THE EFFECTS OF EQUINE FOLLICLE STIMULATING HORMONE (eFSH) ON MARE FERTILITY

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ABSTRACT

A series of experiments were designed to study the effects of a purified equine pituitary extract product containing a high FSH to LH ratio (eFSH) on superovulation and reproductive performance in mares. A significance level of $P < 0.05$ was used for the data analyses.

The treatment protocol included twice daily administration of 12.5 mg eFSH beginning at a follicular diameter of ≥20 or 25 mm. The treatment was stopped when a preovulatory-sized follicle was detected (≥35mm), and subsequently human chorionic gonadotropin (hCG) was administered to induce ovulation(s). The eFSH treatment significantly stimulated the ovaries of cycling and vernal transitional mares. This resulted in the development of multiple preovulatory-sized follicles, increased the number of ovulations, and enhanced donor embryo recovery rates. In mares which ovulated, approximately 70% of embryo recovery attempts resulted in the recovery of ≥1 embryo. However, incidences of ovulation failure and non-ovulatory follicles were significantly higher compared to control mares. Furthermore, there were significant variations in the superovulatory response to eFSH among cycling and vernal transitional mares in the same study, and among different studies, in terms of number of ovulations, number of embryos and embryo per ovulation rates.

Administration of eFSH significantly modified reproductive tract variables (tone and edema) and serum concentrations of progesterone (P4) and estradiol-17β (E2) on the days that oocyte maturation, fertilization, and early embryonic development were expected to occur. The administration of eFSH was also significantly associated with lower quality scores in a proportion of embryos recovered, and lower than expected pregnancy rates in recipients which received an embryo recovered from eFSH-treated cycling donor mares as compared to embryos from non-stimulated control mares. Moreover, eFSH treatment did not significantly increase pregnancy rate per estrous cycle in mares intended to carry their own pregnancy; however, the incidence of twin pregnancy tended to increase.

The effects of estrus synchronization regimens employed prior to eFSH treatment initiation were examined in cycling mares. A progesterone and estradiol regimen (P&E) was significantly more efficient than PGF2α administration in diestrus for ovulation synchrony among eFSH-treated mares, with ≥80% of mares ovulating within a 3 day period. The

superovulatory outcomes (proportion of mares that ovulated, number of ovulations and embryo recovery), however, were significantly lower than those obtained with PGF2α administration.

In vernal transitional mares, eFSH treatment resulted in a significantly higher number of preovulatory-sized follicles and a greater number of ovulations, compared to vernal transitional mares treated with deslorelin or porcine-FSH, or as compared to control mares. Most transitional mares (73% to 100%) ovulated after a mean of 5 days of eFSH treatment. These ovulations resulted in pregnancies and/or successful embryo recoveries. Following eFSH treatment in vernal transition, the first inter-ovulatory interval of the breeding season was significantly prolonged (>21 d) in about half of the mares.

In summary, eFSH treatment significantly stimulated follicular growth and multiple ovulations in cycling mares and in vernal transitional mares. The treatment significantly increased reproductive efficiency of cycling mares in terms of embryo recovery rates, and in vernal transitional mares in terms of establishing pregnancies or recovering embryos early in the breeding season. However, the eFSH treatment significantly altered the hormonal environment (E2 and P4), and was associated with modifications in follicular growth, ovulation, and embryo parameters. These aspects should be considered in the development of superovulation protocols for mares in future studies.

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DEDICATION

To Karin and Niv

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LIST OF ABBREVIATIONS

AAEP	American Association of Equine Practitioners
AI	artificial insemination
ANOVA	analysis of variance
B-FSH-ReF-001	Bioniche Animal Health Standard of FSH
CA	California
CL	corpus luteum
CO	Colorado
d	days
Des	deslorelin
E2	estradiol-17β
eCG	equine chorionic gonadotropin
eFSH	equine follicle stimulating hormone
eLH	equine luteinizing hormone
EPE	equine pituitary extract
ET	embryo transfer
FL	Florida
FSH	follicle stimulating hormone
g	grams
GE	General Electric
GnRH	gonadotropin releasing hormone
h	hours
hCG	human chorionic gonadotropin
HSD	Honestly Significant Difference
ICM	inner cell mass
IGF	insulin-like growth factor
i.m.	intramuscular
Inc.	incorporated

IOI	inter-ovulatory interval
IU	international units
i.v.	intravascular
KY	Kentucky
Ltd.	limited
LH	luteinizing hormone
LSD	least significant difference
NC	North Carolina
mg	milligram
MHz	megahertz
ml	milliliter
mm	millimeter
MS-Cycle	mid-season cycle
NY	New York
ng	nanogram
NIH-FSH-P1	National Institutes of Health USA Reference Standard of porcine FSH
Ov	ovulation
P	probability
P4	progesterone
P450 scc	cytochrome P450 side chain cleavage
P&E	progesterone and estradiol
pFSH	porcine follicle stimulating hormone
pg	picogram
PG	prostaglandin
PGF2α	prostaglandin F2α
reFSH	recombinant equine follicle stimulating hormone
reLH	recombinant equine luteinizing hormone
rhFSH	recombinant human follicle stimulating hormone
RIA	radioimmunoassay
SAS	Statistic Analysis Software
SD	standard deviation

SEM	standard error of the mean
Tx	treatment
VEGF	vascular endothelial growth factor
µg	microgram
USA	United States of America
USD	United States' Dollar
WCVM	Western College of Veterinary Medicine
3βHSD	3 beta-hydroxysteroid dehydrogenase

1. GENERAL INTRODUCTION

Mares are seasonal, polyestrous, long day breeders. Periodic development of follicular waves occurs continuously in the breeding season during most of the post-natal life of the mare. Different types of follicular waves, with variable levels of activity within waves, may develop under specific physiological conditions. Follicular waves are influenced by factors such as the stage of estrous cycle, season, pregnancy, age, breed and individual animal (Ginther et al., 2004a). Changes in gonadotropin levels (follicle-stimulating hormone; FSH and luteinizing hormone; LH) and in the sensitivity of follicles to circulating gonadotropins, largely explain these physiological variations in follicle development.

Horses have an effective follicle selection mechanism; therefore, only one (occasionally two, rarely thress) antral follicle(s) of a major ovulatory follicular wave usually becomes dominant and ovulates, while the remaining follicles (subordinates) regress (Ginther, 2000; Ginther et al., 2004c). Anovulatory follicular waves also occur and are defined as 'anovulatory major waves' when a dominant follicle develops, and 'anovulatory minor waves' when a dominant follicle does not develop. The key selection event during a major wave is a distinctive change in growth rates between the developing dominant follicle and the remaining subordinate follicles, and is known as deviation. In mares, the mean diameters of the two largest follicles at the beginning of deviation are approximately 23.0 and 19.0 mm (Gastal et al., 1997; Gastal et al., 1999b; Gastal et al., 1999c; Ginther, 2000). Apparently, when the largest follicle reaches a decisive developmental stage, rapid development of the deviation mechanism blocks the second-largest follicle, and other follicles, before they reach a similar diameter. Despite the rapid designation of follicle status, subordinate follicles may maintain adequate viability for one or more days after the beginning of deviation so that they can convert to dominant status if the dominant follicle fails or is ablated (Ginther et al., 2003; Ginther et al., 2004a).

Studies indicate that at the beginning of diameter deviation in the mare several intra-follicular factors increase in the largest follicle of the ovulatory wave (Beg and Ginther, 2006). These intra-follicular changes in the future dominant follicle apparently increase the responsiveness of this follicle to systemic concentrations of decreasing FSH and increasing LH, which occur at that time. The other follicles of the wave have the same capacity for dominance, but do not reach a similar preparatory stage before being negatively affected by the changing gonadotropin concentrations

(mainly a reduction in FSH). Thus, the largest follicle alone continues to grow, becomes dominant, and ovulates.

Effective and reliable treatments to induce multiple ovulations or superovulation are of interest to the equine industry because they may increase reproductive efficiency in cycling and seasonally anovulatory mares. Superovulatory treatments may potentially increase the per cycle pregnancy rate in normal or subfertile mares, as well as increase donor embryo recovery rates. Furthermore, superovulatory treatment may stimulate follicular growth and fertile ovulations in seasonally anovulatory mares, and therefore hasten and extend the period of reproductive function in a given year. However, historically, superovulatory treatments have been elusive in the mare and less efficient than in other domestic species (Woods and Ginther, 1983a; Mapletoft et al., 2002; Cognie et al., 2003). Treatment with porcine FSH, eCG, GnRH or inhibin vaccines have had limited success in stimulating multiple ovulations in the mare (McCue, 1996; Squires, 2006a). Equine pituitary extract (EPE), which contains approximately 6–10% LH and 2–4% FSH, has been reported to stimulate multiple ovulations in mares (Ginther, 1992b). However, results varied significantly from study to study as there was no commercially available standardized EPE product. Recently, a purified equine pituitary extract product called equine follicle-stimulating hormone (eFSH) has become available from Bioniche Animal Health Inc. (Niswender et al., 2003). This has created the potential for successful superovulatory treatment in mares.

This thesis summarizes several studies that examined the fertility of mares following treatment protocols which employed twice daily eFSH administration initiated at the approximate time of follicular deviation in mares.

2. REVIEW OF LITERATURE

2.1. Overview of the estrous cycle

Reproductive activity in horses is seasonally dependent, as it is primarily affected by the length of daylight (photoperiod). In mares, an increase in the duration of daylight (long-day photoperiod) activates reproductive activity, while shortening the duration of daylight (short-day photoperiod) triggers its termination. Therefore, the physiologic breeding season, or the ovulatory season, of the mare lasts from early spring, until late summer. During the breeding season the non-pregnant mare will have recurring estrous cycles. The estrous cycle is defined as the period from one ovulation to a subsequent ovulation, with each ovulation being accompanied by signs of estrus and plasma progesterone concentrations below 1 ng/ml (reviewed by Ginther, 1992b; Nagy et al., 2000).

The equine estrous cycle is commonly described as a combination of a follicular phase, or estrus, and a luteal phase, or diestrus (reviewed by Ginther, 1992b). During estrus, the mare is sexually receptive to the stallion, and the genital tract is prepared to accept and transport sperm and an oocyte to the site of fertilization in the ampullary region of the uterine tube (Crowell-Davis, 2007). During the diestrus period the mare is not receptive to the stallion, and the genital tract is prepared to accept and nurture the conceptus. The diestrus period ends with regression of the corpus luteum (CL) which occurs due to prostaglandin F2α (PGF2α) release from the endometrium of the non-pregnant mare (Vernon et al., 1979).

Mean length of the estrous cycle in the mare population during the physiologic breeding season is approximately 21 days, but it can vary greatly (range 18 - 24 days) (reviewed by Ginther 1992b). The length of diestrus remains relatively constant at 14 - 15 days and is not affected by the season (Adams and Bosu, 1988). However, although estrus typically comprises 4-7 days of the cycle, its length is more variable (ranging from 2-12 days or more). In the beginning and at the end of the breeding season the length of estrus may be 7 to 12 days, whereas, around the summer solstice, estrus may last only 3 to 4 days (Hughes et al., 1975; Dowsett et al., 1993). Therefore, the duration of estrus is shortest during the peak of the ovulatory season. The diameter of the largest follicle at the time of luteolysis affects the interval from onset of estrus to ovulation. Larger

follicles present at CL regression typically ovulate sooner, thus shortening the associated estrus period (Palmer, 1978).

In the mare, growth of antral follicles during the estrous cycle occurs in wave-like patterns (Pierson and Ginther, 1985; 1987; Ginther et al., 2004a; Ginther et al., 2004c). Most mares were shown to have one or two follicular waves per cycle. The types of follicular waves that develop in mares are major waves (characterized by dominant and subordinate follicles) and minor waves (largest follicle does not attain the diameter of a dominant follicle). Based on transrectal palpation, a single major follicular wave was proposed initially for the equine estrous cycle (Ginther, 1979). The wave of follicles dissociates about 6 days before ovulation into a single growing preovulatory follicle and several regressing follicles. Subsequently, the initial transrectal palpation findings were substantiated by ultrasound, based on grouping of follicles into diameter categories (Pierson and Ginther, 1987) and tracking of individual follicles (Sirois et al., 1989; Ginther, 1990a). There are profound breed differences in wave patterns during the estrous cycle (reviewed by Ginther, 1992b). In some breeds (e.g. Quarter-Horses, Ponies), usually only one major wave develops in late diestrus and culminates in the estrous ovulation. In other breeds (e.g. Thoroughbreds), a secondary major wave frequently develops in early diestrus, and the dominant follicle may be anovulatory (more common), or occasionally ovulatory (diestrous ovulation) (Hughes et al., 1973). Minor follicular waves have been demonstrated in mares (Ginther and Bergfelt, 1992; Ginther, 1993). In all horse breeds, a major ovulatory wave begins at mid-cycle and one follicle (occasionally two, rarely three) becomes dominant and ovulates. Follicular dynamics and the selection phenomenon of a dominant follicle will be further discussed in a later section.

2.2. Endocrine regulation of the estrous cycle

The endocrinology of the estrous cycle involves a delicate balance among hormones produced by the pineal gland, hypothalamus, pituitary gland, ovaries, and endometrium. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which is released in brief pulses into the hypothalamic-pituitary portal system, and stimulates the synthesis and release of the gonadotropins, FSH and LH, from the anterior pituitary gland (Irvine and Alexander, 1994). Thus, GnRH secretion elicits the release of both LH and FSH from the pituitary, but apparently the ratio of LH/FSH reaching the circulation is influenced by GnRH pulse frequency and by physiologic

feedback from inhibin, estrogens, and progesterone. The gonadotropins enter the systemic circulation and, at the level of the ovaries, FSH stimulates follicular recruitment and growth, and LH stimulates maturation of the follicles, maturation of the oocytes, production of estrogens, ovulation, and luteinization of the CL (Ginther, 1992b). The estrogen produced by maturing follicles has a positive feedback effect on LH release in the presence of low circulating progesterone concentrations. Inhibin and estrogen produced by growing follicles have a negative feedback effect on release of FSH. Progesterone produced by the CL has a negative feedback on the release of LH (Miller et al., 1980).

The following few sections provide detailed information regarding the aforementioned hormones of reproduction; other regulatory factors will be presented in future sections.

2.2.1. Gonadotropin-releasing hormone

Gonadotropin-releasing hormone is a decapeptide (comprised of 10 amino acids), and is a pivotal regulatory hormone controlling reproductive biology. In response to the appropriate environmental, hormonal, and neuronal cues, the axons of the neurosecretory cells in the medial basal cells in the hypothalamus release GnRH in a pulsatile manner into the hypothalamic-pituitary portal system, which transports the GnRH to the anterior pituitary (Lee et al., 2008). The hormone binds and activates the GnRH receptor on the surface of pituitary gonadotrope cells leading to a cascade of hormonal events throughout the pituitary and the reproductive system. (Ginther, 1992b). Low-frequency pulses of GnRH stimulate synthesis and release of FSH; and high-frequency GnRH pulses stimulate synthesis and release of LH (Irvine and Alexander, 1994). During diestrus, progesterone released from the CL suppresses the high frequency of GnRH release. During estrus, after luteolysis occurs and circulating progesterone concentrations are low, GnRH pulse frequency is markedly increased as the estrogens produced by maturing follicles increase and positively stimulate the hypothalamus to release GnRH.

2.2.2. Luteinizing hormone

The pituitary gonadotropins, LH and FSH, are ubiquitous among vertebrate species. These glycoproteins are heterodimeric and composed of noncovalently associated α- and β- subunits (Bousfield et al., 1996). The α-subunit of the various gonadotropins has an identical amino acid

sequence within a species, whereas the β-subunit is unique for each hormone (LH, FSH, hCG, eCG) and determines its functional roles. The β-subunit of equine LH consists of 149 amino acids, and the α-subunit contains 96 amino acids. The pulsatile discharges of LH are in turn attributable to pulsatile discharges of GnRH. The half-life of equine LH in the circulation was reported to be one hour during the first hour, and 4 to 5 hours thereafter (Ginther et al., 1974).

In many species, increases and decreases in LH are rapid, with maximum circulating concentrations detected a few hours before ovulation (preovulatory LH surge). In the mare, however, the progressive increase and decrease in LH release lasts many days, with maximum concentrations frequently occurring a day or two after ovulation (Evans and Irvine, 1975; Miller et al., 1980; Adams and Bosu, 1988). The profile of circulating LH concentrations during the estrous cycle can be summarized as follows: 1) LH remains low during diestrus; 2) begins to increase a few days before the onset of estrus; 3) increases progressively thereafter to maximum values shortly after ovulation; and 4) decreases progressively over the next 4 to 6 days to the low diestrous values (Miller et al., 1980; Ginther, 1992b).

The LH pulsatility was detected during the preovulatory period in pituitary venous blood (Alexander and Irvine, 1987), with frequency of pulses varying from approximately one pulse per two hours early in the LH surge, to almost one pulse per hour at the time of ovulation. During mid-diestrus LH pulse frequency was reported to be markedly lower (4-6 pulses per day). The increased LH pulse frequency in estrus is attributed to increased GnRH pulse frequency which occurs due to 1) decreased progesterone concentrations (luteolysis) and removal of its negative feedback, and 2) increased positive stimulatory effect of estrogens produced by maturing follicles (Freedman et al., 1979a; Miller et al., 1980; Miller et al., 1981).

2.2.3. Follicle Stimulating Hormone

As opposed to LH, information regarding FSH in the mare is lacking. The β-subunit of equine FSH consists of 111 amino acids, and the α-subunit, identical to that of LH, contains 96 amino acids (Bousfield et al., 1996; Saneyoshi et al., 2001). Surges of FSH stimulate the occurrence of minor and major waves during the estrous cycle (Ginther, 1993; 2000). In some mares, two broad surges of FSH at 10- to 12- day intervals were described; one occurring during late estrus - early diestrus, and the other during late diestrus with a peak at 10 to 13 days before ovulation (Evans

and Irvine, 1975; Foster et al., 1979). In other mares, only one FSH surge can be detected in late diestrus. However, mean hormonal profiles of FSH are commonly obscured, and FSH surges are not obvious. This is because there is significant variation among mare estrous cycles in the number of FSH surges and in the time these surges occur (Ginther, 1992b). In addition, two surges of FSH occur more frequently during estrous cycles early in the ovulatory season, whereas one surge occurs more frequently late in the season (Turner et al., 1979a). Therefore, although the generalization of high mean diestrous levels and low estrous levels of FSH is documented, the high mean diestrous levels do not result from a consistent pattern among individuals.

In ovariectomized mares FSH concentrations are higher than those of intact mares in any stage of the estrous cycle (Freedman et al., 1979a). This is because throughout the estrous cycle the concentrations of FSH are regulated mainly by negative feedbacks. The decrease in FSH levels in late diestrus, which coincides with the increase in LH, is attributed mainly to the negative effect of estrogens and inhibin produced by growing follicles on FSH release from the pituitary (Miller et al., 1981). The temporary increase in FSH at late estrus - early diestrus is attributed to the decreased production of estrogens occurring 1 to 2 days before ovulation, and perhaps to the gradual increase in progesterone occurring after ovulation (Miller et al., 1980). Hence, there are close regulatory relationships between FSH, follicular growth, and follicular hormone production. These regulatory relationships are further discussed in section 2.3.

2.2.4. Progesterone

Progesterone is a steroid hormone produced by the CL during the luteal phase of the estrous cycle. It is the major ovarian progestogen, which holds a key role in preparation of the reproductive tract to accept and nurture the conceptus (McKinnon et al., 1988). Circulatory concentrations of progesterone during estrus are well below 1 ng/ml (usually below 0.5 ng/ml) (Evans and Irvine, 1975). Initiation of luteal development occurs immediately after ovulation with luteinization of granulosa cells and production of progesterone. The presence of a high level of LH plays a role in luteal development and may account for the increase in progesterone levels immediately after ovulation (Niswender et al., 2000). Progesterone concentrations are increased significantly within the first 24 hours after ovulation and thereafter continue to increase progressively to the high diestrous values by Days 5 to 7 after ovulation (mean range among 10

reports: 4 to 22 ng/ml) (reviewed by Ginther, 1992b). High progesterone concentrations are then maintained until Day 13 or 14 (approximately 3 days before estrus). During this period of luteal maintenance, the high affinity for LH of the luteal cells allows binding of LH molecules despite the low diestrus levels of LH in the blood. In an absence of an embryo, PGF2α is released by the uterus on Days 13 to 15; it travels through the systemic route to the ovary where it causes luteal demise (Stabenfeldt et al., 1981); progesterone values then decreases rapidly until the low estrous values reached.

During diestrus, progesterone has negative effects on LH release as it does not allow GnRH pulse frequency to increase. Nevertheless, temporal associations suggest a positive effect of progesterone on FSH (Miller et al., 1980).

2.2.5. Estrogens

The follicles are temporary endocrine glands which are associated with production of the ovarian estrogens. In the mare, estradiol-17β is the major estrogen, and approximately 10% or less is estrone sulphate (Ginther, 1992b). Both layers of the follicle wall (granulosa and theca interna) are involved in the pathways for steroidogenesis in the equine ovary. The production of androgens by the thecal cells, followed by their conversion to estrogens by granulosa cells, is known as the two-cell theory of follicular steroidogenesis (Tonetta and diZerega, 1989).

The granulosa and the thecal cells are positively stimulated by FSH and LH, respectively, to produce estrogens in the follicle. In general, production of estrogens is increased as the follicle diameter increases. Variation among mares in the circulatory concentrations of estrogens can be attributed to the variation in gonadotropins and follicular waves, as mentioned above (Ginther, 1992b). The following is a generalization on circulatory estrogen concentrations: beginning 6 to 8 days before ovulation, or approximately at the beginning of estrus, circulating concentrations of estradiol-17β or estrone sulphate increase progressively and reach a peak approximately two days before ovulation. By the time ovulation occurs, the concentrations are significantly decreased; hence, near the end of estrus or within a day or two after ovulation values are as low as basal diestrous values (Ginther et al., 2007b; Ginther et al., 2007c). The increasing estrogen concentrations in estrus have positive effects on GnRH pulse frequency and on circulating LH concentrations (Miller et al., 1981).

