.3. Results

2.3.1. Experiment 1. Effect of bolus treatment with OT on PGFM

One outlier in PGFM concentration in the OT-2.5 group at Minute 1 was omitted from the analyses and figure. Concentrations of PGFM after a bolus injection of vehicle and various doses of OT had an effect of minute and a group-by-minute interaction for Minutes 0 to 45 (Fig. 2.1). The highest PGFM concentration was observed at Minute 4 after treatment in all OT-treated groups. The group-by-minute interaction was represented by greater concentration in each OT-treated group than in the vehicle group at Minutes 3 to 15 but not at Minutes 0, 1, 2, 30, and 45. The peak at Minute 4 was not different among the OT-treated groups. Concentration at Minute 45 was not different from the pretreatment concentration for each OT-treated group. Concentrations did not increase during the hourly samples (not shown).

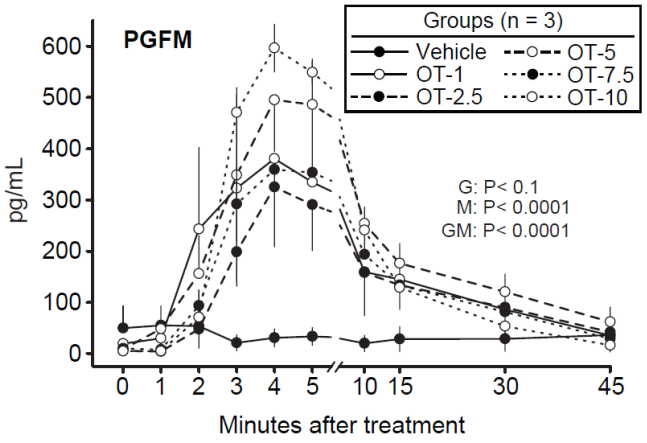

Figure 2.1. Mean ± SEM concentrations of PGF2α metabolite (PGFM) from Minutes 0 to 45 (Minute 0 = bolus treatment) in a vehicle and oxytocin-treated (OT) groups (e.g., OT-1 = OT at 1.0 IU/mare). Probabilities for main effects of group (G) and minute (M) and the interaction of group by minute (GM) are shown. Concentration was greater in each OT-treated group than in the vehicle group at Minutes 3 to 15, but the differences among OT groups were not significant.

2.3.2. Experiment 2. Effect of 2-h infusion of OT on PGFM pulses

Concentration of PGFM had a significant main effect of hour, an effect of group that approached significance, and a significant group-by-hour interaction (Fig. 2.2). The hour effect reflected maximal PGFM concentration at Hours 1 or 2. The approaching group effect reflected greater ($P < 0.05$) concentration in the three groups with the highest doses of OT. The group-by-hour interaction was primarily from greater ($P < 0.05$) PGFM concentration at Hours 1 and 2 in the groups OT-1.25, OT-2.5, and OT-5 than in the other groups.

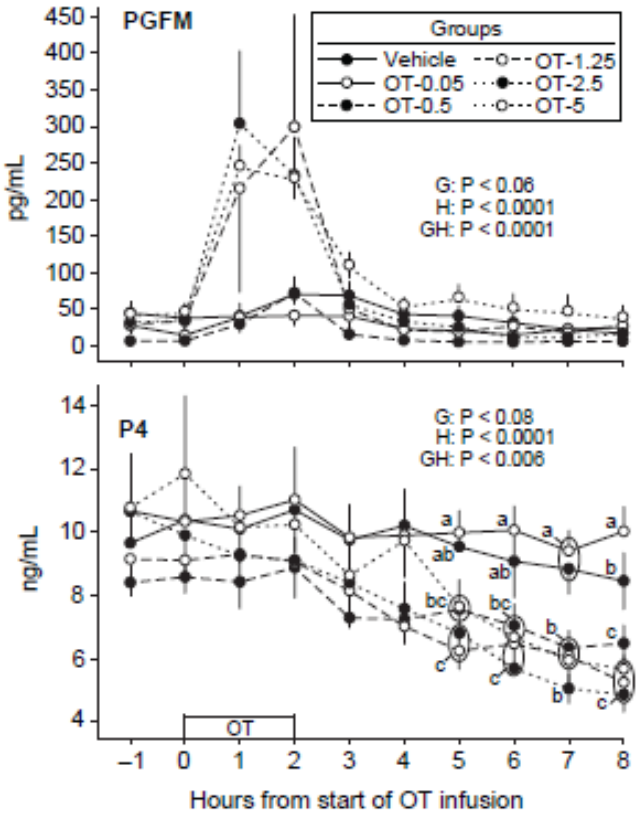

Figure 2.2. Mean ± SEM concentrations of PGF2α metabolite (PGFM) and progesterone (P4) at hourly intervals in a vehicle (n = 8) and oxytocin-treated (OT) groups (n = 4/group) at the indicated dose (e.g., OT-0.5 = OT at 0.5 IU/100 kg). The vehicle and OT were infused for 2 h as indicated above the hour scale. Probabilities for main effects of group (G) and hour (H) and an interaction (GH) are shown. Within each hour, any two means without a common letter (abc) are different ($P < 0.05$); a ring is used to encompass two means that are too close for placement of separate letters.

A PGFM pulse was detected by the CV method in 4 of 8 mares treated with vehicle, compared to 18 of 20 mares treated with OT ($P < 0.04$). Concentrations in the PGFM pulse from 2 h before to 2 h after the peak had significant main effects of group and day and an interaction (Fig. 2.3). The group effect was from greater ($P < 0.05$) concentration in each of the three highest OT doses (combined, 144.8 ± 21.8 pg/mL) than for each of the two lowest doses (combined, 34.2 ± 7.0 pg/mL); the vehicle group was intermediate (75.6 ± 12.2 pg/mL). The interaction was primarily from greater ($P < 0.05$) PGFM concentration at the peak (0 hour) for each of the three highest doses than for each of the other three groups (Table 2.1) and no difference at –2 and 2 h; significant differences among groups at –1 and 1 hours are shown (Fig. 2.3). Amplitude of the pulses from the beginning nadir to the peak was greater in each of the three highest dose groups than in each of the other three groups, but the nadir-to nadir interval was not different among groups (Table 2.1). The nadirs at the beginning and end of a pulse were not significantly different among groups (not shown).

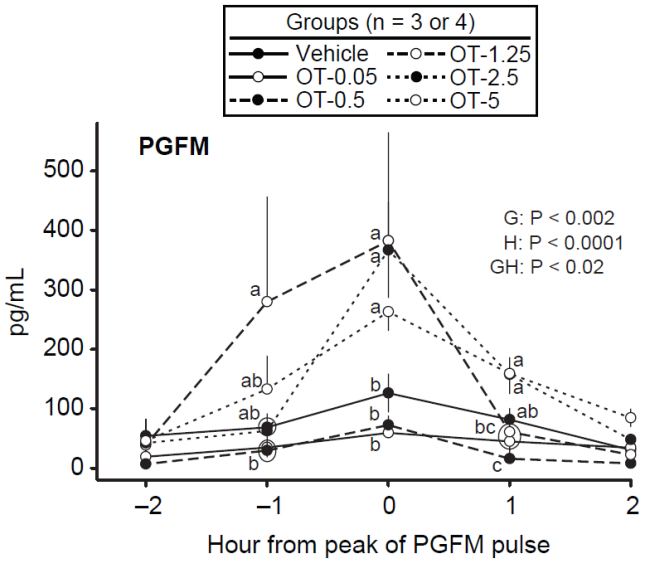

Figure 2.3. Mean ± SEM concentrations of PGF2α metabolite (PGFM) in identified pulses centered on the pulse peak in a vehicle and oxytocin-treated (OT) groups at the indicated dose (e.g, OT-5 = OT at 5 IU/100 kg). Probabilities for main effects of group (G) and hour (H) and an interaction (GH) are shown. Within each hour, any two means without a common letter (abc) are different ($P < 0.05$); a ring is used to encompass two means that are too close for placement of separate letters.

Table 2.1. Mean ± SEM for pulse characteristics of PGFM pulses during an 8 h period of hourly sampling in mares in vehicle and OT-treated groups[a].

	Pulse characteristics			
Group[a]	Peak (pg/mL)	Amplitude (pg/mL)	Mean for all hours (pg/mL)[b]	Nadir-to-nadir interval (h)
Vehicle	126.3 ± 31.9[d]	92.9 ± 18.3[d]	66.7 ± 16.0[cd]	5.3 ± 0.8
OT-0.05	59.3 ± 8.2[d]	42.3 ± 4.3[d]	33.5 ± 7.5[de]	6.0 ± 0.0
OT-0.5	72.5 ± 15.3[d]	66.3 ± 13.5[d]	21.9 ± 6.5[e]	5.5 ± 0.7
OT-1.25	382.4 ± 182.2[c]	349.8 ± 173.4[c]	124.1 ± 58.6[c]	6.3 ± 0.3
OT-2.5	367.0 ± 80.0[c]	336.9 ± 67.3[c]	101.5 ± 14.7[c]	6.8 ± 0.5
OT-5	263.2 ± 31.6[c]	220.0 ± 41.5[c]	112.7 ± 6.9[c]	6.8 ± 0.5
Probability[f]	P < 0.0004	P < 0.0002	P < 0.0003	NS

[a] Each dose of oxytocin (OT) was infused iv during 2 h (e.g., OT-0.05 = 0.05 IU/100 kg of OT) (n = 3 or 4/group).

[b] Differs from the group effect of Figure 2.3 because the figure was limited to –2 to 2 h from the peak, and this table encompasses all hours from nadir to nadir.

[cde] For each column, means of PGFM pulse characteristics with no common superscript letter are different (P < 0.05).

[f] Probability for a difference among groups for each column. Each nadir was not significantly different among groups and is not included.

For P4 concentration, the group effect approached significance and the hour effect and interaction were significant (Fig. 2.2). The approaching group effect averaged over hours reflected no difference among the OT-0.5, OT-1.25, and OT-2.5 groups, but less (P < 0.05) concentration in each of these OT groups (combined, 7.7 ± 0.2 ng/mL) than in the OT-0.05 group (10.2 ± 0.3 ng/mL) and vehicle group (9.7 ± 0.3 ng/mL). Concentration of P4 in each of the OT-0.05 and OT-5 groups was not different from the vehicle group. The interaction of group and hour represented no differences among groups for each of Hours –1 to 2, less P4 in the OT-0.5 group than in the vehicle group at Hour 3, and less P4 in the OT-0.5 and OT-1.25

groups but not in the OT-2.5 and OT-0.5 groups than in the vehicle group at Hour 4. The indicated differences among groups at Hours 5 to 8 contributed to the interaction. Most notably, each of the vehicle and OT-0.05 groups had greater P4 concentration than each of the other four groups at Hours 7 and 8, including the OT-0.5 group. However, the OT-0.5 group had the lowest P4 concentration before OT was infused. Therefore, percentage decrease from Hours –1 to 8 was also analyzed. The percentage P4 decrease was greater ($P < 0.05$) for the three highest doses (43 to 50% decrease) than for the vehicle (11% decrease) and the lowest dose (4% decrease), but the decrease (22%) for OT-0.5 dose was not different from any other group. The proportion of mares with P4 < 1 ng/mL at Hour 48 (Day 15) was as follows: vehicle, 3/8; OT-0.05, 1/4; OT-0.5, 2/4; OT-1.25, 3/4; OT-2.5, 4/4; and OT-5, 2/4. The proportion approached being significantly greater ($P < 0.08$) in the OT-2.5 group than in the vehicle group, but the other OT-treated groups were not different from the vehicle group.

2.3.3. Experiment 3. Effect of FM bolus treatment and OT infusion on P4

One mare from the control group and one mare from the FM/OT group were omitted from the analyses because luteolysis was underway on Day 13.0. The percentage change from Day 13.0 (first FM treatment) was used for P4 concentration because of apparent differences among groups on Day 13.0. Percentage change in P4 concentration on Day 13.3 (8 hours after first FM treatment) to Day 15 had a significant effect of day and a group-by-day interaction (Fig. 2.4). The group-by-day interaction was primarily from greater ($P < 0.05$) percentage P4 decrease in the OT and FM/OT groups than in the control and FM groups on Day 13.3, greater ($P < 0.04$) change in the OT than in the FM group on Day 14, and similarity among groups on Day 15. Actual concentration of P4 decreased ($P < 0.05$) between Days 13.0 and 13.3 in the OT group (12.7 ± 1.6 to 7.5 ± 1.2 ng/mL) and FM/OT group (11.0 ± 2.3 to 7.0 ± 2.5 ng/mL)

but not in the control and FM groups. The day of end of luteolysis and the length of the interovulatory interval were not different among groups.

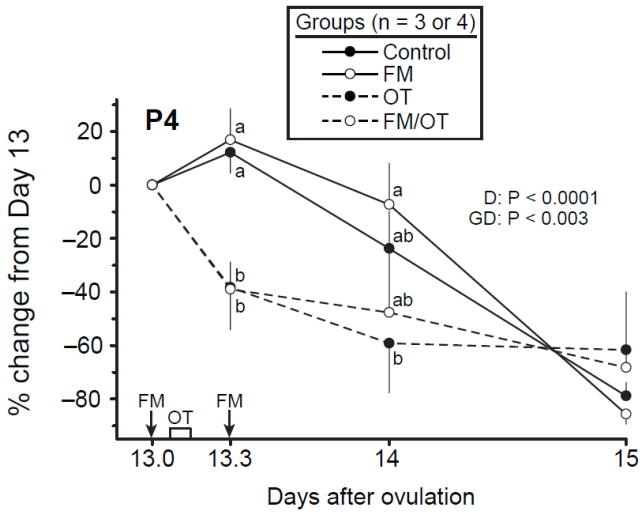

Figure 2.4. Mean ± SEM percentage of change from Day 13 in concentration of progesterone (P4) in control, flunixin meglumine (FM), oxytocin (OT), and FM/OT groups. An FM treatment (1.0 mg/kg) was given on Days 13.0 and 13.3, and OT was infused (2.5 IU/100 kg) during 2 h beginning 2 h after the first FM treatment. Probabilities for main effect of day (D) and an interaction (GD) are shown. Within each day, any two means without a common letter (ab) are different ($P < 0.05$).

2.3.4. Experiment 4. Effect of FM bolus treatment and OT infusion on PGFM and P4

Concentration of PGFM had significant main effects of group and hour and a group-by-hour interaction (Fig. 2.5). The group effect was represented by the following concentrations (pg/mL): control (26.8 ± 2.7^{a}), FM (9.6 ± 0.6^{b}), OT (81.1 ± 24.0^{c}), and FM/OT (69.5 ± 23.2^{c}); means with different superscripts are different ($P < 0.05$). The group-by-hour interaction was primarily from greater ($P < 0.05$) PGFM concentration at Hours 1 and 2 in the OT and FM/OT groups than in the other two groups and greater ($P < 0.05$) concentration at Hours 1, 3, 6, 7, and 8 in the controls than in the FM group; other differences are shown. A PGFM pulse was detected by the CV method in 1 of 5, 0 of 5, 4 of 5, and 5 of 5 mares in the control, FM, OT, and FM/OT groups, respectively ($P < 0.004$). Concentrations in the PGFM

pulses averaged over 2 h before to 2 h after the peak, at the peak, nadirs at the beginning and end of a pulse, and amplitude from nadir 1 to the peak were not different between the OT and FM/OT groups (not shown).

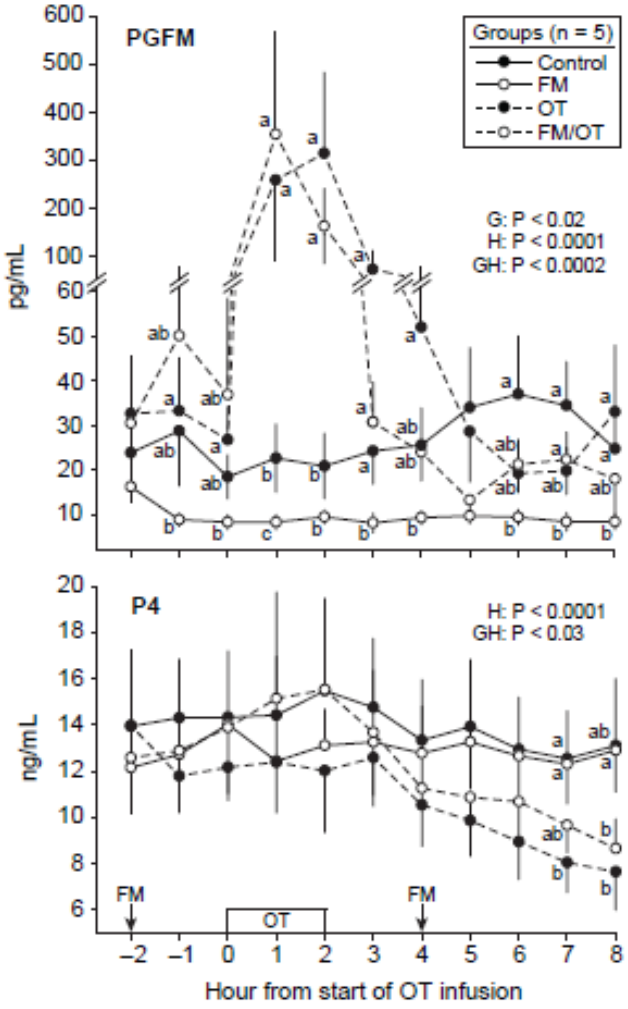

Figure 2.5. Mean ± SEM concentrations of PGF2α metabolite (PGFM) and progesterone (P4) on Day 13 in control, flunixin meglumine (FM), oxytocin (OT), and FM/OT groups. The PGFM concentration is in two different scales. An FM treatment (1.0 mg/kg) was given at Hours –2 and 4 and OT was infused (2.5 IU/100 kg) during 2 h beginning at Hour 0. Probabilities for main effects of group (G) and hour (H) and an interaction (GH) are shown for PGFM and P4. Within each panel and hour, any two means without a common letter (abc) are different ($P < 0.05$).

For P4 concentration, the hour effect and interaction were significant (Fig. 2.5). The interaction of group and hour represented no differences among groups for each of Hours –2

to 6, less P4 in the OT than in the control and FM groups at Hour 7, and less P4 in the OT and FM/OT groups than in the FM group at Hour 8. When analyzed separately, concentration of P4 decreased between Hour 0 and 8 in the OT ($P < 0.007$) and FM/OT ($P < 0.04$) groups but not in the control and FM groups. The first significant decrease ($P < 0.001$) in the control group occurred between Hours 8 and 24 (13.1 ± 2.9 to 9.0 ± 3.1 ng/mL), in the FM group ($P < 0.0008$) between Hours 8 and 24 (12.9 ± 1.8 to 9.7 ± 2.2 ng/mL), in the OT group ($P < 0.003$) between Hours 3 and 4 (12.6 ± 2.1 to 10.5 ± 1.8 ng/mL), and in the FM/OT group ($P < 0.05$) between Hours 2 and 4 (15.5 ± 4.0 to 11.3 ± 2.0 ng/mL).

2.4. Discussion

In farm species, including horses, PGF2α is secreted from the endometrium at the appropriate time and stimulates luteolysis. In cattle and sheep, OT from the CL and hypothalamus binds to OT receptors in the endometrium, arachidonic acid is released from the cell membranes, and PGF2α is secreted [16]. A positive feedback loop between OT and PGF2α plays a critical role in luteal regression in these species [39]. However, in mares the role of OT in the regulation of uterine PGF2α secretion and P4 reduction during luteolysis has not been determined [39]. In the current studies, the effect of exogenous OT on PGFM and P4 concentrations in mares was evaluated. First, a dose and method of OT administration was developed that would stimulate a PGFM pulse that was similar to a natural pulse during luteolysis.

Hypothesis 1, that simulation of a PGFM pulse can be done by infusion of a specific dose of OT during 2 h but not by a single OT bolus dose, was supported. Constant infusion of a specific dose of OT during 2 h stimulated a pulse of PGFM that was similar to reported [6,8] spontaneous pulses during and immediately after luteolysis. The mean of the peak PGFM concentration of the OT-stimulated pulses (~260 to 380 pg/mL) was not different among the

three highest infused doses and was similar to the reported peaks of spontaneous pulses (~200 to 300 pg/mL [8]). However, the base (nadir to nadir) seemed broader for the induced pulses (~6.6 h) than for reported pulses (~5.4 h [6]).

