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Methods to induce earlier onset of cyclicity in transitional mares

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METHODS TO INDUCE EARLIER ONSET OF CYCLICITY IN TRANSITIONAL MARES

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LIST OF ABBREVIATIONS

ng – nanogram
P_4 – progesterone
mL – milliliter
PGF – prostaglandin F
$PGF_{2\alpha}$ – prostaglandin $F_{2\alpha}$
hCG – human chorionic gonadotrophin
mm – millimeter
GnRH – gonadotrophin releasing hormone
LH – luteinizing hormone
FSH – follicle stimulating hormone
PGE_2 – prostaglandin E_2
mRNA – messenger ribonucleic acid
ob – obese
h – hour
µg – microgram
Kg – kilogram
SC – subcutaneous
q 8 h – every eight hours
IV – intravenously
D – dopamine
PO – per os
mg – milligram
IM – intramuscular
IU – international unit
min – minute
g – gravity
MHz – megahertz
VEGF – vascular endothelial growth factor
IGF – insulin growth factor

ABSTRACT

The purpose of this study was to investigate methods to induce earlier onset of cyclicity in transitional mares. Two experiments were conducted evaluating the effect of follicular aspiration to advance the onset of cyclicity, more succinctly define criteria for selection of mares for follicular aspiration and to compare aspiration to deslorelin treatment for initiating cyclicity in transitional mares. In Experiment 1, anestrous mares were assigned to control (n=6) or follicular aspiration (n=11). The control mares were monitored twice weekly, until ovulation was detected. The aspiration mares were similarly monitored until a follicle >35 mm was identified, then transvaginal ultrasound guided follicular aspiration was performed. After aspiration, the mares were monitored for luteal tissue formation.

In Experiment 2, anestrous mares were assigned to control (n=14), follicular aspiration (n=10), or deslorelin (n=12). The control mares were treated as in Experiment 1. The aspiration mares were monitored in the same manner as Experiment 1 but were treated only if uterine edema was present. The deslorelin treated mares were monitored similarly to the aspiration mares, but instead of aspiration the mares were administered deslorelin. In both experiments, plasma was obtained at each examination from all mares to verify a rise in progesterone. In Experiment 1, the time from January 1 to the first rise in serum progesterone was 23.8 days earlier for aspiration treated mares than for control mares (80.5±7.3 and 104.3±8.8 days, mean±SE, for aspiration and control groups, respectively; P=0.024). In Experiment 2, there was no significant difference in time from January 1 to the first rise in serum progesterone between groups (100.4±5.8, 113.0±3.0 and 110±6.7 days, for the aspiration, control and deslorelin groups, respectively,

P=0.328). However, if mares that did not receive a repeat aspiration treatment due to lack of uterine edema are excluded, there was a significant difference between the aspiration and control groups (93.9±6.7 and 113.0±3.0 days, for the aspiration and control groups, respectively, P=0.045). Results of this study indicate that follicular aspiration of a follicle > 35 mm during late transition may be a means to advance the onset of cyclicity in mares.

CHAPTER 1

GENERAL INTRODUCTION

Mares are considered to be seasonally polyestrous animals. Seasonality is influenced primarily by the photoperiod. It is the pineal gland that processes the photoperiod signals and subsequently regulates GnRH secretion (Daels and Hughes 1993). The season for cyclicity for most mares is from the spring to fall. As mares begin to cycle after winter anestrus, they go through a period of transition in the late winter through early spring months. This spring transition period is characterized by a resurgence of follicular activity, irregular estrous behavior and resumption of secretion of gonadotrophins and ovarian steroid hormones. When mares are in transition, ovulations are highly unpredictable, thus frustrating breeding management. The mean total duration from first detection of follicular growth in the spring to first ovulation is ~60 days (Sharp and Davis 1993). Spring transition ends when the mare has the first ovulation of the season.

Although the natural breeding season occurs from spring through early fall, many horse registries heavily favor foals born as close as possible to January 1st. Mares must therefore conceive early in the year, outside the normal breeding season. Methods have been sought to advance the onset of cyclicity by shortening winter anestrus or spring transition. Providing 16 hours of light by adding light at the end of the day, starting on December 1st, generally stimulates ovulation 3 months after the beginning of the treatment. Administration of GnRH as hourly pulses for 8 days has been reported to be successful only in half the mares treated, and is costly and labor intensive (McKinnon et al. 1993). A GnRH analogue, deslorelin, in the form of a slow release subcutaneous

implant, has been reported to induce ovulation in late transition, with 8 of 10 mares ovulating within 3 days after treatment, using an average of 2.1 implants (McKinnon et al. 1996).

Nequin et al. (1993) have reported that daily administration of domperidone (a dopamine antagonist) for 15 to 55 days during anestrus increased ovarian follicular activity. However, variable success was reported when attempting to induce cyclicity in transitional and deep anestrous mares. Sulpiride (another dopamine antagonist) has also been administered during winter anestrus in an attempt to enhance the onset of transition, but was not found to stimulate gonadotropin secretion or ovarian activity (Donadeu and Thompson 2002).

Follicular aspiration has been used for oocyte collection for in vitro oocyte maturation studies, in vitro fertilization, and for other assisted reproductive technologies. Follicular aspiration has resulted in luteinization of follicles in cycling mares during the breeding season (Hinrichs et al. 1991). The effect of follicular fluid aspiration on luteinization has not been thoroughly investigated in transitional mares. Bogh et al. (2000) suggested that repeated follicle aspiration in mares during transition may advance the onset of cyclicity. However, their study was designed to examine steroid concentrations in follicular fluid of transitional and cyclic mares that had been repeatedly aspirated rather than any effect on cyclicity.

The general hypothesis of this thesis was that by aspirating the dominant follicle during the late transitional period in the spring, luteal tissue formation would result, thereby hastening the onset of cyclicity. To address these concerns, two studies were conducted. The objective of the first study was to evaluate the effect of aspiration of the

dominant follicle on the onset of cyclicity in mares during late transition. The objective of the second study was to more succinctly define selection criteria for follicular aspiration and to compare follicular aspiration to deslorelin treatment for initiating cyclicity in transitional mares. The first rise in serum progesterone was used to document the onset of cyclicity in the two experiments.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Seasonality

A seasonal pattern of reproductive activity in the mare ensures that offspring are born in favorable climatic conditions at the appropriate time of the year. The annual change in photoperiod is the primary environmental cue used for the timing of the annual reproductive cycle (Kooistra and Ginther 1975). The seasonal pattern of mares is regulated by daylight or photoperiod. The photoperiod length modulates reproductive activity via the pineal gland through the regulation of GnRH secretion (Hyland 1988; Sharp 1988). The pineal gland signals the hypothalamus through secretion of melatonin. When the day length is short (winter), melatonin released by the pineal gland suppresses GnRH synthesis and release (Hart et al. 1984). When the day length is long (summer), melatonin secretion is reduced and the inhibitory influence of melatonin on GnRH synthesis and secretion is removed (Hyland 1988).

The mare reproductive year has been divided into four phases that correspond to changes in day length (Ginther 1992). The period of peak fertility (breeding season) surrounds the longest day of the year or summer solstice (June 21). The mare then moves into a transitional period (fall transition) of anovulatory activity that coincides with the autumnal equinox (September 21) when day and night are of equal length. Mares then enter a state of sexual quiescence (anestrus) that centers around the shortest day of the year, or winter solstice (December 21). After this period, the mare enters another transitional period (spring transition) of anovulatory ovarian activity that coincides with the vernal equinox (March 21) when day and night are of equal length (Ginther 1992).

Although mares are regarded as a seasonally breeding animal, a small percentage of mares exhibit estrous cycles during the non-breeding season. In one study, only half of the non-lactating mares over the age of 3 years entered winter anestrus, whereas in mares that have foaled, occurrence of anestrus in the following winter was ubiquitous (Guillaume et al. 1995). The occurrence of estrous cycles during the winter months does not reflect an inability to recognize or translate decreasing day length or a presumptive inhibitory melatonin cue (Fitzgerald and Schmidt 1995). Instead, the continuation of estrous cyclicity during the non-breeding season may be characteristic of mares that have not foaled or lactated during the previous breeding season (Guillaume et al. 1995). The mare has an endogenous circannual reproductive rhythm and the main rule of seasonal clues appears to be to synchronize the endogenous rhythm to winter and summer.

2.2 Estrous Cycle of the Mare

The mare's estrous cycle is defined as the period from one ovulation to the subsequent ovulation, with each ovulation being accompanied by signs of estrus and plasma progesterone concentrations below 1 ng/mL (Hughes et al. 1972). The estrous cycle consists of two phases, the follicular phase and the luteal phase. In the follicular phase (estrus), which involves ovulation, the mare is sexually receptive to the stallion (fails to kick and allows the stallion to mount) (Squires 1993), and the genital tract is prepared to accept and transport sperm to the oviducts for fertilization (Daels and Hughes 1993).