2.3. Follicular dynamics and selection of the dominant follicle in the mare

2.3.1. Growth pattern of ovarian antral follicles; development and selection for dominance

In the mare, periodic development of follicular waves continuously occurs and is influenced by factors such as stage of the estrous cycle, season, pregnancy, age, breed and individual. Hence, different types of follicular waves (minor or major, ovulatory or anovulatory) with variable levels of activity within waves may develop under specific physiological conditions (Ginther et al., 2004a). In the mare ovary there are approximately 40,000 primordial follicles and 100 growing follicles (Driancourt et al., 1982; Ginther, 1992b). Antral formation in the growing population of underlying follicles in both horses and ponies occurs when the follicle reaches a diameter of 0.2 to 0.4 mm. Atresia (regression) of follicles is rare until they reach 1 mm (Driancourt, 1979). Unfortunately, the factors influencing the initial stages of follicular development in domestic animals are less known (reviewed by Fortune, 2003). Furthermore, our understanding of dynamics in the population of small follicles (up to about 5 - 10 mm) in the mare is lacking; however, it is believed that this phase in the horse occurs over a prolonged period of time, as was shown in other species (Cahill, 1981; Lussier et al., 1987; Pierson and Ginther, 1987; Ginther, 1992b). It was speculated that small follicles are continuously growing and regressing and thus providing a reservoir for larger follicles (Ginther, 1992b).

Horses have an effective follicle selection mechanism, so that usually only one antral follicle of an ovulatory follicular wave becomes dominant and ovulates (Ginther, 2000; Ginther et al., 2004c). Anovulatory follicular waves also occur and are defined as major waves when a dominant follicle develops and minor waves when a dominant follicle does not develop, as described above. Among farm species, cattle and horses have the most effective selection mechanism as indicated by a greater frequency of single ovulations than in goats, sheep, and swine (Ginther, 2000; Ginther et al., 2003). A distinctive change in growth rates between the developing dominant follicle and the remaining subordinate follicles is the eminent selection event in a follicular wave, and is defined as deviation.

Major and minor follicular waves develop in mares from the stimulation of an FSH surge (Ginther and Bergfelt, 1993; Ginther et al., 2003; Ginther et al., 2005a).The ovulatory wave which

emerges in mid-diestrus is most consistent. However, earlier waves in the estrous cycle may or may not develop a dominant follicle (≥28 mm) or may not be detectable (Ginther et al., 2004a). The ovulatory waves can be studied ultrasonically for approximately 3 days before the peak of the wave-stimulating FSH surge in mares. Mean numbers of 7 – 11 follicles per wave emerge at diameters of 5 – 6 mm over one to several days and enter a common-growth phase of about 6 days (Gastal et al., 1997; Gastal et al., 1999b; Ginther, 2000). The common-growth phase extends from the beginning of wave emergence to the beginning of deviation; the end of the common-growth phase and the beginning of deviation are synonymous. During the common-growth phase, the follicles grow at an approximately similar rate and each follicle has the capacity for future dominance (Gastal et al., 2004; Beg and Ginther, 2006). However, on average, the future dominant follicle emerges one day earlier than the other follicles of the wave; this early emergence results in a size advantage for the future dominant follicle at the end of the common-growth phase. Follicles that emerge late in a wave reach a smaller maximal diameter, and may reach a plateau or maximum diameter at the end of the common-growth phase (Ginther, 2000; Ginther et al., 2003; Gastal et al., 2004; Ginther et al., 2004a).

Studies support the conclusion that all, or most, follicles in the same cohort during the common-growth phase have the potential for future dominance (Ginther, 2000; Gastal et al., 2004; Beg and Ginther, 2006). Ablation of the largest follicle at or shortly after the end of the common-growth phase or beginning of deviation is followed by the establishment of dominance by the second largest follicle in mares (Ginther, 2000). Thus, rather than selection of a dominant follicle, the selection or deviation process more literally involves an action against the remaining follicles. This is an important consideration in developing hypotheses on the nature of the deviation mechanism (Ginther et al., 2003; Ginther et al., 2004a).

Deviation of an individual follicular wave is defined at the examination prior to the first apparent change in the differences in diameter between the two largest follicles (Ginther et al., 1997; Ginther, 2000). In mares, the mean diameters of the two largest follicles at the beginning of deviation are approximately 23.0 and 19.0 mm (Gastal et al., 1997; Gastal et al., 1999b; Gastal et al., 1999c; Ginther, 2000). Apparently, when the largest follicle reaches a decisive developmental stage, rapid development of the deviation mechanism blocks the second-largest follicle, and other follicles before they reach a similar diameter. Thus, the mean difference in diameter between the

two largest follicles at the beginning of deviation indicates that the destiny of the follicles must be established in <1 day in mares (equivalent to a difference of 3 to 4 mm). Despite the rapid designation of follicle status, a subordinate follicle may maintain adequate viability for one day or more after the beginning of deviation so that it may be rescued to convert to dominant status if the dominant follicle fails or is ablated (Ginther et al., 2003; Ginther et al., 2004a). Therefore, in mares, treatment protocols to increase the number of ovulations (superovulation) commonly employ initiation of stimulatory hormonal treatments at the time of deviation; these treatments are given in order to stimulate, or "save", those subordinate follicles to achieve maturation and to ovulate (Niswender et al., 2003; Raz et al., 2005; Raz et al., 2006a; Raz et al., 2006b; McCue et al., 2007a; Squires and McCue, 2007; Raz et al., 2009c; Raz et al., 2009d).

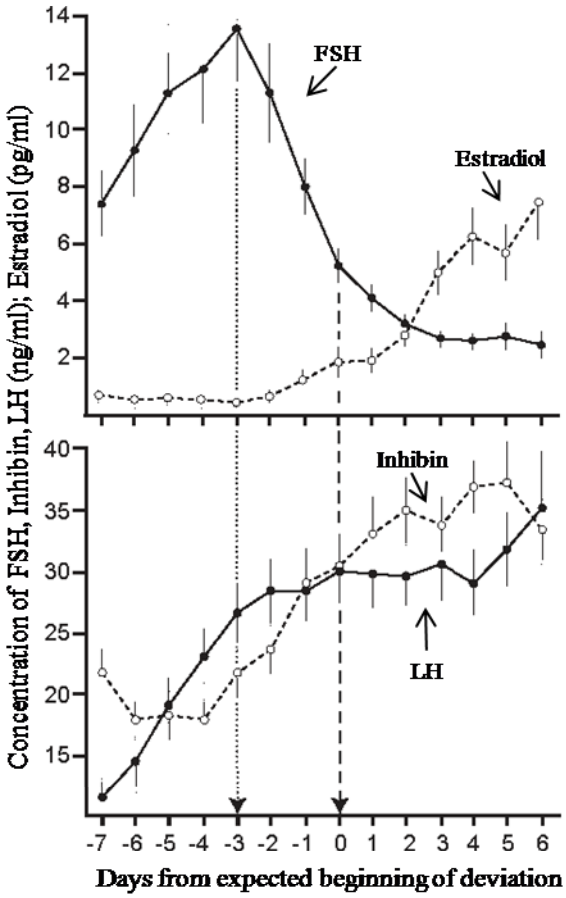
2.3.2. Systemic hormonal aspects of follicular deviation

In mares, the wave-stimulating FSH surge reaches peak concentrations when the largest follicle is about 13 mm (Gastal et al., 1999b; Ginther, 2000; Ginther et al., 2004a). Thereafter, FSH concentrations decrease over several days (Figure 2.1). During the decline in the wave-stimulating FSH surge and before follicle deviation, growth of follicles is dependent on FSH (Checura et al., 2008). However, this FSH decline is necessary for the establishment of deviation, as indicated by a delay or prevention of deviation by administering FSH or by increasing endogenous FSH with an anti-inhibin immunization (Shi et al., 1999; Ginther et al., 2003; McCue et al., 2007a; Squires and McCue, 2007). There is a 3-day interval between peak FSH concentrations and the beginning of deviation. During that 3-day interval, all follicles of the wave continue to require FSH; however, their secretion of estrogens and inhibin contribute to the decline in pituitary FSH release. In association with the beginning of deviation, large follicles are more responsive to declining or low concentrations of FSH than small follicles (Ginther et al., 2001; Ginther et al., 2003). Therefore, the role of FSH after the peak of the surge involves the continuation of growth and development of all follicles before deviation and the developing dominant follicle after deviation. Based on several studies, FSH stimulates the production of estradiol, insulin-like growth factor (IGF)-1, inhibin-A, activin-A, and other factors (Khalid et al., 2000; Campbell and Baird, 2001; Glister et al., 2001; Ginther et al., 2003; Beg and Ginther, 2006). These FSH-stimulated factors have intra-follicular roles in deviation, as will be discussed in section 2.3.3.

Estradiol and inhibin, alone and synergistically, suppress the circulating concentrations of FSH. Circulating estradiol in mares begins to increase at about one day before deviation, and remains elevated until 1 or 2 days before ovulation (Bergfelt et al., 2001). Total inhibin concentrations begin to increase earlier than estradiol, just before the beginning of the declining portion of the wave-stimulating FSH surge (Irvine et al., 2000; Bergfelt et al., 2001; Donadeu and Ginther, 2001; Ginther et al., 2003). After the expected day of deviation, total inhibin remains elevated, which is attributable to the dominant follicle. Experimental ablation of the largest follicle at the expected beginning of deviation prevented a further increase in circulating estradiol and a decrease in inhibin; this resulted in a post ablation FSH increase (Ginther et al., 2003; Ginther et al., 2004a; Ginther et al., 2005a). Hence, the beginning of the FSH decline at 13 mm diameter follicle in mares is coinciding with the beginning of an inhibin increase for both minor and major waves (Bergfelt and Ginther, 1993; Donadeu and Ginther, 2002b). For major waves, inhibin (probably inhibin-A) is produced by multiple follicles before deviation and by the developing dominant follicle after the beginning of deviation and is an FSH suppressant during the entire FSH decline. Estradiol begins to play a greater role in FSH suppression near the beginning of deviation.

In mares, concentrations of the periovulatory LH surge begin to increase during the FSH decline, reach maximal concentrations one day after ovulation and then decline for several days (Ginther, 1992b; Ginther et al., 2004a; Ginther et al., 2005a). Thus, LH and FSH are dissociated during the periovulatory period. It has been shown that the granulosa cells of the future dominant follicle acquire LH receptors before the beginning of diameter deviation (Goudet et al., 1999). Furthermore, experimental reduction of LH encompassing deviation resulted in smaller post-deviation diameter of the largest follicle and lower circulating concentrations of estradiol and total inhibin compared to controls (Gastal et al., 1999a; Bergfelt et al., 2001). These studies indicated that regulation of the production of inhibin, as well as estradiol, depends at least partly on LH, and that there is an LH-mediated survival advantage for the largest follicle of the wave before cessation of growth by the remaining follicles (Ginther et al., 2003; Ginther et al., 2004a).

Figure 2.1 Mean (± S.E.M.) concentrations of four circulating hormones in 15 mares. Data are normalized to the expected beginning of deviation (day 0). The dotted and dashed vertical lines indicate the mean peak of the FSH surge and the beginning of deviation, respectively. FSH continued to decrease after day 0, and estradiol first increased ($P < 0.05$) between days -2 and 0. Inhibin began to increase on the day before FSH began to decrease, and LH increased and formed a plateau that encompassed deviation.



Adopted from Ginther et al., 2004a

2.3.3. Intra-follicular aspects of follicular deviation

Over the last few years our knowledge and understanding of the intra-follicular aspects of follicular deviation in monovular species has increased significantly due to experimental approaches such as follicular fluid sampling (Evans and Fortune, 1997; Mihm et al., 2000; Ginther et al., 2002), follicle ablation (Gastal et al., 1999b; Gastal et al., 2004), and injection of potential stimulatory or inhibitory factors into follicles (Ginther et al., 2004b; Ginther et al., 2004d; Ginther et al., 2005b). Several intra-follicular factors were evaluated and were found to be different between the largest and the second largest follicle before the beginning of deviation. Concentrations of free IGF-I, estradiol, inhibin-A and activin-A are greater in the future dominant follicle than in other follicles before the beginning of deviation (Armstrong and Webb, 1997; Ginther et al., 2004a; Beg and Ginther, 2006). However, studies confirmed that free IGF-I is the key factor for the initiation of deviation in horses.

In mares, the concentrations of free IGF-I increase differentially in the future dominant follicle before the beginning of diameter deviation concomitant with the increase in estradiol (Donadeu and Ginther, 2002a; Ginther et al., 2004a). In addition, when the largest follicle was ablated at the beginning of deviation, free IGF-I began to increase in the second-largest follicle 12 hours before the beginning of experimental diameter deviation between the two largest retained follicles, and was the first noted change among the potential intra-follicular gonadotropin-enabling factors (Ginther et al., 2002). Concentrations of estradiol, inhibin-A and activin-A also increased differentially in the largest retained follicle, but only after experimental deviation had begun, indicating a key role for the IGF-I system in the initiation of the selection mechanism in mares.

The IGF system includes IGF-I and -II, IGF-binding protein (IGFBPs), and IGFBP proteases (Spicer, 2004). Free IGF-I stimulates granulosa cell proliferation and synergizes with gonadotropins to promote differentiation of follicle cells (Spicer and Echternkamp, 1995). *In vitro* effects of IGF-I in cattle include increased proliferation of granulosa cells and estradiol production (Glister et al., 2001), enhanced sensitivity of granulosa cells to FSH (Monget and Monniaux, 1995; Spicer and Echternkamp, 1995), increased secretion of inhibin-A, activin-A and follistatin from granulosa cells (Glister et al., 2001), and enhanced LH stimulation of androgen synthesis from theca cells (Stewart et al., 1995).

The IGFBPs exert a pivotal role in the regulation of IGF bioavailability by selectively binding the IGFs and making them unavailable to their receptors (Armstrong and Webb, 1997; Beg and Ginther, 2006). The IGFBPs are inhibitory to gonadotropin-induced follicular growth and differentiation and inhibit the actions of IGFs at the level of target cells (Spicer and Echternkamp, 1995; Monget et al., 1996). Thus, changes in intra-follicular IGFBPs lead to changes in IGF bioavailability and the up or down regulation of gonadotropin actions on follicular cells (Ginther et al., 2004a). Proteolytic activity for BP-2 (Mazerbourg et al., 2003), BP-4 (Mazerbourg et al., 2000), and BP-5 (Bridges et al., 2002) has been reported in dominant follicles during the follicular phase in mares. The IGFBP proteases degrade the binding proteins and thus increase the bioavailability of IGF-I in follicles (Mazerbourg et al., 2000; Spicer, 2004; Beg and Ginther, 2006). A greater proteolytic activity of such proteases was reported in the future dominant follicle at the beginning of deviation and was temporally associated with greater concentrations of free IGF-I (Ginther et al., 2005b).

About a day before the beginning of deviation, when the largest follicle is approximately 18-19 mm in diameter, the follicular fluid estradiol concentrations begin to increase differentially in the future dominant follicle (Gastal et al., 1999c; Donadeu and Ginther, 2002a). Studies indicated that an increase in estradiol, either systemically or locally in the follicle, was not a prerequisite to the initiation of diameter deviation in mares, even though estradiol increases differentially in the future dominant follicle before the beginning of natural deviation. Instead, the intra-follicular role for estradiol is consistent with an increase in granulosa-cell content of steroidogenic acute regulatory protein and the enzymes P450 scc, 3βHSD, and aromatase as the follicle grows from 20 – 24 mm (equivalent to the beginning of deviation) to ≥30 mm (dominant size) (Belin et al., 2000; Ginther et al., 2004a).

In horses, inhibin-A and activin-A concentrations begin to increase in the future dominant follicle but not in the future subordinate follicles at the beginning of diameter deviation, and this difference continues after the beginning of deviation (Donadeu and Ginther, 2002b; a; Ginther et al., 2004a; Beg and Ginther, 2006). Inhibin enhances the LH-induced androgen production in theca cells (Hsueh et al., 1987; Knight and Glister, 2001). Activin induces granulosa cell proliferation; increases FSH receptor expression, granulosa cell steroidogenesis, basal and gonadotropin-stimulated aromatase activity and estradiol production; and also delays the onset of

luteinization and atresia (Knight and Glister, 2001). However, studies show that this increase in inhibin-A and activin-A in the largest follicle is a late response which occurs about 24 h after the increase in IGF-I (Ginther et al., 2002); therefore, it was concluded that the increases in inhibin-A and activin-A do not play a role in the initiation of the deviation process in horses, but are rather a consequence of the process (Ginther et al., 2004a; Beg and Ginther, 2006).

Vascular endothelial growth factor (VEGF) stimulates mitosis of endothelial cells, increases vascular permeability and angiogenesis (Reynolds and Redmer, 1998). In the follicles of cattle and pigs, an increase in VEGF correlated with an increase in their diameter (Barboni et al., 2000; Berisha et al., 2000). When cattle (Schams et al., 2001) and monkey (Martinez-Chequer et al., 2003) granulosa cells were exposed to IGF-I *in-vitro*, synthesis and secretion of VEGF increased. In horses, there is an early increased vascularity in the future dominant follicle (Beg and Ginther, 2006). Follicular fluid VEGF concentrations were reported to be higher in the largest follicle than in the second largest follicle on the day after the beginning of diameter deviation (Ginther et al., 2004a; Ginther et al., 2004d); however, the earlier temporal relationships before deviation have not been studied in horses. An increase in vascularity would give the follicle an advantage to receive preferential supply of growth factors, gonadotropins, steroid precursors and other nutrients required for its continued development. Therefore, follicle-produced VEGF is a candidate for a role in vascular/follicle interrelationships during diameter deviation in the mare, but such a role has not been adequately demonstrated to date (Beg and Ginther, 2006).

There is limited information concerning the expression of key receptors in the ovarian granulosa and theca cells in horses. However, it is clear that the expression of such receptors is important for the autocrine and paracrine actions of local ovarian factors as well as endocrine actions of systemic hormones. In cattle, estradiol and progesterone receptors are present in both granulosa and theca cells (Rosenfeld et al., 1999), and they are upregulated in dominant follicles (Schams and Berisha, 2002). Changes in the expression of estradiol and progesterone receptors have not been studied with reference to the beginning of diameter deviation; however, it is clear that these receptors are expressed in association with follicle growth and development. The expression pattern of IGF-I receptors in relation to deviation is not known; however, the expression of IGF-I binding sites in cattle were shown to increase from primary to large antral follicles (Wandji et al., 1992). The induction of LH receptors in granulosa cells is one of the early

events in selection of a single dominant follicle in cattle (Bao et al., 1997; Beg and Ginther, 2006). Compared to smaller follicles, the LH-receptor protein content in equine granulosa cells was greater when the follicles were 15–19 mm in diameter than in smaller follicles, but the results were equivocal with regards to the temporality of differential LH receptor acquisition and the beginning of deviation (Goudet et al., 1999). Changes in FSH receptors activity have not been reported for mares. In cattle, no difference in the expression of FSH receptors was found between small and large follicles (Bao et al., 1997).

In summary, studies indicated that at the beginning of diameter deviation in the mare several intra-follicular factors increase in the largest follicle of the ovulatory wave (Beg and Ginther, 2006). However, to date, the IGF system is the only mechanism with a demonstrated positive effect on the initiation of deviation. The intra-follicular changes in the future dominant follicle apparently increase the responsiveness of this follicle to decreasing FSH and increasing LH concentrations. The other follicles of the wave have the same capacity for dominance, but do not reach a similar preparatory stage before being negatively affected by the changing gonadotropin concentrations. Thus, the largest follicle alone continues to grow and becomes dominant.

2.4. Reproductive seasonality in the mare

2.4.1. Physiology and endocrinology of seasonality in the mare

Animals have developed strategies for seasonal breeding that ensure that their offspring is born at the appropriate time of the year. In horses, as in many other species, the circannual rhythm of reproduction is cued primarily by photoperiod changes (reviewed by Ginther, 1992b; Nagy et al., 2000). The mare is a seasonal, polyestrous, long day breeder that typically experiences a 2 - 3 month period of anestrus during winter. The period of seasonal anestrus is characterized by minimal hypothalamic GnRH secretion, curtailed gonadotropin secretion, persistently low circulating levels of progesterone and estradiol, and relatively inactive ovaries (no follicles greater than 15 mm in diameter) (Garcia and Ginther, 1976; Oxender et al., 1977a; Hart et al., 1984; Johnson and Becker, 1988; Ginther, 1992b). Following winter solstice (December 21), anestrous mares gradually reacquire sexual competence during a prolonged phase (60 - 120 days) called vernal or spring transition. This transition phase is characterized by a series of stages or events that direct the increase of GnRH and gonadotropin secretion, resurgence of follicular development,

estrous behavior and finally ovulation (Sharp, 1980b; Ginther, 1992b; Nagy et al., 2000). During the early transition phase the number of follicles with a diameter ≥20 mm in the mare's ovaries increases, and ovaries usually contain several developing and atretic follicles (Turner et al., 1979b; a; Ginther, 1990b; 1992a). During the late transition phase most mares develop 1 - 3 anovulatory follicular waves each characterized by a large dominant follicle (≥30mm); follicles continue to emerge and regress until one is ultimately favoured to be the ovulatory follicle (Turner et al., 1979b; Ginther, 1990b; 1992a). Developing follicles during anestrus and the early transition period may be steroidogenically incompetent (particularly in their ability to produce estradiol), thus limiting their ability to undergo normal development and maturation (Davis and Sharp, 1991).

Increasing length of daylight plays a major role in the resurgence of sexual competence, and the exact month that a mare will experience the first ovulation of the breeding season depends on the geographical latitude at which she lives; genetics, nutrition, climate, and other environmental factors may also play a role (Sharp, 1980b; Ginther, 1992b; Niekerk and Niekerk, 1997; Nagy et al., 2000; Gentry et al., 2002). The mechanism whereby gonadotropin and presumably GnRH secretion is decreased during the anestrous period is not well understood in mares. However, it is believed that photoperiod is the main environmental signal that is translated to an endocrine signal in the pineal gland, which secretes melatonin during the phase of darkness. Studies support the concept that photoperiod and the rhythm of melatonin secretion entrain the endogenous circannual rhythm but do not influence reproductive activity directly (reviewed by Nagy et al., 2000). Although the effect of photoperiod is well documented, the mechanism of action of melatonin has not been studied in horses. Studies in other species show that melatonin does not influence GnRH-secretion directly, but acts through a complex network of interneurons involving a number of different neurotransmitters, and its target sites appear to be located within the hypothalamus (Malpaux et al., 1999). In horses, specific melatonin binding was found in the pars tuberalis of the pituitary, in the median eminence of the hypothalamus, and in the suprachiasmatic nucleus (near the optic chiasm) (Stankov et al., 1991).

The content of gonadotropin releasing hormone (GnRH) within the hypothalamus varies with season; levels are lowest in December (Northern Hemisphere), but are not different when compared among the months of March, July and October (Hart et al., 1984). One of the earliest endocrine changes in response to increased photoperiod following winter solstice is the rapidly

enhanced GnRH content in the hypothalamus (Johnson and Becker, 1993). However, restoration of pituitary LH content lags behind the seasonal increase in hypothalamic GnRH content by 1 - 2 months despite the finding that the concentration of pituitary GnRH receptors does not differ with respect to the month (Hart et al., 1984) or the reproductive state (Silvia et al., 1986). Serum LH concentrations are correlated with pituitary LH content; thus circulating levels are lowest during the period of anestrus and do not noticeably increase until several days prior to the first ovulation of the breeding season (Freedman et al., 1979b; Turner et al., 1979b; Silvia et al., 1986; Donadeu and Ginther, 2002b).