In contrast to the response to OT infusion, bolus treatment in various doses induced a relatively short burst of PGFM that did not resemble a natural pulse. The burst reached maximum in 4 min compared to an ascending portion of a natural pulse of 2 to 3 h [6,8]. Although the maximal value at 4 min did not differ among the OT doses, the range of means (~300 to 600 pg/mL) encompassed the means for the OT-induced and reported spontaneous pulses. However, each of the ascending and descending portions of a spontaneous PGFM pulse require about 2.5 h, whereas the corresponding portions of the burst from a bolus treatment were 4 and 41 min, respectively. Clearly, a PGFM pulse can be approximately simulated by a 2-h iv infusion of OT, but not by a bolus iv treatment. In this regard, the same assay system [8,38] was used for both the current and reported studies. In a previous study using an iv bolus of OT, the peak was reached in 10 min [21] or later than in the current study. However, the reported treatment was done on Days 0 and 2. The current study was done on a day (Day 13) that was close to the expected day of luteolysis (Days 14 or 15 [2]) and the beginning of an increase in number of uterine OT receptors [22–24].

Hypothesis 2, that OT induction of a simulated PGFM pulse stimulates a decrease in P4, was supported. Each of the three highest OT doses produced prominent PGFM pulses that were similar to natural pulses and induced a P4 decrease or percentage P4 decrease by Hour 8. This was shown by the average concentration/group and by the percentage P4 decrease between Hours –1 and 8. These results indicated that OT is an intermediary for secretion of PGF2α in mares, as previously shown for ruminants [16].The current results apparently represent the first firm demonstration of the luteolytic effect of exogenous OT in mares. In a previous study, chronic sc administration of OT beginning on Day 10 was associated with early luteolysis in 2 of 5 mares compared to 0 of 4 controls; however, the number of mares was small and the

proportion of mares with early luteolysis was not significant [28]. The current successful demonstration is attributable to the infusion of physiologically compatible concentrations of exogenous OT, so that the resulting PGFM pulse was similar to a natural pulse during luteolysis. The induced decrease in P4 on Day 13 was not associated with a reduced length of the IOI, which is attributable to the reported necessity of multiple pulses of PGF2α for a continued decrease in P4 or complete luteolysis in mares [35].

On an observational and preliminary basis, the highest infused dose of OT (5 IU/100 kg) may have been less effective in reducing P4 concentration than the lower doses of 1.25 and 2.5 IU as indicated by the following: (1) apparent beginning of a mean P4 decrease (Hour 4) was later than for the doses of 1.25 and 2.5 IU (Hour 2) and (2) P4 concentration for the highest dose (5 IU) averaged over hours was not different from the vehicle and 0.05 IU dose and approached being greater than for the doses of 1.25 and 2.5 IU. Less effectiveness of a high dose of OT than a lower dose in stimulating a luteolytic PGFM pulse may be related to the induction of a persistent CL with chronic [28] or daily administration of OT in high doses (e.g., 60 IU/mare [29–31]). In comparison, the three highest doses in the current project were equivalent to an average of 5, 10, and 20 IU/mare. The daily injection of pharmacologically high doses OT prevents luteolysis in association with suppression of PGF2α by a decrease in endometrial expression of cyclooxygenase-2 and not by a decrease in OT receptor expression [31] or differences in OT binding capacity of the endometrium [30].

Hypothesis 3, that OT exerts a luteolytic effect only through stimulation of PGF2α secretion, was not successfully tested. Support required that OT would not have a partial luteolytic effect after the prevention of the OT stimulation of PGF2α secretion by inhibiting cyclooxygenase enzymes with FM. This expectation was based on an *in vitro* report that a cyclooxygenase inhibitor (indomethacin) impeded the secretion of PGF2α in OT-stimulated equine endometrial explants [40]. In experiment 3, P4 decreased in both OT and FM/OT groups 6 h after the beginning of OT infusion. However, blood samples were not available until 8 h

after FM treatment. Therefore, experiment 4 was done to obtain hourly samples to determine whether at least a partial P4 decrease would occur in the FM/OT group, despite inhibition of PGFM pulses. The effectiveness of FM in lowering PGFM concentrations on Day 13 (before the beginning of luteolysis) was verified within the experiment. The PGFM was lower in the FM group than in the controls, even though PGFM concentrations and pulses were minimal in controls on Day 13. However, FM did not block the PGFM pulse and P4 reduction of the 2-h infusion of 2.5 IU of OT. That is, the prominence of the OT-induced PGFM pulses and the P4 decrease were similar between the OT and FM/OT groups. The P4 concentration began to decrease sooner and was lower in the OT and FM/OT groups at Hours 7 or 8 than in the control and FM groups. The FM did not suppress the negative effect of PGF2α (based on PGFM) on P4 concentration and therefore the hypothesis was not tested.

The reason for the inability of FM to prevent OT stimulated PGF2α secretion was not determined. The inhibition of PGF2α secretion by FM is dose dependent [41]. The dose of FM was the same in the present and previous studies [7] and effectively inhibited PGF2α secretion in mares. In cattle and sheep, OT releases arachidonic acid (PGF2α precursor) from the phospholipid bilayer of endometrial cell membranes [16]. In addition, in vitro studies with endometrial epithelial cells have shown that OT has a dose-dependent positive effect on PGF2α production and gene expression for cyclooxygenase 1 and 2 [42,43]. The cyclooxygenase enzymes are rate-limiting in prostaglandin synthesis [44]. Therefore, it is speculated that OT may have increased the availability of cyclooxygenase, thereby overcoming the potential inhibitory effect of FM on cyclooxygenase. Further studies will be needed.

2.5. Conclusions

An increase in PGFM concentration occurred after an iv bolus treatment with OT on Day 13. Concentration of PGFM reached a peak within 4 min after treatment and returned to

pretreatment concentration in 45 min; the PGFM burst did not resemble a natural PGFM pulse. Infusion of OT (1.25, 2.5, or 5 IU/100 kg) during 2 h on Day 13 stimulated a PGFM pulse similar to the peak of a spontaneous PGFM pulse during luteolysis. Lower doses were not effective. The induced PGFM pulses stimulated a decrease in P4 concentration within 8 h after the beginning of OT infusion. Treatment with a cyclooxygenase inhibitor did not prevent the OT-induced PGFM pulses and the decrease in P4. Results demonstrated for the first time in mares that a physiologic dose and method of OT administration induces a PGFM pulse and a decrease in P4.

2.6. Acknowledgments

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3. Role of PGF2α in luteolysis based on inhibition of PGF2α synthesis in the mare.

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Abstract

The effects of inhibition of PGF2α synthesis on luteolysis in mares and on the incidence of prolonged luteal activity were studied in controls and in a group treated with flunixin meglumine (FM), a PGF2α inhibitor (n = 6/group). The FM was given every 8 hours (1.0 mg/kg) on each of Days 14.0 to 16.7. Concentration (pg/mL) of PGFM, a metabolite of PGF2α, averaged over 8 hours of hourly blood sampling at the beginning of each day was lower in the FM group than in the controls on Day 14 after ovulation (6.7 ± 1.3 vs 13.8 ± 2.9, $P < 0.05$), Day 15 (15.0 ± 3.9 vs 35.2 ± 10.4, $P < 0.10$), and Day 16 (21.9 ± 5.7 vs 54.7 ± 11.4, $P < 0.03$). Concentration (ng/mL) of progesterone (P4) was greater in the FM group than in the controls on Day 14 (10.1 ± 0.9 vs 7.7 ± 0.9, $P < 0.08$), Day 15 (9.2 ± 1.0 vs 4.3 ± 1.0, $P < 0.008$), and Day 16 (5.6 ± 1.6 vs 1.2 ± 0.4, $P < 0.02$). The interval from ovulation to the beginning of a decrease in P4 and to the end of luteolysis (P4 < 1 ng/mL) were each delayed ($P < 0.03$) by approximately 1 day in the FM group. Intervals involving the luteal phase were long (statistical outliers, $P < 0.05$) in two mares in the FM group, indicating prolonged luteal activity. Results supported the hypotheses that: (1) inhibition of PGF2α synthesis interferes with luteolysis in mares and (2) inhibition of PGF2α at the expected time of luteolysis may lead to prolonged luteal activity.

Keywords: Mares; Luteolysis; PGFM pulses; Progesterone; Prolonged luteal activity

3.1. Introduction

The decrease in progesterone (P4) or regression of the corpus luteum (CL) at the end of diestrus is a pivotal event during the estrous cycle in farm species including mares (reviewed in [1–4]). A 40-year chronology of research findings on the nature of luteolysis in mares has been reported [5]. Luteal terminologies before, during, and after the end of luteolysis are preluteolysis, luteolysis, and postluteolysis, respectively, and in mares, luteolysis occurs on Days 14 to 16 (Day 0 = ovulation) [6]. The transition between preluteolysis and luteolysis is manifested within 1 hour [7], based on P4 concentrations in blood samples collected hourly. The end of luteolysis and beginning of postluteolysis is commonly defined as a P4 decrease to < 1 ng/mL [6].

Luteolysis is a response to the secretion of PGF2α from the endometrium in the absence of an embryo [1,2]. In mares, as in other farm species, circulating concentrations of a PGF2α metabolite (PGFM) is often used to represent changes in PGF2α concentrations [8], owing to the short half-life of PGF2α [9]. Classical approaches to demonstrating that PGF2α is the luteolysin in farm species are: (1) administering PGF2α, (2) assaying circulating PGF2α concentrations, and (3) blocking PGF2α secretion. In mares: (1) luteolysis occurs after administration of PGF2α [10]; (2) at the expected time of luteolysis, an increase in PGF occurs in the uterine vein [11] and an increase in PGFM occurs in the systemic circulation [6,12,13]; and (3) removal of the site of PGF2α secretion (uterus) results in maintenance of the CL [14,15]. Administration of an inhibitor of PGF2α biosynthesis (flunixin meglumine, FM) has been done in ruminants [16], but systemic administration has not been used as an experimental approach for the study of luteolysis in mares. However, FM in mares prevents PGF2α secretion into the uterine lumen [17]. Systemic treatment with FM has been used to inhibit prostaglandin synthesis in mares during the follicular phase to induce hemorrhagic anovulatory follicles [18] and after insemination to alter uterine contractions [19]. The FM acts on the cyclooxygenase

enzyme in the endometrium [20] and thereby blocks PGF synthesis [21]. Phenylbutazone also inhibits the synthesis of PGF2α and has been used in attempts to alter CL function but without success [22].

Complete luteal regression in mares involves secretion of PGF2α in more than one pulse during the length of the luteolytic period, based on the P4 response to simulated PGFM pulses [23]. Therefore, PGFM pulses are an important consideration in the study of the luteolytic mechanism. Pulses of PGFM occur about every 9 hours during luteolysis and encompass 4 or 5 hours from beginning to ending nadirs [24]. During preluteolysis, PGFM concentrations remain below assay sensitivity or the identified pulses are small (e.g., 30 pg/mL peak) [6]. Pulse prominence increases considerably during luteolysis and postluteolysis (e.g., 300 pg/mL). The peak of a small (e.g., 45 pg/mL) pulse occurs at or within 1 hour of the transitional hour between preluteolysis and luteolysis and may be similar in prominence to the previous pulse during preluteolysis [6]. Oxytocin increases in synchrony with the small PGFM pulse at the hour of transition and during prominent pulses of luteolysis but does not increase during the small pulses before the transitional pulse [25]. Concentrations of P4 decrease linearly within the hours of a PGFM pulse [13,26] and between the PGFM nadirs of adjacent pulses [24]. Luteal blood flow begins to decrease during the descending portion of a PGFM pulse [26].

Prolonged luteal activity or luteal persistence occurs in mares (reviewed in [1,2,27]). Persistent CL can result from impairment of uterine PGF2α secretion by severe uterine pathology [28]; this form of luteal persistence has been termed uteropathic [29]. Spontaneous [27,30] or idiopathic [29] persistent CL in mares that do not have known uterine pathology has also been reported, and the mean length of the luteal phase is approximately 2 months [30]. The incidence has been reported as 25% of estrous cycles [30] and as 8 to 10% but with a higher incidence (20 to 35%) near the end of the ovulatory season [31]. Although the pathogenesis of idiopathic luteal persistence is unknown, an impairment in the secretion of

PGF2α has been suspected [27,29,30]. Prolonged P4 production also occurs when a secondary CL forms from ovulation during diestrus, and the CL is too immature to respond to PGF2α secretion [29,30].

Inhibition of PGF2α biosynthesis has not been used in mares to study the role of PGF2α in luteolysis, and therefore FM was used in the current experiment during the expected time of luteolysis (Days 14 to 16). Hypotheses were: (1) inhibition of PGF2α synthesis interferes with luteolysis in mares and (2) inhibition of PGF2α at the expected time of luteolysis may lead to prolonged luteal activity. The effect of FM on circulating P4 was based on concentrations when assessed daily or every 8 hours and by number and prominence of PGFM pulses in hourly samples.

3.2. Materials and methods

3.2.1. Mares

Mares were mixed breeds of nonlactating large ponies and apparent pony-horse crosses, aged 4 to 18 years and weighed 400 to 600 kg. The mares had not been bred for at least 3 years. Abnormalities of the reproductive tract were not detected by ultrasonographic examinations [32]. The experiment was done in the summer (June). The mares were housed under natural light in an open shelter and outdoor paddock and were maintained by free access to primarily grass hay, trace-mineralized salt, and water. All mares remained healthy and in good body condition throughout the study. Mares were handled according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

3.2.2. Protocol

Mares were assigned randomly to a control group and an FM-treated group (n=8 mares/group). Mares in the FM-treated group were given an iv treatment of FM (1.0 mg/kg) every 8 hours, whereas the mares in the control group were not treated. The dose of FM approximated the dose of 1.1 mg/kg that was recommended by the manufacturer and was experimentally used to block the role of PGF2α in uterine contractions [19]. Treatments were given on each of Days 14, 15, and 16 at 7 AM, 3 PM, and 11 PM (8-hour intervals). Days 14 to 16 are the expected days of functional regression of the CL [6].

Mares were examined daily from Day 11 until ovulation by gray-scale and color-Doppler ultrasonography for determining luteal area (cm^2) at the maximal cross-sectional plane, diameter of the two largest follicles in each ovary, endometrial score from 1 to 4 (minimal to maximal) as an estimate of the extent of endometrial edema [32], and percentage of CL with color-Doppler signals for blood flow of the CL [33]. The percentage of the CL with color-Doppler signals of blood flow was estimated from a scan of the entire CL. A duplex B-mode (gray scale) and color-Doppler ultrasound instrument (Aloka SSD 3500; Aloka American, Wallingford, CT) equipped with a linear-array 7.5 MHz transducer was used. Blood samples were collected into heparinized vacutainer tubes from a jugular vein. Samples were taken every day from Day 11 to ovulation and every 8 hours from Day 13 at 7 AM, 3 PM, and 11 PM (Days 13.0, 13.3, and 13.7, respectively) until Day 17.0 (Fig. 3.1). In addition to 8-hour samples, blood samples were taken each hour for 8 hours on each of Days 14, 15, and 16 beginning at 7 AM. The first sample in each 8-hour set was designated Hour 0 and was taken just before FM treatment.

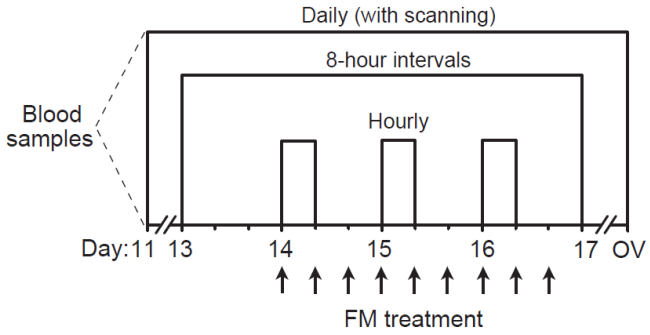

Figure 3.1. Diagram of experimental protocol. Flunixin meglumine (FM,1.0 mg/kg) was given every 8 hours for a total of 9 treatments from Day 14 at 7:00 AM until Day 16 at 11:00 PM. Daily ultrasound examinations and blood sampling were done from Day 11 postovulation until ovulation. Blood samples were collected every 8 hours from Day 13 at 7:00 AM until Day 17 at 7:00 AM. Three sets of hourly samples for 8 hours were collected on Days 14, 15 and 16 from 7:00 AM until 3:00 PM. Ov, ovulation.

Prolonged luteal activity was indicated if a statistical outlier occurred in the interval from ovulation to the beginning of luteolysis or from ovulation to the end of luteolysis (P4 < 1 ng/mL). The day of the beginning of luteolysis was assigned to the 8-hour interval when P4 began to decrease without a subsequent transient increase. A reported minimal length of prolonged luteal activity is 30 days from ovulation to a decrease of P4 to < 1 ng/mL [31].

Pulses of PGFM were identified on each of Days 14 to 16 in an 8-hour set of hourly blood samples. To distinguish between fluctuations and pulses, the intra-assay coefficient of variation (CV) was used as described [34]. Briefly, a PGFM fluctuation was an increase and decrease encompassing at least four hourly values, including nadirs. A fluctuation with a CV that was at least three times greater than the mean intra-assay CV was defined as a pulse. The peak of an identified PGFM pulse was designated as 0 hour.

3.2.3. Hormone assays

Blood samples were immediately placed in ice water for 10 min after collection, followed by centrifugation (2,000 X *g* for 10 min). The plasma was decanted and stored (–20º C) until assayed. Plasma samples were assayed for PGFM by an ELISA that was developed and validated in our laboratory for use in mare plasma [6]. Plasma P4 concentrations were determined with a solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure has been validated and described for mare plasma in our laboratory [35]. Plasma concentrations of estradiol-17β were measured by a double-antibody radioimmunoassay kit (Double Antibody Estradiol, Diagnostic Products Corporation, Los Angeles, CA, USA), as described and validated in our laboratory for mare plasma [36]. The intra- and interassay CV and sensitivity, respectively, were 12.8%, 19.0%, and 4.1 pg/mL for PGFM and 8.3%, 4.7%, and 0.03 ng/mL for P4. The intra-assay CV and sensitivity for estradiol were 5.7% and 0.78 pg/mL, respectively.

3.2.4. Statistical analyses

Data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were transformed to natural logarithms or ranks. Statistical outliers ($P < 0.05$) for intervals were determined by the Dixon test. Determination was made of the main effects of group and time (daily, 8-hour intervals, and hourly) and the interaction of group and time. An inadequate number of PGFM pulses on Days 14 (n = 1) and 15 (n = 0) in the FM group was managed by partitioning the mares into day/group combinations of Day 14/control, Day 15/control, Day 16/control, and Day 16/FM. The six day/group combinations were also used in the analyses of PGFM and P4 for the 8-hour sets of hourly samples each day. The

statistical analyses were done by using SAS PROC MIXED (Version 9.2; SAS Institute Inc., Cary, NC, USA). Owing to repeated measures in each mare, a repeated statement was used to account for autocorrelation by modeling the first-order autoregressive or spatial power between sequential measurements. Student's unpaired *t*-tests were used for comparing differences between the control and FM groups at each examination, and Student's paired *t*-tests were used to compare times within a group. Discrete characteristics between groups were compared by Student's unpaired *t*-tests. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to ≤ 0.10 indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

3.3. Results

Two mares in the control group and two in the FM group were omitted from the analyses. Luteolysis in these four mares was complete (P4 < 1 ng/mL) or underway (P4 1.7 to 3.4 ng/mL) before the first treatment on Day 14. The remaining mares (n = 6/group) were in preluteolysis and were used in the analyses. The probabilities for a main effect of group or a day/group combination and time (daily, 8-hour intervals, hourly) and the interaction for differences that were significant or approached significance are shown in the figures or tables. Other probabilities for differences are given in the text.

Daily concentrations of PGFM had only a day effect that approached significance primarily from greater concentration averaged over groups for Day 16 than for Day 14 (Fig. 3.2). The percentage change from Day 11 was used for P4, CL area, and endometrial score because of apparent differences between the two groups for the 3 days before the first treatment. Significant day effects averaged over groups represented decreases after Day 14 in percentage change in P4 concentration and CL area and in percentage of CL with blood-flow signals. Significant day effects representing increases after Day 14 were obtained for estradiol

concentration and percentage change in endometrial score. Although only a day effect was significant for percentage change in CL area and percentage change in endometrial score, days of a difference ($P < 0.05$) between groups are shown (Fig. 3.2). A group effect that approached significance for percentage change in P4 concentration and percentage of CL with blood-flow signals reflected greater values in the FM group averaged over Days 14 to 21 for P4 and for blood flow. Days of a difference ($P < 0.05$) between groups are shown (Fig. 3.2).

Concentrations of PGFM at 8-hour intervals on Days 13.0 to 17.0 had only a day effect (Fig. 3.3). Concentrations were greater ($P < 0.05$) averaged over the two groups on Days 15.0, 15.3, 16.3, and 16.7 than on Day 14.0. Percentage change in P4 concentrations had significant effects of group and day and an interaction that approached significance. The interaction was represented by greater ($P < 0.05$) concentration in the FM group than in the controls on each of Days 14.7 to 17.0; there was an approaching greater ($P < 0.09$) concentration on Day 14.3. These differences accounted for the group effect.