The regular pattern of estrous cycles relies on the delicate balance among hormones produced by the pineal gland, hypothalamus, pituitary gland, ovaries and endometrium. The hypothalamus produces a decapeptide hormone, GnRH, which is released into the

hypothalamic-hypophyseal portal system, and stimulates the synthesis and release of the gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland (Sharp 1988). These hormones act on the ovaries, with FSH being responsible for follicular maturation and production of estrogen and LH for ovulation and luteinization of the corpus luteum (CL). The growing follicles produce estrogen, which has a positive feedback on LH release (i.e., promotes further LH release) in the presence of low circulating progesterone concentration. Inhibin, produced by the granulosa cells of the growing follicles, has a negative feedback effect on the release of FSH (i.e., inhibits FSH release) (Bergfelt and Ginther 1985). After ovulation, a CL forms and secretes the hormone progesterone, which has a negative feedback effect on LH release from the anterior pituitary gland.

In the luteal phase (diestrus) the mare is not receptive to the stallion and the genital tract is prepared to accept and nurture the conceptus. The diestrous period ends with regression of the corpus luteum and initiation of the next follicular phase (Daels and Hughes 1993). The average length of the estrous cycle in the mare is 21 days, with estrus comprising 5 to 7 of these days. The length of diestrus remains relatively constant at 14 to 15 days and is not affected by the season, where as the length of estrus is more variable, ranging from 2 to 12 days, being longer in duration early in the breeding season (Ginther and Wentworth 1975).

The follicular phase of the estrous cycle is characterized by follicular growth with estrogen production, which is responsible for the estrual behavior that mare exhibits when teased by a stallion (winking of vulvar lips, tail raising, squatting and urinating, failure to kick and receptivity to stallion) (Squires 1993). Follicular development occurs

throughout the estrous cycle and large follicles (> 30 mm) can occur even in diestrus. Only the largest (dominant) follicle will ovulate and the remainder become atretic. The time of ovulation is variable among mares but the majority of ovulations occur 48 hours before the end of estrus. Follicular diameter at the time of ovulation ranges from 30 to 70 mm and usually approximates 40 to 45 mm, although smaller or larger follicles sometimes ovulate (Ginther and Wentworth 1975).

The luteal phase is initiated at ovulation by the formation of a progesterone-secreting corpus luteum. Progesterone concentrations are more than 1ng/ml during diestrus, during which mares rarely show estrous behavior (Hughes et al. 1972). The life span of the CL depends on the release of endogenous prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium of the non-pregnant uterus between days 13 and 16 post-ovulation (Neely et al. 1979). The $PGF_{2\alpha}$ is absorbed into the uterine venous drainage, enters the circulation, and reaches the ovaries by a systemic route. The $PGF_{2\alpha}$ then causes luteolysis and a subsequent drop in progesterone concentration, which releases the block to LH secretion. Follicular maturation and behavioral signs characteristic of the follicular phase of the estrous cycle ensue.

2.3 Ovulation

The FSH secreted from the anterior pituitary gland acts on the ovaries to stimulate follicular growth and development. When FSH binds to its receptors on the granulosa cells, it causes the conversion of testosterone to estradiol. The FSH also stimulates the development of LH receptors on granulosa cells which is an important step in the preparation of the follicle for ovulation. The granulosa cells in the growing follicles produce estrogen, which has a positive feedback on LH release (i.e., promotes further LH

release). Increased estradiol leads to the pre-ovulatory surge of LH, which is critically important because it initiates a series of biochemical events that lead to ovulation. The LH binds to receptors on theca cells of the follicle wall and the net effect is the conversion of cholesterol to testosterone. Testosterone then diffuses out of the theca interna cells to granulosa cells. The preovulatory surge of LH, at the follicular level, causes an increase in prostaglandin E_2 (PGE_2) and histamine production (Murdoch et al. 1986). The PGE_2 and histamine increase local blood flow and cause hyperemia in the follicular wall. Accompanying this local hyperemia, the theca interna becomes edematous because of the increased vascular permeability induced by histamine. The edema increases hydrostatic pressure around the follicle, which may facilitate its eventual rupture. The follicle also produces angiogenic factors which promote the growth of new blood vessels.

Following the LH surge, the theca cells begin to produce progesterone instead of testosterone. Progesterone leads to the synthesis of collagenase by the theca interna cells. Collagenase causes the breakdown of collagen, a major component of connective tissue, which makes up the tunica albuginea, the outer connective tissue covering the ovary (Smith et al. 1999). At the same time as follicular wall digestion by collagenase, the granulosa cells increase the secretion of follicular fluid, which increases the fluid volume inside the follicle and subsequently increasing the pressure inside the follicle which is driven against the follicular wall. In cattle, this leads to stigma formation at the apex of the follicle, which will push forward due to the increase in the pressure within the follicle and further weakening of the apex of the follicle.

In addition to PGE_2 production, $PGF_{2\alpha}$ is also produced locally by the ovary. Prostaglandin $F_{2\alpha}$ causes contractions of the smooth muscle components of the ovary, leading to the rupture of the lysosomes contained within the granulosa cells, releasing their enzymes that cause further connective tissue deterioration at the apex of the follicle in cattle. All these events eventually lead to the mechanical rupture of the follicular wall and release of the follicular contents (ovulation).

The purported role of PGE_2 is to help the follicle remodel itself into a corpus luteum after ovulation. This occurs through activation of plasminogen (tissue reorganization factor), which causes dissolution of blood clots and dissolving of the coagulum of the corpus hemorrhagicum (Dow et al. 2002). Plasminogen activating factor converts plasminogen into its active form, plasmin. Ovarian targets for plasmin include fibrin, fibrinogen, types 3 and 4 collagen, fibronectin, laminin and proteoglycans (Dow et al. 2002).

From studies on the ovulation process in rats and humans, the ovulation process can be compared with an inflammatory response (Brannstorm et al. 1993; Hellberg et al. 1991; Tanriverdi et al. 2003; Hill et al. 1987) in which cells of the immune system are recruited to the ovary and participate in the process of tissue remodeling. It has been established that estrogen contributes to regulating recruitment of these cells to the site of tissue remodeling involved in each ovulation event, particularly macrophages, neutrophils and T lymphocyte populations (Brannstorm et al. 1993; Hellberg et al. 1991). Estrogen receptor transcripts have been identified in mature peripheral T lymphocytes (Tanriverdi et al. 2003), which have also been recovered from follicular fluid (Hill et al. 1987). Therefore, estrogen plays an important role in the ovulatory process as it regulates

gonadotropin production and release due to its dual role as a positive and negative hypothalamic-pituitary-gonadal regulator.

2.4 Reproductive Tract Changes During the Estrous Cycle

2.4.1 Follicular Phase (Estrus)

The presence of large follicles on the ovaries can be determined by palpation per rectum with or without the aid of ultrasound. However, follicles are also present during diestrus, so they alone are not a reliable indicator of estrus. The presence of follicles on the ovaries, associated with estrous behavior during teasing with a stallion, a soft and relaxed cervix and uterus, and edematous endometrial folds observed on ultrasound exam are indicative of estrus. On ultrasound exam, edematous endometrial folds have the appearance of a “wagon wheel” or “orange slices”. Uterine edema is due to secretion of estrogen from growing follicles and used as a managerial tool for monitoring progress toward ovulation (Pelehach et al. 2002). It is important to remember that the decline in edema score from peak values is a better indicator of ovulation time than presence of edema alone (Pelehach et al. 2002).

2.4.2 Luteal Phase (Diestrus)

During diestrus, mares usually do not show signs of estrous behavior when teased with a stallion and a corpus luteum can be visualized on an ovary by ultrasound examination. On palpation, the uterus is toned and the cervix is narrow and tightly closed due to the effect of progesterone secreted from the CL (Daels and Hughes 1993).

2.5 Fall Transition (Autumn Transition)

During the fall, the majority of mares in the Northern Hemisphere go through a transition period that is characterized by erratic estrous cycles. During this period, mares

may undergo follicular development but the follicles fail to ovulate. Failure of these follicles to ovulate is associated with the lack of an LH surge (Snyder et al. 1979). Corpus luteum function (King et al. 1988) and follicular estrogen production (Weedman et al. 1993) are compromised several cycles before this point. The end result of autumn transition is the beginning of the functional hypothalamic-pituitary decline that characterizes the anestrous period. The mechanisms by which mares lose reproductive competence are not understood (Snyder et al. 1979).

2.6 Anestrus

Anestrus implies an absence of ovarian activity and consequent lack of regular periods of estrus (Allen 1977). This condition occurs in the winter (winter anestrus) when daylight hours are shorter in length. During this period the hypothalamic-pituitary axis is essentially non-functional. The GnRH secretion is greatly reduced and the pituitary fails to release significant amounts of gonadotropins (Hart et al. 1984). The pituitary content of LH is low, but the FSH content remains unchanged, so anestrous mares will have small ovaries with only one or two small follicles (< 15mm) (Watson et al. 2002).