During anestrus, LH secretion reportedly occurs in a pulsatile pattern with an estimated frequency of one pulse per 24 h period. Approximately 2 weeks prior to the first seasonal ovulation the frequency of LH pulses increases to four to six pulses per 24 h, and is maximal during the periovulatory LH surge (up to almost 2 pulses per hour) (Alexander and Irvine, 1987; Fitzgerald et al., 1987b). The majority of LH pulses are associated with increased secretion of hypothalamic GnRH (Alexander and Irvine, 1987; Sharp and Grubaugh, 1987).

The secretion of follicle stimulating hormone (FSH) also occurs in a pulsatile pattern (Alexander and Irvine, 1987). During the middle of the anovulatory season, circulating FSH concentrations are inhibited by the short photoperiod (Freedman et al., 1979a). During the ovulatory season, FSH is stimulated by the long photoperiod but rhythmically inhibited by the ovaries. Seasonal effects on circulating FSH are mediated by changes in GnRH secretion, and negative effects on FSH are mediated by the ovarian follicular products, estradiol and inhibin (Freedman et al., 1979a; Ginther, 1992b). Follicular production of estradiol and inhibin is reduced during the anovulatory season and an increase in their production does not occur until the late transition (Davis and Sharp, 1991; Peltier et al., 1998; Donadeu and Ginther, 2002b; Watson and Al-Zi'abi, 2002; Donadeu and Ginther, 2003). Serum FSH concentrations are modestly increased between 60 and 35 days before the first seasonal ovulation (compared with winter anestrous levels), then gradually decline until the first ovulation (Freedman et al., 1979a; Silvia et al., 1986). The increased levels of FSH during the later part of the transition phase are associated with an increased incidence of hypothalamic GnRH secretion (Sharp and Grubaugh, 1987). The decreasing levels of FSH at the late resurgence are attributed to the FSH inhibitory factors (estradiol and inhibin) from follicles that grow at that stage (Miller et al., 1981; Bergfelt and Ginther, 1985;

Donadeu and Ginther, 2003). A few follicular waves emerge at that period of time; each is stimulated by surges in circulating FSH (Donadeu and Ginther, 2002b). However, when FSH levels decline, an LH surge does not occur due to inadequate LH content in the pituitary; as a result, follicles stop growing, regress, and a new wave then develops (Ginther, 1992b). If an estrogen-competent dominant follicle (>30 mm) develops when LH pituitary content is adequate, the follicle will enhance circulating levels of LH through estrogens. The resulting LH surge induces ovulation and thereby ends the anovulatory season.

As with most seasonal species, pituitary and serum concentrations of prolactin are lowest in mares during the winter months, and they rise with increasing photoperiod in spring. The functional importance of a seasonal prolactin profile is not clear, but it appears to play a role in seasonal reproductive recrudescence and hair coat changes in the horse (Ginther, 1992b). Relationships between levels of prolactin, FSH, and LH in the serum and the pituitary have been reported for horses (Thompson et al., 1986). A rise in prolactin preceding the rise in LH that is associated with the first ovulation is a temporal indication that prolactin may be part of the hormonal cascade leading to the onset of the ovulatory season (Ginther, 1992b). However, administration of prolactin, or of dopamine antagonists which increase prolactin, to anovulatory mares did not always yield conclusive results with regard to the role of prolactin in seasonal reproductive recrudescence (Thompson et al., 1997b; Donadeu and Thompson, 2002a).

2.4.2. Methods available to induce ovulation and cyclic ovarian activity early in the year

A circannual rhythm in mares entrained by environmental cues ensures foaling during spring and summer. Unlike most other species, the breeding season and parturition season of mares overlap because of the approximately 11-month gestation period. However, an artificial breeding season has been imposed for many breeds, encouraged by the use of January 1 (Northern Hemisphere) or August 1 (Southern Hemisphere) as the official birth date of foals in order to be eligible for registration in their respective associations. This results in economic pressure to breed mares as early as possible in the year to have an age advantage over foals born later in the year. Horses that are born early in the year have an advantage and perform better than horses born later (Langlois and Blouin, 1997; 1998; 2007). Therefore, the arbitrary birth date has stimulated researchers to understand the mechanisms of reproductive seasonality in mares and develop

methods for induction of an early onset of the breeding season. Artificially increased photoperiod has long been recognized as a means of returning seasonally anestrous mares to reproductive competence sooner than would occur with natural day length (Burkhardt, 1947; Ginther, 1992b). However, increasing daylength with artificial light does not shorten transition but merely causes it to begin earlier in the year (Freedman et al., 1979a; b; Palmer et al., 1982). Whether transition is brought about by natural or artificial light, it is a lengthy process lasting 6 to 12 weeks, and there is considerable variation in the interval to ovulation among mares (Sharp, 1980a; Ginther, 1992b; Nagy et al., 2000). Horse breeders would benefit from management protocols that would not only shift the beginning of the ovulatory season to a point earlier in the year, but would also shorten the length of vernal transition.

2.4.2.1. Artificial Light

A variety of artificial lighting regimens have been used in mares to hasten the onset of ovarian activity in early spring; and at present, this is the most reliable method to bring mares from deep anestrus into vernal transition (Ginther, 1992b; Nagy et al., 2000). The most traditional supplemental lighting regimen is a 14.5 - 16 h fixed-length photoperiod (combination of natural and artificial light) that is applied starting at the winter solstice (December 21), or earlier, without a gradual transition from short to long days (McCue et al., 2007c). For most mares, follicular activity is expected to significantly increase within only a few weeks, and ovulation is expected to occur approximately 8 – 12 weeks after the beginning of treatment. Although this regimen is very effective, such a long photoperiod can involve considerable expense and inconvenience. Therefore, other common and effective lighting regimens include 1) extension of the natural daylight with additional artificial light starting 30 minutes before darkness for approximately 3 hours; 2) providing photostimulation for 1 to 2 h during a photosensitive phase 9.5 h after the beginning of darkness (so called “pulse lighting”); or 3) gradual increase of artificial light 30 minutes every week, starting at winter solstice, until a total of 16 h light (combined natural and artificial) is reached.

To effectively gain an advantage over the natural photoperiod, artificial photoperiod regimens should begin no later than winter solstice (Palmer et al., 1982; Scraba and Ginther, 1985). Initiation of the regimen as early as 5 to 7 weeks before winter solstice may be of further

advantage (Sharp, 1980a; Nagy et al., 2000). Traditionally, the recommended intensity of the light source is around 100 lux (approximately one foot-candle) and the treatment is continued beyond the first ovulation, until the natural long day of late spring (Ginther, 1992b). However, it was reported that 14.5 h of 10 lux light applied for only 35 days beginning at winter solstice was sufficient to advance the ovulatory season without a negative effect on reproductive cyclicity after artificial light was discontinued (Nagy et al., 2000).

As mentioned above, artificial light regimens do not shorten transition but merely advance it to begin earlier in the year (Freedman et al., 1979a; b; Palmer et al., 1982). Therefore, to achieve higher efficiency, artificial light regimens are commonly combined with other, mainly hormonal, treatments when mares are in the vernal transitional phase (Nequin et al., 1990; Nagy et al., 2000; McCue et al., 2007b).

2.4.2.2. GnRH and GnRH analogues

Several studies demonstrated that GnRH or its analogues may increase endogenous pituitary LH and FSH release, and may induce ovulation in seasonally anestrous mares (Nequin et al., 1990; McCue et al., 2007b). Native GnRH has been reported to be effective when administered 2 - 3 times daily by injection (Evans and Irvine, 1977; Fitzgerald BP, 1987; Minoia and Mastronardi, 1987), in hourly pulses using externally mounted infusion or peristaltic pumps (Johnson, 1987; Johnson and Becker, 1988; McCue et al., 1991), or by constant infusion using subcutaneously implanted osmotic minipumps (Hyland et al., 1987; Ainsworth and Hyland, 1991). Agonists of GnRH have been administered by twice daily boluses (Fitzgerald BP, 1987; Ginther and Bergfelt, 1990; Harrison et al., 1990; McCue et al., 1991; McCue et al., 1992b), constant release long-term implants (Allen et al., 1987; Harrison et al., 1990; Turner and Irvine, 1991; Fitzgerald et al., 1993), or repeated administration of short-term implants (McKinnon et al., 1997; Nickerson et al., 1998).

From the available data, it appears that the efficacy of GnRH treatment depends in part on the stage of anestrus and is related to follicle size at the onset of treatment, as well as on the mode of administration (Nagy et al., 2000; McCue et al., 2007c). Typically, mares in transition with follicles >25 mm in diameter are more likely to respond than mares with follicles < 15 mm in diameter. Mares in deep anestrus may fail to respond to continuous administration of GnRH but pulsatile systems appear effective during early and late spring.

The ovulations following GnRH treatments of anestrous mares are generally considered to be similar to spontaneous ovulations with regards to CL formation and function (progesterone production), and subsequent fertility (Nagy et al., 2000; McCue et al., 2007c). However, occasional failures of normal CL formation or function have been reported (Evans and Irvine, 1977; Turner et al., 1979a; Hyland et al., 1987; Nickerson et al., 1998). Furthermore, occasionally, mares may fail to continue having estrous cycles in a typical manner when their first ovulation of the breeding season was induced by GnRH, and they may return to anestrus (Johnson, 1987; Ginther and Bergfelt, 1990; McCue et al., 1992b; McKinnon et al., 1997; McCue et al., 2007b). This may be most common if ovulation was induced very early before the onset of the natural breeding season, or following administration of long-term implants or repeated use of short term implants that potentially may down-regulate GnRH receptors (McKinnon et al., 1997; Nickerson et al., 1998; Farquhar et al., 2001; Johnson et al., 2002b; McCue et al., 2007b) .

Currently, regimens that employ GnRH and its analogues do not have an important place in the management of anestrous mares in practice due to cost, the preferred modes of administration, and limited commercial drug availability.

2.4.2.3. Equine pituitary extract and purified equine FSH

Equine pituitary extract (EPE), containing eFSH and eLH, has been reported to induce ovulation in seasonally anestrous mares (Douglas et al., 1974; Lapin and Ginther, 1977; Woods and Ginther, 1982; Coy et al., 1999). In a favoured reproductive status, it appears that the majority of anestrous mares successfully ovulate following EPE treatment, and that approximately 50% of those ovulating mares have ≥2 ovulations. A study by Woods and Ginther (1982) showed that the efficiency of EPE to induce ovulation in anestrous mares increased progressively as the diameter of the largest follicle at the onset of treatment increased. Transitional mares with small follicles (20-25 mm in diameter) at the onset of EPE treatment experienced a lower ovulation rate (2 of 7 mares, 29%) and longer interval to ovulation (28.4 ± 5.3 d) compared to transitional mares with larger follicles (30 - 35 mm), all of whom ovulated in a much shorter interval (9.1 ± 1.0 d) from treatment initiation. Higher efficiency of EPE treatment in transitional (follicles >25 mm in diameter) as compared to deep anestrous (follicles < 15 mm in diameter) mares was also reported

by Coy et al. (1999). There is limited information regarding fertility in anestrous mares following EPE - induced ovulation (Woods and Ginther, 1982; McCue et al., 2007b).

Equine pituitary extract products are not available commercially and for this reason, are not used in practice. However, a purified equine-FSH product (eFSH®), with a ratio of FSH:LH of 10:1 (personal communication with Dr. Duncan K. Hockley, Bioniche Animal Health Ltd.), has recently become available (Niswender et al., 2003). Niswender et al. (2004) reported that treatment of transitional mares with daily administration of 12.5 mg of eFSH stimulated follicular development and advanced the first ovulation of the year. In that study, conducted in Colorado, USA, mares (n = 20) were managed under routine fixed artificial photoperiod from the first week of December, and were randomly assigned to one of two treatment groups at the onset of transition (follicle ≥25 mm). In the control group, mares were observed during a 15-day period, but treatment was not given. In the eFSH group, a twice daily eFSH treatment was initiated, and when one or more follicles ≥35 mm were detected, the eFSH treatment was discontinued and human chorionic gonadotropin (hCG) was administered to induce ovulation. Administration of eFSH followed by hCG resulted in ovulation in 8/10 (80%) of the mares, while none of the control mares ovulated within the 15-day observation period. In the eFSH group mean interval from treatment initiation to ovulation was 7.6 ± 2.4 days in mares that successfully ovulated; and in 5 out of 8 ovulating mares ≥2 ovulations were detected. Mares used in that study were not bred and subsequent fertility was not evaluated (Niswender et al., 2004). However, a recent study conducted in Sao-Paulo, Brazil, by Peres et al. (2007) reported successful embryo recovery following eFSH treatment of late-transitional donor mares. Further studies on the effects of eFSH on transitional anovulatory mares were conducted as part of this dissertation and will be presented in later chapters (Raz et al., 2009a; Raz et al., 2009b; Raz et al., 2009d).

2.4.2.4. Progestogens

The effects of native progesterone and synthetic progestins on seasonally anovulatory mares have been widely examined (reviewed by Ginther, 1992b; Nagy et al., 2000; McCue et al., 2007b). The outcome of the treatment depends on the stage of anestrus and ovarian activity at the beginning of treatment. Overall, it appears that daily progestogen administration does not consistently induce ovulation in deep anestrus or early transition, but ovulation may occur within

15 days after the end of treatment when applied during late vernal transition (Nagy et al., 1998a; Nagy et al., 1998b). This effect in late transition is attributed to the ability of progestogens to synchronize the onset of ovarian activity and to decrease the incidence of prolonged estrus and anovulation which are common at the late transitional phase (Palmer, 1979; Squires et al., 1979; Squires et al., 1983; Alexander and Irvine, 1991). Progestogens have also been combined with estradiol-17β but there is no sufficient data to suggest that this combination is more effective in inducing ovulation in anestrous mares than progestogens alone (Taylor et al., 1982a; Wiepz et al., 1988).

The mechanism of action of progestogens during the late transitional phase is not completely understood, but it seems reasonable to assume that their administration might have mild positive effect on FSH secretion from the pituitary (Miller et al., 1980; Turner et al., 1981; Thompson et al., 1984). Whatever the mechanism may be, it is clear from the available data that mares must be well into transition to be able to respond to progestogens treatment.

A combination strategy commonly used by practitioners is based on a classic light treatment to induce cyclic ovarian activity, combined with a period of progestogen with or without estradiol treatment to synchronize or time ovulation at the end of the light treatment. This method has allowed the reduction of the variable interval from start of light treatment to first ovulation (Palmer, 1979; Nagy et al., 2000; McCue et al., 2007c; b).

2.4.2.5. Ovulation-inducing agents

Administration of ovulation-inducing agent, such as hCG or deslorelin acetate, is commonly used to induce ovulation in the late transitional phase, when mares are expressing behavioural estrus and have a dominant follicle (≥35 mm in diameter). The treatment can be used alone or with combination with other treatments mentioned in this section (Nagy et al., 2000; McCue et al., 2007b). As mentioned above, most mares develop 1 - 3 major follicular waves during the late transitional phase, and each is characterized by a large dominant follicle; however, LH secretion may not be sufficient to induce ovulation (Turner et al., 1979b; Ginther, 1990b; 1992a). Therefore, administration of an ovulation-inducing agent when a large dominant follicle is present in a transitional mare may shorten time to ovulation. If an ovulation-inducing agent is not

administered, the follicle may regress without ovulation, a new follicular wave eventually develops and the ultimate result is a natural transitional period.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone with LH activity which is commonly used by veterinary practitioners (Samper, 2008). It stimulates the ovary directly, and increases the likelihood of successful ovulation, regardless of the ability of the hypothalamic-pituitary-ovarian axis to generate an endogenous LH surge. Administration of hCG is effective in a proportion of late transitional mares if given at an appropriate time (behavioural estrus and large dominant follicle present) (Colbern et al., 1987; Carnevale et al., 1989). Deslorelin acetate is a potent GnRH agonist which was reported to be effective in inducing ovulation in late transitional mares (Farquhar et al., 2000). A recombinant equine LH (reLH) product has recently been reported to be a reliable and effective ovulatory agent in cycling mares (Yoon et al., 2007); however, the efficiency of reLH in inducing ovulation in transitional mares has not yet been tested.

2.4.2.6. Prolactin, and dopamine antagonists

Pituitary and serum concentrations of prolactin are lowest in mares during the winter months, and they rise with the increasing photoperiod in spring (Thompson et al., 1986; Ginther, 1992b). However, the functional importance of seasonal prolactin profile in the mare is not fully understood (Nagy et al., 2000). Limited data from other species suggest that prolactin exerts its effect on the ovary by increasing the number of gonadotropin receptors and thus mediating the effect of circulating gonadotropins (Advis et al., 1981; Klemcke et al., 1981; Klemcke et al., 1984; Wiepz et al., 1988). Prolactin receptors have been demonstrated on granulosa cells in pigs and hamsters (Oxberry and Greenwald, 1982; Bevers et al., 1988), but no information is available on the presence of prolactin receptors in the equine ovary. Limited data, however, suggest that administration of ovine (Nequin et al., 1993) or porcine (Thompson et al., 1997b) prolactin may hasten the first ovulation in seasonally anoestrous mares.

Pituitary production of prolactin is primarily regulated through inhibition by the neurotransmitter dopamine (Cross et al., 1995). Dopamine concentration in the cerebrospinal fluid is higher during the anovulatory period than during the breeding season (Melrose et al., 1990). The effects of several D2-dopamine antagonists on seasonally anestrous mares have been examined, with domperidone and sulpiride being the most commonly reported. Administrations of dopamine

antagonists to mares resulted in increased endogenous prolactin secretion (Besognet et al., 1996; 1997; Donadeu and Thompson, 2002b; Duchamp and Daels, 2002), and hastened the first ovulation of the year (Besognet et al., 1996; McCue et al., 1999; Duchamp and Daels, 2002; Daels, 2006). However, the effect of dopamine antagonists on the onset of reproductive activity is highly variable among experiments and individual mares and raises questions about the relative role of dopamine in the regulation of seasonal reproduction (Nagy et al., 2000). Although D2-dopamine antagonist administration results in increased plasma prolactin concentrations, pituitary gonadotropin (FSH and LH) secretion does not seem to be altered (Donadeu and Thompson, 2002a); instead, it was speculated that treated mares have increased numbers of gonadotropin receptors in their ovaries and therefore are more sensitive to endogenous gonadotropins (Donadeu and Thompson, 2002a; Daels, 2006). A study conducted by King et al. (2005), indicated that equine ovarian tissues indeed possess D1- and D2- dopamine receptors (in medulla, cortex, and granulose/theca), which suggests that dopamine antagonists can act directly on ovarian tissues through their interaction with dopamine receptors. Furthermore, an inverse temporal, seasonally dependent relationship between ovarian D2-dopamine receptors and FSH receptors in mares was recently reported; however, the functional significance of this relationship deserves further study (King et al., 2008).

Dopamine antagonists may not be effective under all management conditions and the duration of treatment may be prolonged, depending on the stage of anestrus at the initiation of treatment. In vernal transitional mares, ovulation may be induced after 12–22 days of treatment, however, in deep anestrous mares a prolonged period of treatment, up to 2 months, is required (Nagy et al., 2000; Daels, 2006; McCue et al., 2007b). Therefore, it is recommended to apply dopamine antagonists in vernal transitional mares or include an artificial photoperiod prior to administration of dopamine antagonist.

2.5. Equine embryo transfer

2.5.1. Introduction

Since the first successful embryo transfer (ET) in horses (Oguri and Tsutsumi, 1972) the use of embryo transfer and the development of embryo technologies for the horse have increased significantly (Squires et al., 2003a). Embryo transfer is now accepted by many horse breed

registries. It is a valuable tool for 1) increasing the number of progeny from genetically valuable mares; 2) obtaining foals from performance mares without interrupting their sporting careers; 3) obtaining foals from mares incapable of carrying a pregnancy to term; 4) obtaining foals from young (two-year-old) mares without jeopardizing their normal growth and development; 5) producing foals from mares that foaled late in the year, while still allowing them to conceive early in the next breeding season; 6) obtaining embryos for genetic cryopreservation for easier international trade; and 7) research.

The penetration of ET into the horse breeding industry is lagging behind the cattle industry. Following the development of ET techniques for horses during the 1970s, the unwillingness of most horse breed registries to allow multiple registrations of foals per mare for a given year seriously limited the use of ET. This situation has changed dramatically over the last few years. In 2002, the two of the largest breed registries in the United States, the American Quarter Horse Association and the American Paint Horse Association approved unlimited registration of foals from any mare during a given year using ET. However, most damaging to the commercial expansion of ET in horses were the findings that the induction of superovulation in mares was not efficient (reviewed by McCue, 1996; Squires, 2006a), and that equine embryos were poorly tolerant of cryopreservation (reviewed by Squires et al., 1999). In addition, the great variations in follicular growth and estrous length among mares make it difficult to adequately synchronize estrous cycles of donor and recipient mares (Ginther, 1992b), which may add labour and cost.

Embryo transfer is the cornerstone of many of the more advanced assisted reproductive techniques (e.g. *in-vitro* fertilization, cloning, gene manipulation) that are not yet sufficiently developed for large-scale commercial implementation (Hinrichs and Choi, 2005; Galli and Lazzari, 2008). Although ET is now fairly widespread in clinical equine practice, it continues to attract scientific interest because techniques that could further improve efficiency (e.g. superovulation and embryo cryopreservation) are still only marginally successful (Stout, 2006). Currently, single embryo recovery attempts are most common in equine ET programs. A 50% embryo recovery rate per ovulation and a 50–65% pregnancy rate per transferred embryo are expected, for a total success rate of 25% to 35% (Squires et al., 1999; Squires et al., 2003a; Stout, 2006). There is clearly a need to increase the success rate of equine ET.

2.5.2. The basics of nonsurgical embryo recovery and transfer

Equine embryo recovery is most commonly performed on Day 7 or 8 post ovulation since the selective transport of the embryo through the mare's oviduct takes place between approximately 130 and 142 h (day 5 to 6) postovulation (Freeman et al., 1991). The primary indication for recovering embryos on Day 6 is for cryopreservation (Squires et al., 2003a). Embryos are not routinely collected on day 9, because their large size may increase the risk of physical damage to them during collection and transfer, and because their transfer success rate is generally lower for than Day 7 or 8 embryos (McKinnon and Squires, 1988a; Squires and Seidel, 1995; Carnevale et al., 2000).