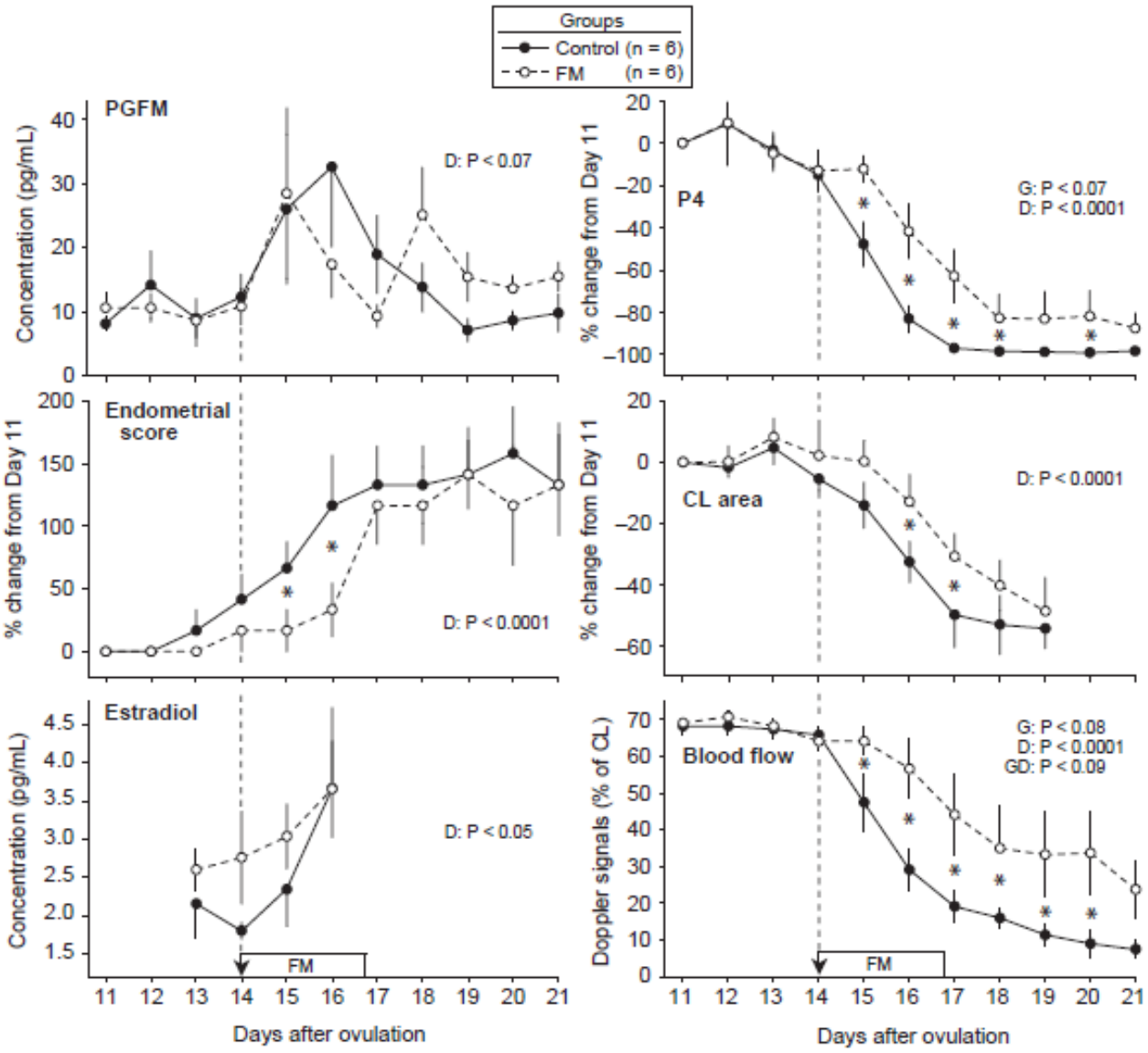

Figure 3.2. Mean ± SEM concentrations of PGFM; percentage change from Day 11 in concentration of progesterone (P4), endometrial score (1 to 4 for increasing edema), and cross-sectional area of the CL; concentrations of estradiol; and percentage of CL with color-Doppler signals of blood flow from Days 11 to 21 in controls and flunixin meglumine (FM) treated mares (1.0 mg/kg). Arrow with broken line indicates first treatment and the rectangle indicates the period of treatment (every 8 hours). Probabilities for main effects of group (G) and day (D) and the interaction (GD) are shown. An asterisk (*) indicates a day with a difference between groups ($P \leq 0.05$).

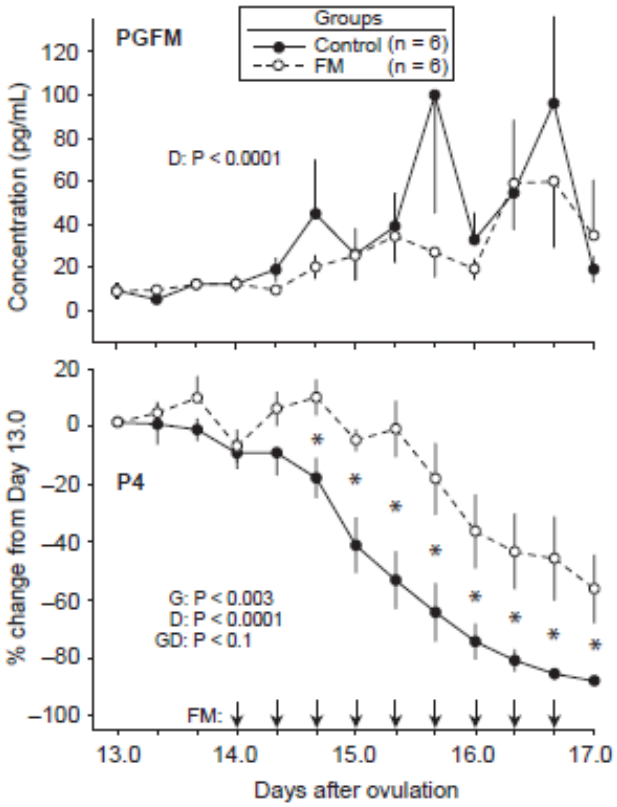

Figure 3.3. Mean ± SEM concentration of PGFM and percentage change from Day 13.0 in progesterone (P4) every 8 hours from Days 13.0 to 17.0. The arrows indicate that flunixin meglumine (FM, 1.0 mg/kg) was given every 8 hours from Day 14.0 to 16.7. Probabilities for main effects of group (G) and day (D) and the interaction (GD) are shown. An asterisk (*) indicates a day with a difference between groups ($P \leq 0.05$).

Concentrations of PGFM at hourly intervals for Hours 0 to 8 had significant day/group combination and hour effects and an interaction of combination and hour that approached significance (Fig. 3.4). The combination effect represented progressively greater concentrations averaged over hours within each of the six day/group combinations and greater concentrations in the control group than in the FM group averaged within each day as shown (Table 3.1). The interaction represented differences among the six combinations of group and day as shown (Fig. 3.4). Concentration was lower ($P < 0.05$) in the FM group than in the controls at Hours 2, 3, 5, and 6 on Day 14 and at Hours 3 to 5 on Days 15 and 16. Other differences among the six combinations of days and groups were complex and are shown (Fig. 3.4). Concentrations of P4 at hourly intervals for Hours 0 to 8 had significant main effects of combination and hour without an interaction (Fig. 3.4). The changes within each combination

for the 3 days and between groups within each day were in the opposite direction to the changes for PGFM as shown (Table 3.1). When the two groups were compared within each day, the hour effect averaged over groups was significant from a decrease over hours for Day 15 ($P < 0.01$) and for Day 16 ($P < 0.0001$); there was no interaction for either day.

Table 3.1. Mean ± SEM for PGFM and P4 concentrations averaged over 8 hours of hourly blood sampling on each of Days 14 to 16 for the control and FM groups.

Day	Control group	FM group	Probability
PGFM (pg/mL)			
14	13.8 ± 2.9^{a}	6.7 ± 1.3^{a}	$P < 0.05$
15	35.2 ± 10.4^{ab}	15.0 ± 3.9^{ab}	$P < 0.10$
16	54.7 ± 11.4^{b}	21.9 ± 5.7^{b}	$P < 0.03$
P4 (ng/mL)			
14	7.7 ± 0.9^{x}	10.1 ± 0.9^{x}	$P < 0.08$
15	4.3 ± 1.0^{y}	9.2 ± 1.0^{xy}	$P < 0.008$
16	1.2 ± 0.4^{z}	5.6 ± 1.6^{y}	$P < 0.02$

^{ab} Means for PGFM within the control group or within the FM group with no common superscript letters are different ($P < 0.05$).

^{xyz} Means for P4 within the control group and within the FM group with no common superscript letters are different ($P < 0.05$).

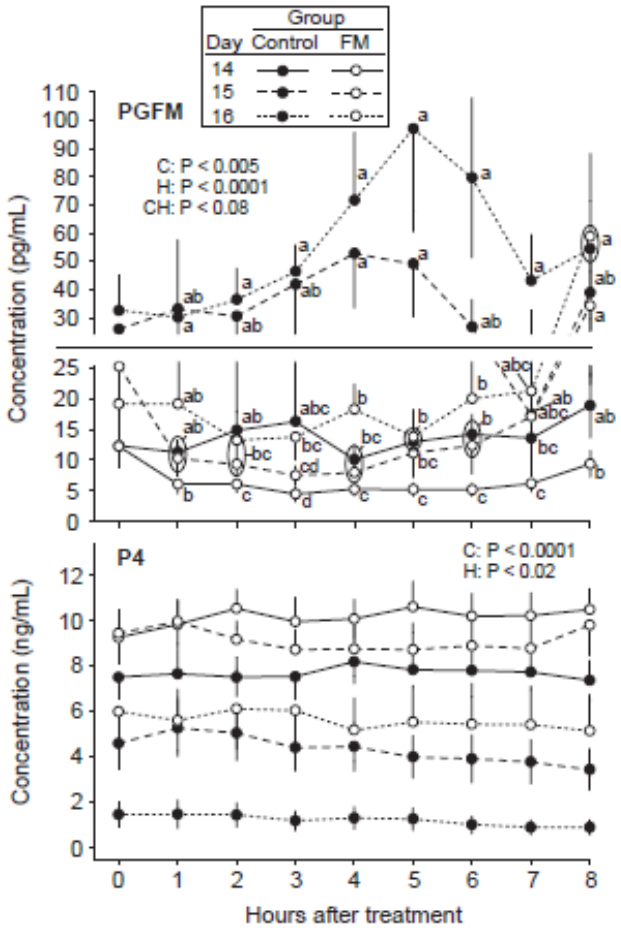

Figure 3.4. Mean ± SEM concentrations of PGFM and progesterone (P4) at hourly intervals for 8-hour sets at the beginning of each of 3 days for the controls and flunixin meglumine (FM) treated groups, using six combinations of day and group as shown. The PGFM concentrations are in two different scales on the y-axis. Probabilities for a main effect of combination (C) and hour (H) and the interaction (CH) are shown. Within each hour, means without a common letter (abcd) are different ($P < 0.05$).

In the Day 14/FM and Day 15/FM combinations, only one and no mares, respectively, had a detected PGFM pulse during the 8 hours of hourly samples, and these two combinations were not included in the analyses of pulses. In the remaining four combinations, the mean number of pulses per 8 hours was not different among combinations (Table 3.2). In addition to the expected hour effect when pulses were normalized to the peak, the main effect of the day/group combination was also significant (Fig. 3.5). The combination effect was represented by greater ($P < 0.05$) average PGFM concentration in the Day 16/control than in the Day 14/control and Day 16/FM combinations (Table 3.2). Concentration was greater ($P < 0.05$) at

the peak (0 hour) and the hour before the peak in the Day 16/control than in the Day 14/control and Day 16/FM combinations (Fig. 3.5). The amplitude of the PGFM pulses was greater ($P < 0.05$) in the Day 16/control than in the Day 14/control and the Day 16/FM combinations (Table 3.2). There were no differences among the four combinations for concentration at the nadirs or the interval from beginning to ending nadirs of the PGFM pulse. An apparent ($P < 0.1$) gradual decline in concentration of P4 occurred within the hours of a PGFM pulse in the Day 15/control and Day 16/control combinations but not in the other two combinations (not shown).

Table 3.2. Mean ± SEM concentrations of PGFM in detected PGFM pulses during 8 hours of hourly sampling on the indicated days postovulation in mares in the control and FM groups.

	Control group			FM group	Proba-
Pulse characteristics	Day 14	Day 15	Day 16	Day 16	bility
Number pulses/mare/8 hours	0.7 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	NS
Peak (pg/mL)	23.0 ± 6.8^{a}	85.2 ± 28.2^{b}	136.8 ± 33.1^{b}	36.0 ± 7.7^{a}	P < 0.02
Nadir 1 (pg/mL)	8.4 ± 1.8	30.0 ± 24.3	29.6 ± 7.2	18.9 ± 6.9	NS
Nadir 2 (pg/mL)	15.8 ± 8.6	16.3 ± 4.3	25.2 ± 5.3	11.0 ± 0.8	NS
Amplitude (pg/mL)	14.7 ± 5.3^{a}	55.3 ± 22.7^{ab}	107.2 ± 37.3^{b}	17.1 ± 5.3^{a}	P < 0.06
Mean for all hours	15.4 ± 4.6^{a}	37.8 ± 13.0^{ab}	65.6 ± 11.8^{b}	18.8 ± 4.1^{a}	P < 0.01
Pulse duration (hours)	4.8 ± 0.2	6.6 ± 0.9	6.4 ± 0.8	5.5 ± 0.9	NS

There was only 1 or 0 mares with a PGFM pulse during the 8 hours of hourly sampling in the Day 14/FM and Day 15/FM day/group combinations, respectively.
NS = Neither significant nor approaching significance.

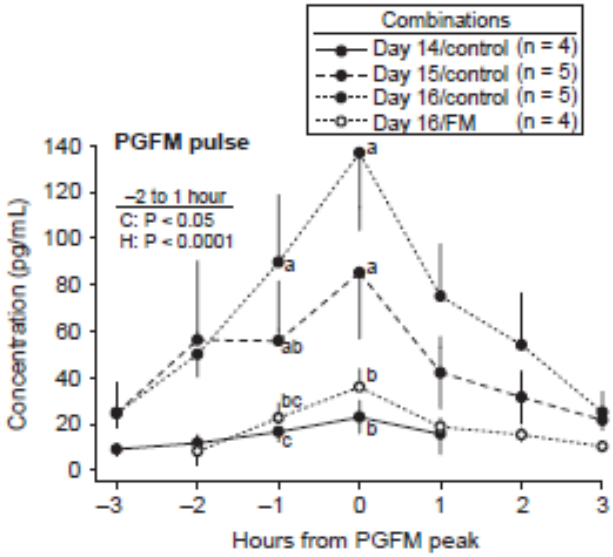

Figure 3.5. Mean ± SEM concentrations of PGFM in identified pulses centered on the pulse peak for four combinations of day and group. Only one and zero pulses were detected in the FM group on Days 14 and 15, respectively, and were not included. Probabilities for a main effect of combination (C) and hour (H) and the interaction (CH) are shown. Within each hour, any two means without a common letter (abc) are different (P < 0.05).

In the FM group, the interval from ovulation to the beginning of luteolysis was a statistical outlier in one mare (21 days; IOI, 28 days) and the interval from ovulation to the end of luteolysis was an outlier in another mare (36 days; IOI, 41 days). These two intervals were used to indicate the occurrence of prolonged luteal activity, and the intervals, but not the PGFM and P4 concentrations, were omitted from the analyses between groups. The mare with an IOI of 41 days had a transient decrease in P4 on an expected day of luteolysis (Fig. 3.6). Concentrations decreased to 1.3 ng/mL on Day 18 and then increased to > 4 ng/mL. Changes in cross-sectional area of the CL and percentage of CL with blood-flow signals were similar to the P4 concentration change. The interval from ovulation to the beginning of a progressive and continuous decrease in P4, as determined by averaging data for individuals, and from ovulation to the end of the luteal phase (P4 < 1 ng/mL) were longer in the FM group than in the controls (Table 3.3). The mean beginning of a decrease in P4 concentration based on inspection of figures was Days 14 and 15 for the daily samples (Fig. 3.2) in the control and FM groups,

respectively, and Days 14.3 and 15.3 for the 8-hour samples (Fig. 3.3). The first significant decrease in percentage change in P4 from Day 14.0 in the samples at 8-hour intervals occurred on Day 15.0 in the controls and Day 16.0 in the FM group. The interval from the end of the luteal phase to ovulation was not different between groups (Table 3.3).

Table 3.3. Mean ± SEM length of intervals (days) between events in control and FM groups, based on an 8-hour interval between blood samples.

Interval (days)	Control group (n = 6)	FM group (n = 5)[a]	Probability
Interovulatory (IOI)	23.3 ± 0.7	24.8 ± 9.7	NS
Ovulation to beginning of P4 decrease	14.4 ± 0.1	15.3 ± 0.4	P < 0.03
Ovulation to P4 < 1 ng/mL	16.1 ± 0.2	18.3 ± 0.9	P < 0.02
P4 < 1 ng/mL to ovulation	7.3 ± 0.6	6.3 ± 0.6	NS

[a] A statistical outlier was omitted for the IOI and for intervals from ovulation to the beginning of a P4 decrease and to a P4 concentration < 1 ng/mL.

NS = Neither significant nor approaching significance.

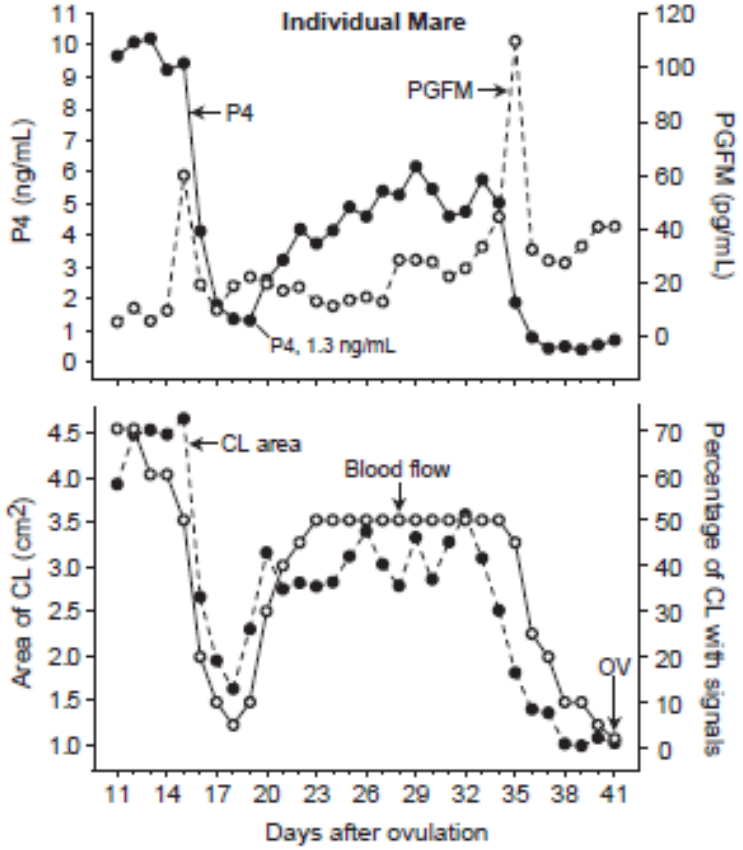

Figure 3.6. Concentrations of progesterone (P4) and PGFM, cross-sectional area of the CL, and percentage of CL with color-Doppler signals of blood flow for an individual mare in the flunixin meglumine group that had prolonged luteal activity and IOI. A transient decrease in P4 began on Day 15, did not decrease to < 1 ng/mL, and then increased beginning on Day 19. A maximal concentration of PGFM occurred near the beginning of the initial transient decrease and the final decrease in P4 near the end of the 41-day IOI. The profile for area of the CL (cm2) and percentage of the CL area with blood-flow signals approximated the profile for P4 concentrations. Study by ultrasonic imaging seemed to indicate that a single CL was involved.