The uterus during anestrus is thin walled, flaccid and difficult to palpate. The cervix is soft and either open or passively closed (Allen 1977). The duration of winter anestrus varies between individual mares and some mares have estrous cycles throughout the year. As mares begin to cycle after anestrus, they go through a period of transition in the spring, a period of anovulatory estrual activity.

2.7 Spring Transition (Vernal Transition)

Spring transition is the period through which mares progress from anestrus during the winter months to normal estrous cycles during the breeding season. It is a period of

renewed sexual function, characterized by a resurgence of follicular development on the ovaries, onset of estrous behavior, and renewed secretion of pituitary gonadotropins and ovarian hormones (Sharp and Davis 1993). During transition, estrous periods are irregular, ovulations unpredictable and breeding mares is frustrating.

Mares develop a series of 3 to 4 large (> 30mm) anovulatory follicles at intervals of ~12 days (Davis et al. 1987; Ginther 1990; Watson et al. 2002). These large anovulatory follicles are steroidogenically incompetent, as reflected by relatively low concentrations of androgens and estrogens in the peripheral circulation (Oxender et al. 1977; Seamans and Sharp 1982). These follicles fail to ovulate because of suppression of GnRH secretion by inhibitory neuronal mechanisms that result in lack of LH stimulation (Aurich et al. 2000; Fitzgerald and Mellbye 1988). It has also been shown that peripheral plasma estradiol concentrations increase from early transition to late transition and again from late transition to the first ovulation of the year in parallel with tissue production and follicular content (Davis and Sharp 1991). Incubation of the follicular wall from preovulatory follicles produces more estradiol than follicles collected during late transition, which in turn secrete more estradiol than early transitional follicles (Davis and Sharp 1991). Androgen production also increased from late transition to the first ovulation, but progesterone production showed no change. This failure of progesterone rise has been explained by a possible failure of the converting enzyme 17-α steroid hydroxylase in early transitional follicles (Davis and Sharp 1991). Therefore, it was suggested that inadequate estradiol production by transitional follicles results from lack of androgen substrate, possibly due to low 17-α steroid hydroxylase enzyme activity in the theca cells (Davis and Sharp 1991).

Resumption of ovarian cyclicity in the spring requires a series of events that begins with an increase in GnRH secretion. This may be mediated by dopamine (Besognet et al. 1996). The increase in GnRH secretion is followed by a rise in gonadotropins and resumption of some degree of follicular activity. When a dominant follicle, which is steroidogenically competent, develops in the ovary, the high circulating concentrations of estradiol feedback positively on the hypothalamic-pituitary axis and induce increased synthesis and secretion of GnRH and/or pituitary LH subunit mRNA expression (Sharp et al. 2001). This results in release of LH in sufficient amounts to cause ovulation (Sharp et al. 1991).

The time course of transition into the breeding season is remarkably consistent (Sharp and Davis 1993). However, there are variations among individual mares and this period cannot be ascertained. Transition ends when the mare has the first ovulation of the season and normal estrous cycles begin. The mean total duration from first detection of follicular growth to first ovulation is approximately ~60 days (Sharp and Davis 1993).

2.7.1 Reproductive Changes during Spring Transition

2.7.1.1 Gonadotropin Releasing Hormone (GnRH)

GnRH secretion is nearly zero during anestrus, and the reestablishment of GnRH secretion is the first change in the hypothalamic-pituitary-ovarian axis during spring transition (Besognet et al. 1996). Increased secretion rates of GnRH are characteristic of spring transition and precede any major changes in gonadotrophins and ovarian hormones (Sharp and Grubaugh 1987). Sharp and Grubaugh (1987) stated that there is a small but significant increase in GnRH secretion detectable shortly after the onset of increasing daylength in winter. This increase in GnRH secretion was reflected by an increase in FSH

concentrations, however, no increase in LH concentration was detected (Sharp and Grubaugh 1987).

2.7.1.2 Follicle Stimulating Hormone (FSH)

The increase in circulating FSH concentrations is the second event in spring transition. The time from the first significant increase in FSH to the first ovulation of the year may be as long as 60 days. Pituitary FSH stores do not change throughout the year and FSH is always available for release (Sharp and Davis 1993). Therefore, any FSH increase is caused by an increase in GnRH secretion, not by increased FSH production.

The increase in FSH secretion causes a resumption of ovarian follicular activity. As follicles start to grow and emerge, eventually a dominant, steroidogenically-competent follicle develops and secretes sufficient quantities of estradiol. The estrogen secreted triggers GnRH synthesis and secretion and/or pituitary LH subunit mRNA expression (Sharp et al. 2001). The mRNA expression results in the release of LH in sufficient amounts to cause ovulation (Sharp et al. 1991).

2.7.1.3 Luteinizing Hormone (LH)

During the anovulatory season, circulating LH concentrations remain essentially baseline, reflecting the reduced pituitary LH stores that characterizes the anestrous period. However, there is a gradual increase in the number of secretory events (pulses), which are irregular in timing and low in frequency (a maximum of just over three pulses per 12-hour period) (Hart et al. 1984). The increase in LH secretion is the last event of spring transition and is followed shortly by the first ovulation of the year (Davis and Sharp 1991). Although GnRH secretion is increased from anestrus through the transitional phase and stimulates available pituitary LH stores, it is ineffective in eliciting

a significant LH response. This failure of LH response is due to the paltry amount of LH available in the pituitary (Silvia et al. 1986). This is has been shown to be true by a lower LH response in anestrous mares than cycling mares when given GnRH. Estrogen secretion may be an important signal in the regulation of LH synthesis and secretion (either directly or indirectly through increased GnRH secretion), because the surge in estradiol (and androstenedione) precede the secretion of substantial amounts of LH by a matter of a few days (Sharp and Davis 1993). Estrogen administered to ovariectomized ponies at the equivalent time of spring transition resulted in both increased pituitary content and peripherally circulating concentrations of LH by 1 week after the start of treatment (Sharp et al. 1991). Further, after 2 weeks of exogenous estradiol administration, pituitary content of LH was significantly elevated in treated mares.

2.7.1.4 Follicular Development

As a result of increased circulating FSH concentrations, an increase in follicular development occurs. Pony mares develop an average of 3.7 ± 0.9 consecutive anovulatory 30 mm follicles during spring transition (Davis and Sharp 1991). Usually, horse mares similarly develop a series of 3 to 4 large (> 30mm) anovulatory follicles at intervals of ~12 days (Davis et al. 1987; Ginther 1990; Watson et al. 2002). These follicles enlarge, fail to ovulate, and remain at ~30 mm for a week, and then subsequently regress, still fluid filled as assessed by ultrasonography. The total time from first detection of follicle growth to first ovulation is ~55 to 60 days (Sharp and Davis 1993). These follicles are steroidogenically incompetent, as circulating estrogen (estradiol and estrone) concentrations remain relatively low throughout the period of folliculogenesis (Davis and Sharp 1991). Furthermore, follicular fluid estrogen and androgen

concentrations are both significantly lower in the first 2 or 3 anovulatory transitional follicles (Oxender et al. 1977; Seamans and Sharp 1982). Mares may show estrous behavior during spring transition, although having low estrogen concentrations, due to the hypersensitivity of the mare's nervous system to estrogen. In addition to estrous behavior, during spring transition mares exhibit uterine edema detectable by ultrasound (Watson et al. 2003). Estrogen concentrations produced from anovulatory follicles during spring transition are low compared to estrogen concentrations produced from ovulatory follicles during the breeding season (Watson et al. 2003). This may infer a hypersensitivity of the uterus to low estrogen concentrations during spring transition and therefore the exhibition of uterine edema.

2.8 Factors Affecting Cyclicity in Mares

2.8.1 Photoperiod

The mare is characterized as a long day breeder, because increasing day length (photoperiod) in the spring causes the mare to cycle. The retina of the eye is stimulated by light. This photoreception is transferred by a nerve tract to a specific area of the hypothalamus, known as the suprachiasmatic nucleus. From the suprachiasmatic nucleus another nerve travels to the superior cervical ganglion (Grubaugh et al. 1982). These presynaptic neurons cause the postganglionic neurons to fire. The postganglionic neurons synapse with inhibitory neurons that make contact with cells in the pineal gland (pinealocytes). Studies on the mare have shown that both superior cervical ganglionectomy (Grubaugh et al. 1982; Kilmer et al. 1982) and pinealectomy alter the ability of mares to respond to a stimulatory photoperiod (Sharp et al. 1979).

The pinealocytes secrete the hormone melatonin (Sharp et al. 1979). During the daylight hours (increased photostimuli) the light sensed by the retinal cells of the eye activates an excitatory neural pathway at the level of the pineal gland where inhibitory neurons continue to fire, thus inhibiting the release of melatonin from pinealocytes. Since melatonin inhibits GnRH secretion, the increased day-length inhibiting melatonin causes an increase in GnRH secretion, and thus promotes cyclicity. The opposite occurs in response to decreased day-length (photoperiod) during which the synthesis and secretion of melatonin inhibits cyclicity (Sharp et al. 1979).