Non-surgical embryo recovery is a straightforward procedure in the mare, and has been widely described in the literature (McKinnon and Squires, 1988a). Briefly, a large-bore Foley-type catheter is introduced through the donor mare's cervix into the uterine body, the cuff is inflated with air or flushing medium, and the catheter is then retracted untill the cuff occludes the internal os of the cervix. Thereafter, the entire uterus is filled repeatedly with 1 to 2 L of warm (35-37°C) embryo recovery medium for a total of 4 to 8 L per recovery attempt. The fluid is allowed to flow back out through the catheter and is passed through a 0.75 µ embryo filter. Some operators perform manual trans-rectal uterine massage, and administer oxytocin during the procedure in order to facilitate fluid recovery (Stout, 2003; Hudson and McCue, 2004; Stout, 2006). When the fluid recovery is complete, the small retained volume of embryo recovery medium in the filter is transferred to a gridded Petri dish. Embryos are then indentified using a stereomicroscope (magnification range of 10x to 50x), scored for quality (1-excellent, 2- good, 3-fair, 4-poor) according to their morphology (McKinnon and Squires, 1988b), and are subjectively assessed for their developmental stage (morula/ early blastocyst/ expanded blastocyst). Embryo quality assessment will be further discussed in section 2.5.4.

Following identification, the embryo is "washed" by transferring it sequentially through 3 to 5 drops of embryo holding medium. The embryo holding medium is a better environment for the embryo in case of any delay in performing the transfer, and the washing steps help dilute out any microorganisms either introduced during embryo recovery or already present in the uterus of the donor mare (McKinnon and Squires, 1988a; Moussa et al., 2002; Moussa et al., 2003). Embryos can be transferred immediately after recovery ('fresh embryo'), or cooled for 12 to 36 h in order to

be transported prior to transfer ('cooled transported embryo') (Carnevale et al., 1987; Carney et al., 1991). Regardless of whether the embryo is fresh, cooled-transported, or frozen-thawed, the most commonly used transfer is by a non-surgical trans-cervical technique (Scherzer et al., 2008). The transfer of the embryo to the recipient mare is usually performed with a 'cassou' type transfer pipette, or insemination pipette. The choice of pipette type is largely a question of the operator's preference, with emphasis that the pipette should be adequate for the relatively large size of the equine embryo (Table 2.1). Transfer should be performed in a gentle clean manner such that the embryo will be placed in the uterine lumen of the recipient without the accompaniment of contaminants picked up from the recipient's vulva or vagina, and with minimal trauma to the cervical canal and endometrium. The equine embryo can be deposited either in the uterine body or in one of the uterine horns because the utero-ovarian control of luteolysis is systemic, which is distinct from ET in the cow, in which the embryo should be deposited ipsilateral to the CL due to local utero-ovarian control of luteolysis (Ginther, 1967; Mapletoft et al., 1976; McKinnon and Squires, 1988a). Recipient mares are commonly evaluated for pregnancy status approximately one week after embryo transfer and thereafter as for any pregnant mare, if pregnancy was established.

2.5.3. Estrus and ovulation synchronization

Demand for a reliable means of predicting or regulating estrus and ovulation in the mare has increased with the use of assisted reproductive techniques such as embryo transfer, artificial insemination, and other forms of appointment breeding (Squires et al., 2003a; Samper, 2008). In embryo transfer programs, donor and recipient mares' ovulations need to be closely aligned, and synchrony is a critical factor affecting pregnancy rates (McKinnon and Squires, 1988a; Squires et al., 1999; Carnevale et al., 2000). Numerous studies have established that recipients ovulating between one day before and 3 days after the donor are equally likely to become pregnant after receiving an embryo (Allen and Rowson, 1975; Squires and Seidel, 1995; Allen, 2005; Stout, 2006); outside this window of acceptable synchrony, recipient pregnancy rates may drop substantially.

Hormonal regimens used to synchronize estrus and ovulation in the mare include administration of prostaglandins (Douglas and Ginther, 1975b), and administration of progestogens alone or in combination with estradiol (Blanchard and Varner, 1995). These

regimens are commonly combined with administration of ovulation inducing agents such as hCG or GnRH analogue (Samper, 2008). Ultrasound-guided follicle aspiration/ablation has also been described as a method for synchronization of follicular wave emergence and ovulations; however, it may not be practical in the equine industry because the procedure is invasive (Bruck et al., 1992b; Bergfelt et al., 2007).

An alternative approach described for ensuring adequate synchrony of a recipient is the use of an ovariectomized or seasonally anestrous mare treated with progestogens, where treatment is initiated soon after the donor has ovulated (Hinrichs and Kenney, 1987; Hinrichs et al., 1987; Carnevale et al., 2000). This approach may be particularly useful early in the season when relatively few cycling recipients are available (Carnevale et al., 2000; Pessoa et al., 2005).

2.5.3.1. Estrus synchronization with prostaglandin

Prostaglandin F2α (PGF2α) and its analogues are the most affordable and widely used treatment to shorten the luteal phase and synchronize estrus in mares. The effectiveness of PGF2α in causing luteolysis in the mare and in other species is well established (Allen et al., 1974; Oxender et al., 1974; Douglas and Ginther, 1975b; Miller et al., 1976). The equine CL is sensitive to a single injection of prostaglandin by day 5 or 6 after ovulation, with some mares responding even sooner (Douglas and Ginther, 1975a; Bergfelt et al., 2006). Following PGF2α administration, most mares will show signs of estrus within 2 - 6 days. However, the time to estrus and ovulation varies significantly, depending on the follicular dynamics of the ovary. The great variations among mares in the number of follicular waves per cycle, time of follicular wave emergence, and estrous length, contribute to the variation in response to PGF2α administration. The diameter of the largest follicle at the time of luteolysis affects the interval to estrus and the interval from the onset of estrus to ovulation: larger follicles present at CL regression typically ovulate sooner, thus shortening the associated estrus period (Palmer, 1978). Therefore, although the mean interval to ovulation after single PGF2α treatment is reported to be between 7 and 10 days, ovulation may occur anywhere within 0 to 17 days (Douglas and Ginther, 1975b; Lofstedt, 1988; Blanchard and Varner, 1995; Raz et al., 2005). A protocol that may provide somewhat better synchronization among mares is the administration of two PGF2α injections 14 days apart, with the aspiration that all mares would be in diestrus at the time the second PGF is given (Blanchard and Varner, 1995).

This protocol, however, takes up a substantial portion of the breeding season, and may not be practical in all circumstances.

When PGF2α is used for estrus synchronization, it is common to have a ratio of at least two recipient mares for each donor mare. Also, it is strongly advised that mares are examined on the day they are given prostaglandin to determine the anticipated time frame for the onset of estrus and ovulation.

2.5.3.2. Estrus synchronization with steroid hormones: progesterone & estradiol

Artificial luteal phases have been used in attempts to control estrous cycles in mares (Blanchard and Varner, 1995). These treatments have been adapted from the cattle industry where they generally have good results (Bo et al., 2002; Martinez et al., 2002; Colazo et al., 2006; Colazo et al., 2007). However, in mares, progestogens alone do not work well for estrus synchronization (Lofstedt and Patel, 1989). Protocols include administration of progestogen (most commonly altrenogest) for 10 to 15 days, and PGF2α administration on the last day of the regimen (Daels et al., 1996). Following cessation of the treatment, most mares show estrus in 3 to 5 days, and ovulation usually occurs 9 to 11 days after the end of treatment. However, there is wide variability in the interval to ovulation among mares. This is because the time of ovulation depends on the stage of follicle growth when the treatment ends, and because the follicular phase is long and variable among mares. In addition, progestogens are not particularly effective at suppressing ovulation in mares, and mares may ovulate during the treatment (Daels et al., 1996). Evidence for this phenomenon can also be found in the fact that mares can ovulate spontaneously during the diestrus (Allen et al., 1974) and of course, they may ovulate or frequently luteinize follicles during pregnancy (forming accessory corpora lutea) when serum progesterone concentrations are very high (Hughes et al., 1973; Murphy and Martinuk, 1991). Therefore, progestogen regimens are most commonly used to suppress estrous behaviour, but not for estrus or ovulation synchronization (Crowell-Davis, 2007).

The administration of progesterone and estradiol-17β (P&E), daily for 10 days, followed by administration of prostaglandin on the last day of the P&E treatment, and hCG when a large preovulatory follicle is subsequently detected, is one of the most predictable hormonal regimens to synchronize ovulation in mares used in embryo transfer programs, particularly in situations when

only one or two recipients per donor are to be used (Blanchard and Varner, 1995; Stout, 2006). The P&E regimen is usually initiated at unknown or random stages of the estrous cycle. It provides better negative feedback on gonadotropin release than progestogen alone, which inhibits follicular development (Loy et al., 1981; Taylor et al., 1982b; Bristol et al., 1983). When the exogenous P&E therapy is discontinued, there is less diversity in follicular maturation, and the timing of ovulation can be more closely synchronized (Evans et al., 1982; Plata-Madrid et al., 1992). With this protocol, more than 75% of mares ovulate between 9 to 12 days after the last P&E injection.

Prostaglandin is always given at the end of the P&E treatment to lyse any pre-existing CLs and any CLs that may have formed during P&E treatment (Bristol et al., 1983; Blanchard and Varner, 1995). Corpora lutea formed after such ovulations might be too immature to undergo luteolysis after prostaglandin treatment on the final day of P&E treatment and with a 15-day life span; the progesterone from those CLs could interfere with the expected ovulation on day 9 to 12 after the end of treatment. The P&E treatment seldom fails to give good results but this phenomenon would explain some failures.

2.5.3.3. Estrus synchronization following follicular ablation

Transvaginal ultrasound-guided aspiration of antral follicles was initially developed as a non-surgical and repeatable procedure to collect ooctyes from horses (Bruck et al., 1992a; Squires and Cook, 1996) and cattle (Pieterse et al., 1988). Ultrasound-guided follicle aspiration/ablation has been used extensively as an approach to study the nature of follicular dynamics in horses and cattle, without the potential confounding effects of exogenous steroids (Ginther et al., 2001; Ginther et al., 2003; Ginther et al., 2004a), and to enhance ovulation synchronization among mares (Bergfelt and Adams, 2000; Bergfelt et al., 2007).

To date, there are two studies that critically examined the use of follicle aspiration/ablation for ovulation synchronization among mares. In a study reported by Bergfelt and Adams (2000), follicle aspiration/ablation at random stages of the estrous cycle resulted in renewed follicular growth within 3 days in 90% of the mares, and mean interval to ovulation of 13 d (ranged 7 to 17 d). In a more recent study (Bergfelt et al., 2007), commercial application of follicle ablation was compared to a conventional P&E synchronization regimen in mares. All follicles ≥10 mm in

diameter were ablated using trans-vaginal ultrasound-guided aspiration; four days after follicle ablation, two doses of PGF2α were given 12 h apart, and a single dose of hCG was given at a fixed time, 6 d after PGF2α treatment (expected follicle ≥30 mm). The rational for PGF2α administration was that follicle ablation in some mares results in an elevation in progesterone concentration presumably through the formation of luteal tissue, and that other mares have preexisting luteal tissue. Ultrasound-guided follicle ablation resulted in a significant reduction (39%) in the interval from the start of the synchronization regimen to ovulation compared to conventional P&E synchronization regimen. Furthermore, the degree of ovulation synchronization among mares was comparable to that of the conventional P&E synchronization regimen, with >80% of the mares ovulating within a 4 day period in both groups. The authors concluded that although ultrasound-guided follicle ablation may not be practical in all circumstances, it precludes the conventional 10-d regimen of P&E and is an efficacious and feasible, non-steroidal alternative for ovulation synchronization in mares during the estrous cycle.

2.5.3.4. Induction of ovulation with human chorionic gonadotropin (hCG), deslorelin, or recombinant equine luteinizing hormone (reLH)

To achieve ovulation at a predictable time, ovulation-inducing agents, such as hCG, GnRH analogue deslorelin acetate, or recombinant LH (rLH), may be given to mares expressing estrus signs and having a dominant follicle (≥35 mm). The treatment can be used alone, or following other hormonal regimens which were mentioned above.

Human chorionic gonadotropin is a hormone used as an ovulating inducing agent (Samper, 2008). The recommended dose of hCG ranges between 750 to 5000 IU in normally ovulating mares, and the most common dose given is between 2000 to 2500 IU (Harrison et al., 1991; McCue et al., 2007d; Morel and Newcombe, 2008). Approximately 75% of hCG-induced ovulations occur between 24 and 48 h after treatment (Duchamp et al., 1987; Barbacini et al., 2000); about 12% of mares ovulate within the first 24 h of treatment, whereas others ovulate >48 h post-treatment. Most of the mares that ovulate later than 48 h ovulate by 72 h (Samper et al., 2002). The use of hCG improves the efficiency of a breeding program by reducing the duration of estrus, decreasing the number of breedings at each estrus and providing a means of synchronizing ovulation with a stallion's breeding schedule (Vanderwall et al., 2001; Morel and Newcombe,

2008). Repeated use of hCG in the mare, however, has been reported to result in antibody formation, which has been variably reported to result in reduced responsiveness (Roser et al., 1979; Duchamp et al., 1987; Green et al., 2007). Season may also influence responsiveness to hCG and the interval to ovulation (Ginther, 1992b).

Deslorelin acetate is a potent GnRH analogue which induces pituitary LH release and ovulation. It is available in some countries as a biodegradable, short-term implant (Ovuplant™) containing 2.2 mg of deslorelin (McKinnon et al., 1993), or as a compounded liquid pharmaceutical product (Stich et al., 2004; Samper, 2008). Based on recent studies, induced ovulations with deslorelin occur predictably between 38 and 42 h after treatment (Vanderwall et al., 2001; McCue et al., 2007d). However, some mares treated with a deslorelin implant showed, a delayed return to the next natural estrus, which was attributed to prolonged stimulation, GnRH receptor downregulation, and reduction of gonadotropin secretions (Farquhar et al., 2001; Johnson et al., 2002b). This apparently may be prevented by removing the implant shortly after ovulation (Farquhar et al., 2002; McCue et al., 2002).

Recombinant equine LH product (Ovi-Stim™) has recently become available (Jablonka-Shariff et al., 2007; Yoon et al., 2007). It is a single chain gonadotropin that is of lower molecular weight than the two-chain hCG and therefore may be considered less antigenic. Plasma testosterone concentrations in Quarter Horse breeding stallions treated with reLH were fourfold higher compared to saline-treated controls (Jablonka-Shariff et al., 2007). When mares were treated with 0.75 or 0.9 mg of reLH, ovulation rates were 90 and 80%, respectively, which were similar to hCG treatment (85.7%) (Yoon et al., 2007), Samper 2008). It was therefore concluded that reLH is a reliable and effective ovulatory agent that does not significantly alter endogenous hormone profiles or affect interovulatory intervals.

2.5.4. Morphology and development of the equine embryo

In the mare, shortly after ovulation (Day 0), the unfertilized oocyte is picked up by the infundibulum of the oviduct (uterine tube) and is presumed to be rapidly transported to the ampullary isthmic junction where fertilization takes place (reviewed by Ginther, 1992b; Vanderwall, 1996). The first cleavage division typically occurs by 24 h postovulation, with subsequent divisions occurring in 12 to 24 h intervals (Bezard et al., 1989; Vanderwall, 1996).

After the first cleavage division, individual cells are termed blastomeres. In the oviduct, continued cell divisions, and later, tight junction formations, lead to the formation of a compact morula. The compact morula is a compact mass of ≥32 blastomeres, which is the last developmental stage typically found in the oviduct. There is selective transport of the embryo through the mare's oviduct (Vanderwall, 1996). In the case of failed fertilization, the unfertilized oocyte is normally retained in the oviduct and does not reach the uterine lumen (Betteridge and Mitchell, 1972; Onuma and Ohnami, 1975; Flood et al., 1979). In case of fertilization and normal development in the oviduct, the transportation of the equine embryo through the mare's oviduct takes approximately 5 to 6 days postovulation (Freeman et al., 1991). This selective transport of the fertilized oocyte that forms the embryo is associated with prostaglandin E2 production by the embryo (Weber et al., 1991b; a). The rhythmic changes in the oviduct and uterus, including gross, histologic, contractile and secretory changes, are under the control of ovarian steroids, and are in physiologic harmony with the stage of the estrous cycle and developmental transitions of the oocyte and embryo (Ginther, 1986; 1992b).

The earliest normal embryonic developmental stage harvested from the uterus is a morula. After entering the uterus, active division of blastomeres continues, and the morula develops to a blastula. This development of the blastula is characterized by the formation of a fluid-filled cavity (blastocoele) within the morula, and rearrangement of cells into two distinct populations: 1) trophoblast, a single layer of cells of ectodermal origin which line the blastocoele, and eventually contribute to the formation of the placenta; and 2) the inner cell mass (ICM), which is a population of cells at one pole that projects into the blastocoele, and is the forerunner of the embryo proper. During the initial stages of blastocoele development, the conceptus is referred to as an early blastocyst. Once the blastocoele is fully formed, the size of the conceptus starts to increase dramatically (Table 2.1), and the conceptus is referred to as an expended blastocyst (Squires 1985). After the blastocoele, the ICM, and the trophoectoderm are formed, the endoderm begins to develop.

When equine embryos enter the uterus they are at the late morula or early blastocyst stage of development and are still surrounded by the zona pellucida (Betteridge et al., 1982; Flood et al., 1982). Soon after, coinciding with the formation of the blastocoele, an acellular glycoprotein capsule is formed as a thin layer between the trophectoderm and the zona pellucida (Flood et al.,

1979; Betteridge et al., 1982; Vanderwall, 1996). During the next 24 h, the zona pellucida decreases markedly in thickness before rupturing to allow the blastocyst, now completely enclosed within its capsule, to hatch (Flood et al., 1982). The capsule first becomes visible around Day 6.5, thickens until at least Day 11, and disappears after Day 22 (Flood et al., 1982; McDowell et al., 1988; Oriol et al., 1993). The capsule plays a significant role in embryonic survival (Tremoleda et al., 2003; Stout et al., 2005). It is believed that the capsule provides mechanical support to maintain the spherical shape of the conceptus (Ginther, 1984; Bousquet et al., 1987; Allen and Stewart, 2001; Betteridge, 2007), is involved in normal maternal recognition of pregnancy (McDowell et al., 1988; Oriol et al., 1993), and protects the embryo from intrauterine microorganisms and against maternal immunological recognition and attack (Crossett et al., 1998; Herrler et al., 2000; Betteridge, 2007).

In embryo transfer programs, morphologic evaluation of the recovered embryos is performed routinely using a stereomicroscope (magnification range of 10x to 50x) in order to assess embryonic viability. Morphologic features include shape, color, size, developmental stage (morula/ early blastocyst/ expanded blastocyst), compactness of blastomeres, presence of extruded and damaged blastomeres, size of the perivitelline space, and damage to the zona pellucida or to the capsule (McKinnon and Squires, 1988b; a). McKinnon and Squires (1988b) described a scoring system of 5 categories (Table 2.2: 1- excellent, 2- good, 3- fair, 4- poor, 5- unfertilized or dead). However, a four-point scoring system it is now most commonly used, which eliminates category 5 (unfertilized or dead); unfertilized oocytes were removed from the scoring system, and dead or severely degenerated embryos are included in category 4 (Vanderwall, 1996; Squires et al., 1999; Carnevale et al., 2000; Stout, 2006).

Table 2.1 Diameter of equine embryos recovered from the uterine lumen on days 6 - 9 post ovulation.

Day Post-Ovulation	Number of embryos	Embryo Diameter (mm)	
		Mean	Range
6	121	0.208	0.132-0.756
7	144	0.406	0.136-1.460
8	142	1.132	0.120-3.980
9	41	2.220	0.730-4.520

*Adopted from Squires et al., 1985a

Table 2.2 Classification system used to grade equine embryo quality**

Grade	Description
1	**Excellent**: an ideal embryo; spherical; with cells of uniform size, color, and texture.
2	**Good**: minor imperfections, such as a few extruded blastomeres; irregular shape; or trophoblastic separation
3	**Fair**: definitive, but not severe problems; presence of extruded blastomeres; degenerate cells; or collapsed blastocoele.
4	**Poor**: severe problems; collapsed blastocoele; numerous extruded blastomeres; degenerated cells but with a viable-appearing embryonic mass.
5	**Unfertilized or dead**: unfertilized oocytes or totally degenerate embryos.

**Adopted from McKinnon and Squires, 1988b

Light microscopic evaluation only reveals gross abnormalities in embryo morphology. However, it is a valuable tool to evaluate the likelihood of pregnancy in the recipient (McKinnon and Squires, 1988b; a; Squires et al., 1999), and the incidence of subsequent pregnancy loss (Carnevale et al., 2000). The vast majority of equine embryos recovered from mares with single ovulations are of good morphological quality (>90% grade 1 or 2)(McKinnon and Squires, 1988a; Squires et al., 1999; Carnevale et al., 2000; Squires et al., 2003a; Stout, 2006). It was reported that embryos graded as 3 or 4 (fair to poor) were less likely to result in pregnancy than grade 1 or 2 embryos (Squires et al., 1999; Carnevale et al., 2000). In addition, pregnancies resulting from embryos with a quality score of 2 or less are more likely to fail by Day 50 (Carnevale et al., 2000). Furthermore, Carnevale et al. (2000) found that morulae or very small blastocysts recovered on Day 7 or 8 post ovulation were less likely to result in a viable pregnancy, thereby emphasizing that developmental retardation may be a sign of poor embryo quality even if gross morphology is otherwise reasonable (Squires et al., 1999; Carnevale et al., 2000). Possible predispositions to reduced embryo quality may include: unresolved post breeding endometritis, advanced maternal age, genetic or developmental embryonic defects, abnormalities affecting the environment in the oviduct or uterus responsible for nourishment, as well as treatment protocols and embryo handling (Squires and Seidel, 1995; Squires et al., 1999; Carnevale et al., 2000; Stout, 2006; Paccamonti and Carnevale, 2008).

2.5.5. Factors affecting the success of embryo recovery

In cattle embryo transfer programs, response to superovulatory treatment by the donor cow is probably the most important factor affecting embryo recovery (reviewed by Mapletoft et al., 2002). However, in mares, the vast majority of embryos are being recovered from non-superstimulated mares, as superovulatory treatments are less effective. Important factors affecting the likelihood of recovering an embryo from a donor mare are the breeding management and the intrinsic fertility of the donor mare and stallion (Squires et al., 1999; Stout, 2003). Embryo recovery is usually higher in young fertile donor mares as compared to aged mares (>15 years), or as compared to those with a history of subfertility (Squires et al., 1982; Vogelsang and Vogelsang, 1989; Squires et al., 1999; Squires et al., 2003a). When artificial insemination is employed, stallion fertility, semen dose, quality and preservation further influence embryo recovery (Squires et al., 1985b; Colenbrander et al., 2003); the use of frozen-thawed semen, in particular, is less

efficacious than that of chilled transported or fresh semen (Stout, 2003; Stout, 2006). The day post ovulation on which the embryo recovery attempt is performed influences embryo recovery, with lower embryo recoveries reported on Day 6 as compared to Day 7 or 8 (Iuliano et al., 1985; Squires et al., 1985b; Squires et al., 1999); this is because the equine embryo may be still in the oviduct on Day 6. Furthermore, a recent study indicated that exercising donor mares in a hot and humid environment may be associated with reduction in embryo recovery, and reduced embryo quality (Mortensen et al., 2009).