3.4. Discussion

3.4.1. Inhibition of PGFM

Treatment with FM during the expected luteolytic period (Days 14 to 16) in mares inhibited the secretion of PGF2α, as indicated by a metabolite (PGFM) and as previously reported for cattle [16] and sheep [37]. In heifers, three treatments with FM at 8-hour intervals during preluteolysis (Day 16) effectively inhibited PGF2α for about 24 hours from first treatment [16]. Similar results were obtained in the current study in mares, except that each FM treatment at 8-hour intervals was effective for only 7 hours. This was indicated by a return to approximately the concentration in controls between Hours 7 to 8 on Days 15 and 16. There was no indication that the 1-hour lapse in PGF2α suppression resulted in a change in P4 concentration at the end of each 8-hour set of hourly sampling. That is, the PGFM was reduced from the low concentration in controls on Day 14 and from the greater concentration in controls on Days 15 and 16.

Collection of blood samples at 1-day or 8-hour intervals was ineffective in demonstrating the FM-induced reduction in PGF2α concentration. The increase in PGFM, especially after Day 15 (day effect) likely reflected an increase in prominence of PGFM pulses and the chance occurrence of sampling during a major portion of a pulse in individuals. The day effect was demonstrated effectively by using the average for the hourly samples in the 8-hour set for each day.

The increase in prominence of the PGFM pulses increased over days as indicated by peak concentration, amplitude, and overall mean concentration, agrees with the report [6] that the prominence of PGFM pulses increases from preluteolysis to postluteolysis. The effectiveness of FM in reducing the number of PGFM pulses on Days 14 and 15 was shown by the detection of one pulse in only one mare and on only one day. Although the number of pulses

in the FM-treated group on Day 16 was similar to the number in the controls on each of Days 14 to 16, the prominence of the Day-16 pulses in the treated group was low. The PGFM pulses in the controls on Days 14 to 16 and the pulses in FM-treated mares on Day 16 originated from a base that was similar among days, as indicated by the similarity between groups and among days in the concentrations at the nadirs and in duration of the base of the pulse.

The effectiveness of FM treatment in reducing the PGFM concentration on each of Days 14, 15, and 16 was indicated by the lower concentration averaged over the 8-hour sets of hourly sampling at the beginning of each day. The mean concentration in the FM-treated mares was 48%, 43%, and 40% of the concentration in the controls on Days 14, 15, and 16, respectively. The mean concentration increased progressively over Days 14 to 16, in both the controls and FM-treated group. The percentage increase in the controls between Days 14 and 15 (39%) and between Days 15 and 16 (64%) seemed similar to the corresponding percentage increase in the FM group (48% and 68%). The similarity between groups in the percentage change in PGFM concentrations among days indicated similar effectiveness of the FM over Days 14 to 16. In contrast, the presence of only one mare with a PGFM pulse on Day 14 and the absence of pulses on Day 15 in the FM group and a similar number in the controls and FM group on Day 16 indicated a loss of FM effectiveness on Day 16. However, the pulses were considerably less prominent on Day 16 in the FM group than on Day 16 in the controls. The relative effectiveness of FM on different days cannot be adequately assessed for the current study because of the continuous treatment over days. Further study would be needed that includes treatment that is limited to each day.

3.4.2. Progesterone response to inhibiting PGF2α

Hypothesis 1, that inhibition of PGF2α synthesis interferes with luteolysis, was supported. Support was from the following: (1) an approaching group effect for P4 in the daily

samples, with greater concentration (represented by percentage change) in the FM group during Days 15 to 18; (2) a group effect and approaching interaction for P4 in the samples collected every 8 hours, with greater concentration in the FM group on Days 14.7 to 17.0; (3) greater concentration of P4 in the FM group averaged over the hourly samples for each of Days 15 and 16; and (4) longer intervals from ovulation to the beginning and to the end of luteolysis in the FM group. The analyses for PGFM and P4 used all 12 mares, but the analyses for length of intervals did not include the outliers for the beginning and for the end of luteolysis. If the outliers had been included, the mean lengths would have been even longer in the FM group. After excluding the outliers, the beginning of luteolysis and the end of luteolysis were each delayed approximately 1 day by FM treatment that began before the end of the preluteolytic period, based on the samples collected every day or every 8 hours. The similar 1-day delay in the beginning and in the end of luteolysis and the approximately parallel decrease in P4 between groups on Days 15.3 to 17.0 indicated that the effect of the reduction in PGF2α was exerted at the beginning of luteolysis, rather than during the later portion of luteolysis. These results were unexpected but suggest that PGF2α exerts its major effect at the initiation of luteolysis, and this could serve as rationale for further studies. This is apparently the first demonstration in mares that inhibiting PGF2α interferes with luteolysis. The longer IOI in the FM group was attributable to the longer luteal phase in that the interval from the end of luteolysis to the end of the IOI was not different between groups

Luteolysis or the period of decreasing P4 encompassed several days in the controls and in the FM group when outliers were included and daily samples were used. A luteolytic period of 2 to 4 days has been reported for daily samples in nontreated mares [7,38]. The prolonged period of luteolysis for daily samples and samples collected every 8 hours is illusionary, however, owing to the beginning of luteolysis on different days in individuals [7]. Based on hourly sampling, the length of luteolysis in mares is 23 hours rather than several days [6,7]. Critical study involving frequent blood sampling (e.g., hourly) is needed, especially for detailed

study on the beginning of luteolysis. The current study with daily sampling provides guidance on when to do the frequent sampling.

3.4.3. Prolonged luteal activity

Hypothesis 2, that inhibition of PGF2α at the expected time of luteolysis may lead to prolonged luteal activity, was supported. The support was from two mares in the FM group that met the definition of prolonged luteal activity on the basis of statistical outliers involving the luteal phase. Reservation is indicated, however, because the small number of mares in each group (n = 6) precluded an analysis on a difference in frequency of prolonged luteal activity between the two groups. In addition, the prolonged luteal activity (21 and 36 days) was shorter than for other reports [27,30].

The mare with a 21-day interval from ovulation to the beginning of luteolysis and a 28-day IOI was deficient in PGFM and was the only mare in either group with PGFM concentrations that were at assay sensitivity for all samples from Day 11.0 to Day 17.3. The role of FM in the continuation of the low PGFM concentrations in this mare cannot be determined in that the low concentrations also occurred for all six samples collected before treatment. In the mare with the 36-day interval to the end of luteolysis and a 41-day IOI, an increase in PGFM and the beginning of a P4 decrease occurred at the expected time on Day 15, based on daily samples. A pulse of PGFM was not detected during hourly sampling on Days 14 to 16. The P4 decreased to 1.3 ng/mL by Day 19, but an immediate increase or resurgence prevented designating the day of the end of luteolysis (Fig. 3.6). Area of the CL and percentage of CL with blood-flow signals followed the P4 profile. Although not commented upon, a similar transient decrease in P4 on approximately the day of the expected end of luteolysis followed by resurgence is apparent in published P4 profiles in several of 12 cases of spontaneous prolonged luteal activity in nonbred Thoroughbred mares [30]. Ovulation was not

detected near the current and reported transient depressions in P4 concentrations. In the current study, the CL before and after the transient P4 depression to almost 1 ng/mL was apparently the same structure. The structure was in the same location in the ovary each day, and the largest follicle combined for both ovaries was 16.2 mm on the days before and during the resurgence (Days 17 to 19), compared to a preovulatory follicle diameter of ~ 45 mm [2]. Luteinization of a follicle in the same area of the ovary seems unlikely but cannot be ruled out.

Resurgence of P4 concentrations has been reported previously under several conditions. Apparent partial resurgence of the CL from the previous estrous cycle after P4 decreased to < 1 ng/mL has been reported during early pregnancy in a pony [39]. The resurgence was indicated by an increase in cross-sectional area of the CL from the previous cycle and by an increase in P4 that reached unusually high concentrations on approximately Days 5 to 10 of pregnancy. In both ponies and horses, the size of the primary CL and P4 concentrations resurge after Day 30 of pregnancy [39,40]. Treatment of mares with a low dose (0.5 mg) of PGF2α results in P4 resurgence, but not when a second treatment is given the next day [41]. Resurgence in P4 concentration occurs when mares are treated with a single 5-mg dose of PGF2α on Days 2 or 3 [42]. Resurgence was reported in three heifers in association with a nonprominent PGFM pulse [43]. It is not known if the resurgence of the CL involves an increase in number of luteal cells or an increase in size of the extant luteal cells. In this regard, the small luteal cells may differentiate into large luteal cells [44] under the influence of LH [45]. Further study is needed to confirm that resurgence after luteolysis nears completion is a phenomenon that may occur naturally.

The mechanism involved in spontaneous or idiopathic prolonged luteal activity is not known, but impaired PGF2α secretion has been suspected [27,29,30,46]. Previous studies with a prolonged luteal phase induced by altrenogest treatment indicated that mares with prolonged luteal activity had a lower response to oxytocin treatment compared to controls, perhaps from inadequate oxytocin receptors in the endometrium [47]. The mechanism may be similar to that

described for pregnant mares, wherein concentrations of oxytocin receptors are considerably lower in pregnant than in cycling mares [48]. Paradoxically, repeated administration of oxytocin during diestrus in the mare prolongs luteal function [49,50]. Speculatively, the syndrome of prolonged luteal activity may involve low numbers of oxytocin receptors, and the prolonged luteal activity associated with chronic oxytocin treatment may involve down-regulation or saturation of the oxytocin receptors. The mechanism that leads to the prolonged luteal activity syndrome and the potential role of oxytocin needs to be studied.

3.5. Conclusions

Secretion of PGF2α is needed for luteolysis in mares, based on a 1-day delay in both the beginning and end of luteolysis in association with inhibition of PGF2α by FM. An increase (statistical outliers) in the length of the luteal phase may occur (2 of 6 mares) after inhibition of PGF2α secretion on Days 14.0 to 16.7. One of the mares with prolonged luteal activity had a transient decrease in P4 on the expected days of luteolysis, but a resurgence in P4 concentration occurred before P4 decreased to < 1 ng/mL.

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3.7. References

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4. Hormonal, luteal, and follicular changes during initiation of persistent corpus luteum in mares

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Abstract

Mares with persistent corpus luteum (PCL) with no known etiology (idiopathic) were matched with mares with an interovulatory interval (IOI) of apparent physiologic length, so that ovulation at the beginning of each PCL and IOI occurred during the same month (n = 6 /group). Blood samples were collected daily from Days 12 to 22 (Day 0 = ovulation). Mean progesterone (P4) decreased in both groups on Days 14 and 15 and then diverged with a continued decrease in the IOI group and the beginning of constant and greater ($P < 0.05$) P4 concentration on each day in the PCL group. Before P4 divergence between groups, P4 in the PCL group decreased either abruptly (apparent incomplete luteolysis) or gradually. Concentration of PGFM (a metabolite of PGF2α) was not different between groups and reached maximum on mean Day 15 in each group. After the divergence in P4 between groups, LH and estradiol (E2) remained low in the PCL group. There was no indication that an increase in a luteotropic effect of LH in the PCL group accounted for the divergence in P4. Differences in prolactin between groups were inconclusive. The hypothesis that secretion of PGF2α at the time of expected luteolysis is defective in mares with idiopathic PCL was not supported. The hypothesis that E2 concentration before expected luteolysis is greater in mares with PCL than without PCL was not supported; however, a difference on Day 12 approached significance ($P < 0.06$) and tentatively indicated greater E2 in the PCL group before the beginning of luteolysis.

Keywords: Corpus luteum; Estradiol; Persistent CL; PGF2α; Progesterone;

4.1. Introduction

The corpus luteum (CL) is a transient endocrine gland essential to the establishment and maintenance of pregnancy by progesterone (P4) production (mare reviews [1–5]). Functional and structural regression or luteolysis of the CL occurs in the absence of a viable embryo. In the mare, luteolysis begins on average on Day 14 (Day 0 = ovulation) and based on hourly blood sampling lasts 23 hours [6]. In many species, including mares, CL regression utilizes multiple pulses of prostaglandin F2α (PGF2α) that are secreted by the nongravid uterus [7]. Circulating concentrations of a PGF2α metabolite (PGFM) is often used to represent changes in PGF2α concentrations [8], owing to the short half-life of PGF2α [9]. The base of a PGFM pulse from nadir to nadir is about 5 h in mares, and the peak of sequential pulses occurs about every 9 h [7].

A CL that fails to regress during the expected time of luteolysis in a nonpregnant mare is termed persistent CL (PCL) [10,11]. The condition is reported to occur in 8 to 10% of estrous cycles during the peak of the ovulatory season and about 25% during the autumn months [11]. Persistent CL can be related to ovulation late in diestrus, embryonic loss after the first luteal response to pregnancy, and uterine pathology such as pyometra (reviews [1,10,12,13–14]). The term uteropathic PCL is appropriate when the condition can be attributed to a uterine abnormality or pathology [10]. However, PCL also occurs without any known etiology and has been named idiopathic PCL [10] or spontaneous PCL [11,15]. Idiopathic or spontaneous can be replaced by an appropriate term when a specific nonuterine cause becomes known.

Length of the luteal phase as indicated by continued secretion of P4 is prolonged about 2 mo in mares with PCL [15,16]. Sequential mean P4 concentration for multiple mares with PCL and a statistical comparison of P4 concentrations between mares with and without PCL are limited. However, P4 profiles in individual mares with PCL have been published [15–18]. Based on individual profiles, Stabenfeldt and coworkers [15] stated, "… progestin levels in

mares with prolonged luteal activity follow the characteristic pattern for normal animals until about day 13 or 14 postovulation…followed by a plateauing of progestin levels around 4 to 5 ng/ml plasma".

The secretion of PGF2α was initially reported to be the luteolytic factor in mares based on termination of diestrus following administration [19] and by PGF concentrations in the uterine vein of nonpregnant versus pregnant mares [20]. The luteolytic role of PGF2α in mares has been confirmed (reviews [21,22]). Idiopathic PCL has been attributed to failure of PGF2α secretion at the appropriate time [11,17]. However, for each of these reports the conclusion that idiopathic PCL results from a failure of PGF2α secretion apparently was based on an individual mare. Inhibition of PGF2α secretion at the time of expected luteolysis prolonged the luteal phase (P4 > 1 ng/mL) but only for 21 and 36 days in two of eight mares (statistical outliers) [23]. In addition, one of the mares had a history of 5/21 (31%) ovulations that were followed by PCL (unpublished). Clearly, the involvement of defective PGF2α secretion in idiopathic PCL has not been critically established. The uterus does not lose the ability to secrete or the CL to respond to PGF2α as demonstrated by the termination of PCL after an increase in PGF2α concentration from intrauterine infusion of saline [11,17].

Ovulation sometimes occurs during PCL [16,24], but full estrous behavior does not [16,25]. The follicular wave in PCL that corresponds to the ovulatory follicular wave in an interovulatory interval (IOI) in mares has not been characterized. In one mare with PCL, a small follicle was detected by transrectal palpation at the end of diestrus but then regressed and no other follicles were detected [16]. Another report indicated that follicular growth was initiated before Day 14 during an IOI and at mean Day 19 during spontaneously prolonged cycles [26]. Individual profiles for P4, LH, and estradiol (E2) for PCL have been described in two mares [16]. Concentrations of E2 fluctuated widely from day to day, and in one mare the high LH concentrations associated with estrus appeared to remain high during a portion of the PCL. In another study, mean urinary estrogen concentration was greater during Days 5 to 10

in mares that developed PCL than in mares that did not. [25]. Concentration of E2 were also reported to be similar between the early luteal phase and the expected time of luteolysis in mares with PCL, contrasting with an increase in E2 between the two luteal phases in IOIs [26]. Prolactin increases during luteolysis during the IOI and during the expected time of luteolysis in mares with PCL [11]. However, a role for prolactin in PCL should be further considered, owing to the demonstrations that prolactin in mares is involved in reproductive seasonality [18], prolactin activity increases during luteolysis [27], pulses of prolactin and PGFM are synchronized [27,28], and PGF2α stimulates prolactin rather than prolactin stimulating PGF2α [29].

Studies on circulating hormone concentrations in mares with PCL have considered hormone profiles in individual mares with PCL, usually without comparison to mares without PCL. Concentration of LH has been reported only for individuals and characterization of follicular waves, cross-sectional area of the CL, and the extent of blood flow in the CL have not been considered.

The current study compared mares with and without idiopathic PCL, using daily concentrations of P4, PGFM, E2, LH, and prolactin beginning on Day 12 until Day 22 so that the period of luteolysis in mares without PCL would be encompassed. In addition, luteal dimensions and blood flow and the characteristics of the dominant follicle were included. The hypotheses were (1) secretion of PGF2α at the time of expected luteolysis is defective in mares with idiopathic PCL and (2) E2 concentration before expected luteolysis is greater in mares with PCL than without PCL. Rationale was not adequate for hypothesizing whether a luteotropic factor was involved in PCL.

4.2. Materials and methods

4.2.1. Mares

Nonlactating mares of unknown breeding, aged 4 to 14 y, and weighing 360 to 520 kg were used in the northern temperate zone. The wide range of weight and body conformation indicated an individual mare could be described as a horse or a pony-horse cross. Abnormalities of the reproductive tract were not detected by transrectal ultrasonic scanning [30]. The mares had not been bred for at least 3 y. The ovulation at the beginning of an IOI or PCL occurred during September to October, 2012 and May to July, 2013. Mares were housed in an open shelter and outdoor paddock and were maintained by free access to primarily grass hay, trace-mineralized salt, and water. The mares were housed under natural light during spring, summer, and fall (April through November in the northern hemisphere) and under artificially extended photoperiod during the winter (December through March) as described [1]. All mares remained healthy and in good body condition throughout the study. Mares were handled according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

4.2.2. Protocol

Mares were examined daily by transrectal ultrasound with a duplex B-mode (gray scale) and color-Doppler ultrasound scanner (Aloka SSD 3500; Aloka American, Wallingford, CT, USA) equipped with a linear-array 7.5 MHz transducer. The scanner was used for determining the day of ovulation, luteal area (cm^2) at the maximal cross-sectional plane, percentage of CL with power-Doppler signals for blood flow [31], diameter of largest follicle, and endometrial score. The percentage of CL with power-Doppler signals of blood flow was estimated from a

scan of the entire CL as described and validated [31]. The endometrial score (1 to 4, minimal to maximal) was based on anechoic areas as indicators of the extent of endometrial edema [30,32].

Mares in a PCL group (n = 6) were selected that had continuation of the luteal phase until the end of experiment on Day 28, as indicated by daily determination of CL area (cm^2), percentage of CL with Doppler blood-flow signals, and circulating concentration of P4. An IOI group (n = 6) was randomly selected with the beginning of the IOI in the same month as the ovulation at the beginning of each PCL. Comparisons were made on Days 12 to 22 between the IOI group and PCL group for concentrations of P4, PGFM, LH, and prolactin; Days 12 to 20 for E2; and Days 11 to 22 for diameter of the largest follicle, area (cm^2) and blood-flow signals of the CL, and endometrial edema. Statistical comparisons between groups ended on Day 22 in that the ending ovulation in the IOI group occurred on or after Day 22 in five IOIs and on Day 18 in the remaining IOI. Data for the PCL group were shown until Day 28.

4.2.3. Hormone assays

Blood samples were collected into heparinized vacutainer tubes by venipuncture of a jugular vein. Samples were immediately placed in ice water for 10 min, followed by centrifugation (2,000 X *g* for 10 min). The plasma was decanted and stored (–20 ºC) until assayed. The assay procedure for each hormone or factor has been validated and described for mare plasma in our laboratory as indicated. Plasma samples were assayed for P4 by a solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) [33]. The intra- and interassay CV and sensitivity for P4 were 6.4%, 8.2%, and 0.02 ng/mL, respectively. Plasma samples were assayed for PGFM by an ELISA [6]. The intra- and interassay CV and sensitivity were 8.9%, 10.1%, and 4.8 pg/mL, respectively. Plasma concentrations of LH were determined

by RIA [34]. The intra-assay CV and sensitivity were 4.8% and 0.2 ng/mL, respectively. The concentrations of E2 were measured by a double-antibody RIA kit (Double Antibody Estradiol, Diagnostic Products Corporation) [34]. The intra-assay CV and sensitivity were 9.6% and 0.08 pg/mL, respectively. Plasma samples were assayed for prolactin by RIA [34]. The intra-assay CV and sensitivity were 8.7% and 0.3 ng/mL, respectively.

4.2.4. Statistical analyses

Data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were resolved by transformation to natural logarithms or ranks. A value defined as an extreme outlier within both a day and within an animal by the interquartile range analysis was removed from the analyses and figures. The statistical analyses were done by using SAS PROC MIXED (version 9.3; SAS Institute Inc., Cary, NC, USA). A repeated statement was used to account for autocorrelation by modeling the first-order autoregressive between sequential measurements. Determination was made of the main effects of group and day and the interaction of group and day. When a significant interaction was found, student's unpaired t-tests were used for comparing differences between IOI and PCL groups within a day. A probability of $P \leq 0.05$ indicated that a difference was significant and a probability of $P > 0.05$ to ≤ 0.10 indicated that significance was approached. Differences that approached significance were included on a preliminary basis for potential consideration in future studies. Data are presented as the mean ± SEM, unless otherwise indicated.