Sharp et al. (1980) found that serum melatonin concentrations were highest during anestrous periods. Melatonin serum concentrations are higher during the spring than in the fall (Diekman et al. 2002; Sharp et al. 1980). In cycling mares, Diekman et al. (2002) found that melatonin secretions were greater during the night than the day. Diekman et al. (2002) concluded from their study on melatonin concentrations in cycling and noncycling mares that changes in serum concentrations of melatonin do not appear to be useful as a cue to regulate cyclic activity in the mare throughout the seasons.

Thus, photoperiod is the most important environmental signal and entrains the hypothalamic-pituitary-ovarian axis. Most of the work on advancement of the breeding season in mares has focused on manipulation of the photoperiod.

2.8.2 Temperature

Palmer (1979) proposed that the light sensitive phase 9.5 to 10 hours after dusk, which would usually correspond to the nightly minimum temperature, is an important signal in the timing of the onset of cyclicity. Field data from Thoroughbred mares in the United Kingdom suggested that spring transition is slowed by cold weather (Allen 1987).

However, Cooper and Wert (1975) stimulated an earlier onset of the breeding season using artificial lights, in freezing winter conditions.

2.8.3 Age

Ginther (1974) found that the age of mares was not significantly correlated with the length of anestrus, the breeding season, or the frequency of estrous behavior during anestrus (out of breeding season) but found it is more common for older mares not to cycle during the breeding season. Carnevale et al. (1997) reported that less follicular activity occurred in old mares than in young mares during spring transition and the same occurs in mares older than 20 years of age during the breeding season.

Reduced follicular activity in old mares during spring transition and during the breeding season has been explained due to depletion of the primordial follicles and a consequent reduction in the number of viable follicles (Carnevale et al. 1994). It also has been reported that the first ovulation of the year often occurs 2 weeks later in mares > 19 years of age than in mares < 13 years of age (Vanderwall and Woods 1990).

2.8.4 Nutrition

Nutrition and body condition may contribute to the onset of cyclicity and first ovulation in mares. Ginther (1974) compared the interval from January 1 to the onset of the breeding season between mares that lost weight and ones that gained weight during that period. The results showed that the mares that lost weight had a delayed onset of the breeding season compared with those that gained weight. Henneke et al. (1984) observed that the average interval to first ovulation was significantly longer in mares with body condition scores less than 5 (scale from 1 to 9) compared with mares having body condition scores > 5. High energy intake hastened the onset of first ovulation in

transitional mares with low amounts of body fat, but did not appear to benefit mares in moderate or fat condition (Henneke et al. 1984; Kubiak et al. 1987).

Increasing the plane of nutrition ~3 weeks prior to the breeding season stimulates ovarian activity (van Neikerk and van Heerden 1972), however Carnevale et al. (1997) found that increasing the plane of nutrition prior to the breeding season did not hasten the first ovulation of the year more than maintaining mares in fat body condition. Henneke et al. (1984) found that improving the condition of thin mares at the time of the breeding season enhanced reproductive performance. Moderate or fat mares cycled earlier, required fewer cycles per pregnancy and had higher conception rates than thin mares (Henneke et al. 1984). Kubiak et al. (1987) studied the influence of energy intake and percentage of body fat on the reproductive performance of non-pregnant mares and found that flushing thin mares prior to the breeding season should be abandoned. It was suggested that increasing the plane of nutrition would only be necessary for those mares that, for some uncontrolled reason, enter the breeding season in a thin condition.

2.8.5 Leptin

Leptin is a protein encoded by the Ob gene in adipose cells and thought to play a role in the regulation of food intake and metabolism (Barash et al. 1996). This adipocyte derived hormone plays a key role in body weight homeostasis. Leptin has recently emerged as a relevant neuroendocrine mediator in different systems, including the reproductive axis. Leptin provides information to the brain on energy status and may serve as a signal to the reproductive axis indicating a nutritional status adequate for the onset of cyclicity (Barash et al. 1996). Interest in the role of leptin in reproduction was initiated after the demonstration that infertile ob/ob mutant mice, which lack the ability to

produce leptin, could be made fertile with leptin injections (Chehab 1997; Chehab et al. 1996).

The inhibition of reproductive activity by melatonin during a decreasing photoperiod interval may be modified by energy availability in the Syrian Hamster (Barash et al. 1996). This response is signaled to the hypothalamic-pituitary axis by a change in the circulating concentration of leptin (Schneider et al. 1998). This was illustrated through the exposure of mares to a single day of feed restriction, which resulted in a significant decrease in serum leptin concentrations (McManus and Fitzgerald 2000). However, serum prolactin, LH and FSH concentrations were not significantly altered by feed restriction (McManus and Fitzgerald 2000). An apparent association between leptin and reproductive activity in the mare was inferred from the observation that in mature mares, higher amounts of body fat were associated with high circulating concentrations of leptin during the summer and autumn months when mares were reproductively active (Fitzgerald and McManus 2000). In pony mares, circulating leptin concentrations were found to be dependent on age, gender and body condition score (Buff et al. 2002). Concentrations are high in horses between 5 and 12 years of age and are higher in males than females. Fat pony mares tend to have greater serum concentrations of leptin than thin pony mares (Buff et al. 2002). Fitzgerald and McManus (2000) found that mature, fat mares were more likely to have estrous cycles during the winter than young, lean mares. Results from another study have shown that mares with high body condition score fail to exhibit reproductive quiescence, but those which lose body condition exhibit anestrus (Gentry et al. 2002).

Therefore, the occurrence of seasonal anestrus is determined, in part, by metabolic signals. Fitzgerald et al. (2002) administered recombinant human leptin (50 μg/Kg, SC, q 8 hours) to lean, anestrous mares and used FSH as an index of GnRH secretion. This treatment did not result in FSH secretion, however, a similar dose of leptin administered to feed restricted, steroid treated wethers resulted in a marked increase in pulsatile gonadotrophin secretion (Nagatani et al. 2000). Fitzgerald et al. (2002) hypothesized that one aspect of the long-term regulation of seasonal reproductive rhythms, specifically in anestrous mares, is the recognition of the availability of metabolic fuels before perception of a change in photoperiod. Therefore, energy availability may need to reach a critical value before presumptive inhibitory day-length signal initiates termination of the breeding season (Fitzgerald et al. 2002).

2.8.6 Neurotransmitters

The absence of reproductive activity during the winter is the result of a suppression of GnRH secretion induced by several inhibitory neuronal systems within the hypothalamus (Nagy et al. 2000). Opioid neurons have been implicated in the seasonal suppression of GnRH secretion from the hypothalamus by acting on the pituitary to reduce its responsiveness to GnRH (Davison et al. 1998). This effect has been demonstrated by an immediate increase in LH secretion after administration of naloxone, an opioid antagonist, to anestrous mares (Aurich et al. 1994). However, no changes in LH secretion were observed when a high dose of naloxone (2 mg/Kg) was administered to anestrous mares (Sharp et al. 1985). Irvine et al. (1994) reported that the naloxone-induced increase in gonadotrophin secretion is dose dependent and the response curve is bell shaped. Opioid inhibitory effect is increased during anestrus (endogenous levels are

high) and the occurrence of cyclic activity during anestrus is associated with a decrease in the inhibitory effect of opioids on the hypothalamic-pituitary axis (Davison et al. 1998; Turner et al. 1995).

2.8.7 Catecholamines

Catecholaminergic neurons have been implicated in the seasonal suppression of GnRH secretion. Xylazine (α-2-adrenergic receptor agonist), increases both FSH and LH pulse frequency when administered to seasonally anestrous mares. Meyer and Goodman (1986) noted a significant increase in the frequency of pulsatile LH secretion after sedation of mares with xylazine, and suggested the inhibitory adrenergic mechanism may be responsible for the seasonal suppression of pulsatile LH secretion.

Clenbuterol (β_2-adrenergic receptor agonist), has been described as a repartitioning agent, which reduces fat deposition, increases energy expenditure in rats (Reeds et al. 1988) and regulates leptin gene expression (Li et al. 1997). Daily administration of clenbuterol (3.2 μg/Kg) to mares from October through January and in another group from June through September resulted in a decrease in body fat and circulating concentrations of leptin, however, the timing and proportion of mares exhibiting anestrus was not modified (McManus and Fitzgerald 2003).

2.8.8 Dopamine and Dopamine Antagonists

Dopamine is a catecholamine synthesized in the adrenal medulla. It has the same catechol and amino groups as epinephrine and noradrenaline. Dopamine concentrations in the cerebrospinal fluid of anestrous mares are higher than in mares during the breeding season (Melrose et al. 1990). The mechanism by which dopamine influences seasonality in the mare is still unclear. Dopamine has been reported to exert a tonic inhibition on

reproductive activity during seasonal anestrous in mares (Besognet et al. 1995). Besognet et al. (1996) studied dopaminergic regulation of gonadotropin secretion in anestrous mares and found that dopamine inhibits FSH pulsatile secretion.