2.5.6. Factors affecting the success of embryo transfer (recipient pregnancy rate)

It is recommended that recipients are relatively young (3-12 years), in good health and body condition, and with healthy reproductive organs (Squires et al., 1985b; McKinnon and Squires, 1988a; Squires et al., 1999; Stout, 2006). Old recipient mares are predisposed to endometrial degeneration, which may compromise the mare's ability to carry pregnancy to term, and theretofore should not be used (Squires et al., 1985b; Vogelsang and Vogelsang, 1989; Ricketts and Alonso, 1991; Rambags et al., 2003). Assuming proper selection of recipient mares, embryo quality and donor-recipient ovulation synchrony are probably the two most important factors affecting the likelihood of pregnancy in the recipient mare following ET. As mentioned above (section 2.5.4.), embryo quality, as assessed by light microscopic gross morphology, is correlated with transfer success; the transfer of grade 1 or 2 embryos is significantly more likely to result in pregnancy as compared to transfer of grade 3 or 4 embryos (Squires et al., 1999; Carnevale et al., 2000). The use of recipients ovulating between one day before and 3 days after the donor is the most commonly recommended window of acceptable synchrony (McKinnon and Squires, 1988a; Squires et al., 1999; Carnevale et al., 2000); outside this window recipient pregnancy rates may drop substantially (for more information please refer to section 2.5.3.).

The techniques used for trans-cervical embryo transfer may also be an important factor if the transfer results in bacterial contamination of the progesterone-dominated uterus (Allen, 2005), and if PGF2α (Kask et al., 1997) or oxytocin (Handler et al., 2003) are released following excessive dilation or manipulation of the cervix. Therefore, it is not uncommon for operators to routinely treat recipient mares with systemic antibiotics pre- and post transfer, non-steroid anti-inflammatory drugs before transfer, or progestogens for a few weeks after transfer (Allen, 2005;

Stout, 2006). However, the high pregnancy rates achieved by experienced operators in the absence of any supportive treatments suggest that such treatments may be unnecessary if technique is performed properly (Squires et al., 1985b; Carnevale et al., 2000; Jasko, 2002; Stout, 2006). However, a recent study indicated that embryo transfer induces a subclinical endometritis in recipient mares which can be prevented by treatment with non-steroid anti-inflammatory drugs such as meclofenamic acid or flunixin meglumine (Koblischke et al., 2008). Further studies should be performed to identify factors affecting the likelihood of pregnancy establishment in recipient mares following embryo transfer.

2.6. Induction of multiple ovulations (superovulation) in the mare

Effective and reliable treatments to induce multiple ovulations or superovulation are of interest to the equine industry because they may increase reproductive efficiency in cycling and seasonally anovulatory mares. Superovulatory treatments may potentially increase embryo recovery and success rates of embryo transfer programs. Superovulatory treatment may be beneficial for other types of assisted reproductive technologies in the horse, including oocyte transfer and gamete intrafallopian transfer (Hinrichs, 1998; Squires et al., 2003a). Multiple ovulations may also increase the per-estrous cycle pregnancy rate in normal or subfertile mares as superovulatory treatments may increase the number of oocytes available for fertilization and may increased the amount of progesterone produced by the ovaries, which may be advantageous for the establishment of pregnancy (Bergfelt et al., 1992). Increasing the ratio between the number of oocytes and number of spermatozoa may also increase pregnancy rates in mares bred to a stallion with oligozoospermia, or subfertility (Squires et al., 2003b; Logan et al., 2007a). Furthermore, superovulatory treatment may potentially stimulate follicular growth and fertile ovulations in seasonally anovulatory mares, and by that hasten and extend the period of reproductive productivity (e.g. embryos, foals) in a given year.

Historically, superovulatory treatments have been elusive in the mare and less efficient than in other domestic species (Woods and Ginther, 1983a; Mapletoft et al., 2002; Cognie et al., 2003). Over more than 35 years, a number of treatments have been tested for their ability to stimulate the development of multiple follicles in either cyclic or seasonally anovulatory mares, mostly with limited success; these include equine chorionic gonadotropin (Day, 1939; 1940; Allen, 1982;

McCue, 1996), GnRH (Johnson and Becker, 1988; Ginther and Bergfelt, 1990), active or passive immunization against inhibin (McCue et al., 1992a; McKinnon et al., 1992; Terhaar et al., 1997), porcine pituitary FSH preparations (Irvine, 1981; Squires et al., 1986; Fortune and Kimmich, 1993), and recombinant human FSH (Tharasanit et al., 2006). To date, however, gonadotropins extracted from equine pituitary glands and purified equine FSH (eFSH) have proven to be the most effective stimulants of recruitment of multiple dominant follicles and ovulation (reviewed by Squires and McCue, 2007).

2.6.1. Equine chorionic gonadotropin (eCG)

Equine chorionic gonadotropin is produced by the trophoblastic cells in the endometrial cups of the pregnant mare, and is believed to act as an LH-like hormone to induce supplementary ovulation and/or luteinization of follicles (Murphy and Martinuk, 1991; Hoppen, 1994). In other species, eCG has a dual LH:FSH activity, and it is widely used to superstimulate animals. However, in the mare, eCG exhibits only LH activity. In spite of the amino acid homology between eCG and equine LH (eLH), the LH-like activity of eCG in the mare is much lower than that of eLH. Unfortunately, eCG injected even in large amounts has not been successful in inducing superovulation in mares (Day, 1939; 1940; Allen, 1982; McCue, 1996).

2.6.2. GnRH and GnRH analogues

A higher incidence of multiple ovulations following GnRH or GnRH analogue treatment regimens employed in seasonal anovulatory mares have been reported by some investigators (Johnson and Becker, 1988; Ginther and Bergfelt, 1990); but not by others (Palmer and Quellier, 1988; Harrison et al., 1990; McCue et al., 1992b; McKinnon et al., 1997). Johnson and Becker (1988) reported that the administration of GnRH in an intravenous pulsatile pattern stimulated multiple ovulations in seasonally anovulatory mares (two to seven ovulations in 11 of 14 mares treated with 20 or 100 μg/h); ovulation rate was related to the dose of GnRH administered, with the highest dose (100 mg/h) resulting in 3.5 ovulations. Ginther and Bergfelt (1990) reported that vernal transitional mares receiving a GnRH agonist twice daily at a dose of 100, 200, or 400 mg exhibited multiple ovulation (2 or 3 ovulations) rates of 24%, 32%, and 37%, respectively.

However, administration of GnRH to cycling mares was not effective in inducing multiple ovulations (Squires et al., 1989).

2.6.3. Immunization against inhibin

Inhibins are gonadal glycoprotein hormones selectively and potently inhibiting FSH secretion from the pituitary gland (Medan et al., 2007). Passive (Nambo et al., 1998) and active (McCue et al., 1992a; McKinnon et al., 1992; Terhaar et al., 1997) immunization of mares against inhibin has been shown to induce multiple ovulations; this effect was attributed to the increase in FSH concentrations that followed the reduction in circulating inhibin. Following intravenous administration of 100 or 200 mL of inhibin antiserum per mare, mean ovulation numbers were 3.75 and 4.5 ovulations per mare, respectively (Nambo et al., 1998). Following active immunization against the α-subunit of inhibin, the mean number of ovulations varied from 1.4 to 2.8 ovulations per mare. In one study Day 7 embryo recovery rate tended to be higher in immunized mares (1.6 embryos) than in control mares (0.7 embryos) (McCue et al., 1992a).

Despite these encouraging results, passive and active immunization against inhibin are unacceptable approaches to induce multiple ovulations in the equine industry (Squires and McCue, 2007). Administration of inhibin antiserum may carry significant biosecurity risk, and it is not a practical method. The use of active immunization of mares against inhibin required multiple inoculations over several weeks; and adverse reactions, ranging from mild tissue swelling to abscessation, occasionally occurred at the injection site (Squires, 2006b). In addition, one study reported abnormal ovarian function in some of the immunized mares, such as delayed ovulations, anovulatory haemorrhagic follicles, follicle atresia, and ovulation without behavioural estrus; this appeared to be more frequent in the animals with high titres (Terhaar et al., 1997). Additional research may further explore the potential benefit of immunization against inhibin in mares.

2.6.4. Porcine FSH

Different forms of porcine FSH (pFSH), obtained from pig pituitaries collected at an abattoir, have been reported to mildly increase the number of ovulations (Irvine, 1981; Squires et al., 1986; Fortune and Kimmich, 1993) and the rates of embryo recovery in cycling donor mares (Veselinovic et al., 1994; Krekeler et al., 2006), as compared to non-treated controls. Irvine (1981)

treated four mares with pFSH and reported 1.7 ovulations per treated mare, and 1.0 ovulation per control untreated mare. Squires et al. (1986) compared the response of mares to injection of equine pituitary extract (EPE; primarily FSH and LH) to that of pFSH (6.4 and 6.8 days of treatment, respectively); mares treated with pFSH obtained only 1.6 ovulations, compared to 2.2 ovulations for mares given EPE. Similar differences were reported by Veselinovic et al. (1994), who found an average of 1.25 ovulations per pFSH-treated mare, 3.25 ovulations per EPE-treated mare, and 1.0 ovulation per control mare. Fortune and Kimmich (1993) evaluated various doses (8 – 32 mg, twice daily) of pFSH for induction of multiple ovulation in mares, and reported mean ovulation numbers that ranged from 1.5 to 1.8 per mare, with 50–83% of mares having double ovulations. Recently, Krekeler et al. (2006) treated donor mares with a commercial preparation of pFSH (25 mg, twice daily, Folltropin®-V, Bioniche Animal Health Inc.) and obtained 2.3 ovulations and 1.3 embryos per mare. However, the use of pFSH as a superovulatory treatment is not widely accepted in the equine industry since the results were moderate, and inferior to those obtained with gonadotropins of equine-origin (EPE and equine FSH) (Squires et al., 1986; Veselinovic et al., 1994; Squires and McCue, 2007).

The effect of pFSH on vernal transitional mares was recently evaluated by us (Raz et al., 2009a); the results are presented in Chapter 11.

2.6.5. Recombinant human FSH

A recent study investigated the effects of an LH-free FSH preparation, recombinant human follicle stimulating hormone (rhFSH), on follicle development, ovulation, and embryo production in mares (Tharasanit et al., 2006). Mares were treated twice daily with 450 or 900 I.U. rhFSH starting on day 6 after ovulation, coinciding with PGF2α analogue administration. When the dominant follicle(s) exceeded 35 mm, ovulation was induced with hCG; embryos were recovered on day 7 after ovulation. As compared to control, neither dose of rhFSH altered the number of days before the dominant follicle(s) reached 35 mm, the number of follicles of any size class (10-25, 25-35, >35 mm) at ovulation induction, the pre- or post-ovulatory estradiol-17β or progesterone concentrations, the number of ovulations, or the embryo yield. It was therefore concluded that rhFSH, at the doses used, was insufficient to stimulate multiple follicle development in mares.

2.6.6. Equine pituitary extract, and equine FSH

Most studies on the induction of multiple ovulations in the mare have utilized equine pituitary extract prepared according to the procedures described by Braselton and McShan (1970) and later by Guillou and Combarnous (1983). This later procedure yielded approximately 6 g of crude pituitary extract/kg of pituitaries, which contained approximately 6–10% LH and 2–4% FSH.

Early studies signaled the superovulatory potential of EPE when averages of 1.7 – 3.8 ovulations per cycle were induced in mares treated during either the breeding season or the spring transitional period (Douglas et al., 1974; Lapin and Ginther, 1977). Woods and Ginther (1983a) examined several EPE treatment protocols in cycling mares; overall, 70% of the 112 EPE-treated mares had multiple ovulations, for an average of 3.0 ovulations per mare. They reported that ovulation rate was lower when EPE treatment was initiated at day 19 than when it was initiated at day 11 or 15 post-ovulation; and that including hCG as ovulation induction agent in the protocol was beneficial as it decreased the interval to ovulation(s).

Initial attempts to improve the effectiveness of EPE for induction of superovulation in cyclic mares focused on a once daily frequency of EPE treatment; however, a later study conducted by Alverenga et al. (2001) presented a modification of the EPE administration regimen to encompass twice daily injections beginning on day 5–6 after ovulation (i.e. before the emergence of the mid-cycle dominant follicle); this was reported to markedly improve the ovulatory response (7.1 ovulations/cycle) and embryo recovery (3.5 embryos/cycle) in donor mares. However, it was not known from that study whether the increased response was due to the frequency of injection or the total amount of hormone injected daily (25 mg versus 50 mg). A subsequent study which compared once versus twice daily EPE administration yielded less dramatic results (Scoggin et al., 2002); mares were administered either 25 mg of EPE once or twice daily, or 12.5 mg of EPE once or twice daily. The number of ovulations per mare was higher in mares administered 25mg of EPE twice daily (4.7 ovulations /cycle). However, the number of embryos recovered per mare was higher in mares given 12.5 mg of EPE twice daily (2.6 embryos/cycle). Based on these studies, it is recommended that EPE is administered twice daily to mares.

There is conflicting information regarding the effect of EPE on equine embryo viability, with some studies suggesting that embryos collected from superovulating mares were less viable than

those from single-ovulating mares (Woods and Ginther, 1982; 1983b; Woods, 1984). Furthermore, ovulation and embryo recovery per ovulation in studies that utilized EPE varied significantly, possibly due to failure of fertilization, abnormal oocyte transfer, or perhaps high embryonic loss in the oviduct (Douglas et al., 1974; Woods and Ginther, 1982; Palmer et al., 1993; Alvarenga et al., 2001; Carmo et al., 2006; Squires and McCue, 2007). This variation, at least in part, may be related to the fact that the amount of LH in the EPE varies considerably and, in many studies, the exact FSH to LH ratio was not reported. In cattle, a high concentration of LH in the FSH preparation generally results in a poor ovulatory response (Kanitz et al., 2002; Mapletoft et al., 2002). Thus, two studies were conducted in which EPE was purified further to provide a lesser contamination of LH in the FSH preparation. Hofferer et al. (1993) and Rosas et al. (1998) compared the ovulatory response of mares given EPE to that of mares given an enriched FSH preparation containing less LH contamination. There were no differences in the ovulatory response (Hofferer et al., 1993; Rosas et al., 1998) or in embryo recovery (Rosas et al., 1998) between mares administered the enriched FSH preparation as compared to EPE. Thus, further research should be conducted to clarify the effect of superovulatory treatment on follicular growth, ovulation, and embryo viability and development.

Equine pituitary extract was not commercially available until recently. In 2003, a purified equine pituitary extract product called equine follicle-stimulating hormone (eFSH®, Bioniche Animal Health Inc., Athens, GA, USA) became available (Niswender et al., 2003); this product was developed to induce a successful superovulatory treatment in mares. The present thesis presents results of several studies conducted at the University of Saskatchewan to examine the effects of eFSH on the fertility of mares (Raz et al., 2005; Raz et al., 2006a; Raz et al., 2006b; Raz et al., 2009a; Raz and Card, 2009; Raz et al., 2009b; Raz et al., 2009c; Raz et al., 2009d). Prior to the initiation of our work, there were only two published studies in which eFSH® treatment was utilized (Niswender et al., 2003; Niswender et al., 2004).

In a report by Niswender et al. (2003), cycling mares were treated by twice daily injection of eFSH in two experiments. In the first experiment, eFSH treatments were initiated 5 or 6 days after ovulation, and PGF2α was administered on the second day of eFSH treatment. The eFSH treatment ceased when the majority of follicles reached a diameter ≥35 mm, at which time an ovulation induction agent was administered. The eFSH treatment was utilized in a total of 20

mares; 5 mares were treated with a standard dose of eFSH (12 mg, twice daily) followed by hCG (SD-eFSH-hCG), 5 mares were treated with a standard dose of eFSH followed by deslorelin (SD-eFSH-Des), and 10 mares were treated with a double dose of eFSH (25 mg, twice daily) followed by deslorelin (DD-eFSH-Des). Twenty-nine control mares were used, in which eFSH was not given, but deslorelin was administered as an ovulation inducting agent. All mares were bred with semen from 1 of 4 stallions, and pregnancy status was determined ultrasonographically per rectum at 14 to 16 days after ovulation. Mean (±SEM) number of ovulations per mare was 3.4 ± 0.7, 1.8 ± 0.7, 3.3 ± 0.9, and 1.1 ± 0.1 ovulations for the SD-eFSH-hCG, SD-eFSH-Des, DD-eFSH-Des, and control groups, respectively. The proportions of mares which became pregnant were not reported in this study. Instead, mean embryo number ("pregnancies per mare") were reported as 1.8 ± 0.7, 0.8 ± 0.5, 0.6 ± 0.3, and 0.6 ± 0.1 embryos/mare, respectively. Mean embryo number in the 5 mares in the SD-eFSH-hCG group was significantly higher than the control; however, eFSH treatment in the other 15 eFSH-treated mares did not increase the number of embryos obtained. In the second experiment by Niswender et al. (2003), donor mares (n =16) were used in two consecutive estrous cycles, in which the first cycle served as a control, while the eFSH treatment protocol was utilized in the second cycle (twice daily 12 mg of eFSH, followed by hCG). The number of ovulations (3.6 ± 0.5 vs. 1.0 ± 0.0 ovulations/cycle) and the number of embryos recovered (1.9 ± 0.3 vs. 0.5 ± 0.1 embryos/cycle) were significantly higher in mares treated with eFSH.

In a study conducted by Niswender et al. (2004) the effect of eFSH treatment in vernal transitional mares was examined. Treatment with eFSH (12 mg twice daily) was initiated when a follicle ≥25 mm was detected and ceased when one or more follicles reached ≥35 mm diameter, at which time hCG was administered. Administration of eFSH followed by hCG resulted in ovulation in 8/10 (80%) of the eFSH-treated mares. Mean ovulation number was 2.5 ± 1.7 per mare. In 5 out of the 8 ovulating mares more than one ovulation was detected. Fertility was not evaluated in that study as mares were not bred.

Information regarding further studies which examined the use of eFSH as superovulatory treatment will be included in the subsequent chapters (Raz et al., 2005; Raz et al., 2006a; Raz et al., 2006b; Welch et al., 2006; Logan et al., 2007b; Peres et al., 2007; McCue et al., 2008; Raz et al., 2009a; Raz and Card, 2009; Raz et al., 2009b; Raz et al., 2009c; Raz et al., 2009d).

2.6.7. Recombinant equine FSH

Novel single-chain recombinant equine gonadotropins have recently been developed (Aspen Bio Pharma, Castle Rock, CO, USA). Currently there is a single preliminary report about the effect of recombinant equine FSH (reFSH) in mares, which infers that reFSH treatment in mares increases the number of preovulatory-sized follicles and ovulations (Niswender et al., 2007). Using a recombinant equine follicle stimulating hormone product to stimulate follicular development may offer the advantage of using a pure FSH product without the protein contaminants or LH activity of equine pituitary extract. However, further studies should be conducted to determine the effect of this reFSH product in mares. Currently, reFSH is not commercially available.

3. GENERAL HYPOTHESIS

Purified equine follicle stimulating hormone (eFSH) treatment

1- stimulates follicular growth and multiple ovulations in cycling mares and in vernal transitional mares.
2- increases reproductive efficiency in terms of pregnancy and donor embryo recovery rates.
3- alters the hormonal environment, follicular growth, ovulation, and embryo parameters.

4. GENERAL OBJECTIVE

The overall objective of the experiments reported herein was to examine the effects of equine FSH treatments on the fertility of mares.

Specific objectives for each section of this thesis are indicated in the introduction preceding each study.

5. FOLLICULOGENESIS, EMBRYO PARAMETERS, AND POST-TRANSFER RECIPIENT PREGNANCY RATE IN eFSH-TREATED DONOR MARES

This study was presented at the 52nd Annual Conference of the American Association of Equine Practitioners in 2006

5.1. Abstract

Induction of multiple ovulations, or superovulation, may potentially increase the efficiency of equine embryo transfer programs. The objectives of this study were to investigate the effects of eFSH on folliculogenesis, ovulations, embryo recovery, embryo quality, and subsequent recipient pregnancy rates. Draft-cross donor mares (n = 12) were used during the physiologic breeding season in Canada. On the first cycle, donor mares served as controls; and on the second cycle, a twice daily eFSH treatment was initiated when a follicle ≥25 mm in diameter was detected by trans-rectal ultrasonographic examination and ceased when a follicle reached ≥35 mm in diameter. On both cycles, donor mares were treated with hCG when a follicle ≥35 mm was detected, and subsequently were artificially inseminated with fresh semen. Embryo recovery attempts were performed 8 days after ovulation. Each embryo was transferred non-surgically, trans-cervically to the uterine body of a synchronized recipient mare, and pregnancy status was evaluated 7 - 10 days later by trans-rectal ultrasonographic examination. In the control cycle, all donor mares (12/12, 100%) developed at least one follicle ≥35 mm in diameter, and ovulated subsequent to hCG administration. In the eFSH cycle, all donor mares (12/12, 100%) developed at least one follicle ≥35 mm in diameter after 5.3 ± 0.8 d of eFSH treatment; however, two mares failed to ovulate. The number of preovulatory follicles (≥30 mm) at the time of hCG administration was significantly higher in the eFSH cycle (eFSH: 3.8 ± 0.6 vs. Control: 1.4 ± 0.1 follicles/cycle). In mares that ovulated, the number of ovulations was higher ($P < 0.05$) in the eFSH cycle (2.8 ± 0.5 ovulations/cycle; vs. 1.4 ± 0.1 ovulations/cycle). Mean number of embryos per recovery attempt was higher ($P < 0.05$) in the eFSH cycle (1.5 ± 0.3 vs. 0.5 ± 0.2 embryos), however, mean embryo morphology grade (1-excellent, 4-poor) tended to be lower (2.7 ± 0.2 vs. 1.8 ± 0.3, $P = 0.06$). The transfer of 6 embryos recovered from the control cycle resulted in a pregnancy in 4 (67%)

recipient mares. The transfer of 15 embryos recovered from the eFSH cycle resulted in a pregnancy in 5 (33%) recipient mares. In summary, the eFSH treatment stimulated the ovary, and resulted in a greater number of recovered embryos; however, the recovered embryos tended to have a lower embryo morphological grade as compared to control, and the recipient pregnancy rate per transferred embryo was lower than anticipated in commercial embryo transfer programs. The number of recipient pregnancies that resulted from the eFSH treatment was not different from that obtained with the control treatment.