4.3. Results

Daily concentration of P4 had significant main effects of group and day and a group-by-day interaction (Fig. 4.1). The group effect was from greater concentration of P4 averaged

over Days 12 to 22 in the PCL group (8.9 ± 0.6 ng/mL) than in the IOI group (3.3 ± 0.7 ng/mL). The group-by-day interaction was represented by no differences in P4 between groups on Days 12 to 14 and greater concentration in the PCL group on Days 15 to 22. Concentration of P4 decreased until the end of luteolysis (P4 < 1 ng/mL [15]) on Day 16 in the IOI group, whereas P4 concentration did not differ during Days 15 to 28 in the PCL group. On Days 15 to 22, P4 concentration in the PCL group in individuals was 46 to 54% of the concentration on Day 12. Concentration of P4 in individual mares in the IOI and PCL groups is shown (Fig. 4.2). A pronounced 1-day decrease in P4 was apparent in each IOI mare beginning on Day 13, 14, or 15. In two mares in the PCL group, an abrupt 1-day decrease in P4 occurred similar to the decrease at the beginning of luteolysis in the IOI group. In three mares, the P4 decrease was gradual from Day 12 or 13 to Day 16 or 17. In the other mare, the P4 decrease fluctuated and did not fit adequately into either subgroup.

Concentration of PGFM had an effect of day from increases and decreases averaged over groups with the maximal concentration in each group on Day 15 (Fig. 4.1). The group effect, interaction, and maximal concentration were not significantly different and did not approach significance between groups.

Concentration of E2 had an effect of day and a group-by-day interaction (Fig. 4.1). The group-by-day interaction was represented by no significant differences in E2 between groups on Days 12 to 18 and greater concentration in the IOI group on Days 19 and 20. The concentration approached being greater (P < 0.06) in the PCL group on Day 12. The first significant increase in E2 concentration occurred between Days 12 and 13 in the IOI group and between Days 14 and 15 in the PCL group. Concentration of E2 increased until Day 20 in the IOI group, whereas concentration in the PCL group did not differ over Days 15 to 20.

Concentration of LH had an effect of group and group-by-day interaction after one extreme outlier on Day 18 was removed from the IOI group (Fig. 4.1). The effect of group was from greater LH concentration averaged over Days 12 to 22 in the IOI group (3.7 ± 0.3 ng/mL)

than in the PCL group (1.6 ± 0.1 ng/mL). The group-by-day interaction was represented by no differences between groups on Days 12 to 15 and greater concentration of LH in the IOI group on Days 16 to 22. The first significant increase occurred between Days 14 and 16 in the IOI group and continued to increase until Day 22. In the PCL group, concentration of LH remained low and did not differ over Days 12 to 18 and decreased ($P < 0.05$) from Days 18 to 21.

Concentration of prolactin had an approaching significant group-by-day interaction after two extreme outliers were removed from the IOI group on Days 14 and 15 and one from the PCL group on Day 23 (Fig. 4.1). The group-by-day interaction was from no differences between groups on Days 12 to 13 and Days 15 to 21 and greater concentration of prolactin in the IOI group on Days 14 and 22.

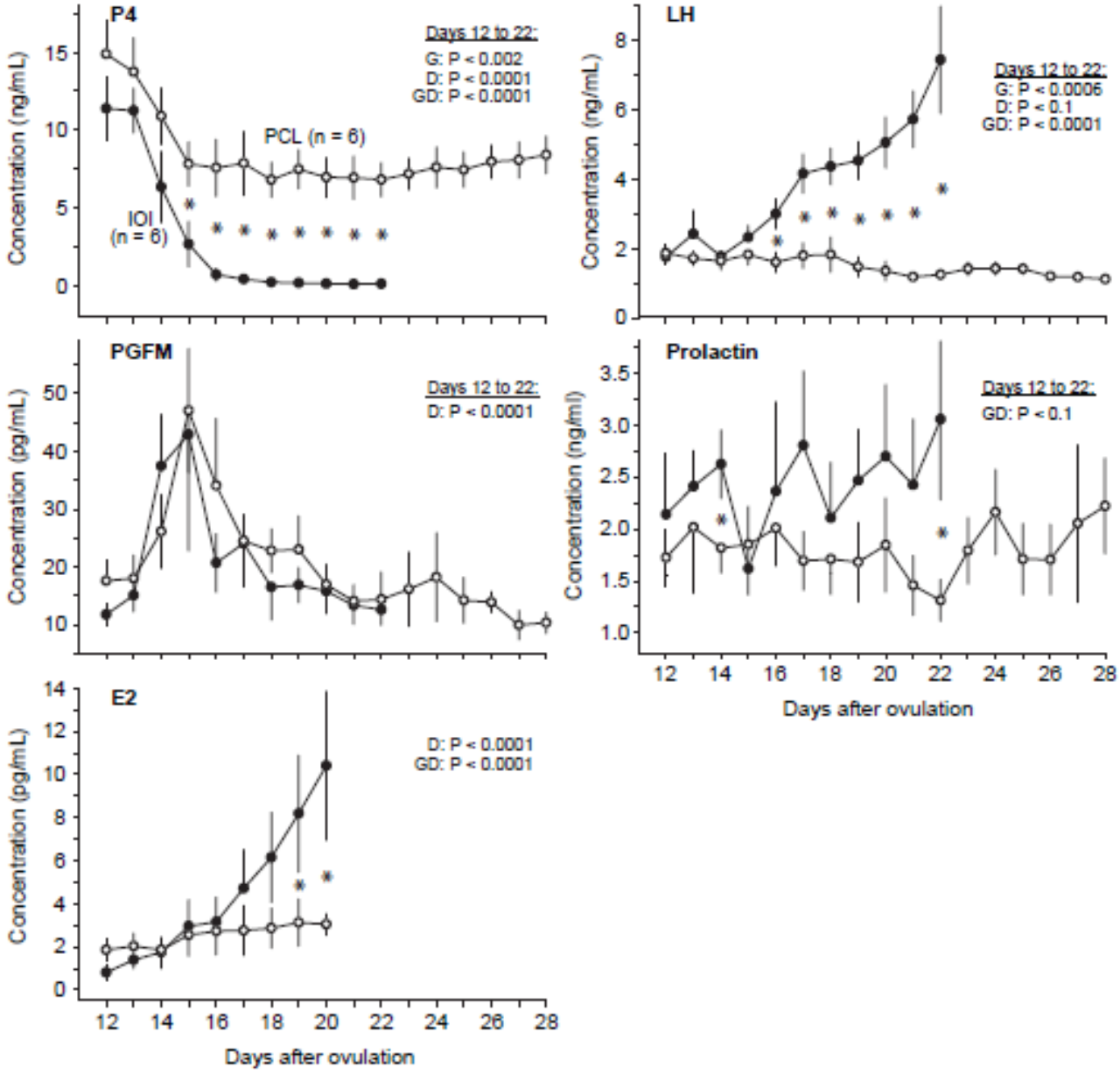

Figure 4.1. Mean ± SEM concentrations of progesterone (P4), PGFM, estradiol (E2), LH, and prolactin in the IOI group and PCL group. Probabilities for main effects of group (G) and day (D) and the interaction (GD) are shown. An asterisk (*) indicates a day with a difference ($P < 0.05$) between groups.

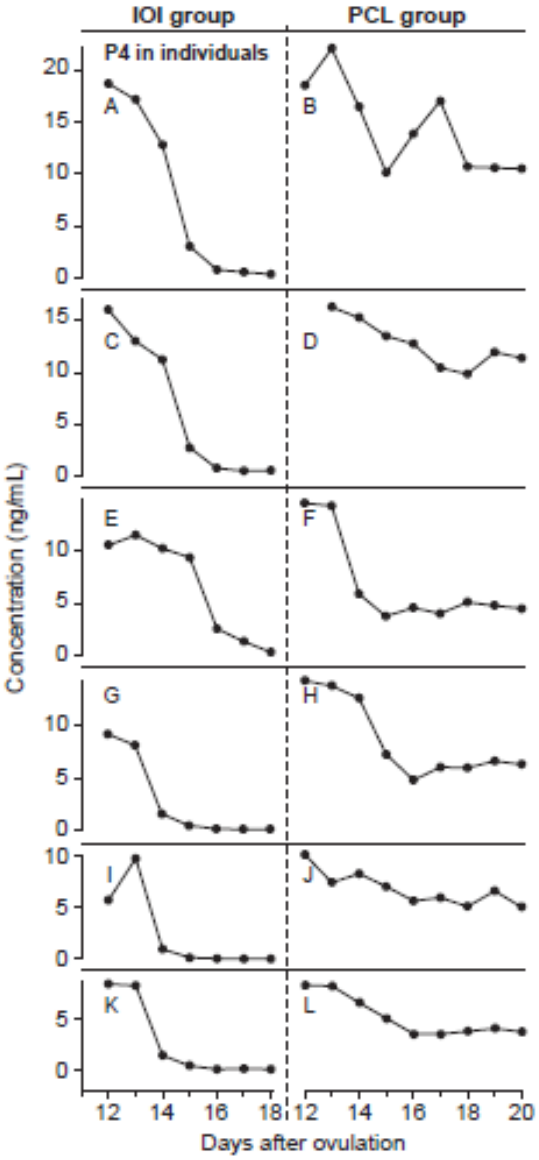

Figure 4.2. Concentration of progesterone (P4) in 12 individual mares in the IOI group (A, C, E, G I, K) and PCL group (B, D, F, H, J, L). Mares are paired between groups according to maximal concentration on Days 12 or 13. A pronounced 1-day decrease (a portion of luteolysis) is apparent in each IOI mare. A pronounced decrease (apparent initial portion of luteolysis) are shown for three mares (B, F, H) and a gradual decrease for three mares (D, J, L) in the PCL group; however, concentrations were irregular in one mare (B). Compared to the IOI mares, P4 in the PCL mares decreased only about 50%.

Cross-sectional area of CL had significant effects of group and day and a group-by-day interaction (Fig. 4.3). The effect of group was from greater CL area averaged over Days 11 to 19 in the PCL group (4.1 ± 0.1 cm2) than in the IOI group (3.0 ± 0.2 cm2). The group-by-day interaction was represented by no differences between groups on Days 11 to 15 and greater

area of CL in the PCL group on Days 16 to 19. The first significant decrease occurred between Days 13 and 14 in the IOI group and between Days 14 and 15 in the PCL group. Area of CL decreased progressively until Day 19 in the IOI group, whereas area of CL did not differ in the PCL group over Days 15 to 19 and increased ($P < 0.05$) between Days 19 to 21.

Daily percentage of CL with blood-flow signals had significant effects of group, day, and a group-by-day interaction (Fig. 4.3). The effect of group represented greater percentage of CL with blood-flow signals averaged over Days 11 to 18 in the PCL group (49.0 ± 2.0%) than in the IOI group (36.2 ± 3.5 %). The group-by-day interaction was represented by no significant differences between groups on Days 11 to 15 and greater percentage of CL with blood-flow signals in the PCL group on Days 16 to 18. The first significant decrease in percentage of CL with blood-flow signals occurred in both groups between Days 13 and 14. Blood-flow signals decreased until Day 18 in the IOI group but did not differ over Days 15 to 18 in the PCL group. The percentage of CL with blood-flow signals in the PCL group approached being greater ($P < 0.1$) on Day 28 than on Day 15.

Daily diameter of the largest follicle had a significant effect of day and a group-by-day interaction (Fig. 4.3). The group-by-day interaction reflected no differences between groups on Days 11 to 15 with a progressively increasing disparity between groups, so that diameter of the largest follicle was greater in the IOI group than in the PCL group on Day 21. The growth rate of the largest follicle on Days 11 to 21 was greater ($P < 0.006$) in the IOI group (2.4 mm/day) than in the PCL group (1.4 mm/day). In addition, the maximum diameter reached by the largest follicle was greater ($P < 0.01$) in the IOI group (48.6 ± 2.8 mm) than in the PCL group (38.5 ± 2.4 mm). The diameter of the largest follicle in the PCL group did not differ over Days 22 to 28.

Score for endometrial edema had a significant effect of group and day and a group-by-day interaction (Fig. 4.3). The group effect represented greater endometrial score averaged over Days 11 to 22 in the IOI group (2.7 ± 0.1) than in the PCL group (1.6 ± 0.1). The group-by-

day interaction was represented by no significant differences between groups on Days 11 to 15 and greater endometrial score in the IOI group on Days 16 to 22. The first significant increase in endometrial score was observed in both groups between Days 12 and 13. Endometrial score significantly increased until Day 15 in each group. The scores did not differ over Days 15 to 22 in the IOI group, whereas in the PCL group the score decreased ($P < 0.05$) from Days 16 to 21.

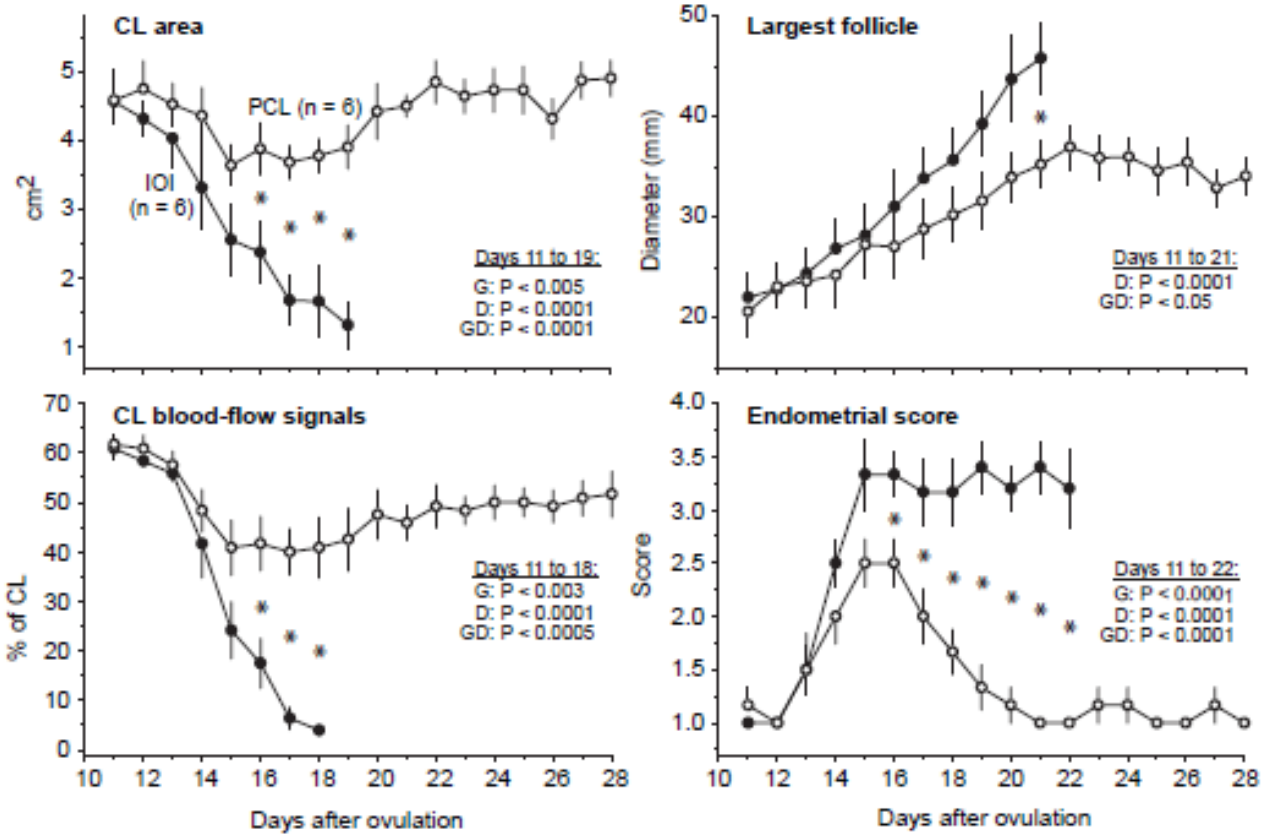

Figure 4.3. Mean ± SEM for area of CL, percentage of CL with blood-flow signals, diameter of the largest follicle, and endometrial score in the IOI group and PCL group. Probabilities for main effects of group (G) and day (D) and the interaction (GD) are shown. An asterisk (*) indicates a day with a difference ($P < 0.05$) between groups.

4.4. Discussion

Several precautions were taken to allow critical statistical comparisons between mares that subsequently did and did not develop apparent idiopathic PCL. In the three years before and during the current study, the mares were ≤ 14 y of age and therefore less likely to have the reproductive complications of senescence [36–38]. The mares were not bred, precluding the effects of early embryonic death on PCL [39]. In addition, uterine complications (e.g., metritis, pyometra) were not likely involved in that even small intraluminal fluid collections (indicators of metritis [30,40]) were not detected at any time during the three ovulatory seasons. Therefore, the PCLs were not attributable to uterine pathology (uteropathic PCL) and the PCLs were considered idiopathic [10]. Body condition or health complications were not apparent throughout the 3 y. Unique aspects of the study were: (1) exclusion of IOIs and PCLs with more than one CL or with the development of a hemorrhagic anovulatory follicle [30] and (2) matching of each PCL with an IOI according to the month of ovulation at the beginning of the PCL or IOI during what was considered part of the ovulatory season (May to October for these mares). Matching of months minimized confounding that may have occurred because of seasonal effects on the mare reproductive system [1,41].

Concentration of P4 before expected luteolysis was similar between mares with and without PCL, as indicated by no difference in P4 concentration between the IOI and PCL groups on Days 12 to 14. However, concentration before Day 12 was not available. The mean decrease in P4 during luteolysis involved Days 13 to 17 in the IOI group. In the PCL group, the mean decrease in P4 concentration discontinued on Day 15 at about 50% of the concentration on Day 12 and became constant. The similarity in P4 concentration between IOIs and PCLs until Day 14 and the plateau of lesser concentration beginning on Day 15 for PCLs seems consistent with reported P4 profiles in individual PCL mares [15]. The constant P4 concentration in individuals beginning on mean Day 15 in the PCL group in the current study

was preceded by either a 1-day abrupt decline or a gradual decline. Although confirmation is needed, these results indicated that P4 concentration in some mares with idiopathic PCL may begin to decline abruptly as for the beginning of luteolysis, whereas in others the decline may be gradual as reported for P4 of an IOI (Days 8 to 14 [42]). Apparently, PCL can begin by an initial period of partial luteolysis in some mares and a continuation of the gradual preluteolytic decrease in other mares. This is a novel observation in that only the apparent plateau in P4 concentration was observed previously in individual mares with PCL [15].

During the PCL, the area of the CL and percentage of blood-flow signals of the CL followed the same pattern as the P4 profile; divergence between IOI and PCL groups occurred on Day 15. This observation shows that functional changes in circulating concentration of P4 relate to structural changes in CL cross-sectional area and vascularity as expected. In this regard, the structural changes were determined before P4 concentration was known, thereby assuring objectivity.

Hypothesis 1 that secretion of PGF2α at the time of expected luteolysis is defective in mares with idiopathic PCL was not supported. This was indicated by the similarity (no significant differences) in daily PGFM concentration between groups, including a maximum concentration on Day 15 in each group. The daily concentrations of PGFM, as for LH and prolactin, did not consider the distinct pulses of the hormones that have been demonstrated in hourly blood samples [6,27]. The concentrations in daily samples depends on whether a pulse was underway when the sample was taken, but even at the nadirs the concentration is greater during luteolysis than before luteolysis [7]. It is expected therefore that the daily samples provided a useful overview. Results of the current daily samples encourage further study using more frequent sampling. A deficiency in PGF2α secretion has been observed in individual mares with uteropathic PCL [13]. The comparison of the IOI and PCL groups in the current study did not support the apparent supposition [11,17] that idiopathic PCL is attributable to a

deficiency in PGF2α secretion. However, further study with more mares and more frequent sampling is needed.

Hypothesis 2 that E2 concentration before expected luteolysis is greater in mares that develop PCL was not supported. Lack of support was from the similar concentration between groups on Days 12 to 15 or before divergence in P4 between groups. The main rationale for the hypothesis was the report that urinary estrogens are higher on Days 5 to 10 in mares that develop PCL [25]. A report on the associations among follicles, luteal function, and plasma E2 found no difference between the early (Days 2 to 5) and late (1 to 3 days before luteolysis) luteal phases in mares that developed PCL [26]. However, although apparently not analyzed or commented upon, tabulated data indicates that E2 on Days 2 to 5 may have been greater in a group that developed PCL (24.7 ± 1.8 pg/mL) than in a group that did not develop PCL (20.9 ± 0.7 pg/mL) [26]. Whether E2 is involved in the PCL syndrome has not been resolved by challenges with E2 administration. Several studies have indicated that exogenous estrogen does not alter the life span of the CL in mares [43–45], as it does in other species [1], which would be consistent with the conclusion that E2 does not play a role in the stimulation of idiopathic PCL. Nevertheless, greater urinary estrogen concentration during the early luteal phase [25] and the approaching greater plasma E2 concentration on Day 12 in the PCL group in the current study indicate that further study on a role of E2 in PCL development is needed, especially for Days 0 to 12.