The response of seasonally anestrous mares to the administration of dopamine D_2-antagonists has been reported to be highly variable. Nequin et al. (1990) found that administration of dopamine D_2-antagonists to anestrous mares did not induce acute changes in LH and FSH secretion. In another study, Nequin et al. (1993) reported that administration of the dopamine antagonist domperidone during anestrus increased ovarian follicular activity. Nagy et al. (2000) showed that dopamine D_2-antagonists induce cyclicity only when mares have already entered transition.

Dopamine antagonist administration results in an increase in prolactin secretion, which may exert its effect on the ovary by increasing the number of gonadotropin receptors, thus mediating the effect of circulating gonadotropins (Bennett-Wimbush et al. 1998). Long term treatment with dopamine D_2-antagonists (sulpiride, domperidone, perphenazine) induced ovarian cyclicity in seasonally anestrous mares (Bennett-Wimbush et al. 1998). In vernal transition, ovulation was induced after 12 to 22 days of treatment with domperidone (1.1 mg/Kg; PO) or sulpiride (0.5 mg/Kg; IM) (Bennett-Wimbush et al. 1998). When using dopamine D_2-antagonists in deep anestrous mares, 50 to 60 days are required to induce ovarian activity.

Sulpiride, used during winter anestrus to enhance the onset of transition in mares, was not found to stimulate gonadotrophin secretion or ovarian activity (Donadeu and Thompson 2002). From these and other studies (Bennett-Wimbush et al. 1998; Donadeu and Thompson 2002), dopamine D_2-antagonists appear to only induce ovulation when

mares are in transition. This explains the unpredictability of the interval from the initiation of treatment to first ovulation and the failure of treatment in mares kept under unfavorable environmental conditions.

2.9 Methods to Advance the Onset of the Breeding Season

The natural breeding season occurs from spring through early fall, however, in the Northern Hemisphere, modern Thoroughbred horse breeding demands foals to be born as close to January 1 as possible. Mares therefore must conceive early in the year outside the normal breeding season. Several methods have been used to advance the onset of the breeding season either by shortening winter anestrus, thus advancing the onset of spring transition, or by shortening the spring transition.

2.9.1 Artificial Lighting

The use of photoperiodic stimulation to influence the mare's reproductive rhythm is dependent on the photoperiodic history, the state of refractoriness to photoperiodic changes, and the existence of a photosensitive phase during the night (Sharp and Ginther 1975). Follicular activity increases ~2 weeks after the initiation of the artificial photoperiod and ovulation occurs 6 to 12 weeks after the onset of treatment (Grubaugh et al. 1982).

To correspond more closely with the horse registries' (mandated birthday of January 1), horse breeders have used artificial lighting programs to stimulate early onset of the breeding season (Burns et al. 1982; Cooper and Wert 1975). Anovulatory mares exposed to gradually increasing photoperiod during the winter ovulated earlier than mares kept under natural day length (Loy 1968; Sharp et al. 1975). Lighting programs consisting of a fixed photoperiod of 16 hours of light from the start of treatment advanced

the onset of the ovulatory season by two months (Kooistra and Ginther 1975; Oxender et al. 1977). Conventional light treatment should be started in December by adding light at the end of the day, with 16 hours of light and 8 hours of darkness until the natural day length exceeds 14.5 hours (Palmer et al. 1982; Scraba and Ginther 1985; Sharp et al. 1975). If the increased amount of light was started later (i.e., January/February), mares may not ovulate any earlier than mares under natural light (Grubaugh et al. 1982).

Based on experiments using artificial lighting, light must be present 9.5 hours after the end of the main light phase in order for the mare to recognize a long-day stimulus (Palmer et al. 1982). To take advantage of the photosensitive phase of the mare, 1 to 2 hours of light can be applied 9.5 hours after the beginning of darkness but the time of the flash must be adjusted to the changing onset of darkness (Daels 2000).

Keeping pony mares on 15.5 hours of light from the summer solstice through the following winter delayed termination of the ovulatory season; 30% of treated mares compared with 0% of the control mares remained cyclic throughout the year (Kooistra and Ginther 1975). In another study, exposure to natural day length plus an additional 2.5 hours of light after sunset shortened the interval to onset of the ovulatory season (Sharp et al. 1981).

Mares exposed to more than 20 hours of light do not demonstrate an increase in ovarian activity, indicating that a certain amount of darkness is required to ensure cyclicity (Palmer et al. 1982; Sharp and Ginther 1975). The recommended intensity of light is approximately 100 lux, in a 12 foot ×12 foot stall. Mares ovulating under light treatment conditions must remain under a lighting program until the day length exceeds 14 hours to ensure continuation of cyclicity.

2.9.2 Gonadotropin Releasing Hormone (GnRH)

Reduced GnRH synthesis and storage in the hypothalamus (Hart et al. 1984; Strauss et al. 1979) and decreased quantities of LH in the anterior pituitary (Hart et al. 1984) during winter is the basic reason for sexual quiescence in anestrous mares. Therefore, it would be expected that administration of gonadotropins to anestrous mares would reinitiate reproductive capacity.

A single injection of GnRH in seasonally anovulatory and ovulatory mares causes a transient increase in circulating concentrations of LH (Ginther and Wentworth 1974), whereas constant infusions result in continuing LH release (Garcia and Ginther 1975). Constant hourly GnRH infusion for 28 days (100 ng/Kg; SC via an osmotic minipump) induced ovulation and fertile estrus in 50% of mares treated during deep or shallow seasonal anestrus (Hyland et al. 1987). However, mares in shallow seasonal anestrus were more likely to respond to GnRH infusion than deep anestrous mares. In another study, GnRH was used as daily injections to try to induce ovulation in anestrous mares, but only induced the development of pre-ovulatory follicles. Only hourly injections or constant infusions of GnRH were found to induce ovulation (Turner and Irvine 1991).

Minoia and Mastronardi (1987) showed that thrice daily administration of 0.5 mg GnRH for 7 or 7.5 days induced normal follicular maturation and normal luteinization in anestrous mares. There was a difference in response between individual treated mares from time of treatment to time of showing behavioral estrus. This difference was attributed to the depth of anestrus at the start of treatment. From these studies using GnRH, it has been shown that administration as hourly pulses or daily injections is not

cost effective, is labor intensive and results in a variable response to treatment between mares, especially mares in deep anestrus.

2.9.3 GnRH Agonists

GnRH agonists were used as injections or slow releasing implants to induce estrus and ovulation in anestrous and transitional mares. Deslorelin (a GnRH analogue) has been used to induce ovulation in cycling mares (McKinnon et al. 1993; Meinert et al. 1993). Meyer et al. (1990) reported an increased frequency of ovulation and decreased interval to ovulation when anestrous mares were given a slow release implant of the GnRH analogue goserelin acetate. However, in another study, incidence of ovulation was not high enough (15%) to be of managerial value when using 28-day goserelin acetate implants (Mumford et al. 1994).

Harrison et al. (1990) administered buserelin (a GnRH agonist), twice daily (40 μg, IM, q 12 hours) for 28 days, or as subcutaneous implants designed to release 100 μg/day to transitional mares for 28 days. Some mares (45%) ovulated between day 10 and 25 in response to the twice daily injections of buserelin after initiation of treatment, however 60% of mares ovulated 4 to 30 days after implant treatment. Mumford et al. (1994) administered 950 μg of goserelin (a GnRH agonist) as a bolus injection in early transitional mares that had previously failed to ovulate during winter anestrus in response to administration of a goserelin implant for 28 days. Their results showed an increase in circulating LH concentration. The same results were seen when the GnRH agonist was combined with estradiol treatment (Mumford et al. 1994). In addition, estradiol given to mares in early transition did not increase circulating LH concentrations when compared

with controls, GnRH agonist and GnRH agonist plus estradiol treated mares (Mumford et al. 1994).

The GnRH analogue deslorelin in the form of a slow release subcutaneous implant, has been shown to be effective in increasing LH and hastening ovulation in cycling mares (McKinnon et al. 1993; Meinert et al. 1993; Mumford et al. 1995; Squires et al. 1994). Results from the use of the deslorelin (2.2 mg, SC) in cycling mares suggested a dose dependent response with maximal effect in 12 to 24 hours and duration of 3 to 4 days after implantation. Deslorelin also has been reported to induce ovulation in 8 of 10 mares during late transition when mares had ≥ 30mm follicles, estrus behavior and uterine edema. Mares ovulated within 3 days after treatment using an average of 2.1 implants per mare (McKinnon et al. 1996). Mares in early transition were implanted every other day until ovulation occurred or 6 implants used, resulting in ovulation in 6 of 11 mares within 10 days after treatment (McKinnon et al. 1996). It was concluded from their study that accurate, timed ovulation was achieved during late transition using implants of deslorelin. In the latter study, ovulation was confirmed based on ultrasound examination; however progesterone concentrations were not measured.