5.2. Introduction

The use of embryo transfer and the development of embryo technologies for the horse have increased steadily over the past three decades (Squires et al., 2003a). Embryo transfer offers distinct advantages to mare owners who wish to maximize the number of foals from a particular mare, and also may be useful for mares when carrying a foal to full term is neither desirable nor possible. However, the high cost of equine embryo transfer dictates that only genetically superior animals should be used as donors. Contributing to the expense of embryo transfer are the unique biological features and technical problems that must be overcome in mares. Currently, single embryo recovery attempts are common in equine-embryo transfer. A 50% embryo recovery rate per ovulation and a 50 – 65% pregnancy rate per transferred embryo are expected in commercial embryo transfer operations, for a total success rate of 25% to 40% per-cycle (Squires et al., 1999; Squires et al., 2003a; Stout, 2006). Economically, there is a need to increase the success rate of equine embryo transfer.

Mares that spontaneously double ovulate during a given cycle have higher embryo recovery rates than single ovulating mares (Squires et al., 1987b). Therefore, the induction of multiple ovulations, or superovulation, may potentially increase the efficiency and decrease the cost of embryo transfer by increasing embryo collection rates (McCue, 1996). Superovulation could also be beneficial for other types of assisted reproductive technologies in the horse, including oocyte transfer and gamete intrafallopian transfer (Hinrichs, 1998; Squires et al., 2003a). However, historically, superovulatory treatments have been elusive in the mare and less efficient than in other domestic species (Woods and Ginther, 1983a; Mapletoft et al., 2002; Cognie et al., 2003). Porcine FSH, eCG, GnRH and inhibin vaccines have been of limited success in stimulating

multiple ovulations (McCue, 1996; Squires, 2006a). Equine pituitary extract has been reported to stimulate multiple ovulations in mares but was not commercially available. Recently, a purified pituitary extract product called equine follicle-stimulating hormone (eFSH) has been investigated and was reported to increase ovulation rate and embryo-recovery rate of cycling donor mares (Niswender et al., 2003).

The objectives of this study were to investigate the effects of eFSH on folliculogenesis, number of ovulations, embryo recovery, embryo quality, and subsequent recipient pregnancy rates as compared with control. We hypothesised that 1) eFSH treatment would increase number of preovulatory follicles, ovulations, and embryo recovery rate, and that 2) post transfer recipient pregnancy rate would be similar for embryos recovered from eFSH-treated donor mares to those recovered from controls.

5.3. Materials and methods

5.3.1. Animals and reproductive tract examinations

Twelve donor mares and thirty-seven recipient mares obtained from a commercial herd were used during the physiologic breeding season (May to August) in the research facility of the University of Saskatchewan in Canada, which is located at 52°07′ latitude in the Northern Hemisphere. All mares were of a body condition score of at least 5 on a 9 point assessment system, and had no signs of systemic disease or lameness. Donor mares (n =12) were draft-cross type, aged 4 to 16 years (Mean ± S.E.M: 8.9 ± 1 years). Recipient mares (n = 37) were draft-cross or Quarter Horse-cross type, aged 4-10 years. Mares were kept in outdoor paddocks with sheds, and were maintained on alfalfa and grass hay with access to water and trace-mineralized salt, in accordance with the University of Saskatchewan's Institutional Animal Care and Use Committee. Examinations of the reproductive tracts were performed using a trans-rectal approach and included manual palpation followed by an ultrasonographic examination. A B-mode ultrasonographic scanner (GE Ausonics, Universal Ultrasound, Bedford Hills, NY, USA) equipped with a 5.0 MHz linear-array transducer was used to measure follicle diameters, and to detect ovulation(s).

5.3.2. Experimental design and treatments

To synchronize estrus, donor mares were administered intramuscularly a compounded product containing 150 mg of progesterone and 10 mg of estradiol-17β (P&E) in sesame oil (WCVM Veterinary Pharmacy, University of Saskatchewan, Saskatoon, SK, Canada), daily, for 8 - 11 days. The P&E treatments were initiated on a predetermined date, regardless of the physiologic status of the mare (estrus/diestrus). Prostaglandin F2α (PGF2α, 5 mg s.c., Lutalyse®, Pharmacia Animal Health, Orangeville, ON, Canada) was administered on the final day of P&E regimen. Donor mares were then used in two consecutive cycles. The first cycle served as a control to the second cycle, in which eFSH was administered.

In the first control cycle, mares were monitored into estrus, and when a follicle ≥35 mm in diameter was detected, hCG (Chorulon®, Intervet Canada Ltd, ON, Canada; 2000 IU, i.m.) was administered to induce ovulation. Donor mares were artificially inseminated 24 h after hCG was administered, and again every 48 h until ovulation (Day 0 = day of ovulation) with fresh semen collected from a stallion of proven fertility. A minimum dose of 5×10^8 progressively motile spermatozoa extended 1:1 with Kenney extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA, USA) was used to inseminate the mares. Eight days after ovulation, embryo recovery attempts were performed. Mares received PGF2α just after the first embryo recovery attempt was completed, and were assigned to the second, eFSH cycle.

In the eFSH cycle, donor mares were examined daily. When a follicle ≥25 mm in diameter was detected in a mare, a twice daily eFSH treatment (12.5 mg i.m., eFSH®, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) was initiated. The eFSH treatment was ceased when at least one follicle ≥35 mm in diameter was detected, at which time hCG was administered. Subsequently, the mares were bred and embryo recovery attempts were performed as in the control cycle.

In both cycles, donor mares with post-breeding uterine fluid accumulations were treated as needed using uterine saline lavage and/or antibiotics (intra-uterine or systemic) and/or oxytocin injections.

5.3.3. Embryo recovery, handling and transfer

Embryo recovery attempts were performed 8 d after ovulation using a routine nonsurgical trans-cervical technique as described elsewhere (McKinnon and Squires, 1988a). A total of 4 L of embryo flush medium (ViGro Complete Flush Solution®, Bioniche Animal Health Canada Inc.) was used per mare, and mares were administrated oxytocin (40 IU i.v., Oxytocin®, Vêtoquinol N.-A. Inc., Lavaltire, QC, Canada) before the procedure was completed to facilitate flush recovery. Embryos were identified using a stereomicroscope, enumerated, and rinsed 3 times in Holding Media (Vigro Holding Plus®, Bioniche Animal Health Canada Inc.). Embryos were scored for quality (1-excellent, 2- good, 3-fair, 4-poor) according to their morphology as described by McKinnon and Squires (McKinnon and Squires, 1988b), and were subjectively assessed for age according to their developmental stage (morula/ early blastocyst/ expanded blastocyst) and size.

Embryos were held at room temperature and transferred within 1 h of recovery. Each embryo was aspirated into a sterile 0.5 ml straw with 0.25 ml of holding medium, loaded into a flexible Universal pipette (Minitube, Ingersoll, ON, Canada.), and transferred into the uterine body of a recipient mare using a non-surgical, trans-cervical approach as described elsewhere (McKinnon and Squires, 1988a). Recipient mares were synchronized using P&E, and/or PGF2, and hCG, as was needed to align their ovulations with the donor mares as described previously (Loy et al., 1981; Blanchard and Varner, 1995; Raz et al., 2005). Recipients that ovulated in a range of 1 day before to 2 days after the donor mares' ovulations were used. Pregnancy status in recipient mares was evaluated 7-10 days after embryo transfer by trans-rectal ultrasonographic examination. Pregnant recipient mares were allowed to carry the pregnancy to term.

5.3.4. Statistical analyses

Statistical analyses were performed with analytic computerized software (Statistix 8 Student Edition, Analytical Software, Tallahassee, FL, USA). Continuous data, such as number of preovulatory follicles, number of ovulations, and number of embryos, were compared between cycles using paired-t test analyses. Fisher Exact Test analyses were used to compare proportional data such as the proportion of mares which ovulated, proportion of mares with multiple ovulations, proportion of mare cycles with large non-ovulatory follicles, overall proportion of large follicles from which ovulation failed to occur, proportion of successful embryo recoveries, and proportion

of recipient mares which became pregnant. Categorical data, such as embryo morphological grade and embryo age, were compared with Kruskall–Wallis non-parametric one-way ANOVA. Differences were considered significant at $P < 0.05$. Results are presented as mean ± S.E.M.

5.4. Results

5.4.1. Folliculogenesis and ovulation

In the control cycle, all donor mares (12/12, 100%) developed at least one follicle ≥35 mm in diameter, and ovulated subsequent to hCG administration. In the eFSH cycle, all donor mares (12/12, 100%) developed at least one follicle ≥35 mm in diameter after 5.3 ± 0.8 d of eFSH treatment; however, two mares failed to ovulate (Table 5.1). The number of preovulatory follicles (≥30 mm) at the time of hCG administration was significantly higher in the eFSH cycle (3.8 ± 0.6 follicles/cycle; range 1 - 8 follicles/cycle) as compared to the control cycle (1.4 ± 0.1 follicles/cycle; range 1 - 2 follicles/cycle). Mean number of ovulations per cycle tended to be higher ($P = 0.09$) in the eFSH cycle (2.3 ± 0.5 ovulations/cycle; range 0 - 6 ovulations/cycle), as compared to control cycle (1.4 ± 0.1 ovulations/cycle; range 1 - 2 ovulations/cycle). When the two mares that failed to ovulate in the eFSH cycle were excluded from the analysis, the mean number of ovulations was significantly higher in the eFSH cycle (2.8 ± 0.5 ovulations/cycle; range 1 - 6). Multiple ovulations (≥2) were recorded in 5/12 (42%) mares in the control cycle, and in 9/10 (90%) ovulating mares in the eFSH cycle ($P < 0.05$). In the 5 mares in which double ovulations were recorded in the control cycle, ovulations were detected at the same day (three mares), within 24 h (one mare), or within 48 h (one mares). In the 9 mares in which multiple ovulations were recorded in the eFSH cycles, ovulations were detected at the same day (five mares), within 48 h (three mares), or within 72 h (one mare).

In the control cycle, all preovulatory follicles that were observed on the day of hCG administration, ovulated within 72 hr. However, in the eFSH cycle non-ovulatory large follicles were observed in 6/12 (50%) of the mares ($P < 0.05$); two mares completely failed to ovulate, and in 4 mares at least one large non-ovulatory follicle (1.5 ± 0.3 non-ovulatory large follicles) was observed in addition to the normal ovulation(s). In these mares, a large non-ovulatory follicle was defined as a preovulatory follicle (≥30 mm) that was observed on the day of hCG treatment, but from which ovulation failed to occur within 3 days of the first ovulation. In the overall per follicle

comparison, ovulation failed to occur from more ($P < 0.05$) preovulatory follicles in the eFSH cycle (17/45, 38% non-ovulatory large follicles) as compared with the control cycle (0/17, 0% non-ovulatory large follicles).

5.4.2. Embryo production

In the control cycle, a total of 6 embryos were recovered in 5/12 (42%) successful embryo recovery attempts (Table 5.2). In the eFSH cycles, a total of 15 embryos were recovered in 9/10 (90%) successful embryo recovery attempts. Embryo recovery rate per ovulation was not significantly different between the groups, and mean number of embryos per recovery attempt was significantly higher in the eFSH cycle (1.5 ± 0.3 embryos) as compared with the control cycle (0.5 ± 0.2 embryos). However, mean embryo morphology grade tended ($P = 0.06$) to be poorer following the eFSH treatment (2.7 ± 0.2 out of 4; range 2-4) as compared to control (1.8 ± 0.3 out of 4; range 1-3). Mean embryo age tended ($P = 0.06$) to be lower following the eFSH treatment (7.2 ± 0.1 d; range 6.5-8.5 d) as compared to control (7.8 ± 0.3 d; range 7.0-8.5 d).

5.4.3. Recipient pregnancy rates

The transfer of 6 embryos recovered from the control cycle resulted in a pregnancy in 4 (67%) recipient mares. The transfer of 15 embryos recovered from the eFSH cycle resulted in a pregnancy in 5 (33%) recipient mares ($P > 0.05$). All pregnancies resulted from a transfer of embryos with a morphology grade of 1 or 2. Eight of the nine pregnant recipients carried the pregnancy to term; one recipient mare died during her pregnancy from a health problem not related to the pregnancy.

Table 5.1 Ovarian response in cycling mares treated with twice daily eFSH followed by hCG administration, as compared to control. Results are presented as mean ± SEM or percentages (%).

	Control (n = 12 mare cycles)	eFSH (n = 12 mare cycles)
Duration of eFSH treatment (d)	---------	5.3 ± 0.8
No. of preovulatory follicles (≥30 mm)	1.4 ± 0.1 [a]	3.8 ± 0.6 [b]
Proportion of mares in which ovulations occurred	100% (12/12)	83% (10/12)
[1]No. of ovulations	1.4 ± 0.1 [a]	2.8 ± 0.5 [b]
[1]Proportion of mares with ≥2 ovulations	42% (5/12) [a]	90% (9/10) [b]
Proportion of mares with large non-ovulatory follicle(s)	0% (0/12) [a]	50% (6/12) [b]
Proportion of preovulatory follicles failing to ovulate	0% (0/17) [a]	38% (17/45)[b]

[ab] Values within a row with different superscripts are significantly different ($P < 0.05$)

[1]Values were calculated for ovulating mares only

Table 5.2 Embryo production of cycling mares ovulating subsequent to treatment with twice daily eFSH followed by hCG administration, as compared to controls. Mares were artificially inseminated with fresh semen while in estrus, and embryo recovery attempts were performed 8 d after ovulation.

	Control (n = 12 mare cycles)	eFSH (n = 10 mare cycles)
Embryo number	0.5[b] ± 0.2	1.5[a] ± 0.3
Successful embryo recovery attempts	42% (5/12) [a]	90% (9/10) [b]
[1]Embryo morphology grade (1-excellent; 4-poor)	1.8 ± 0.3	2.7 ± 0.2
[1]Embryo assessed age (d)	7.8 ± 0.3	7.2 ± 0.1
Recipient pregnancy rate per transferred embryo	67% (4/6)	33% (5/15)

[ab] Values within a row with different superscripts are significantly different ($P < 0.05$)

[1] $P < 0.06$

5.5. Discussion

Treatment with eFSH resulted in a greater number of preovulatory follicles, a greater number of ovulations, and a greater number of recovered embryos; however, recipient pregnancy rate per transferred embryo was lower than anticipated, and the final number of pregnancies resulting from the eFSH cycle was not different than that in the control cycle. Treatments in the study were not randomized to avoid possible carry-over effects of eFSH treatment. The P&E estrus synchronization regimen, which was used prior to treatment initiation of the control cycle, is commonly used in private practice (Blanchard and Varner, 1995). The P&E regimen aims to suppress follicular growth, but after the treatment is stopped follicular growth is fairly predictable, and ovulations are expected to be in synchrony among treated mares (Loy et al., 1981; Evans et al., 1982; Plata-Madrid et al., 1992). Prior to the second, eFSH cycle, only PGF2α was administered to the donor mares in order to cause luteolysis of their corpus luteum, and to allow follicular growth and ovulation. These differences between the two cycles perhaps affected follicular recruitment. In addition, due to our study design, we cannot exclude possible negative carry-over effects from the control cycle to the eFSH cycle.

In the control cycle, the number of ovulations (1.4 ovulations/mare) was higher than the anticipated 1.2 ovulations/mare when hCG is used to induce ovulation (Ginther, 1992b). This could be related to the draft type of the mares and possible genetic predisposition for double ovulations (Ginther, 1992b; Macpherson and Reimer, 2000); also, the P&E estrus synchronization regimen could have possibly altered subsequent endogenous gonadotropin concentrations and ovarian sensitivity to endogenous or exogenous hormonal stimulation. The eFSH treatment protocol used in this study was intended to promote the development of a higher number of preovulatory follicle(s), responsive to ovulation-induction factor, by bypassing the hypothalamic-pituitary axis and stimulating the ovary directly. The dose and frequency of eFSH treatment used in the present study were according to the manufacturer's recommended protocol at the time the study was conducted, and as previously reported (Niswender et al., 2003). The rationale for initiating eFSH treatment at a follicle size of 25 mm was that the treatment is costly and the administration of the hormone should be based on the approximate time of follicular deviation in mares (Squires and McCue, 2007). Administration of hCG was included in the treatment regimens of both cycles for ovulation induction, as it is commonly used in the equine industry. However, it

is now recommended to postpone hCG administration following twice daily eFSH treatments in a 30-36 h period after the last eFSH treatment; this delay period is termed ‘coasting’. The ‘coasting’ period has been reported to be beneficial in superstimulated donor cows, and may also be advantageous when estrous cycling mares are treated with eFSH (Welch et al., 2006). The rationale for the ‘coasting’ period is to allow the FSH concentration to decrease during the final maturation phase of the follicles.

As was anticipated, eFSH treatment resulted in ovarian stimulation, and most mares had multiple ovulations. The number of preovulatory follicles and the number of ovulations were comparable to those reported previously following eFSH treatment (Niswender et al., 2004; Raz et al., 2005; Squires and McCue, 2007). However, two mares failed to ovulate following eFSH treatment, and other mares had non-ovulatory large follicles present. There are reports of a proportion of eFSH-treated mares that completely failed to ovulate, or mares that experienced large non-ovulatory follicles accompanying normal appearing ovulations (Niswender et al., 2003; Raz et al., 2005; Raz et al., 2006a; Logan et al., 2007b; Niswender et al., 2007). In addition, in the current study, as in most other studies, the number of ovulations was less than the number of the preceding preovulatory follicles in eFSH-treated mares, but not in controls. Ovulation failure in eFSH-treated mares may be related to insufficient LH-like stimulation, alteration of the hormonal environment, alteration of the number of LH receptors, or changes in the affinity of hormone receptors to gonadotropins in preovulatory large follicles in superstimulated mares. Another explanation could be the unique structure of the mare ovary in which follicles develop in the interior cortex of the ovary and ovulations only occur through the limited area of the ovulation fossa. Further research is needed to identify possible alterations in hormonal environment, follicular dynamics, and oocyte maturation following superstimulation of mares with eFSH.

In the control cycle, embryo recovery rate, embryo parameters (morphology grade and assessed age), and post transfer recipient pregnancy rate, were as commonly expected in embryo transfer programs when non-superstimulated donor mares are used. In the eFSH cycle, the number of recovered embryos was significantly greater, as was anticipated due to the greater number of ovulations. Lower mean embryo age following the eFSH treatment was also anticipated, as some mares had ovulations that were spread over up to 3 days, but embryo recovery attempts were scheduled to be 8 days after the first detected ovulation. Interestingly, embryo morphology grade

tended to be lower following eFSH treatment, as compared to control; and although more embryos were transferred to recipient mares following the eFSH cycle, it did not result in more pregnancies. Pregnancy rate per transfer embryo was lower than expected for embryos that were recovered from the eFSH treated donor mares, but this was not statistically significant as compared to control, conceivably due to small sample size and lack of power.

In other species, hormonal treatments have been reported to negatively alter quality and viability of some proportion of embryos recovered from females treated to induce ovarian superstimulation (Moor et al., 1985; Barati et al., 2006; Kelley et al., 2006; Lee et al., 2006). In mares, treatment with equine pituitary extract has been suggested to have negative effects on embryo viability (Woods and Ginther, 1983b; Woods, 1984). However, there is very limited information on embryo quality and post-transfer recipient pregnancy rate of embryos recovered from eFSH-treated donor mares. In another study we obtained a recipient pregnancy rate of only 35% after the transfer of a total of 26 embryos recovered from eFSH treated donor mares (Raz et al., 2005). There is only one other study in which embryos recovered from eFSH-treated donor mares were transfered to recipients. In that study, Hudson et al. (2006) transferred embryos after vitrification (n = 20 embryos) or after cooling for a short time followed by vitrification (n = 20 embryos), which resulted in an overall recipient pregnancy rate of 70%. However, only embryos with a grade of 1 to 2 and with <300 μm diameter were selected in their study, from a total of 148 embryos that were recovered from eFSH-treated donor mares on day 6.5 after ovulation or on day 8 after hCG was administrated. Further research is needed to investigate if eFSH treatments in mares alter the hormonal environment, the secretions and contractility of the oviduct and uterus, transfer of gametes, embryo viability, and early embryonic development.

In summary, the eFSH treatment stimulated the ovary, and resulted in a greater number of preovulatory follicles, a greater number of ovulations, and a greater number of recovered embryos in most treated mares; however, the recovered embryos tended to have a lower embryo morphological grade as compared to control, and the recipient pregnancy rate per transferred embryo was lower than anticipated. The number of recipient pregnancies that resulted from the eFSH treatment was not different from the control treatment. In the development of superovulation protocols, the cost-benefit ratio should be evaluated to determine the effect on reproductive efficiency. Further studies are indicated to clarify the effect of eFSH on mare fertility.

6. EVALUATION OF TWO ESTRUS SYNCHRONIZATION REGIMENS IN eFSH-TREATED DONOR MARES

This study was presented at the 51st Annual Conference of the American Association of Equine Practitioners in 2005.

6.1. Abstract

The rise in the use of embryo transfer increased the demand for a reliable means of regulating estrus and ovulation in the mare. The objectives of this study were to investigate and compare folliculogenesis, number of ovulations, embryo recovery, embryo quality, and recipient pregnancy rates using two estrus synchronization methods (P&E vs PGF2α) combined with subsequent eFSH treatment in an embryo transfer program. Donor mares (n = 12) were used in two estrous cycles, using a randomized crossover design. In the P&E group, donor mares were administered a once daily compounded product containing 150 mg of progesterone and 10 mg of estradiol-17β (P&E), intramuscularly, for 10 days; and PGF2α was administered on the last day. In the PG group, donor mares were administered PGF2α 5 days after spontaneous ovulation. In both treatment groups, a twice daily eFSH treatment was initiated when a follicle ≥20 mm in diameter was detected by trans-rectal ultrasonographic examination and ceased when a follicle reached ≥35 mm in diameter, at which time hCG was administered to induce ovulation. Donor mares were artificially inseminated with fresh semen, and embryo recovery attempts were performed 7 or 8 days after ovulation. Each embryo was transferred non-surgically, trans-cervically into the uterine body of a synchronized recipient mare, and pregnancy status was evaluated 7 - 10 days later by trans-rectal ultrasonographic examination. The P&E regimen resulted in higher degree of ovarian synchrony among treated donor mares, as all mares in that group ovulated 9 to 12 days after the last steroid treatment. In both groups the number of ovulations was higher than expected in non-superstimulated mares; however there was a tendency ($P < 0.06$) for more ovulations in the PG group (mean ± S.E.M: 2.5 ± 0.4 ovulations) as compared to the P&E group (1.5 ± 0.3 ovulations). The number of embryos recovered (P&E: 0.9 ± 0.3 as compared with PG: 1.4 ± 0.3 embryo/recovery attempt) and recipient pregnancy rate per transferred embryo (4/9, 44% as

compared with 4/15, 27%) were not statistically different between the groups; however, the later was lower than anticipated in non-superstimulated mares. We concluded that the P&E regimen is more reliable for regulating estrus and ovulation in eFSH treated mares than the PG regimen; however, the tendency for lower number of ovulations in the P&E group may remove any advantage of this regimen when it is combined with eFSH treatments.