An increase in E2 in the IOI group beginning before Day 16 agrees with previous findings that plasma E2 begins a progressive increase on Day 13 [46] and that E2 increases before the follicle deviation of Days 16 or 17 [47,48]. The divergence in E2 concentrations between groups beginning on Day 16 represented increasing concentration in the IOI group but not in the PCL group, consistent with a report that E2 did not increase during late diestrus in mares with PCL [26]. These findings for E2 apply to mares in which PCL has already been established and thereby represent the consequences of persistent P4 concentration rather than

for a role of E2 as an etiologic factor. In a study of hourly blood samples, E2 did not begin to increase until 5 h after the beginning of luteolysis [6]. Thus, if an increase in E2 is involved in PCL development, the increase apparently would be transient and would occur well before luteolysis.

In regard to the characterization of circulating concentration of LH, the beginning of an increase in LH on Day 14 in the IOI group and a progressive increase until Day 22 are consistent with the reported characteristics of the ovulatory LH surge in mares [49]. The surge reaches a peak the day after ovulation and therefore the peak was not expected to be included in the current study; ovulation at the end of the IOI occurred on mean Day 22. The LH increase during an IOI began 2 days before the end of luteolysis (P4 < 1 ng/mL), similar to previous reports [6,48,50]. Reports on LH concentration during PCL are contradictory and are only available for individual mares; both high [16] and low [18] concentration of LH have been observed. In the current study, absence of an increase in LH in the PCL group involved Days 12 to 22, contrasting with the LH increase in the IOI group. The low LH concentration in the PCL group, however, did not likely contribute to or reflect the initiation of the PCL on Day 15 in that LH was not lower in the PCL group until Day 16, the day after the mean divergence in P4 concentration in the two groups. The maintenance of greater P4 concentration in the PCL group may have played a role in the low LH. In this regard, P4 treatment has been shown to depress LH concentration during the IOI in mares [51].

Concentration of prolactin was considered in the current study, despite previous failures to find a luteolytic or luteotropic role of prolactin in mares [52]. In addition, a previous study did not find an effect of prolactin concentration in association with the development of PCL [11]. Concentrations of PGFM, LH, and FSH in addition to P4 were not affected during a reported increase in prolactin [52]. A role for prolactin was nevertheless considered in the current study because of its role in reproductive seasonality in mares [53,54] and the report that pulses of prolactin are in synchrony with pulses of PGFM, apparently representing the

stimulation of prolactin by PGF2α [27]. Therefore, if a deficiency of PGF2α had a role in the etiology of idiopathic PCL, concentration of prolactin might also have been different. However, the current results on a role of prolactin in PCL were inconclusive. That is, the statistical interaction of group and day only approached significance from apparent lower concentration in the PCL group than in the IOI group on Day 13 and Day 22. In addition, the current study and previous reports have not identified an apparent luteotropic factor or mechanism that may be involved in the prevention of luteolysis in some PCL mares and the early cessation of luteolysis in other PCL mares. Despite the current negative findings, luteotropic involvement of prolactin as well as LH in PCL development should be further considered, owing to the absence of a firm demonstration that defective secretion of the luteolysin (PGF2α) plays the primary role in the development of idiopathic PCL.

The dominant follicle in the PCL group that corresponds to the preovulatory follicle in the IOI group was inhibited. The diameter of the largest follicle began to increase similarly in the two groups on Days 11 to 15 but began to diverge between groups on Day 16 and attained a lower diameter in the PCL group on Day 21. The slower growth rate and smaller maximal diameter of the largest follicle in the PCL group are attributable to the lesser concentration of LH in the PCL group. Circulating LH is needed for the continued growth of the largest follicle after the beginning of deviation in mares [51]. In the current study divergence in LH between groups began within a day after the reported diameter of the largest follicle at deviation (22 to 25 mm; [47,55]). In a previous study, follicle emergence was delayed in mares that developed spontaneous PCL [26]. In contrast, a negative effect on follicles in the current study was indicated by slower growth of the largest follicle but not by a delay in day of emergence.

Endometrial edema initially increased in both the IOI and PCL groups in synchrony with an increase in E2 and the decrease in P4 in each group on Days 12 to 15. After the divergence in P4 concentration between groups, endometrial edema began to decrease on Day 16 in the PCL group in temporal association with P4 persistence and lack of an increase in E2.

These results are consistent with the concept that endometrial edema during estrus is a consequence of increasing circulating E2 and decreasing P4 [56].

4.5. Conclusions

An initial decrease in P4 occurred during apparent idiopathic PCL and reached a stable concentration on mean Day 15. The P4 decrease began abruptly in two individuals in the PCL group as during luteolysis in the IOI group, but apparent luteolysis was incomplete. The P4 decrease was gradual in three PCLs. Concentration of P4 began to diverge between the IOI and PCL groups on Day 14 and was greater in the PCL group on Days 15 to 22. Although concentration of E2 approached being greater ($P < 0.06$) in the PCL group on Day 12, divergence between groups did not begin until Day 16, so that concentration was not greater in the IOI group until Days 19 and 20. Concentration of LH remained low in the PCL group and was greater in the IOI group on Days 16 to 22. There was no indication that an increase in LH could have acted as a luteotropin to account for the discontinuation of a P4 decrease in the PCL group. Prolactin concentrations were equivocal in that an interaction between groups only approached significance.

4.6. Acknowledgments

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4.7. References

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5. Long-term characteristics of idiopathic persistent corpus luteum in the mare

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Abstract

Persistent CL (PCL; n = 10) in mares was studied daily from Day 20 (Day 0 = ovulation) to the ending ovulation. In addition, the 10 days before ovulation at the end of a PCL were compared with the end of an interovulatory interval (IOI; n = 28) during the same months. Concentration of P4, cross-sectional area of CL, and percentage of CL with Doppler signals of blood flow during PCLs remained constant from 64 to about 33 days before the end of luteolysis and then decreased linearly. Concentration of LH between Day 20 and beginning of the ovulatory LH surge increased linearly. A dominant follicle developed on average every 15 days throughout each PCL. Novel transient P4 depressions were detected with the P4 nadir at a day of maximal diameter of a dominant follicle. At the apparent beginning of luteolysis before the ending ovulation, P4 concentration in PCLs (5.0 ± 0.5 ng/mL) was less ($P < 0.0001$) than in IOIs (9.3 ± 0.6 ng/mL). Concentration of LH began to increase 2 days before the end of luteolysis in each group, but concentration on the day of the ending ovulation in PCLs (3.7 ± 0.3 ng/mL) was less ($P < 0.005$) than in IOIs (8.9 ± 1.8 ng/mL). In a separate survey of PCLs (n = 23) and IOIs (n = 352), frequency of PCL (6.1%) differed significantly among mares indicating repeatability. These original and critical comparisons between PCLs and IOIs should provide hypotheses for further study.

Keywords: Corpus luteum; Dominant follicle; LH; Persistent CL; Progesterone.

5.1. Introduction

In farm animals, including horses, the corpus luteum (CL) forms at the site of ovulation. The CL secretes progesterone (P4) which prevents another period of estrus and ovulation until after functional and structural CL regression or luteolysis. The control of the CL and the nature of luteolysis in mares have been reviewed [1–5]. In mares, luteolysis begins on average at Day 14 (Day 0 = ovulation) and is completed in 23 h, based on hourly blood sampling [6]. A P4 concentration decrease to < 1.0 ng/mL is used to define the end of luteolysis [7].

A CL that is maintained beyond the expected time of luteolysis in a nonpregnant mare is termed persistent CL (PCL) [8]. The condition can affect 8–10% of interovulatory intervals (IOIs) during the peak of the ovulatory season and 25% during the transition into the anovulatory season [9–11]. The PCL syndrome has been associated with uterine pathology (e.g., pyometra) [12] and with embryonic loss after the first luteal response to pregnancy [13]. The term uteropathic PCL is appropriate when the condition can be attributed to a uterine abnormality or pathology [14]. When the etiology is unknown and cannot be attributed to the uterus or to embryonic death, the terms idiopathic PCL [14] and spontaneous PCL [7,15] have been used.

Mares with PCL have a P4 concentration > 1.0 ng/mL for about 60 days (range: 35 to 95 days) [7]. Uteropathic PCL is attributable to a defect in endometrial secretion of the luteolysin prostaglandin F2α (PGF2α) [12]. Idiopathic PCL also has been attributed to defective PGF2α secretion, based on concentrations of P4 and PGFM (a metabolite of PGF2α) in a few individual mares [11,16].

Studies on the characteristics of idiopathic PCL have considered concentrations of P4, LH, PGFM, or estradiol (E2) in individual mares and have focused on the expected transition between the luteal and follicular phases [11,16–18]. Long-term characterization of hormonal changes during PCL (e.g., Day 20 to ovulation) have been limited to P4 [7–19]. Detailed

descriptions of variations in P4 concentration, ovulation, and estrous behavior are available for individuals [7,15,19]. Concentration of P4 in PCL decreases to an intermediate plateau beginning on about Day 14 [7]. An abrupt decrease in P4 concentration (luteolysis) occurs after the intermediate plateau in PCL mares, whereas a continuing gradual decrease occurs in hysterectomized mares [7,20]. After Day 20, mares with PCL appeared to have low concentration of LH [15,19], and in one mare LH concentration increased for 9 days before luteolysis [19]. Follicular growth occurs during PCL with some of the follicles reaching ovulatory size. Ovulation may occur [7,19], but estrous behavior during PCL has not been reported [17].

Consideration has not been given to the long-term characterization of follicular waves (e.g., frequency of major waves, maximum diameter of the dominant follicle) and the CL (e.g., cross-sectional area, extent of blood flow) during PCL. In the current study, daily concentrations of P4, LH, and FSH were determined, but E2 was considered only for selected portions of major follicular waves. Characteristics of dominant follicles and luteal dimensions and blood flow were included. In addition, normalization to luteolysis or to ovulation at the end of a PCL compared to the end of an IOI has not been used for critical retroactive comparison of mares with and without PCL. The current study compared mares with and without PCL retroactively from the end of luteolysis and from ovulation at the end of an IOI or PCL.

Although the IOI and PCL were compared, the results are considered observational in that there was inadequate rationale for developing hypotheses for comparing P4, FSH, LH, and E2 concentrations, follicles, and CL between the end of an IOI and the end of a PCL.

5.2. Materials and methods

5.2.1. Mares

Mixed breeds of 28 nonlactating mares aged 3 to 18 years and weighing 300 to 600 kg were used in the northern temperate zone. The wide range of weight and body conformation indicated that individual mares could be described as a horse (riding type) or a pony-horse cross. Abnormalities of the reproductive tract were not detected by transrectal ultrasonic scanning [21]. The mares had not been bred for at least 3 years. The experiment characterized PCL after Day 20 and was done from July 2012 through November 2013. Mares were housed in an open shelter and outdoor paddock and were maintained by free access to primarily grass hay, trace-mineralized salt, and water. The mares were housed under natural light during spring, summer, and fall (March through November) and under artificially extended photoperiod during the winter (December through February) as described [3]. All mares remained healthy and in good body condition throughout the study. Mares were handled according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

5.2.2. Protocol

Mares were examined daily by transrectal ultrasound with a duplex B-mode (gray scale) and color-Doppler ultrasound scanner (Aloka SSD 3500; Aloka American, Wallingford, CT, USA) equipped with a linear-array 7.5 MHz transducer. The scanner was used for determining the day of ovulation, luteal area (cm2) at the maximal cross-sectional plane, endometrial score from 1 to 4 (minimal to maximal) as an estimate of the extent of endometrial edema [21,22], and percentage of CL with power-Doppler signals for blood flow [23,24]. The percentage of

CL with power-Doppler signals of blood flow was estimated from a scan of the entire CL as described and validated [25]. Identity of each follicle ≥ 15 mm was maintained from day to day [21].

Regression of the CL was detected by a decrease in CL area and an associated decrease in percentage of CL with blood-flow signals. A cluster analysis indicated that a 20-day length of luteal phase (ovulation to P4 < 1.0 ng/mL) best distinguished between a mare with a PCL and a mare with an IOI. Criteria for assigning 28 IOIs to the IOI group were: (1) length of luteal phase was less than 20 days, (2) the IOI was a control in a previous experiment, (3) a single ovulation at the beginning of the IOI, (4) ovulation at the end of the IOI occurred during April to October, and (5) daily ultrasound scanning records and blood samples were available from Day 12 to ovulation. Criteria for assigning 10 PCLs to the PCL group were: (1) length of luteal phase was 20 or more days, (2) a single ovulation occurred at the beginning of the PCL, (3) no other ovulations or hemorrhagic anovulatory follicles [3,21] were detected between the ovulation at the beginning and end of the PCL, and (4) ovulation after the end of the PCL occurred during April to October. The criterion for ovulation at the end of the IOI or PCL during April to October was used to minimize confounding from season when data were considered retroactively from the end of the luteal phase or from the ending ovulation.

All of 10 PCLs and 28 IOIs were used for comparing groups retroactively from 4 days before the end of luteolysis and from 10 days before ovulation at the end of IOI or PCL. Seven of the 10 PCLs with an interval of at least 64 days retroactively from the end of the luteal phase were used for long-term characterization of P4 concentration, CL characteristics, and LH concentration. Three shorter intervals (CL life span of 22 to 26 days postovulatory) were excluded to minimize data distortion from shorter intervals. A dominant follicle of a follicular wave in PCLs during the interval from Day 20 to ovulation was defined as a follicle that reached 30 mm [26]. A major follicular wave was characterized by a dominant follicle and a

minor follicular wave by a largest follicle that did not attain the diameter of a dominant follicle [27]. The number of major follicular waves after Day 20 in PCLs was determined.

Transient P4 depressions were identified by their relationship to a linear regression line for each PCL from Day 20 until 2 days before the end of luteolysis. The P4 concentration at the nadir of a selected depression was ≥ 3 ng/mL and was below the regression line. The maximal concentrations of the descending and ascending portions were above the regression line. Prominent transient P4 depressions (n = 16) were selected before locations of follicular waves and concentrations of E2 were known. A total of 16 transient P4 depressions were identified in the seven PCLs with the longer CL life span. The three PCL mares without a detected P4 depression had PCLs of short duration. The nadir of the depression was used as a normalization point for diameter of the largest follicle and E2 concentration. Concentrations of E2 were available from 4 days before to 4 days after the nadir of the transient P4 depression.

5.2.3. Hormone assays

Blood samples were collected into heparinized vacutainer tubes by venipuncture of a jugular vein. Samples were immediately placed in ice water for 10 min, followed by centrifugation (2,000 X *g* for 10 min). The plasma was decanted and stored (–20 ºC) until assayed. The assay procedure for each hormone has been validated and described for mare plasma in our laboratory as indicated. Plasma samples were assayed for P4 by a solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) [28]. The intra- and interassay coefficients of variation (CV) and sensitivity for P4 were 5.7%, 8.1%, and 0.02 ng/mL, respectively. Plasma concentrations of FSH and LH were determined by RIA [29]. The intra- and interassay CV and sensitivity were 9.7%, 12.8%, and 1.8 ng/mL for FSH and 9.6%, 7.9%, and 0.2 ng/mL for LH. The concentrations of E2 were measured by a double-antibody RIA kit

(Double Antibody Estradiol, Diagnostic Products Corporation) [29]. The intra- and interassay CV and sensitivity were 5.9%, 7.1%, and 0.06 pg/mL, respectively.

5.2.4. Statistical analyses

The SAS PROC CLUSTER (version 9.3; SAS Institute Inc., Cary, NC, USA) program used the day of the end of luteolysis for dividing the intervals between the beginning and ending ovulations into the experimental groups (IOI and PCL). In the PCL group, multiple linear regression based on SAS PROC REG were used for long-term (64 days) characterization of P4, area and blood-flow signals of CL, and LH normalized for each factor retroactively to the day before preovulatory changes appeared to begin near the end of luteolysis. The regressions ended 2 days before the end of luteolysis for P4 and area and blood-flow signals of CL and 10 days before the end for LH. For comparing the IOI and PCL groups normalized to the end of luteolysis and to the ending ovulation, data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were resolved by transformation to natural logarithms or ranks. The statistical analyses were done by using SAS PROC MIXED. A repeated statement was used to account for autocorrelation by modeling the first-order autoregressive between sequential measurements. Determination was made of the main effects of group and day and the interaction of group by day from 4 days before to 5 days after the end of luteolysis and Days –10 to 0 normalized to the ending ovulation. When a significant interaction of group by day was found, Student's unpaired t-tests were used for comparing differences between IOI and PCL groups within each day. A probability of $P \leq 0.05$ indicated that the difference was significant, and a probability of $P > 0.05$ to ≤ 0.10 indicated that significance was approached. Differences that approached significance were included on a preliminary basis for potential consideration in further studies. Data are presented as the mean ± SEM, unless otherwise indicated.

5.2.5. Survey

In addition to long-term characterizations of PCL and comparisons of IOI and PCL groups in the main experiment, daily ultrasound scanning records from February 2012 to November 2013 were surveyed. The length of an IOI and PCL and month of ovulation at the beginning and end of the IOI or PCL were recorded. The frequency of PCL in the herd was determined. A total of 375 intervals between ovulations (23 PCLs and 352 IOIs) were obtained from an average of 21 mares during the 22 months (462 mare/months). The repeatability of PCL in individuals was expressed as the percentage of intervals between beginning and ending ovulations with formation of a PCL versus an IOI. Body weight, height at the withers, and body-mass index [30] were determined. Body-mass index was used to determine if PCL frequency in individuals was affected by apparent conformation differences among types of mares (horse and pony-horse crosses) and was calculated as body weight (kg)/[height(m)^2]. The repeatability of PCLs in individual mares and the frequency of the beginning and ending ovulation of a PCL during each climatic season were evaluated by chi-square.

5.3. Results

5.3.1. PCL characteristics

The length of intervals from ovulation to the end of luteolysis (luteal phase), from the end of luteolysis to ovulation (follicular phase), and from ovulation to ovulation are shown for the PCL and IOI groups (Table 5.1). The length of the luteal phase was different between groups, but the length of the follicular phase was not.

The P4 concentration in the PCL group for 64 to 2 days before the end of luteolysis was best characterized by a quadratic regression with two segmented linear components from 64 to

33 and 32 to 2 days as shown (Fig. 5.1). The linear component for P4 concentration from 64 to 33 days was constant as indicated by a nonsignificant linear regression and the component from 32 to 2 days was represented by a linear decrease ($P < 0.0001$). The decrease in the PCL group (0.09 ng/mL per day) from 32 to 2 days was less ($P < 0.0001$) than the decrease in the IOI group (0.74 ng/mL per day) from 6 to 2 days preceding the apparent luteolytic period. Cross-sectional area of CL was also best characterized by a quadratic regression with two segmented linear components. Blood-flow signals in the CL were best characterized by a cubic regression with three segmented linear components. Concentration of LH between 64 and 10 days before the end of luteolysis in the PCL group increased at a rate of 0.005 ng/mL per day and was best characterized by a linear regression; concentration remained low (range of minimal and maximal means 0.51 ± 0.11 to 0.99 ± 0.22 ng/mL).