Mares in deep winter anestrus have a poor response to treatment with GnRH or GnRH agonists to induce ovulation (Evans and Irvine 1979; McCue et al. 1991). Multiple injections are required, treatment is labor intensive and costly, and therefore not of practical value. Furthermore, mares returned to the anestrous state, due to failure of the mare to maintain the corpus luteum resulting from the induced ovulation, after ceasing GnRH treatment (Evans and Irvine 1979; McCue et al. 1991) . Fitzgerald et al. (1993) concluded that constant administration of GnRH agonists may induce ovulation in mares

during seasonal anestrus, however, the percentage of mares ovulating and the lack of reproducibility of effect indicated that this approach was inappropriate for use as a reliable method to manipulate breeding activity in commercial broodmares.

2.9.4 Progesterone and Progestagens

Progesterone inhibits GnRH pulse frequency and stimulates pulse amplitude. Serum FSH concentrations in mares in shallow anestrus are increased by treatment with the synthetic progestagen allyl trenbolone (Alexander and Irvine 1991). These effects are mediated through a decrease in GnRH frequency, increasing the FSH to LH concentration ratio.

Progesterone and progestagens are used to decrease the signs of estrus during transition in anestrous mares; however, there is some disagreement on the ability of progestagens to hasten the time to ovulation. Progestagens may only synchronize the onset of ovarian activity without hastening the time to ovulation (Alexander and Irvine 1991). Daily progesterone administration does not induce ovulation in deep anestrous mares, however if it is administered near the end of transition, ovulation occurs within 15 days after the end of treatment. However, it does not advance the mean day of the first ovulation of the breeding season compared with untreated controls (Alexander and Irvine 1991). It was hypothesized that progesterone altered ovarian activity by changing FSH and LH secretion patterns but had no consistent effect on gonadotropin secretion patterns and follicular development in transitional mares (Alexander and Irvine 1991).

Margaret and Irvine (1979) used GnRH in combination with progesterone treatment to induce follicular development and ovulation in seasonally acyclic mares. Their study showed that LH concentrations were lower and shorter in duration in GnRH-

treated mares resulting in failure of follicular maturation and corpus luteum development. In addition, progesterone increased baseline FSH concentrations in GnRH treated mares and stimulated follicular development in mares not treated with GnRH.

2.9.5 Follicular Aspiration

Follicular aspiration, used for research and assisted reproductive techniques, has resulted in luteinization of 30 to 44 mm aspirated follicles when performed on cyclic mares during the breeding season (Hinrichs et al. 1991). Bogh et al. (2000) noted in a small group of transitional mares that had undergone repeated aspiration of all follicles > 8 mm, all mares ovulated within 12 days of the last aspiration. Although there were no control mares with which to compare, it appeared that the onset of cyclicity had been advanced. Alvarenga et al. (1999) aspirated all follicles > 10 mm from the ovaries of transitional mares once a > 30 mm follicle was detected, and concurrently administered hCG. Aspiration resulted in luteinization in only 3 of 8 mares within 7 days. However, it is not clear from the study design (Alvarenga et al. 1999) if the aspirated follicles were of pre-ovulatory size and if the aspiration was performed during early or late transition.

CHAPTER 3

FOLLICULAR ASPIRATION TO INDUCE EARLIER ONSET OF CYCLICITY IN TRANSITIONAL MARES

3.1 Introduction

Follicular aspiration has been used for oocyte collection for in vitro oocyte maturation and in vitro fertilization, and other assisted reproductive technologies. Follicular aspiration has resulted in luteinization of follicular structures when performed on cycling mares during the breeding season (Hinrichs et al. 1991). The effect of follicular fluid aspiration on luteinization has not been thoroughly investigated in transitional mares.

Bogh et al. (2000) have proposed that repeated follicle aspiration of mares during transition may advance the onset of cyclicity. However, the study was designed to examine steroid concentrations in follicular fluid of transitional and cyclic mares that had been repeatedly aspirated rather than any effect on cyclicity. Alvarenga et al. (1999) aspirated all follicles > 10 mm from the ovaries of transitional mares once a > 30 mm follicle was detected and concurrently administered hCG. Aspiration resulted in luteinization in only 3 of 8 mares within 7 days.

We hypothesized that aspiration of the dominant follicle during the late transitional period would induce formation of luteal tissue, thereby hastening the onset of cyclicity. The objective of this experiment was to perform follicular aspiration and compare the time from January 1 to the first rise in progesterone between treated and control mares.

3.2 Materials and Methods

3.2.1 Experimental Animals

The study included 17 light horses (10 to 22 years old) of mixed breed from one herd at the Louisiana State University Embryo Biotechnology Laboratory and one herd of the Louisiana State University School of Veterinary Medicine (latitude 30°22 ′53 ″N). During the study period (mid January to early June), the mares were kept on pasture with no artificial light and supplemented with hay and grain. Mares used in this study were in anestrus, as determined by a 5 week absence of corpora lutea, absence of follicles > 20 mm and plasma progesterone (P_4) concentration remaining at < 1ng/mL.

3.2.2 Experimental Design

Mares were randomly allocated into two experimental groups; (Group I) untreated controls (n = 6) and (Group II) follicular aspiration (n = 11). The mares in the control group (Group I) were examined by transrectal ultrasonography twice weekly until ovulation was detected. The mares in the follicular aspiration (Group II) were examined by transrectal ultrasonography twice weekly until a 35 mm follicle was detected. The follicle was then aspirated by transvaginal ultrasound-guided aspiration. Aspiration of the follicle was performed with the mare standing in stocks under sedation using detomidine hydrochloride (Dormosedan, 20 μg/Kg, IV, Orion Corporation, Espoo, Finland) and Torbugesic (butorphanol, 0.1 mg/Ig, IV, Fort Dodge Animal Health, IA, USA). A transvaginal, curvilinear array, 5 MHz transducer equipped with a 60 cm long, 17-gauge aspiration needle attached to real time ultrasound (Sonovet 600, Medison, Inc., NY, USA) was used for the transvaginal ultrasound-guided aspirations. The transducer was placed in a sterile sleeve, lubricated using sterile lubricant and inserted into the vagina.

By manipulating the ovary manually per rectum and with the other hand guiding the transducer against the vaginal fornix, an assistant punctured the follicle with the needle through the vaginal wall. The follicular fluid was then aspirated using a 60 mL syringe.

The ovaries were examined at 48 hours post-aspiration for evidence of luteal tissue formation. If luteal tissue was not formed, the mares were retreated once a follicle > 35mm was present until luteal tissue formation was confirmed. To confirm that cyclicity had begun; mares that developed a luteal structure were given prostaglandin $F_{2\alpha}$ (Lutalyse 10 mg, IM, Pharmacia and Upjohn Co, Kalmazoo, MI, USA), 7 days after follicle aspiration. Mares were subsequently examined by transrectal ultrasonography for luteolysis and follicular development. Once a 35 mm follicle developed, the mare was then given hCG, (Chorulon, 2,500 IU, IV, Intervet. Inc., Millsboro, DE, USA) to induce ovulation. Mares were then examined for ovulation and subsequent corpus luteum formation.

3.2.3 Blood Collection and Hormone Analysis

On each day of ultrasound examination per rectum, venous blood samples were collected into heparinized tubes, centrifuged for 10 min at 900 × g and the plasma obtained was stored at -20°C for progesterone analysis.

The plasma concentrations of progesterone were measured using commercially available reagents (Diagnostic Systems Laboratory, Webster, TX, USA) that were previously validated for horse plasma. Intra- and interassay coefficients of variation and assay sensitivity were 5%, 9%, and 0.05 ng/mL, respectively.

3.2.4 Statistical Analysis

The mean time from January 1 to P_4 > 1.0 ng/mL was compared among treatment groups using Student t-test. Differences in mean estimates between treatment groups in the time from treatment to the rise in P_4 > 1.0 ng/mL were compared using Student t-test.

3.3 Results

The time from January 1 to the first rise in serum progesterone was 23.8 days earlier for the aspiration group mares than for the control group mares (80.5 ± 7.3 and 104.3 ± 8.8, mean ± SE, for the aspiration and control groups, respectively; P = 0.024) (Table 3.1). The time from detection of a follicle > 35 mm to progesterone > 1.0 ng/mL was shorter for the aspiration group mares (10.0 ± 2.4 and 34.7 ± 7.4 days for the aspiration and control group mares, respectively; P = 0.008). Of the 11 treated mares, 9 formed luteal tissue within 8 days after the first aspiration. Two mares required a second aspiration procedure before developing luteal tissue. When an aspiration procedure resulted in the formation of luteal tissue, progesterone rose to > 1.0 ng/mL in 6.7 ± 0.9 days. Subsequent prostaglandin $F_{2\alpha}$ administration resulted in progesterone declining to < 1.0 ng/mL within 3 days. Administration of hCG during the ensuing estrus induced ovulation in all mares.