6.2. Introduction

Demand for a reliable means of predicting or regulating estrus and ovulation in the mare has increased with the use of assisted reproductive techniques such as embryo transfer, artificial insemination, and other forms of appointment breeding (Squires et al., 2003a). Embryo transfer offers distinct advantages to mare owners who wish to maximize the number of foals from selected mares and also may be useful for mares when carrying a foal to full term is neither desirable nor possible. However, the commercial expansion of embryo transfer in the equine industry is restricted, mainly due to limited success rate and high cost of superovulation (Stout, 2006). In addition, the great variations in follicular growth and estrous length between mares make it difficult to adequately synchronize estrous cycles of donor and recipient mares (Ginther, 1992b).

Hormonal regimens used to synchronize estrus and ovulation in the mare include administration of prostaglandins (Douglas and Ginther, 1975b), and administration of progestagens alone or together with estradiol (Blanchard and Varner, 1995). These regimens are commonly combined with administration of hCG or a GnRH analogue as ovulation inducing agents (Samper, 2008). Prostaglandin F2α (PGF2α) and its analogues are the most widely used hormonal therapy in the horse industry (Allen et al., 1974; Douglas and Ginther, 1975a; Johnson and Becker, 1993; Bergfelt et al., 2006). When PGF2α is administered exogenously, it induces lysis of the corpus luteum (Hyland et al.), thereby allowing the return to estrus. For prostaglandins to effectively terminate the luteal phase, a mature CL must be present that is responsive to prostaglandin. The equine CL exhibits incomplete sensitivity to a single administration of PGF2α until approximately 5 days after ovulation. On average, in the presence of a mature CL, mares return to estrus 5 to 7 days after prostaglandin administration, and ovulation occurs 9 to 11 days after administration (Blanchard and Varner, 1995). However, the interval to ovulation may vary as much as 2 to 15 days from prostaglandin administration, depending on the size and status of

follicles present on the ovaries at the time of administration. This range of response to prostaglandin administration is problematic when donor and recipient mares' ovulations need to be closely aligned for embryo transfer; therefore, when using prostaglandin as estrus synchronization regimen, it is common to have a ratio of 2 to 3 recipient mares to each donor mare (Stout, 2006).

The administration of progesterone and estradiol-17β (P&E), daily for 10 days, followed by administration of prostaglandin on the last day of the P&E treatment, and hCG when a large preovulatory follicle is subsequently detected, is one of the most predictable hormonal regimens to synchronize ovulation in mares used in embryo transfer programs, particularly in situations when only one or two recipients per donor are to be used (Stout, 2006). The P&E regimen provides negative feedback on gonadotropin release, which inhibits follicular development (Loy et al., 1981; Taylor et al., 1982b; Bristol et al., 1983). When the exogenous P&E therapy is discontinued, there is less diversity in follicular maturation, and the timing of ovulation can be more closely synchronized (Evans et al., 1982; Plata-Madrid et al., 1992). With this protocol, greater than 70% of mares ovulate between 9 to 12 days after the last steroid injection.

Historically, superovulatory treatments have been elusive in the mare and less efficient than in other domestic species (Woods and Ginther, 1983a; Mapletoft et al., 2002; Cognie et al., 2003). Recently, a purified pituitary extract product called equine follicle-stimulating hormone (eFSH) has been investigated and was reported to increase the number of ovulations and embryo-recovery rate of cycling donor mares (Niswender et al., 2003). However, information related to the effects of eFSH on mare's fertility is very limited, and the efficiency of this superovulatory treatment combined with the commonly used estrus synchronization regimens still needs to be investigated.

The objectives of this study were to investigate and compare folliculogenesis, number of ovulations, embryo recovery, embryo quality, and subsequent recipient pregnancy rates using two estrus synchronization methods (P&E vs PGF2α) combined with eFSH treatment in an embryo transfer program. We hypothesized that 1) P&E regimen would be more efficient than PGF2α administration for ovulation synchrony among eFSH treated mares, 2) following both estrus synchronization regimes, eFSH treatment would increase the number of preovulatory follicles, number of ovulations, and embryo recovery rate, as compared to that previously reported in single ovulating mares, and 3) the degree of ovarian stimulation, embryo recovery rate, and post transfer

recipient pregnancy rate would be similar subsequent to P&E or PGF2α regimens in eFSH-treated donor mares.

6.3. Materials and methods

6.3.1. Animals and reproductive tract examinations

Mares obtained from a commercial herd were used during the physiologic breeding season (May to August) in the research facility of the University of Saskatchewan in Canada, which is located at 52°07' latitude in the Northern Hemisphere. All mares were of a body condition score of at least 5 on a 9 point assessment system, and had no signs of systemic disease or lameness. Donor mares (n = 12) were draft-cross type, aged 4 to 15 years (Mean ± S.E.M: 8.7 ± 1 years). Recipient mares (n = 36) were draft-cross or Quarter Horse-cross type, aged 3 - 9 years. Mares were kept in outdoor paddocks with sheds, and were maintained on alfalfa and grass hay with access to water and trace-mineralized salt, in accordance with the University of Saskatchewan's Institutional Animal Care and Use Committee. Examinations of the reproductive tracts were performed using a trans-rectal approach and included manual palpation followed by an ultrasonographic examination. A B-mode ultrasonographic scanner (GE Ausonics, Universal Ultrasound, Bedford Hills, NY, USA) equipped with a 5.0 MHz linear-array transducer was used to measure follicle diameter, and to detect ovulation(s).

6.3.2 Experimental design and treatments

Donor mares were used in two estrous cycles, using a randomized crossover design, for a total of 12 estrous cycles per treatment group. In the P&E group, donor mares were administered a once daily compounded product containing 150 mg of progesterone and 10 mg of estradiol-17β (P&E) in sesame oil (WCVM Veterinary Pharmacy, University of Saskatchewan, Saskatoon, SK, Canada), intramuscularly, for 10 days; PGF2α (5 mg s.c., Lutalyse®, Pharmacia Animal Health, Orangeville, ON, Canada) was administered on the final day of P&E regimen. In the PG group, donor mares were administered PGF2α 5 days after spontaneous ovulation. In both treatment groups, a twice daily eFSH treatment (12.5 mg i.m., eFSH®, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) was initiated when a follicle ≥20 mm in diameter was detected in a mare. The eFSH treatment was ceased when at least one follicle ≥35 mm in diameter was detected, at

which time hCG (Chorulon®, Intervet Canada Ltd, ON, Canada; 2000 IU, i.m.) was administered to induce ovulation.

Donor mares were artificially inseminated 24 h after hCG was administered, and again every 48 h until ovulation (Day 0 = day of ovulation) with fresh semen collected from one of two stallions of proven fertility. A minimum dose of 5 x 10^8 progressively motile spermatozoa extended 1:1 with Kenney extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA, USA) was used to inseminate the mares. Donor mares with post-breeding uterine fluid accumulations were treated as needed using uterine saline lavage and/or antibiotics (intra-uterine or systemic) and/or oxytocin injections. Seven or 8 days after ovulation, embryo recovery attempts were performed.

Prostaglandin F2α was administered to all donor mares subsequent to completion of the embryo recovery attempt. Thereafter, in donor mares assigned to the PG treatment first, the P&E treatment was initiated two days after embryo recovery attempt. Donor mares assigned to the P&E treatment first were followed into estrus, and were assigned to the PG treatment 5 days after their spontaneous ovulation was detected.

6.3.3. Embryo recovery, handling and transfer

Embryo recovery attempts were performed 7 or 8 d after ovulation using a routine nonsurgical trans-cervical technique as described elsewhere (McKinnon and Squires, 1988a). A total of 4 L of embryo flush medium (ViGro Complete Flush Solution®, Bioniche Animal Health Canada Inc.) was used per mare, and mares were administrated oxytocin (40 IU i.v., Oxytocin®, Vêtoquinol N.-A. Inc., Lavaltire, QC, Canada) before the procedure was completed to facilitate recovery. Embryos were identified using a stereomicroscope, enumerated, and rinsed 3 times in Holding Medium (Vigro Holding Plus®, Bioniche Animal Health Canada Inc.). Embryos were scored for quality (1-excellent, 2- good, 3-fair, 4-poor) according to their morphology as described by McKinnon and Squires (McKinnon and Squires, 1988b).

Embryos were held at room temperature and transferred within 1 h of recovery. Each embryo was aspirated into a sterile 0.5 ml straw with 0.25 ml of holding medium, loaded into a flexible Universal pipette (Minitube, Ingersoll, ON, Canada.), and transferred into the uterine body of a

recipient mare using a non-surgical, trans-cervical approach as described elsewhere (McKinnon and Squires, 1988a). Recipient mares were synchronized using P&E, and/or PGF2, and hCG, as was needed to align their ovulations with the donor mares as previously described (Loy et al., 1981; Blanchard and Varner, 1995; Raz et al., 2006b). Recipients that had an ovulation that occurred in a range of 3 d before to 1 d after the donor mare's ovulation were used. Pregnancy status in recipient mares was evaluated 8-10 days after embryo transfer by trans-rectal ultrasonographic examination. Pregnant recipient mares were allowed to carry pregnancy to term.

6.3.4. Statistical analyses

Statistical analyses were performed with analytic computerized software (Statistix 8 Student Edition, Analytical Software, Tallahassee, FL, USA). Continuous data, such as number of preovulatory follicles, number of ovulations, interval to ovulation and number of embryos, were compared between cycles using student-t test analysis. Normality of distribution and equality of variances of continuous data were evaluated using the Shapiro-Wilk Test and the Bartlett's Test, respectively. Fisher Exact Test analysis was used to compare proportional data such as the proportion of mares which ovulated, proportion of mares with multiple ovulations, overall proportion of large follicles from which ovulation failed to occur, proportion of successful embryo recoveries, and proportion of recipient mares which became pregnant. Embryo morphological grades were compared with Kruskall–Wallis non-parametric one-way ANOVA. Differences were considered significant at $P < 0.05$. Results are presented as mean ± S.E.M.

6.4. Results

6.4.1. Folliculogenesis and ovulation

In the P&E group, all donor mares completed 10 days of P&E treatment. However, one mare had a 50 mm follicle on the final day of the P&E treatment; therefore, she was not given eFSH, and her data was excluded from the analysis. All other donor mares (11/11, 100%) in the P&E group developed at least one follicle ≥35 mm in diameter after 7.2 ± 1.5 d of eFSH treatment; however, one donor mare failed to ovulate subsequent to hCG administration (Table 6.1). In the PG group, all donor mares (12/12, 100%) developed at least one follicle ≥35 mm in diameter after 8.4 ± 1.1 d of eFSH treatment; however, one donor mare failed to ovulate subsequent to hCG

administration. The interval from PGF2α administration to ovulation was significantly more synchronized among donor mares in the P&E group, as compared to donor mares in the PG group (Figure 6.1).

The number of preovulatory follicles (≥35 mm in diameter) at the time of hCG administration was not significantly different between the groups (P&E: 1.8 ± 0.3 as compared to PG: 2.6 ± 0.5). The number of ovulations tended to be lower in donor mares in the P&E group as compared to the PG group (1.5 ± 0.3 as compared to 2.5 ± 0.4 ovulations, P = 0.06). When mares failing to ovulate were excluded from the analysis, this difference in the number of ovulations was significant (1.6 ± 0.3 as compared to 2.7 ± 0.4 ovulations; P < 0.04). Overall, in the P&E group there was a tendency (P = 0.07) for a higher proportion of preovulatory large follicles from which ovulation failed to occur (4/20, 20% as compared to 1/31, 3%).

6.4.2. Embryo production

In the P&E group, a total of 9 embryos were recovered in 7/10 (70%) successful embryo recovery attempts. In the PG group, a total of 15 embryos were recovered in 8/11 (73%) successful embryo recovery attempts. Mean embryo number per recovery attempt was not significantly different between the groups (0.9 ± 0.3 as compared to 1.4 ± 0.3 embryos). Furthermore, embryo recovery rate per ovulation was not significantly different between the P&E group (9/16, 56%) and the PG group (15/30, 50%). Mean embryo morphology grade was not different for embryos recovered in the P&E group (2.4/4 ± 0.3) as compared to embryos recovered in the PG group (2.6/4 ± 0.2). However, out of a total of 24 embryos recovered in both groups, 8 (33%) embryos were of lower quality (Grades 3 or 4).

Table 6.1 Ovarian parameters and embryo production following two estrus synchronization regimens in eFSH-treated donor mares. Prior to the eFSH treatment donor mares in the PG group were treated with PGF2α in early diestrus; donor mares in the P&E group were treated with daily progesterone and estradiol-17β for 10 days, and PGF2α was administered with the final P&E treatment. A twice daily eFSH treatment regimen, followed by hCG, was given in both groups. Mares were bred, and embryos were recovered and transferred to synchronized recipients 7 or 8 days post ovulation. Results are presented as mean ± S.E.M or percentages (%).

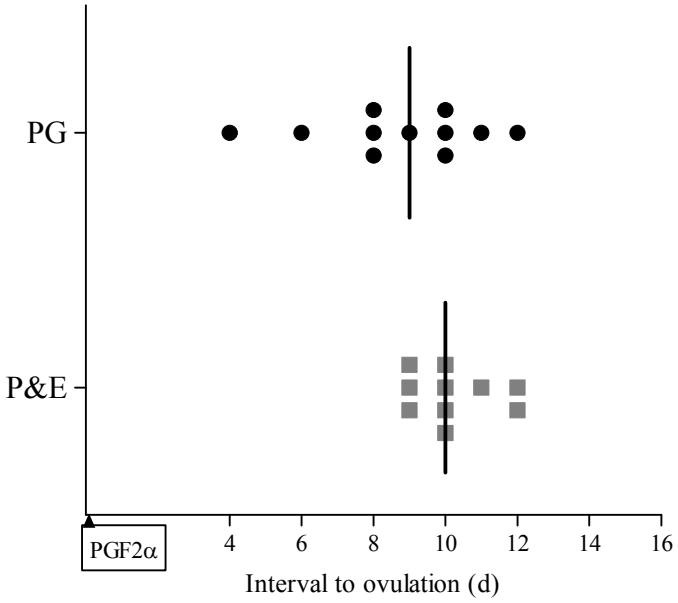
	P&E (n = 11)	PG (n = 12)
Duration of eFSH treatment (d)	7.2 ± 1.5	8.4 ± 1.1
No. of preovulatory follicles (≥35 mm)	1.8 ± 0.3	2.6 ± 0.5
Proportion of mares in which ovulations occurred	91% (10/11)	92% (11/12)
[1]Proportion of mares with ≥2 ovulations	36% (4/11)	75% (9/12)
[2]No. of ovulations	1.6[a] ± 0.3	2.5[b] ± 0.4
[2]Successful embryo recovery attempts	70% (7/10)	73% (8/11)
[2]No. of embryos	0.9 ± 0.3	1.4 ± 0.3
Recipient pregnancy rate per transferred embryo	44% (4/9)	27% (4/15)

[ab] Values within a row with different superscripts are significantly different ($P < 0.05$)

[1] $P < 0.09$

[2] Values were calculated for ovulating mares only

Figure 6.1 Interval to ovulation in eFSH-treated donor mares subsequent to two estrus synchronization regimens. Prior to the eFSH treatment donor mares in the PG group were treated with PGF2α in early diestrus; donor mares in the P&E group were treated with daily progesterone and estradiol-17β for 10 days, and PGF2α was administered with the final P&E treatment. A twice daily eFSH treatment regimen, followed by hCG, was given in both groups. Each dot represents the time of ovulation of an individual mare, in relation to the day of PGF2α administration. Vertical lines represent the median time of ovulations in the group.



6.4.3. Recipient pregnancy rates

The transfer of 9 embryos recovered from the P&E group resulted in pregnancy detection in 4 (44%) recipient mares. The transfer of 15 embryos recovered from the PG group resulted in pregnancy detection in 4 (27%) recipient mares. Overall, recipient pregnancy rate per transfer embryo was 33% (8/24). All pregnant recipient mares carried pregnancy to term.

6.5. Discussion

To our knowledge, there have been no previous studies comparing these two estrus synchronization methods in donor mares allocated to eFSH treatment. As was anticipated, the P&E regimen was more efficient than PGF2α administration for ovulation synchrony among eFSH treated mares. Furthermore, the extent of ovulation synchrony following P&E regimen or PGF2α administration in eFSH-treated mares were similar to those reported previously for non-superstimulated mares (Douglas and Ginther, 1975b; Loy et al., 1981; Taylor et al., 1982b; Bristol et al., 1983; Plata-Madrid et al., 1992; Blanchard and Varner, 1995; Raz et al., 2005). The one mare that did not respond to the P&E treatment by follicular suppression was not treated with eFSH; this is because the superovulatory hormone administration should be based on the approximate time of follicular deviation in mares, which is approximately when the larger follicle diameter is about 23 mm (Ginther, 2000).

Horses are consider to be monotocous species; it is anticipated to obtain an average of 1.2 ovulations per mare when hCG is used to induce ovulation (Ginther, 1992b). In both treatment groups in the current study, the number of preovulatory follicles and the number of ovulations were higher than is expected in non-superstimulated mares, as was expected to occur due to the eFSH treatment. However, there was a tendency for more mares with ≥2 ovulations in the PG group; and the mean number of ovulations was significantly higher in ovulating donor mares in the PG group as compared to the P&E group. This could be related to differences in the number of ovarian follicles that were available for superstimulation at the time of eFSH treatment initiation, as the P&E induced follicular suppression may result in fewer follicles present. In addition, in the P&E group there was a tendency for more preovulatory follicles not to result in an ovulation. The administration of eFSH has been associated with a proportion of mares that fail to ovulate, and also mares with large non-ovulatory follicles accompanying normal appearing ovulations

(Niswender et al., 2003; Raz et al., 2005; Raz et al., 2006a; Logan et al., 2007b; Niswender et al., 2007). The currently recommended eFSH treatment protocol includes the administration of twice daily eFSH, followed by hCG administration in a delayed period of 30-36 h after the last eFSH treatment. This delayed period, termed 'coasting', has been suggested to be advantageous when estrous cycling mares are treated with eFSH (Welch et al., 2006) since it allows the FSH concentration to decrease during the final maturation phase of the follicles. In the current study a 'coasting' period was not utilized, which theoretically could have contributed to ovulation failure. Further studies should be conducted in order to investigate the effect of coasting and superovulatory treatment on follicular maturation and ovulation.

In single ovulating mares it is expected to achieve an average of 0.5 embryos per recovery attempt, and a 50–65% recipient pregnancy rate per transferred embryo (Squires et al., 1999; Squires et al., 2003a; Stout, 2006). In the current study we obtained an overall average of 1.2 embryos per recovery attempt, and a 33% recipient pregnancy rate per transferred embryo. In our study design we did not have a group of non-eFSH treated mares; however, the recipient pregnancy rate per transferred embryo, particularly in the PG group, was lower than anticipated. We speculate that this could have been a sequela to the relatively higher proportion of embryos with low quality. We observed some small for date embryos in both groups, and/or embryos with misshapen wrinkled embryonic capsules; this may indicate arrested embryonic development or devitalisation (McKinnon and Squires, 1988b; Squires et al., 1999; Carnevale et al., 2000). Superovulatory treatments have been reported to negatively affect a proportion of embryos in mares and in other species (Woods and Ginther, 1983b; Woods, 1984; Moor et al., 1985; Barati et al., 2006; Kelley et al., 2006; Lee et al., 2006; Raz et al., 2006b). However, the lower recipient pregnancy rate could have been related to donor mare or recipient mare quality, treatment protocols, and techniques. Developmental competence of embryos resulting from superovulatory treatment should be further investigated.

The two estrus synchronization regimens used in this study are commonly used in commercial equine embryo transfer programs. The P&E regimen requires daily handling of the mares for the administration of the hormonal treatment, which is currently available only through compounding pharmacies; whereas the PG protocol is less intensive, as it is administered once, and less costly. However, in the P&E regimen fewer transrectal examinations are required since the

synchronization results are highly predictable and more reliable than in the PG protocol. In an attempt to synchronize donor and recipient ovulations, the P&E regimen is more trustworthy; nevertheless, the tendency for lower number of ovulations in the P&E group may remove any advantage of this estrus synchronization regimen in eFSH-treated donor mares. The higher numerical recipient pregnancy rate per transferred embryo in the P&E group may indicate that the potential of this estrus synchronization regimen still merits further investigation.

In summary, in this study we compared two estrus synchronization regimens, PGF2α and P&E, in eFSH-treated donor mares. We found that the P&E regimen resulted in a higher degree of ovarian synchrony among treated donor mares. In both groups the number of ovulations was higher than expected in non-superstimulated mares; however there was a tendency for more ovulations in the PG group. Embryo recovery and recipient pregnancy rate per transferred embryo were not different between the groups; however, the later was lower than anticipated in non-superstimulated mares. Overall, from a total of 23 donor estrous cycles in which eFSH was administered, only 8 recipient pregnancies resulted. Further studies are required to investigate the effects of eFSH treatment, estrus synchronization protocols, and their combinations, on the hormonal environment at the time of follicular maturation and ovulation, and on embryonic viability.