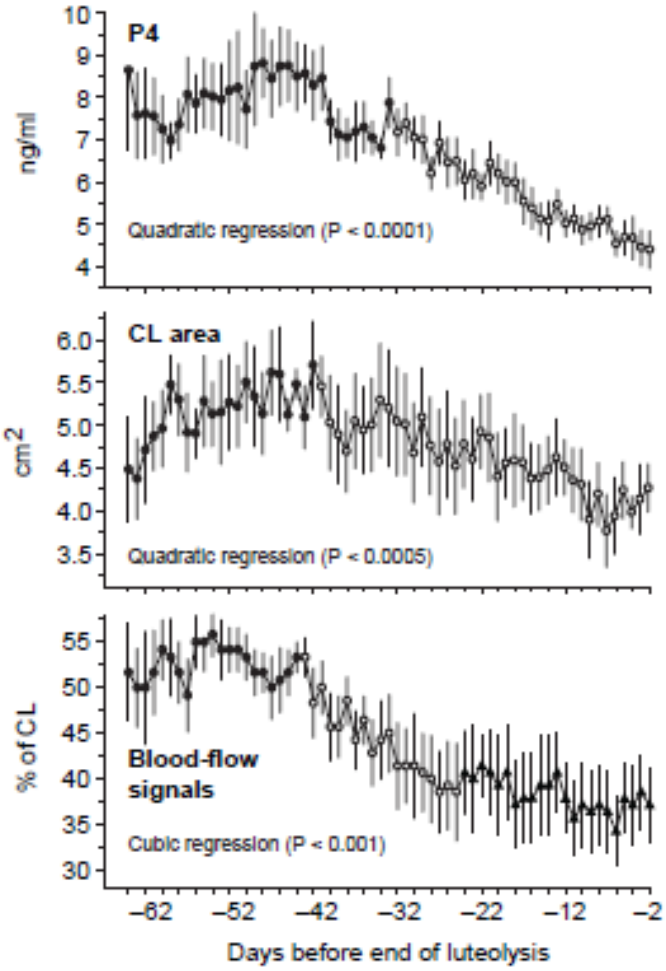

Figure 5.1. Mean ± SEM concentration of progesterone (P4), cross-sectional area of CL, and percentage of CL with blood-flow signals from 64 to 2 days before the end of luteolysis in seven PCLs. Results of multiple regression analyzes for each end point are shown. Linear components for each end point are indicated by different symbols for the means.

Table 5.1. Mean ± SEM length of intervals from ovulation to ovulation, luteal phase, and follicular phase for interovulatory intervals (IOI) and persistent CL (PCL).

	IOI group (n = 28)		PCL group (n = 10)		
End point (days)	Mean	Range	Mean	Range	Probability
Ovulation to ovulation	23.5 ± 2.2	18 to 28	74.0 ± 33.7	28 to 121	P < 0.0001
Luteal phase[a]	16.2 ± 1.2	14 to 19	67.5 ± 32.4	22 to 113	P < 0.0001
Follicular phase[b]	7.3 ± 2.0	3 to 13	6.5 ± 2.6	3 to 11	NS

[a] Ovulation to end of luteolysis

[b] End of luteolysis to ovulation

The ovulations at the beginning and end of each PCL occurred in the same ovulatory season (April to October), except that one PCL of 83 days duration began in January. The characteristics of major follicular waves of PCLs during the interval from Day 20 to ovulation are shown (Table 5.2). A continuous sequence of major waves (maximum diameter of dominant follicle, ≥ 30 mm) occurred throughout all PCLs, except that two of the total of 51 waves were minor waves (maximum diameter of dominant follicle, 25 and 28 mm). In the PCL that began in January, sequential major follicular waves occurred throughout the PCL, including waves that occurred in January and February. An illustration of the sequence of major waves is shown for two of the longer intervals (101 and 73 days) from Day 20 to ovulation (Fig. 5.2).

Table 5.2. Mean ± SEM characteristics of major follicular waves during 10 PCLs from Day 20 to ovulation.

End point	Mean	Range
No. of major waves/PCL	4.9 ± 0.8	1 to 8
Length from Day 20 to ovulation (days)	54 ± 10.7	8 to 101
Intervals between maximum diameters (days)		
Anovulatory to anovulatory wave	15.2 ± 1.4[a]	7 to 21
Last anovulatory to ovulatory wave	10.7 ± 2.0[b]	6 to 19
Maximum diameter (mm)		
Anovulatory waves	43.6 ± 1.7	32.1 to 55.6
Ovulatory waves	46.6 ± 1.9	38.5 to 59.5

[ab] Means for the interval between maximal diameters for anovulatory to anovulatory versus last anovulatory and ovulatory follicular waves are different ($P < 0.04$).

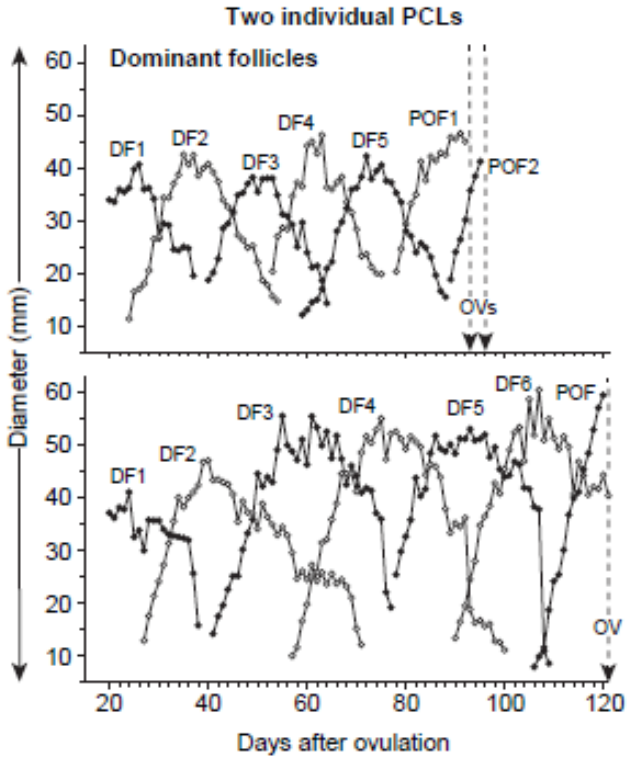

Figure 5.2. Individual profiles of dominant follicles of major follicular waves for two mares with PCL. The interval between ovulations was 93 and 121 days for the two mares, respectively, and the duration of the luteal phase was 89 and 113 days. DF = dominant follicle; OV = ovulation; POF = preovulatory follicle.

5.3.2. Transient depressions in P4

Examples of transient P4 depressions during a PCL are shown (Fig. 5.3). A transient P4 depression occurred 2.1 ± 0.4 times (range 1 to 4) during the persistent concentrations of P4 in PCLs and was not observed during IOIs. The descending and ascending portions of the 16 transient P4 depressions consisted of a mean of 6 and 7 days, respectively (Fig. 5.4). The nadirs had a mean P4 concentration of 5.7 ± 0.4 ng/mL and were located 95 to 25 days before the end of luteolysis. The nadir occurred at the mean maximal diameter of a dominant follicle when the diameter was 39.2 ± 1.6 mm. A depression did not occur during many (24/41, 58.5%) of the major follicular waves. The day effect for E2 when normalized to the nadir of the transient P4 depression was not significant. On a preliminary basis, an increase in E2

concentration approached significance ($P < 0.08$) from 4 to 1 days before the nadir, and decreased ($P < 0.03$) from 1 day before to 2 days after the nadir (Fig. 5.4). The concentrations of LH and FSH did not change significantly during a transient P4 depression, although an increase in FSH between 5 days before the nadir and the day of the nadir approached significance ($P < 0.10$; not shown).

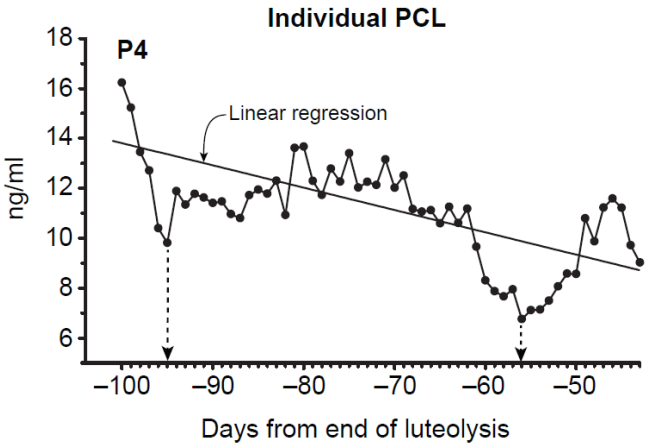

Figure 5.3. Profile of concentration of progesterone (P4) for a mare with PCL. The P4 linear regression was used as an aid for locating P4 depressions. The location of the nadirs of two transient P4 depressions are indicated by the arrow heads on the day scale.

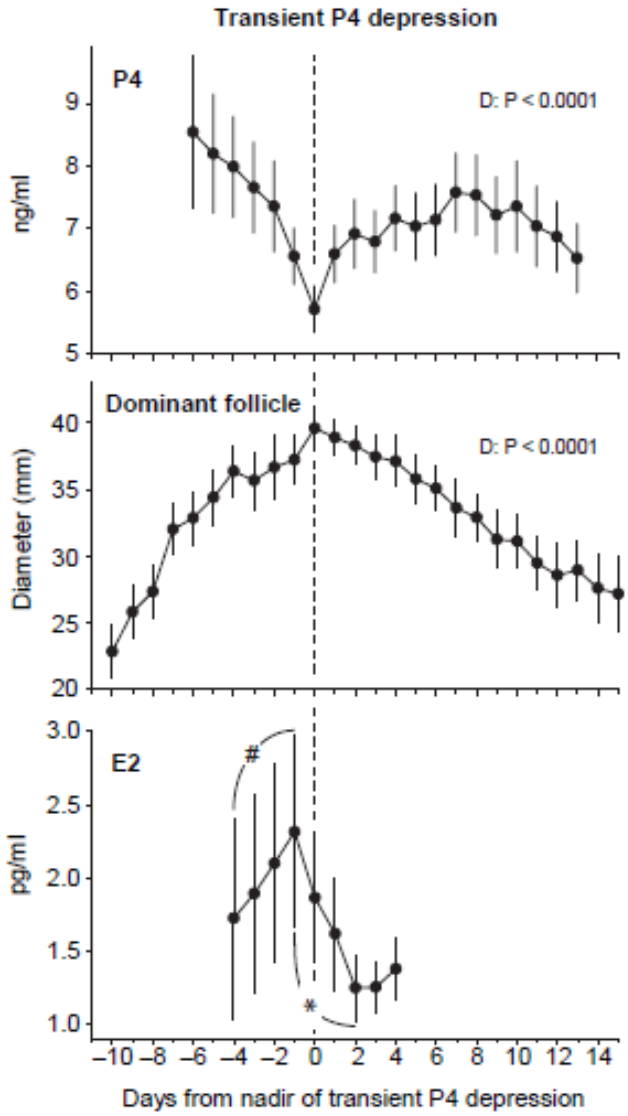

Figure 5.4. Mean ± SEM concentration of progesterone (P4; n = 16), diameter of the dominant follicle (n = 16), and concentration of estradiol (E2; n = 7) normalized to the nadir of a transient depression in P4 concentration during a PCL. Probabilities for main effect of day (D) that were significant are shown. The day effect for E2 was not significant, but on a preliminary basis an asterisk (*) indicates days with a difference ($P < 0.05$) between days, and a hash mark (#) indicates days with an approaching difference ($P < 0.08$).

5.3.3. Normalization to end of luteolysis and to ovulation

Concentrations of P4, LH, and FSH for 4 days before to 5 days after the end of luteolysis are shown (Fig. 5.5). Concentration of P4 had an effect of group and day and a group-by-day interaction. The effect of group was represented by a lower mean P4 concentration in the PCL group (2.2 ± 0.3 ng/mL) than in the IOI group (3.9 ± 0.3 ng/mL). The group-by-day interaction resulted primarily from greater ($P < 0.05$) concentration of P4 in the IOI group 4 to 2 days before the end of luteolysis and no differences between groups on the other days. A decrease ($P < 0.05$) in P4 concentration, apparently representing the day at the beginning of luteolysis, occurred 2 days before the end of luteolysis in each group.

Concentration of LH had a main effect of day and a group-by-day interaction (Fig. 5.5). The group-by-day interaction resulted primarily from less ($P < 0.05$) LH concentration in the PCL group during each day before the end of luteolysis and 1, 4, and 5 days after the end of luteolysis. An increase in concentration of LH began 2 days before the end of luteolysis in each group. A group effect approached significance and on a preliminary basis represented greater LH concentration averaged over 4 days before to 5 days after the end of luteolysis in the IOI group (2.4 ± 0.2 ng/mL) than in the PCL group (1.5 ± 0.1 ng/mL). Concentration of FSH had a main effect of day and a group-by-day interaction (Fig. 5.5). The group-by-day interaction was represented primarily by less ($P < 0.02$) concentration in the PCL group 4 days before the end of luteolysis.

After normalized retroactively from ovulation, concentration of LH was significantly less in the PCL group (1.9 ± 0.1 ng/mL) than in the IOI group (3.3 ± 0.3 ng/mL), as indicated by a group effect (Fig. 5.6). An effect of day on LH concentration resulted from an increase that began on Day –6 averaged over the two groups. Concentration of FSH had a main effect of day and an interaction that approached significance. When each group was analyzed separately, the difference among days was significant for the IOI group but not for the PCL

group. The diameter of the preovulatory follicle normalized to ovulation had significant main effects of group and day (Fig. 5.6). The effect of group was from greater diameter of the preovulatory follicle in the PCL group (38.2 ± 1.0 mm) than in IOI group (32.7 ± 0.6 mm). An effect of day represented increases after Day –10 averaged over groups. The length of the interval from a 20-mm future dominant follicle to ovulation was longer ($P < 0.0006$) in the PCL group (11.8 ± 1.2 days) than in the IOI group (8.9 ± 0.3 days).

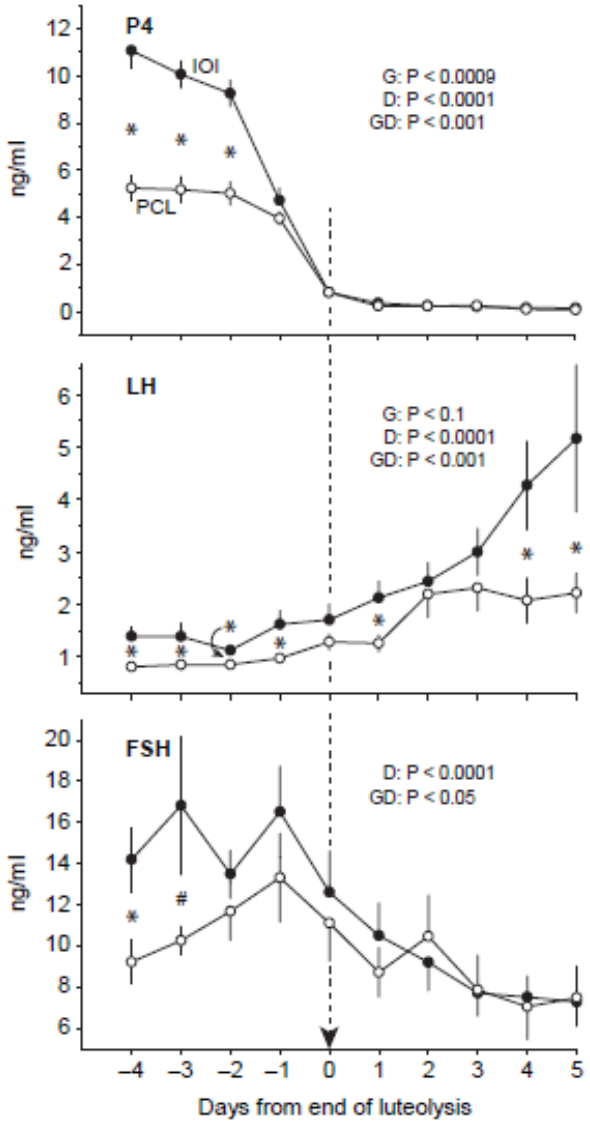

Figure 5.5. Mean ± SEM concentration of progesterone (P4; n = 28 IOIs, 10 PCLs), LH (n = 9 IOIs, 6 PCLs), and FSH (n = 9 IOIs, 6 PCLs) from 4 days before to 5 days after the end of luteolysis. Probabilities for main effects of group (G) and day (D) and the interaction (GD) that were significant or approached significance are shown. An asterisk (*) indicates a day with a difference ($P < 0.05$) between groups and a hash mark (#) indicates a day with an approaching difference ($P < 0.07$).

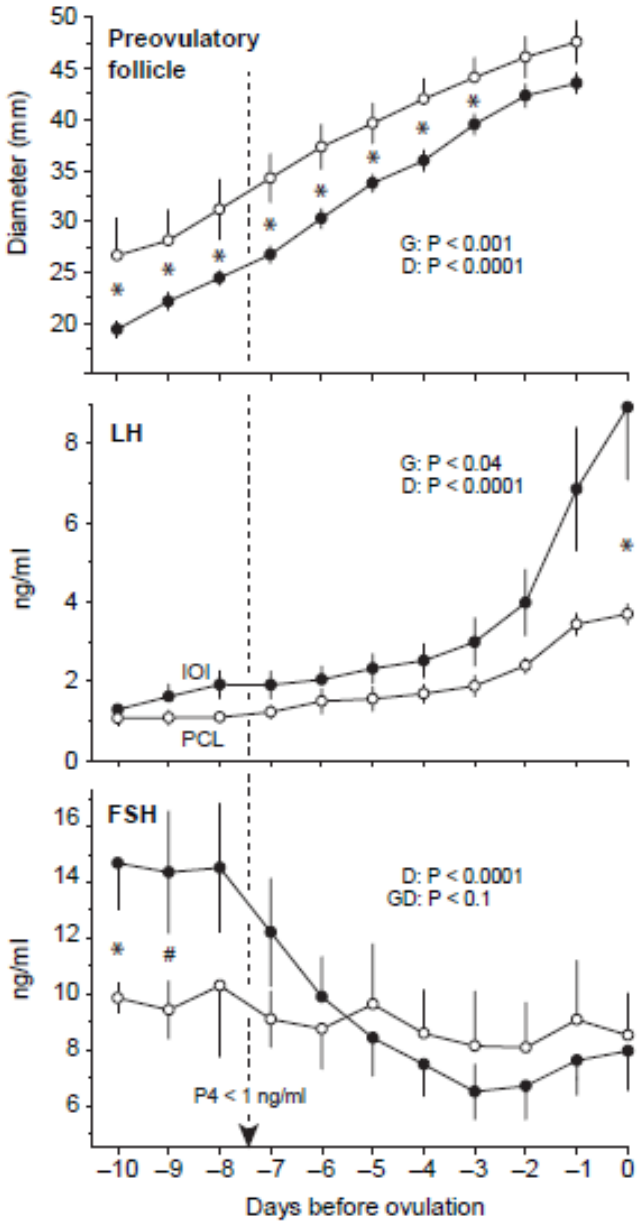

Figure 5.6. Mean ± SEM concentration of LH (n = 9 IOIs, 6 PCLs) and diameter of the preovulatory follicle (n = 28 IOIs, 6 PCLs) for 10 days before ovulation. Probabilities for main effects of group (G) and day (D) that were significant are shown. An asterisk (*) indicates a day with a difference ($P < 0.05$) between groups.

5.3.4. Survey

The frequency of PCL was 6.1% (23/375) of the intervals between ovulations. The development of PCL in the 28 mares during July to November of the following year had measurable repeatability within mares. Repeatability was indicated by a difference ($P < 0.0001$) in frequency ranging from 0 to 75% among mares. The percentage of PCLs for individuals were 75% (one mare), 30 to 31% (three mares), 5 to 12% (five mares) and 0% (19 mares). The mare with the 75% frequency had a PCL during three of four intervals between ovulations. There were no significant effects of age, body weight, height, or body-mass index on the frequency of development of PCL (Table 5.3). Also, there was no difference in the proportion of horses and pony-horse crosses that developed PCL. The probability for development of a PCL based on the beginning ovulation was not different among climatic seasons, even when combinations of two seasons were used (spring and summer compared to fall and winter). However the proportion of PCLs ending in each season differed ($P < 0.03$); the frequency of PCLs ending during spring, summer, fall, and winter in relation to the total number of intervals between ovulations was $3/120^{a}$ (2.5%), $7/134^{a}$ (4.9%), $5/97^{a}$ (5.2%), and $4/20^{b}$ (20%), respectively; frequencies with different superscript letters are different ($P < 0.05$).

Reproductive events other than the single ovulation at the beginning of the PCL occurred in seven of the 23 PCLs as shown (Table 5.4). Profiles of P4 concentration, CL area, CL blood-flow, and diameter of the dominant follicles for a mare that ovulated on Day 45 despite a P4 concentration of 6.7 ng/mL during the PCL are shown (Fig. 5.7). The first major anovulatory wave of a PCL that corresponded to the ovulatory wave of an IOI was followed by a second ovulatory wave with ovulation on Day 45. A 14.1 ng/mL increase in P4 concentration occurred after the second ovulation, and concentration remained at 6.6 to 20.7 ng/mL until the apparent beginning of luteolysis.

Table 5.3. Mean ± SEM for mare characteristics in IOI and PCL[a]. Survey.