Table 3.1: Effect of follicular aspiration on time interval (d) to rise in progesterone (P_4) (mean ± SE).

Treatment	Days from January 1 to P_4 > 1.0 ng/ml	Days from follicle ≥ 35mm to P_4 > 1.0 ng/ml	Days after treatment to P_4 > 1.0 ng/ml	Number of treatments
Control	104.3[a] ± 8.8	34.7[a] ± 7.4	-	-
Aspiration	80.5[a] ± 7.3	10.0[a] ± 2.4	6.7 ± 0.9	1.2 ± 0.1

[a] Values in columns differ (P < 0.05)

3.4 Discussion

A previous study indicated that follicular aspiration during the breeding season resulted in the formation of luteal tissue (Hinrichs et al. 1991). Alvarenga et al. (1999) aspirated all follicles > 10 mm from the ovaries of transitional mares once a > 30 mm follicle was detected and concurrently administered hCG. Aspiration resulted in luteinization in only 3 of 8 mares within 7 days. In the present study, only the dominant follicle was aspirated. Because the transitional period is characterized by waves of follicular growth and regression, it was hypothesized that by waiting until the follicle reached at least 35 mm in diameter that luteinization would be more likely to result. In fact, 9 of 11 mares responded to the first aspiration, usually within less than a week. Interestingly, although not used as a criteria for treatment in this study, each mare that underwent follicular aspiration had ultrasonographic evidence of uterine edema (score of > 2 on a scale of 0 to 4) at the time of aspiration. However, Watson et al. (2003) have recently reported that uterine edema is not a reliable indicator of follicular competence or ovulation.

The exact time period from aspiration to serum progesterone could not be ascertained in our study because mares were only examined every 2 to 4 days after treatment. Nevertheless, an increase in progesterone, indicating the presence of functional luteal tissue, was noted on average within a week after aspiration. Furthermore, the onset of cyclicity was confirmed because each mare responded to subsequent prostaglandin injection with luteolysis and ovulation. Because the average estrous cycle is ~21 to 22 days in length, advancing the day of first rise in progesterone by over 3 weeks would provide an additional breeding opportunity during the breeding season. Results of this

study indicate that follicular aspiration of a follicle > 35 mm during late transition may be a means to advance the onset of cyclicity in mares.

CHAPTER 4

FOLLICULAR ASPIRATION OR DESLORELIN (OVUPLANT) TREATMENT TO ADVANCE THE ONSET OF CYCLICITY IN TRANSITIONAL MARES

4.1 Introduction

Advancing the onset of cyclicity has long been a goal in the equine industry. Follicular aspiration has resulted in luteinization in cycling mares, and Experiment 1 indicated that follicular aspiration of the dominant follicle could advance the onset of cyclicity when performed in mares during late transition. Administration of GnRH as hourly pulses for 8 days has been used, however, this method has been reported to be successful in only half the mares treated, and is costly and labor intensive (Sharp and Davis 1993). A GnRH analogue, deslorelin, in the form of a slow release subcutaneous implant, has been reported to induce ovulation in late transition, with 8 of 10 mares ovulating within 3 days after treatment using an average of 2.1 implants (McKinnon et al. 1996). Mares during transition develop anovulatory large follicles (> 30mm) and some of these follicular waves are associated with the presence of edema in the uterus. It has been suggested that edema in the uterus is associated with a steroidogenically competent follicle secreting estrogen and would indicate conditions more likely for luteinization.

It was hypothesized that in addition to the presence of a > 35mm follicle for follicular aspiration and deslorelin treatment, uterine edema could be used as an additional criterion that would enhance the outcome.The objective of this study was to use more succinctly defined criteria for selection for treatment and to compare follicular aspiration to deslorelin treatment as methods to hasten ovulation in transitional mares.

4.2 Materials and Methods

4.2.1 Experimental Animals

The study included 36 light horses (10 to 22 years old) of mixed breed from one herd at the Louisiana State University Embryo Biotechnology Laboratory and one herd of the Louisiana State University School of Veterinary Medicine (latitude 30°22 ′53 ″N). During the study period (mid January to early June) the mares were kept on pasture with no artificial light and supplemented with hay and grain. Mares used in this study were in anestrus, as determined by a 5 week absence of corpora lutea, absence follicles > 20 mm and plasma P_4 concentration remaining at < 1.0ng/mL.

4.2.2 Experimental Design

Mares were randomly allocated into three groups; (Group I) untreated controls (n = 14), (Group II) follicular aspiration (n = 10) and (Group III) deslorelin (n = 12).The mares in the control group were examined by transrectal ultrasonography twice weekly until ovulation was detected. The mares in the follicular aspiration group were examined by transrectal ultrasonography twice weekly until a follicle ≥ 35 mm was detected and uterine edema was present (score ≥ 2 on a scale of 0 to 3 as follows; 0 = none, 1 = trace, 2 = moderate, 3 = extreme). The follicle was then aspirated by transvaginal ultrasound-guided aspiration.

Aspiration of the follicle was performed with the mare standing in stocks under sedation using detomidine hydrochloride (Dormosedan, 20 µg/Kg, IV, Orion Corporation, Espoo, Finland) and butorphanol (Torbugesic, 0.1 mg/Kg, IV, Fort Dodge Animal Health, IA, USA). A transvaginal, curvilinear array 5 MHz transducer equipped with a 60 cm long, 17-gauge aspiration needle attached to real time ultrasound (Sonovet

600, Medison, Inc., NY, USA) was used for the transvaginal ultrasound-guided aspirations. The transducer was placed in a sterile sleeve, lubricated using sterile lubricant and inserted into the vagina. By manipulating the ovary manually per rectum and with the other hand guiding the transducer against the vaginal fornix, an assistant punctured the follicle with the needle through the vaginal wall. The follicular fluid was then aspirated using a 60 ml syringe.

The ovaries were examined using ultrasonography at 48 hours post-aspiration for evidence of luteal tissue formation. To confirm that cyclicity had begun; mares that developed a luteal structure were given prostaglandin $F_{2\alpha}$ (Lutalyse, 10 mg, IM, Pharmacia and Upjohn Co, Kalmazoo, MI, USA), 7 days after follicle aspiration. Mares were then examined by transrectal ultrasonography for luteolysis and subsequent follicular development. Once a follicle $\geq$ 35 mm developed, the mare was then given human chorionic gonadotrophin (Chorulon, 2,500 IU, IV, Intervet. Inc., Millsboro, DE, USA) for induction of ovulation. Mares were then examined for ovulation and subsequent corpus luteum formation.

The mares receiving deslorelin treatment were similarly examined using the same schedule as mares in the aspiration treatment. When a follicle $\geq$ 35 mm and uterine edema $\geq$ 2 were observed, the mares were then treated with the GnRH analogue, deslorelin (Ovuplant, 2.1 mg, Fort Dodge Animal Health, IA, USA), implanted in the vestibular submucosa. The follicle was examined 48 hours post-deslorelin treatment for ovulation and luteal tissue formation. After 48 hours, the implant was removed using a sterile technique. The mares that did not ovulate after deslorelin treatment were retreated based on the same criteria of follicle size and uterine edema used previously, until

ovulation occurred. All mares that ovulated were treated in the same fashion as the aspiration mares.

4.2.3 Blood Collection and Hormone Analysis

Blood samples were obtained into heparinized tubes from all mares at each time of ultrasound examination per rectum. The blood samples then centrifuged for 10 min at 900 × g and the plasma obtained was stored at -20°C for P_4 analysis.

The plasma concentrations of P_4 were measured using commercially available reagents (Diagnostic Systems Laboratory, Webster, TX, USA) that were previously validated for horse plasma. Intra- and interassay coefficients of variation and assay sensitivity were 5%, 9%, and 0.05 ng/mL, respectively.

4.2.4 Statistical Analysis

The time from January 1 to $P_4 > 1.0$ ng/mL was compared among treatment groups using Kaplan-Meier methods where the probability function of increase in P_4 to > 1.0 ng/mL over time was generated for each treatment group using the log-rank test. Where there was a significant difference in the probability functions ($P \leq 0.05$), ad hoc comparisons were made between groups correcting the significance level to $P \leq 0.017$ such that type I error was maintained at 0.05. Proc LIFETEST (SAS v 8.0, SAS Institute, Cary, NC) was used for analysis.

4.3 Results

The probability functions were not significantly different among groups ($P > 0.05$). Of the 10 aspiration group mares, 7 mares formed luteal tissue within 2.8 days after the last follicle aspiration and 3 mares did not respond to the first aspiration. Four of the 7 mares that responded required a second aspiration and one mare required a third

aspiration before developing luteal tissue. An average of 1.6 ± 0.2 aspirations was required for these mares. The other 3 mares that did not respond to the first aspiration developed a follicle > 35mm but without uterine edema. These mares were not retreated and ovulated on their own. When an aspiration procedure resulted in the formation of luteal tissue, progesterone rise to > 1.0 ng/mL in 5.9 ± 1.2 days. When only comparing the 7 mares that responded to aspiration with the control group mares, the probability function generated by the control group mares was significantly different from the aspiration group mares (113.0 ± 3.0 and 93.8 ± 6.6 days; mean ± SE for the control and aspiration group mares; respectively, P = 0.0016). The deslorelin group mares was not significantly different from the control group mares (P = 0.411) or from the aspirate group (P = 0.076) (Table 4.1).

The deslorelin group mares formed luteal tissue within 2.6 ± 0.2 days after the last treatment. Of the 12 deslorelin group mares, 2 mares required 2 treatments and one required a third treatment before developing luteal tissue. An average of 1.3 ± 0.2 treatments was required for these mares. When luteal tissue formed in mares after deslorelin treatment, P_4 concentrations rose to > 1 ng/mL in 5.8 ± 0.4 days (Table 4.1).

Table 4.1: Effect of follicular aspiration or deslorelin treatment on time interval (d) to rise in progesterone (P_4) (mean ± SE).

Treatment	Days from January 1 to P_4 > 1.0 ng/mL	Days from follicle ≥ 35mm to P_4 > 1.0 ng/mL	Days after treatment to P_4 > 1.0 ng/mL	Number of treatments
Control	113.0[a] ± 3.0	19.2[a] ± 3.9	-	-
Aspiration	100.4[a] ± 5.8	13.1[a] ± 3.8	5.9 ± 1.2	1.6 ± 0.2
Deslorelin	110.0[a] ± 6.7	9.3[a] ± 1.8	5.8 ± 0.4	1.3 ± 0.2

[a] Values within column are not significantly different.

4.4 Discussion

Follicular fluid may be an important regulator of receptor induction, luteinization, and progesterone secretion during follicular maturation (Hinrichs et al. 1991). During the ovulatory process, rupture of the follicle, release of the follicular fluid and collapse of the follicular wall are the major physical-mechanical events. The cellular constituents of the follicular wall, granulosa and theca cells, transform under the effect of LH into large and small luteal cells, respectively.

In many species, including the horse, granulosa cells removed from pre-ovulatory follicles luteinize in vitro without additional gonadotropin stimulation (Channing 1969; Channing et al. 1982). It has been hypothesized that luteinization cannot occur unless a luteinization inhibitor is removed from the granulosa cells (Channing 1972). This removal may occur during follicular maturation and ovulation, or when cells are cultured in vitro without follicular fluid (Channing 1972).

Hinrichs et al. (1991) evaluated the effect of aspiration of the pre-ovulatory follicle on luteinization, corpus luteum function and peripheral plasma gonadotropin concentrations in cyclic mares. In that study, aspiration of follicular fluid from pre-ovulatory follicles in cyclic mares was associated with significantly early luteinization, as determined by the time to rise in progesterone values. These findings support the idea that follicular fluid from small- to medium-size follicles may contain a luteinization inhibitor, and indicate that the presence of follicular fluid during the final days of follicular maturation is not essential for development of a functional CL (Hinrichs et al. 1991).

Alvarenga et al. (1999) aspirated all follicles > 30 mm from the ovaries of transitional mares and concurrently administered hCG. Luteal tissue formation occurred within 7 days in only 3 of 8 mares that were aspirated. In Experiment 1 of the present study (chapter 3), follicles > 35 mm on the ovaries of transitional mares were aspirated. Aspiration resulted in luteinization in 9 of 11 mares within less than 7 days, advancing the onset of cyclicity 23.8 days compared to control mares. In reviewing the mare records, all mares had edema score > 2. We hypothesized that using uterine edema as an additional criterion would enhance the outcome by reducing the number of treatments required. However, including uterine edema as a prerequisite for aspiration resulted in there being no difference between the aspiration and control groups in time from January 1 to first rise in P_4.. In experiment 2, some of the mares developed follicles > 35 mm but lacked significant uterine edema which postponed treatment, resulting in 17 missed aspiration opportunities. Furthermore, the three mares that did not respond to the first aspiration developed follicles > 35mm but without any uterine edema. Therefore they were not aspirated and ovulated later on their own. However, if only those mares that developed edema and were therefore aspirated are considered, in Experiment 2 aspiration shortened transition by 19 days which is similar to the results in Experiment 1.

It is commonly believed by veterinary practitioners that edema in the uterus is associated with a steroidogenically competent follicle secreting estrogen and would indicate conditions likely for ovulation during transition. Recently, however Watson et al. (2003) found that uterine edema is not a reliable indicator of follicular competence or ovulation. Therefore, further work is needed to identify characteristics by which to identify follicles likely to ovulate during transition.

McKinnon et al. (1996) used deslorelin to induce ovulation in mares during early and late transition. During early transition, mares were treated with deslorelin every other day until either ovulation had occurred or 6 implants had been administered. Six mares out of 11 (~55%) ovulated in < 10 days. This was different than control mares, as none of the control mares (0/10) ovulated within the same time frame. In late transition, 8 mares out of 10 (80%) ovulated in a mean of 3.7 days after deslorelin treatment, compared to controls where none ovulated within the same period. It was concluded from this study that accurate, timed ovulation in late transition could be achieved using multiple implants (average of 2.1 implants). In the present experiment, however, no significant difference was detected between deslorelin treated and control mares. The deslorelin treatment resulted in luteinization (based on ultrasound and P_4 results) within 2.6 days after the last treatment. Of the 12 deslorelin treated mares, 2 mares were treated twice and one mare was treated three times with an average of 1.3 ± 0.2 treatments before luteinization was observed. McKinnon et al. (1996) administered deslorelin once a follicle > 30 mm was present with visible endometrial folds. In the present work, deslorelin was not administered until the mares had a follicle > 35 mm with uterine edema (score > 2 on a scale of 0 to 4). This requirement for uterine edema may have caused a delay in treatment in some cases as some mares had follicles > 35 mm yet the uterine edema score was < 2.

The question remains as to why large transitional follicles do not ovulate. Numerous reports (Watson and Al-zi'abi 2002; Watson et al. 2003; Watson et al. 2002) have investigated the morphological, molecular and functional characteristics of dominant follicles during spring transition and ovulatory follicles during the breeding season. Results have shown that transitional follicles have a thin and poorly developed

theca cell layer that receives scant blood supply, when compared with ovulatory follicles during the breeding season. Reduced blood supply, in turn, contributes to the steroidogenic incompetence of these follicles. Furthermore, transitional follicles have low vascular endothelial growth factor (VEGF) which is the ovarian angiogenic factor (multifunctional cytokine which stimulates blood vessel formation and enhances microvascular permeability). In addition to low VEGF, concentrations of estradiol and progesterone are significantly lower in follicular fluid of dominant transitional follicles when compared with follicular fluid of ovulatory follicles (Watson and Al-zi'abi 2002; Watson et al. 2003).

The mechanisms by which follicular aspiration leads to luteinization may be due to removal of an ovulation inhibition factor or by stimulating the angiogenesis factors. These mechanisms need to be further investigated to find the mechanisms by which transitional follicles can be stimulated to ovulate.

CHAPTER 5

SUMMARY

In the first experiment, aspiration of the dominant follicle resulted in luteinization and shortening the transitional period in treated mares compared to controls. The time from January 1 to the first rise in serum progesterone was 23.8 days earlier for the treated mares than for the controls. The onset of cyclicity was confirmed because each mare responded to subsequent prostaglandin injection with luteolysis, estrus behavior and ovulation. Because the average estrous cycle is approximately 21 to 22 days long, advancing the date of first rise in progesterone by over 3 weeks would provide an additional breeding opportunity during the breeding season.

Because results of Experiment 1 were encouraging, further efforts to refine the criteria and improve the outcome were conducted in Experiment 2. However, because of the inclusion of the presence of uterine edema as a requirement for treatment, 17 opportunities for aspiration of large follicles were allowed to pass. This resulted in there being no difference between the aspiration and control groups in the time from January 1 to first rise in P_4. However, when mares that were not aspirated (due to lack of uterine edema) were excluded, aspiration shortened transition by 19 days which is very close to the results in the first experiment.

It has been suggested that edema in the uterus is associated with a steroidogenically competent follicle secreting estrogen and thus would indicate conditions likely for ovulation. However, Watson et al. (2003) found that uterine edema is not a reliable indicator of follicular competence or ovulation. Results of Experiment 2 support these conclusions.

McKinnon et al. (1996) used deslorelin to induce ovulation in mares during early and late transition. They concluded that accurate, timed ovulation in late transition can be achieved using multiple implants. In the present experiment, no significant difference was detected between deslorelin treated and control mares.

The results of the present experiments indicate that follicular aspiration may be useful as a means to hasten the onset of cyclicity in transitional mares. However, the mechanism by which removal of the follicular fluid stimulates luteinization is unknown. Further work in this area is needed to elucidate these mechanisms.

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