	IOI group (n = 19)		PCL group (n = 9)	
End point	Mean	Range	Mean	Range
Age (y)	9.2 ± 0.9	3 to 18	9.1 ± 1.0	4 to 14
Body weight (kg)	464.5 ± 18.2	340.2 to 594.0	441.5 ± 21.4	356.1 to 549.8
Height (m)	1.4 ± 0.02	1.2 to 1.5	1.4 ± 0.02	1.3 to 1.5
Body-mass index	235.6 ± 6.4	185.6 to 273.9	223.0 ± 9.1	202.5 to 292.2

[a] Differences between groups were not significant and did not approach significance for any end point.

Table 5.4. Reproductive events other than the ovulation at the beginning of a PCL in seven (A to G) of the 23 individual PCLs. Survey.

PCL	Events
A	Ovulation on Day 45 when P4 was 6.7 ng/mL (Fig. 5.7)
B, C	Hemorrhagic anovulatory follicle on Day 23 or 26
D	Resurgence of CL area (1.7 to 3.3 cm2) from Days 18 to 20
E	Resurgence of CL area (1.9 to 3.3 cm2) from Days 21 to 23 and ovulation on Day 23
F,G	Double ovulation at beginning of PCL

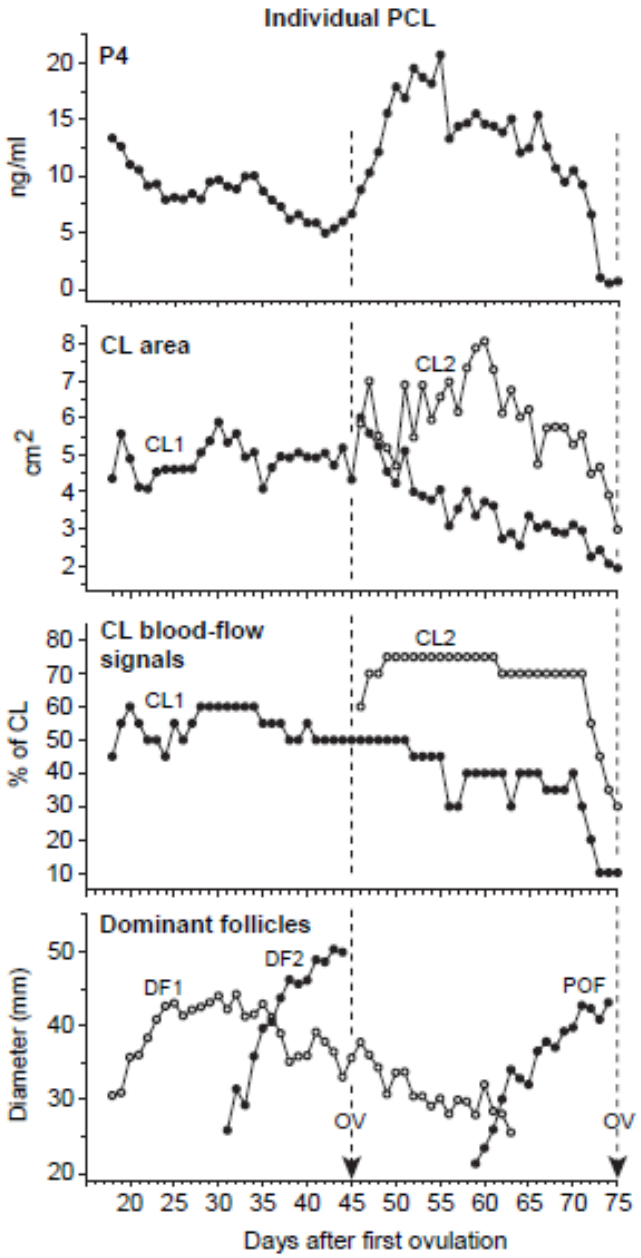

Figure 5.7. Profiles of concentration of progesterone (P4), cross-sectional area of CL, blood-flow signals of CL, and diameter of dominant follicles for an individual mare with an ovulation during the PCL on Day 45. DF = dominant follicle; OV = ovulation; POF = preovulatory follicle.

5.4. Discussion

There are many reports that focus on PCL characteristics in individual mares (reviews: [1,3,9,14,15,31,32]). The current study provides novel information on long-term hormone concentrations and follicle and CL dynamics during PCL. In addition, normalization to the end of luteolysis for comparison between IOIs and PCLs was done for the first time. The PCLs were not attributable to uterine pathology, including the small intraluminal fluid collections of metritis [22,33]. Mares were not bred before the study, thereby excluding early embryonic death as an etiologic factor [13]. Therefore, the PCLs were considered to be idiopathic [14]. Body condition or health complications were not apparent throughout the study. In addition, PCLs with more than one CL or with the development of a hemorrhagic anovulatory follicle were excluded to allow critical statistical comparisons between groups preceding the ovulation at the end of a PCL versus the ovulation at the end of an IOI. Only IOIs and PCLs with an ending ovulation during April to October were used. Assigning the beginning and ending ovulations of PCLs to one of the four climate seasons minimized confounding that may occur, considering that mare reproductive mechanisms are profoundly influenced by season [3,34]. Based on a cluster analysis, a PCL was defined as one that had ≥ 20-day interval from ovulation to the end of luteolysis. The interval from ovulation to ovulation in the IOI group ranged from 18 to 28 days and in the PCL group ranged from 28 to 121 days; the common 28-day interval was attributable to a shorter follicular phase in the PCL than in the IOI.

5.4.1. PCL characteristics

The concentration of P4 for 64 days before the end of luteolysis during a PCL seems compatible with reported P4 profiles for individual PCLs [7]. The current study further indicated that the concentration was constant during the first half of the 64 days before the end

of luteolysis and then gradually decreased. Concentration of P4 during an IOI is characterized by an increase after ovulation until about Day 8 followed by a gradual decrease (preluteolytic period) until the abrupt decrease at the beginning of luteolysis [35]. The mechanism for the long-term P4 decrease in a PCL may be similar to the mechanism responsible for the P4 decrease before luteolysis during an IOI. The P4 decrease before luteolysis may represent the waning of the positive effects of a luteotropin. In this regard, treatment with pituitary antiserum [36], gonadotropins [37], gonadotropin releasing hormone [38], and in vitro culturing of luteal cells [37] indicate that the equine CL requires circulating gonadotropins with luteotropic activity throughout diestrus and the corresponding days of pregnancy. Receptors for LH have been described for equine luteal cells [39,40]. As LH concentrations decline to basal concentrations, the number of LH receptors increases and could have accounted for a luteotropic effect of low concentrations of LH during the gradual decline in P4 in PCL, even though the low LH concentration was slightly increasing. The rate of P4 decrease preceding luteolysis at the end of a PCL was slower than during the few days preceding luteolysis in an IOI, suggesting the presence of a more robust P4-decreasing mechanism preceding luteolysis in an IOI than during the ending portion of a PCL. Further study is needed to determine the mechanism for the long-term P4 decline.

Concentration of LH during PCL gradually increased but was low (e.g., maximal mean, 0.99 ng/mL), compared to the reported peak of the ovulatory LH surge (e.g., 12 ng/mL) [41] for the same assay system. The slight and prolonged LH increase occurred during the decrease in P4 concentration. In this regard, treatment with P4 depresses circulating LH concentration [42]. Therefore, the continuation of P4 concentration during PCL may account for the associated low LH. In this regard, the gradual decrease in P4 was accompanied by a gradual increase in LH.

Changes in P4 concentration during an IOI are related to luteal structural changes that follow a similar pattern [35]. During PCL in the current study, the CL cross-sectional area and

percentage of CL with blood-flow signals were similar to the P4 profile of PCLs, demonstrating the relationship between structural and functional changes. In this regard, the structural characteristics were determined before P4 concentrations were known, assuring objectivity.

Major follicular waves were observed throughout the PCL in the current study, with the exception that two waves in one PCL were minor waves by definition. The major waves occurred even in January and February, which are considered to be part of the anovulatory season; however, only one PCL was involved. To our knowledge, the sequence of major follicular waves throughout a PCL has not been described previously. However, the waves seem compatible with previously observed follicular activity [19,43]. The maximal diameter reached by an anovulatory wave was similar to the maximal diameter of the ovulatory wave and seems consistent with a previous observation [19].

5.4.2. Transient depressions in P4

The demonstration of transient depressions in P4 during the long-term phase of a PCL and the apparent association between a depression in P4 and the dominant follicle of a major wave are novel findings, requiring independent confirmation. Each of the 1 to 4 transient depressions in P4 between Day 20 and the end of the PCL was associated with a major follicular wave. Not all major follicular waves had a concomitant transient P4 depression; mean number of P4 transient depressions per PCL was about half of the mean number of dominant follicles or major follicular waves per PCL. Temporal associations between the P4 depressions and other hormones and factors were not well established. On a preliminary basis, the decreasing portion of a depression occurred during an increase in mean diameter of the dominant follicle, so that the maximum diameter occurred at the P4 nadir. In addition, E2 and FSH tentatively increased (approached significance) during the P4 decrease and diameter increase. A change in LH concentration was not detected during the decreasing P4 concentration and increasing

diameter of the dominant follicle. Further studies are needed to determine the origin of the apparent transient depressions in P4 concentration during a PCL.

5.4.3. Normalization to end of luteolysis and ovulation

The observation that concentration of P4 immediately before luteolysis was less in the PCL group than in the IOI group is consistent with the prolonged gradual decrease in P4 concentration in the PCL group compared to the IOI group. The reason for a less-prominent increase in LH during the ovulatory LH surge in the PCL group than in the IOI group is not known, but may be related to the prolonged P4 decrease. The effects of a long-term exposure of P4 on LH concentration during an eventual ovulatory surge in mares has not been described. In this regard, elevated endogenous P4 concentrations result in reduced frequency of episodic GnRH release and lower circulating concentrations of LH without altering the FSH output [44]. The exposure of the hypothalamus to P4 concentrations during a long period may lead to a refractory period in which GnRH secretion is reduced. Further study will be needed on the potential impacts of long term P4 feedback on hypothalamic function in mares.

The approximately 3-day longer interval from a 20-mm largest follicle to ovulation in the PCL group than in the IOI group indicates a slower growth rate in that the preovulatory diameters (Days –2 and –1) were not different between groups. The longer interval accounts for the greater diameter of the largest follicle in the PCL group on Days –10 to –2. The slower development of the follicle in the PCL group is attributable initially to the lower concentrations of FSH and later to the lower concentrations of LH. Early development of both major and minor follicular waves are associated with an FSH surge that reaches a peak when the future dominant follicle is about 15 mm [45]. After the follicle reaches about 28 mm, LH becomes especially important for continued growth of the dominant follicle [46].

5.4.4. Survey

Reports on the incidence of the PCL in different climatic seasons seem contradictory. The PCL has been reported to be more frequent in the summer [43,47] and in the fall [15]. In the present study, no differences were found in the frequency of the initiation of PCL among seasons. However, more PCLs ended in winter than in the other seasons. The difference among seasons for the ending of a PCL may be related to the seasonality of mare reproductive mechanisms. Most of the mares stopped ovulating during winter, which may have accounted for fewer PCLs ending in the spring. In addition, the variation in length of PCL among mares likely led to PCLs beginning and ending in the same season or in a different season.

The diagnosis of PCL when based primarily on P4 concentration is complicated by the occurrence of other reproductive events after the beginning ovulation that may or may not have contributed to the prolonged delay before luteolysis at the end of the PCL. Such complications have been reported [7,19] and have been reviewed [14]. These complicating events include ovulation toward the end of diestrus when the new CL is not responsive to the luteolysin (PGF2α). In the current study, complicating events occurred during 7 of 23 PCLs and included ovulations, hemorrhagic anovulatory follicles, and resurgence in cross-sectional CL area and percentage of CL blood flow. Apparently, hemorrhagic anovulatory follicles during a PCL have not been previously reported.

Body-mass index, body weight, height, age, and mare type (riding horses, pony-horse crosses) did not have an effect on the frequency of PCL in the current study. However, the number of mares with PCLs and IOIs may have been too few to detect differences in PCL frequencies. Apparently, PCL is rare in ponies. A PCL was not detected during an extensive study with daily monitoring of 14 ponies (mean body weight, 284 kg) for one year [48]. In contrast, PCL has been reported to be as common as 25% of estrous cycles in horse mares [9,15]. The current demonstration that PCL has measurable repeatability among mares is

compatible with previous descriptions for individual mares [7]. Repeatability can be utilized in studying the etiologic factors involved in PCLs that are now considered to be idiopathic.

5.5. Conclusions

Concentrations of P4 in 10 mares with PCL remained constant from 64 to 33 days before the end of luteolysis and then gradually decreased until the beginning of luteolysis. Cross-sectional area of CL and percentage of CL with blood-flow signals followed approximately similar profiles as for the changes in P4 concentrations. Major follicular waves occurred throughout the PCL at an interval of about 15 days between maximal diameters of dominant follicles in adjacent waves. The maximal diameter reached by the dominant follicle was similar between anovulatory waves and the terminal ovulatory wave. A novel transient depression in P4 concentration was observed in association with about 50% of the major follicular waves. In a comparison between 10 PCLs and 28 IOIs during the 10 days before the ending ovulation, diameter of the preovulatory follicle was greater in PCLs than in IOIs. Concentration of P4 before the apparent beginning of luteolysis in PCLs was 45.8% below the concentration in IOIs. During the follicular phase at the end of a PCL, concentration of LH and FSH was less prominent than at the end of an IOI. The concentration of LH in the ovulatory surge on the day of ovulation in PCLs was 58.5% below the concentration in IOIs. In a separate survey, a PCL occurred in 6.1% of the intervals between ovulations (23 PCLs and 352 IOIs), and the frequency of PCL was different among individual mares.

5.6. Acknowledgments

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6. Discussion and conclusion

There were several objectives during this research. The first objective was to determine the role of OT during luteolysis in mares, testing the hypothesis that luteolysis can be induced through the administration of OT. It was previously sugested that OT is the hormone responsible for transition from preluteolysis to luteolysis in mares, since a small preluteolytic PGFM pulse does not initiate luteolysis, but when a similar PGF2α pulse is assotiated with OT luteolysis begins [57]. However, in mares the role of OT in the regulation of uterine PGF2α secretion and P4 reduction during luteolysis has not been determined [72]. In the first set of studies, the effect of exogenous OT on PGFM and P4 concentrations in mares was evaluated. In a first approach, a dose and method of OT administration was developed that would stimulate a PGFM pulse that was similar to a natural pulse during luteolysis. Constant infusion of a specific dose of OT during 2 h stimulated a pulse of PGFM that was similar to reported [52,63] spontaneous pulses during and immediately after luteolysis. In contrast to the response to OT infusion, bolus treatment in various doses induced a relatively short burst of PGFM that did not resemble a natural pulse. The burst reached maximum in 4 min compared to an ascending portion of a natural pulse of 2 to 3 h [52,63]. In adition, OT induction of a simulated PGFM pulse stimulated a decrease in P4. These results indicated that OT is an intermediary for secretion of PGF2α in mares, as previously shown for ruminants [73]. The current results apparently represent the first firm demonstration of the luteolytic effect of OT in mares. The current successful demonstration is attributable to the infusion of physiologically compatible concentrations of exogenous OT, so that the resulting PGFM pulse was similar to a natural pulse during luteolysis. The induced decrease in P4 on Day 13 was not associated with a reduced length of the IOI, which is attributable to the reported necessity of multiple pulses of PGF2α for a continued decrease in P4 or complete luteolysis in mares [56]. In this study, it was not possible to seperatly evalute the effects of OT and PGF2α; therefore, the initiation of

luteolitic mechanism can only be attributed to the action of both hormones. Future studies should attempt to evaluate the role of OT during luteolysis while removing the effect of PGF2α. Furthermore, the evaluation of the role of PGF2α while removing the effect of OT should also be tested. If such studies are possible, this should provide valuable information on the role of each particular hormone during luteolysis and to the knowlegde of the luteolic mechanism.

The mechanism involved in spontaneous or idiopathic prolonged luteal activity is not well understood, but impaired PGF2α secretion has been suspected [1,68,69,71]. In a second study, a cyclooxygenase inhibitor (FM) was administered at the time of expected luteolysis to induce the formation of the PCL. Treatment with FM during the expected luteolytic period (Days 14 to 16) in mares inhibited the secretion of PGF2α, as indicated by a metabolite (PGFM) and as previously reported for cattle [74] and sheep [75]. Inhibition of PGF2α synthesis interfered with luteolysis. Greater P4 concentration in the FM group during Days 15 to 18 and longer intervals from ovulation to the beginning and to the end of luteolysis were observed in the FM group. The beginning of luteolysis and the end of luteolysis were each delayed approximately 1 day by FM treatment. The similar 1-day delay in the beginning and in the end of luteolysis and the approximately parallel decrease in P4 between groups indicated that the effect of the reduction in PGF2α was exerted at the beginning of luteolysis, rather than during the later portion of luteolysis. These results were unexpected but suggest that PGF2α exerts its major effect at the initiation of luteolysis. This is apparently the first demonstration in mares that inhibiting PGF2α interferes with luteolysis. Inhibition of PGF2α at the expected time of luteolysis may also lead to prolonged luteal activity. However; precaution should be taken when making the conclusion that the absence of PGF2α is in the origin of the PCL syndrome since only two mares in the FM group met the definition of prolonged luteal activity on the basis of statistical outliers involving the luteal phase. In addition, the observed length of luteal activity (21 and 36 days) was shorter compared with the reported values of a minimum of 30 days [1,69].

In a third and fourth studies, the characterization and description of the hormonal changes in the development of an idiopathic persistent corpus luteum (PCL) (during the time of expected luteolysis that failed to occur) and during the maintenance of the idiopathic PCL were performed. Concentration of P4 before expected luteolysis was similar between mares with and without PCL. In the PCL group, the mean decrease in P4 concentration discontinued on Day 15 and became constant compared to a continuous decrease between Days 13 and 17 in IOI mares. The similarity in P4 concentration between IOIs and PCLs until Day 14 and the plateau of lesser concentration beginning on Day 15 for PCLs seems consistent with reported P4 profiles in individual PCL mares [69]. In adition, the results indicated that P4 concentration in some mares with idiopathic PCL may begin to decline abruptly as for the beginning of luteolysis, whereas in others the decline may be gradual as reported for P4 of an IOI (Days 8 to 14 [46]). In this study, there was no indication that secretion of PGF2α at the time of expected luteolysis is defective in mares with idiopathic PCL. This was indicated by the similarity in daily PGFM concentration between groups, including a maximum concentration on Day 15 in each group. The comparison of the IOI and PCL groups in the current study did not support the apparent supposition [1,76] that idiopathic PCL is attributable to a deficiency in PGF2α secretion. However, further studies with more mares and more frequent sampling are needed.

During the maintenance of the PCL, the concentration of P4 was constant during the first half of the 64 days before the end of luteolysis and then gradually decreased, which is compatible with reported P4 profiles for individual PCLs [69]. The mechanism for the long-term P4 decrease in a PCL may be similar to the mechanism responsible for the P4 decrease before luteolysis during an IOI. Concentration of LH during PCL gradually increased but was low, compared to the reported peak of the ovulatory LH surge [35]. The slight and prolonged LH increase occurred during the decrease in P4 concentration. During the study, major follicular waves were observed throughout the PCL. Apparently, the sequence of major follicular waves throughout a PCL has not been described previously. However, the waves

seem compatible with previously observed follicular activity [77,78]. The maximal diameter reached by an anovulatory wave was similar to the maximal diameter of the ovulatory wave and seems consistent with a previous observation [78]. The demonstration of transient depressions in P4 during the long-term phase of a PCL and the apparent association between a depression in P4 and the dominant follicle of a major wave are novel findings. Each of the transient depressions in P4 was associated with a major follicular wave, but not all the waves were assotiated with a transient depression. Concentration of P4 before the apparent beginning of luteolysis in PCLs was 45.8% below the concentration in IOIs. During the follicular phase at the end of a PCL, concentrations of LH and FSH were less prominent than at the end of an IOI. The concentration of LH in the ovulatory surge on the day of ovulation in PCLs was 58.5% below the concentration in IOIs. The reason for a less-prominent increase in LH during the ovulatory LH surge in the PCL group than in the IOI group is not known, but may be related to the prolonged P4 decrease. In a separate survey, a PCL occurred in 6.1% of the intervals between ovulations (23 PCLs and 352 IOIs), and the frequency of PCL was different among individual mares. However, no differences were found in the frequency of the initiation of PCL among seasons.

Unfortunatly, it was not possible in the present studies to clearly identify the mechanism leading to the formation of idiopatic PCL in mares. However, a deficiency in PGF2α secretion involved in the formation of idiopatic PCL was not supported. More studies with more frequent samplig during the expected time of luteolysis are nedded to study hormones with a pulsatile secretion. A luteotropic action of LH in the CL has been questionable in runimants; however, in mares the role of LH during luteolysis, and particularly, during the devolepment of idiopatic PCL has not been studied and should be taken into account in future studies.

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