7. EARLY EFFECTS OF eFSH TREATMENT ON HORMONAL AND REPRODUCTIVE PARAMETERS IN MARES INTENDED TO CARRY THEIR OWN PREGNANCY

This study was accepted for publication in Animal Reproduction Sciences (Anim Reprod Sci 2009, 115 76-87. PMID: 19070442)

7.1. Abstract

Superovulatory treatment may potentially increase the embryo recovery rate and the per-cycle pregnancy rate in normal or subfertile mares that are managed properly. However, some studies suggest a possible negative effect of superovulatory treatment on ovarian follicular maturation and embryo viability. Objectives of the present study were to investigate the effects of eFSH treatment in reproductively normal mares in terms of: folliculogenesis, pregnancy rate, early embryonic development, reproductive tract parameters (tone and edema), and serum estradiol-17β and progesterone concentrations. Reproductively sound mares (n = 26) were evaluated daily by transrectal palpation and ultrasonography. Five days after spontaneous ovulation, mares were randomly assigned to one of two treatment groups. In the eFSH group, mares (n = 16 estrous cycles) were administered eFSH twice daily; beginning when a follicle ≥20 mm was detected, and continuing until at least one follicle reached a diameter of ≥35 mm. PGF2α was administered 2 days following initiation of eFSH therapy, and hCG was administered approximately 36 h after cessation of eFSH therapy. In the control group, mares (n = 26 estrous cycles) were administered PGF2α 7 days after spontaneous ovulation, and hCG when a follicle ≥35 mm was detected. All mares were bred with fresh semen, monitored for ovulation (Day 0), and evaluated for pregnancy on Days 11 to 16. Serum estradiol-17β and progesterone concentrations were analyzed using radioimmunoassay on the Day of hCG administration, and Days 8, 11 and 16. Mares treated with eFSH had more follicles ≥30 mm at the time of hCG administration (2.6 ± 0.4 compared with 1.1 ± 0.1; $P < 0.01$), and more ovulations (2.3 ± 0.5 compared with 1.1 ± 0.3; $P < 0.01$). However, pregnancy rates were not significantly different between groups (50%; 8/16 compared with 62%; 16/26). Mean overall daily growth rate of embryonic vesicles from Day 11 to 16 was not

statistically different between the two groups (3.3 ± 0.3 compared with 3.7 ± 0.1 mm/day) (P = 0.2); however, it was more variable (P < 0.01) in the eFSH group (95%CI: 2.6-3.8 mm/day) than in the control group (95%CI: 3.5-3.9 mm/day). Administration of eFSH modified the reproductive tract variables and serum concentrations of progesterone and estradiol-17β on the days that oocyte maturation, fertilization, and early embryonic development are expected to occur. These alterations may be related to the greater incidence of non-ovulatory follicles (25% compared with 0%), fewer embryos per ovulation rate (0.3 ± 0.1 compared with 0.6 ± 0.1), and the lesser than expected pregnancy rates in the eFSH-treated mares.

7.2. Introduction

Effective and reliable treatments to induce multiple ovulations or superovulation are of interest to the equine industry because they may increase reproductive efficiency. Several studies examined the benefit of superovulatory treatments to increase embryo recovery rates in donor mares, when embryo recovery attempts were performed 6 to 8 days post ovulation. However, information regarding the potential benefit of superovulation in circumstances when mares are not used for embryo transfer is very limited (Woods and Ginther, 1983b; Niswender et al., 2003). Superovulatory treatment may potentially increase the per-estrous cycle pregnancy rate in normal or subfertile mares, as more oocytes would be available for fertilization and more embryos will initially be developed in the uterus. Oocyte quality and early embryonic death are significant factors effecting pregnancy rates and foaling rates (Woods, 1989; Carnevale and Ginther, 1995; Carnevale et al., 1999); therefore, increasing the number of oocytes released via ovulation in an estrous cycle may result in a greater proportion of oocytes with acceptable quality, or alternatively increase the likelihood of a surviving embryo. Multiple ovulations are also anticipated to increase the amount of progesterone produced by the ovaries which may be advantageous for the establishment of pregnancy (Bergfelt et al., 1992). Increasing the ratio between the number of oocytes and number of spermatozoa may also increase pregnancy rates in mares bred to a stallion with oligospermia, or that are subfertile (Squires et al., 2003b; McCue et al., 2007a). In addition, developmental competence of embryos resulting from superovulatory treatment may also be assessed by determining embryo number and growth rates in relation to ovulation rates, and may be a useful means of evaluating or comparing different protocols without the expense of embryo recovery.

A drawback for the use of superovulatory treatments in mares intended to carry their own pregnancy would be the increased risk of twin pregnancy which may lead to pregnancy loss. Therefore, early transrectal ultrasonographic detection of twins would be required in mares ovulating from multiple follicles. Twin pregnancies detected prior to Day 16 of gestation, when the embryos are mobile, are most effectively managed by crushing one embryonic vesicle manually, with expected survival rate for the remaining twin exceeding 90% (Bowman, 1986; Pascoe et al., 1987; Macpherson and Reimer, 2000). Therefore, efficacious superovulatory treatment would still potentially increase the likelihood of a successful pregnancy in mares that are managed appropriately.

Historically, superovulatory treatments in mares have been elusive and less efficient than in other domestic species (Betteridge and Rieger, 1993; Mapletoft et al., 2002; Cognie et al., 2003; Baruselli et al., 2006; Squires, 2006a). Recently, availability of a commercial equine FSH preparation (eFSH, Bioniche Animal Health Inc., Athens, GA, USA) has created the potential for successful superovulatory treatment in mares (Niswender et al., 2003). In many studies, eFSH treatment successfully increases the number of ovulations and embryo recovery rates in estrous cycling mares used for embryo transfer (reviewed by McCue et al. 2007a). However, in some studies, superovulatory treatments of mares have been associated with lesser than expected embryo per ovulation rates, alteration of embryo quality, and lesser than expected pregnancy rates after embryos were transfer to recipients (Woods and Ginther, 1984; Alvarenga et al., 2001; Niswender et al., 2003; Peres et al., 2005; Raz et al., 2005; Carmo et al., 2006; Raz et al., 2006b; Logan et al., 2007b). There is a need to identify factors affecting success rate of superovulatory treatments in mares, as those treatments may modify steroid hormonal and reproductive tract milieu in which gametes mature, are transported, are fertilized and develop.

Objectives of the present study were to investigate the effects of eFSH treatment in reproductively normal mares in terms of: folliculogenesis, pregnancy rate, early embryonic development, reproductive tract parameters, and serum estradiol-17β and progesterone concentrations. We hypothesized that eFSH treatment would 1) increase number of ovulations and pregnancy rate, and 2) alter the reproductive tract variables and serum concentrations of progesterone and estradiol-17β.

7.3. Materials and methods

7.3.1. Animals and reproductive tract examinations

Quarter Horse-Percheron cross mares (n = 26), aged 3 to 10 years, with a body condition score of at least 5 on a 9 point assessment system, were used during the ovulatory season (May to August in the Northern Hemisphere). Mares were kept in outdoor paddocks with sheds, and were maintained on alfalfa and grass hay with access to water and trace-mineralized salt, in accordance with the University of Saskatchewan's Institutional Animal Care and Use Committee. Mares had no signs of systemic disease or lameness, and were of good perineal conformation. Examinations of the reproductive tracts were performed using a transrectal approach and included manual palpation followed by an ultrasonographic examination. Uterine and cervical tone were palpated and separately scored from 1 to 4 (1- soft, 2- moderately soft, 3- moderately toned, 4- toned). A B-mode ultrasonographic scanner (GE Ausonics, Universal Ultrasound, Bedford Hills, NY, USA) equipped with a 5.0 MHz linear-array transducer was used to measure the maximum diameter of all follicles ≥20mm, and to detect ovulation. Overall endometrial edema was scored from 0 (no edema, homogeneous grey) to 4 (marked edema with a distinct black and white pattern as modified from Samper (1997).

Before the study was initiated, mares were examined every 1 to 3 days by rectal palpation and ultrasonographic examination, for at least one estrous cycle, to determine health of the reproductive tract and ovarian activity consistent with the breeding season. During the study, mares were examined daily from the time of treatment initiation until 3 days after ovulation(s), and on days 11 to16 after ovulations were detected.

7.3.2. Experimental design and treatment groups

The experimental unit in this study was considered to be a mare cycle. Mares (n = 26) were randomly assigned to one of two treatment groups for a total of 16 estrous cycles when eFSH treatment occurred and 26 estrous cycles for control mares. All mares were used for the control group, and 16 mares were randomly selected to be used in the eFSH group. The 16 mares that were used for both groups were assigned a treatment group using a balanced randomized cross over design; a mare was allocated to the second treatment after the first treatment protocol was

completed, and only after the mare had expressed signs of estrus and ovulation was occurred spontaneously ("rest estrous cycle").

In the eFSH group (n = 16 estrous cycles) mares were treated with eFSH (eFSH®, Bioniche Animal Health Canada Inc., Belleville, ON, Canada; 12.5 mg, i.m.) twice daily. Treatment was initiated ≥5 days after a previous spontaneous ovulation, and when a follicle ≥20 mm in diameter was detected. Mares received prostaglandin F2α (PGF2α, Lutalyse®, Pharmacia Animal Health, Orangeville, ON, Canada; 5 mg s.c.) 2 days after eFSH initiation. The eFSH treatment ceased when a ≥35 mm follicle was detected, and approximately 36 h later human chorionic gonadotropin (hCG, Chorulon®, Intervet Canada Ltd, ON, Canada; 2000 IU, i.m.) was administered. In the control group (n = 26 estrous cycles) mares were administered PGF2α 7 days after ovulation, and were followed into estrus. When a follicle ≥35 mm in diameter was detected, hCG was administered to induce ovulation.

In both treatment groups, mares were artificially inseminated 24 h after a follicle ≥35 mm in diameter was detected, and again every 48 h until ovulation (Day 0 = day of first ovulation) with fresh semen collected from a stallion of proven fertility. A minimum insemination dose of 5×10^8 progressively motile spermatozoa extended 1:1 with Kenney extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA, USA) was used.

7.3.3. Pregnancy diagnosis and completion of treatment

On days 11 to 16 after first ovulation, daily transrectal ultrasonographic examinations were performed to evaluate the mares' pregnancy status (pregnant yes/no) and the number of embryos present. Ultrasonographic images of each embryonic vesicle were frozen three times at the largest subjective image of the vesicle, and for each vesicle the average diameter was recorded. On Day 16, all mares were administered PGF2α . Mares which were used for an additional treatment were allowed to have a "rest estrous cycle" before the second treatment was initiated.

7.3.4. Measurement of serum estradiol-17β and progesterone concentrations

Jugular blood samples were collected into sterile plain vacutainer tubes just prior to hCG administration, and on Days 8, 11, and 16. The blood was refrigerated overnight, the samples

centrifuged, and the sera were separated and stored frozen (-20° C) until hormone assays. Progesterone (P4) and estradiol-17β (E2) concentrations were determined using radioimmunoassays validated for use in horses. For P4, all samples were analyzed in duplicates in one assay, using the Coat-A-Count® Progesterone In-vitro Diagnostic Test Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) (Blight and White, 1983); the intra-assay coefficient was < 5.1%. For E2, samples were analyzed in three assays using a radioimmunoassay developed and validated by the WCVM Endocrinology Laboratory, University of Saskatchewan (Joseph et al., 1992; Bragg Wever et al., 2002); standards (17β-Estradiol, E8875, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) were prepared in charcoal stripped equine serum, and ranged from 1 to 100 pg/ml. Samples from each mare were analyzed in duplicates in the same assay, and the numbers of samples from eFSH and control mares were balanced within an assay. For E2, the intra- and inter-assay coefficients of variation were < 5.3% and < 8.5%, respectively.

7.3.5. Statistical analyses

Statistical analyses were performed with analytic computerized software (Statistix 8 Student Edition, Analytical Software, Tallahassee, FL, USA). Pearson chi-square test analysis was used to compare proportional data such as the proportion of estrous cycles which resulted in a pregnancy, the proportion of mare cycles with large non-ovulatory follicles, and the overall proportion of large follicles from which ovulation failed to occur. The effects of mare and the order of treatments in mares which received the two treatments on the main outcomes (number of ovulations, number of embryos, pregnancy outcome, uterine and cervical variables) were analyzed with the generalized linear mixed model; they were not significant, and therefore were pooled within the residual. Between groups, Kruskal–Wallis non-parametric one-way ANOVA analysis was used to compare the number of preovulatory follicles (≥30mm) at the time of hCG administration, number of ovulations, number of embryos, intervals from PGF2α or hCG administration to the first ovulation, mean number of large non-ovulatory follicles, and serum concentrations of E2 and P4 (on Day of hCG, Days 8, 11, or 16). The general effects of the treatment, the day, and the day by treatment interaction, on uterine and cervical variables were analyzed in a General Analysis of Variance test; comparisons between groups on a specific day (Day of hCG, Days -1, 0, 1, 2, 8, 11-16) were performed with Kruskall–Wallis non-parametric one-way ANOVA.

Embryonic vesicle diameters were not analysed by day, as some mares had asynchronous ovulations, and it was impossible to accurately determine which embryo resulted from which ovulation when there was more than one ovulation. Instead, because equine embryonic vesicles are expected to grow in a linear fashion from day 11 to 16 (Ginther, 1995; Rantanen and McKinnon, 1998; Silva and Ginther, 2006), a value of mean daily growth rate from Days 11 to 16 was determined for each embryonic vesicle. In most pregnant mares, a singleton pregnancy was diagnosed, and mean daily growth rate was determine by averaging the differences of the vesicle's diameters measured on two subsequent days. In three eFSH-treated mares, a twin pregnancy (two embryonic vesicles) was diagnosed; in two of these mares ovulations were asynchronous, the two embryonic vesicles were of distinct sizes and easy to follow individually, and mean daily growth rate was determined for each vesicle; In the third mare, all ovulations were synchronous, and with not being able to follow each embryonic vesicle individually, the mean daily growth rate for each vesicle was calculated from the average diameters of the two vesicles on a given day. Differences in the mean daily embryonic growth rate between the groups were analysed with Kruskall–Wallis non-parametric one-way ANOVA analysis; and Bartlett's Test was used to determine the equally of variances. Differences were considered significant at $P < 0.05$.

7.4. Results

7.4.1. Folliculogenesis and ovarian stimulation

Results are presented as mean ± S.E.M, and in a subsequent order for the eFSH group compared with the control group. The duration of eFSH treatment was 8.9 ± 0.6 days. Two mares in the eFSH group had ovulations before hCG was administrated, therefore, variables which are related to the day of hCG administration were analyzed for 14 out of the 16 estrous cycles in eFSH-treated mares. The intervals from PGF2α to first ovulation (9.3 ± 1 compared with 7.5 ± 0.5 days), and from hCG administration to first ovulation (2.2 ± 0.7 compared with 2.1 ± 0.1 days) were not significantly different between groups. In the eFSH group, there was a greater number of preovulatory follicles (≥30 mm in diameter) at the time of hCG administration (2.6 ± 0.4 compared with 1.1 ± 0.1 preovulatory follicles; $P < 0.05$), and greater number of ovulations (2.3 ± 0.5 compared with 1.1 ± 0.3 ovulations; $P < 0.05$) than the controls (Table 7.1). In 6 of 16 mares in the eFSH group, ovulations were asynchronous; in four mares all ovulations were detected within

24 h (examinations of 2 consecutive days); and in two mares all ovulations were detected within 48 h (examination of 3 consecutive days).

In both treatment groups, all mares had ovulations from at least one follicle; however, in some mares in the eFSH group, a large non-ovulatory follicle was observed in addition to the normal ovulation(s) (Table 7.1). A large non-ovulatory follicle was defined as a preovulatory follicle (≥30 mm) that was observed on the day of hCG treatment, but from which ovulation failed to occur within 3 days of the first ovulation. In the eFSH group, for 4 of 16 (25%) of the estrous cycles at least one non-ovulatory follicle (2 ± 0.4) was observed, whereas in the control group no large non-ovulatory large follicles were observed with any of the estrous cycles ($P < 0.05$). In the overall per follicle comparison, ovulation failed to occur from more preovulatory follicles in the eFSH group than control group (22%, 8/37 compared with 0%, 0/28 non-ovulatory large follicles; $P < 0.05$).

7.4.2. Pregnancy rates and early embryonic development

Pregnancy rates were not significantly different between the eFSH (50%; 8/16) and the control (62%; 16/26) groups (Table 7.2). Also, mean embryo number was not different (0.7 ± 0.2 compared with 0.7 ± 0.1 embryo/mare). However, among the pregnant mares, mean embryo number tended to be greater in the eFSH group (1.4 ± 0.2 compared with 1.1 ± 0.1 embryo/pregnancy; $P = 0.0559$), as the incidence of twin pregnancy (two embryonic vesicles per pregnant mare) tended to be greater than in the control group (38%, 3/8 compared with 7% 1/16; $P = 0.09$). Interestingly, mean embryo number per ovulation was less in the eFSH group compared with the control group, both when all mares were included in the analysis (0.3 ± 0.1 compared with 0.6 ± 0.1 embryo/ovulation), or when only pregnant mares were included (0.6 ± 0.1 compared with 1 ± 0.1 embryo/ovulation) ($P < 0.05$; Table 7.2).

In the eFSH group, five pregnant mares were first diagnosed to be pregnant (at least one embryonic vesicle) on Day 11, and 3 mares on Day 12. All three mares that had a twin pregnancy were first diagnosed on Day 11; in one mare both embryonic vesicles were detected on Day 11; in one mare one embryonic vesicle was first detected on Day 11 and the second on Day 12; and in one mare one embryonic vesicle was first detected on Day 11 and the second on Day 13. In the control group, 14 mares were first diagnosed to be pregnant on Day 11, and two mares were diagnosed on Day 12; in the mare that had a twin pregnancy both embryonic vesicles were

detected on Day 11. During Days 11 to 16, a reduction in the number of embryonic vesicles was not observed in any of the mares. Mean overall daily growth rate from Day 11 to 16 was not statistically different between embryonic vesicles in the eFSH group (3.3 ± 0.3 mm/day) and the control group (3.7 ± 0.1 mm/day; P = 0.2; Figure 7.1); however, it was significantly more variable in the eFSH group (95% CI: 2.6-3.8 mm/day) than in the control group (95% CI: 3.5-3.9 mm/day).

7.4.3. Uterine and cervical variables

Overall, uterine and cervical variables were affected by the day, by the treatment and by the day by treatment interaction (P < 0.01). Generally, uterine and cervical tones were scored less (softer), with greater endometrial edema scores just before and after ovulation, as compared with Days 11 to 16. Uterine and cervical tone increased and endometrial edema decreased from the day of hCG administration to 2 days after the first ovulation (P < 0.01). On these days, mares treated with eFSH had lesser uterine and cervical tone scores, and greater endometrial edema scores than mares in the control group (P < 0.03). When uterine and cervical parameters for both groups on Days 11 to 16 were analyzed, there was no significant day effect. In addition, on these days, endometrial edema and cervical tone were not different between groups; however, the uterus of mares treated with eFSH indicated a greater tone as compared to control mares (P < 0.01).

7.4.4. Serum estradiol-17β and progesterone concentrations

Results from the analysis of serum E2 and P4 concentrations from the eFSH and control groups are presented in Figure 7.2. Mares treated with eFSH had greater serum E2 concentrations on the Day of hCG (51.0 ± 2.3 compared with 15.2 ± 0.2 pg/ml; P < 0.01), and significantly lesser E2 concentrations on Day 16 (6.6 ± 0.2 compared with 11.4 ± 0.2; P < 0.01). Within each group, serum E2 concentrations on the days measured were not different between pregnant and non-pregnant mares (P > 0.1). Serum P4 concentrations were not different between groups on the Day of hCG treatment; however, there were significantly greater concentrations in the eFSH group on Days 8, 11, and 16 as compared with the control group (P < 0.01). Within a group, there were no differences in serum P4 concentrations between pregnant and non-pregnant mares on Day of hCG, and Days 8 and 11. However, in both groups, serum P4 concentrations on Day 16 were greater in pregnant mares (eFSH: 14.5 ± 1.6; Control: 7.1 ± 0.8 ng/ml) compared with non-pregnant mares (eFSH: 2.1 ± 1; Control: 1 ± 0.9; P < 0.02).

Table 7.1 Ovarian variables in mares treated with eFSH as compared to non-treated controls**.** Results are presented as mean ± S.E.M or percentages (%)

	eFSH (n = 16)	Control (n = 26)
No. of preovulatory follicles[1]	2.6 ± 0.4 [a]	1.1 ± 0.1 [b]
No. of ovulations	2.3 ± 0.5 [a]	1.1 ± 0.3 [b]
Proportion of mares in which ovulations occurred	100% (16/16)	100% (26/26)
Proportion of mares with large non-ovulatory follicle(s)	25% (4/16) [a]	0% (0/26) [b]
Proportion of preovulatory follicles failing to ovulate [2]	22% (8/37) [a]	0% (0/28) [b]

[ab]Values within a row with different superscripts are significantly different ($P < 0.05$)

[1]Follicles ≥30 mm in diameter at the time of hCG administration; the mean was calculated from 14 of 16 mares in the eFSH group, and from 26 of 26 mares in the control group.

[2]Proportion of preovulatory follicles from which ovulation failed to occur within 3 days from the first ovulation; was calculated from 14 of 16 mares in the eFSH group, and from 26 of 26 mares in the control group.

Table 7.2 Pregnancy rate (%), embryo number (mean ± S.E.M), and embryo number per ovulation (mean ± S.E.M) in eFSH-treated and control mares, and in pregnant mares within each group.

	eFSH	Control
Pregnancy rate	50% (8/16)	62% (16/26)
No. of embryos	0.7 ± 0.2	0.7 ± 0.1
Embryo per ovulation	0.3 ± 0.1 [a]	0.6 ± 0.1 [b]
Embryo number per pregnancy[1]	1.4 ± 0.2	1.1 ± 0.1
Embryo per ovulation in pregnant mares	0.6 ± 0.1 [a]	1.0 ± 0.1 [b]

[ab]Values within a row with different superscripts are significantly different ($P < 0.05$)

[1] $P = 0.06$

Figure 7.1 Box-and-whisker plots of daily growth rate of embryonic vesicles (Days 11 to 16) in eFSH-treated and control mares. The median (central black line) is shown with the 25% and 75% percentile ranges (box depth) and the maximum and minimum (T-bars).

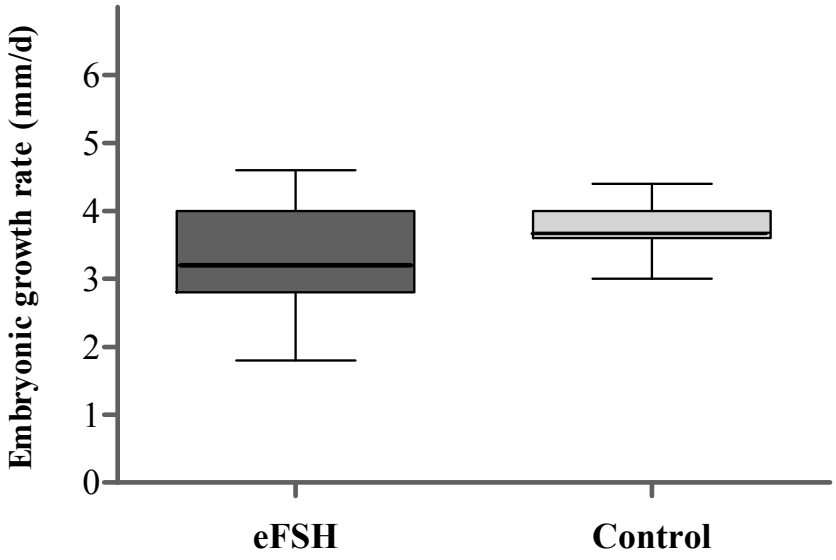
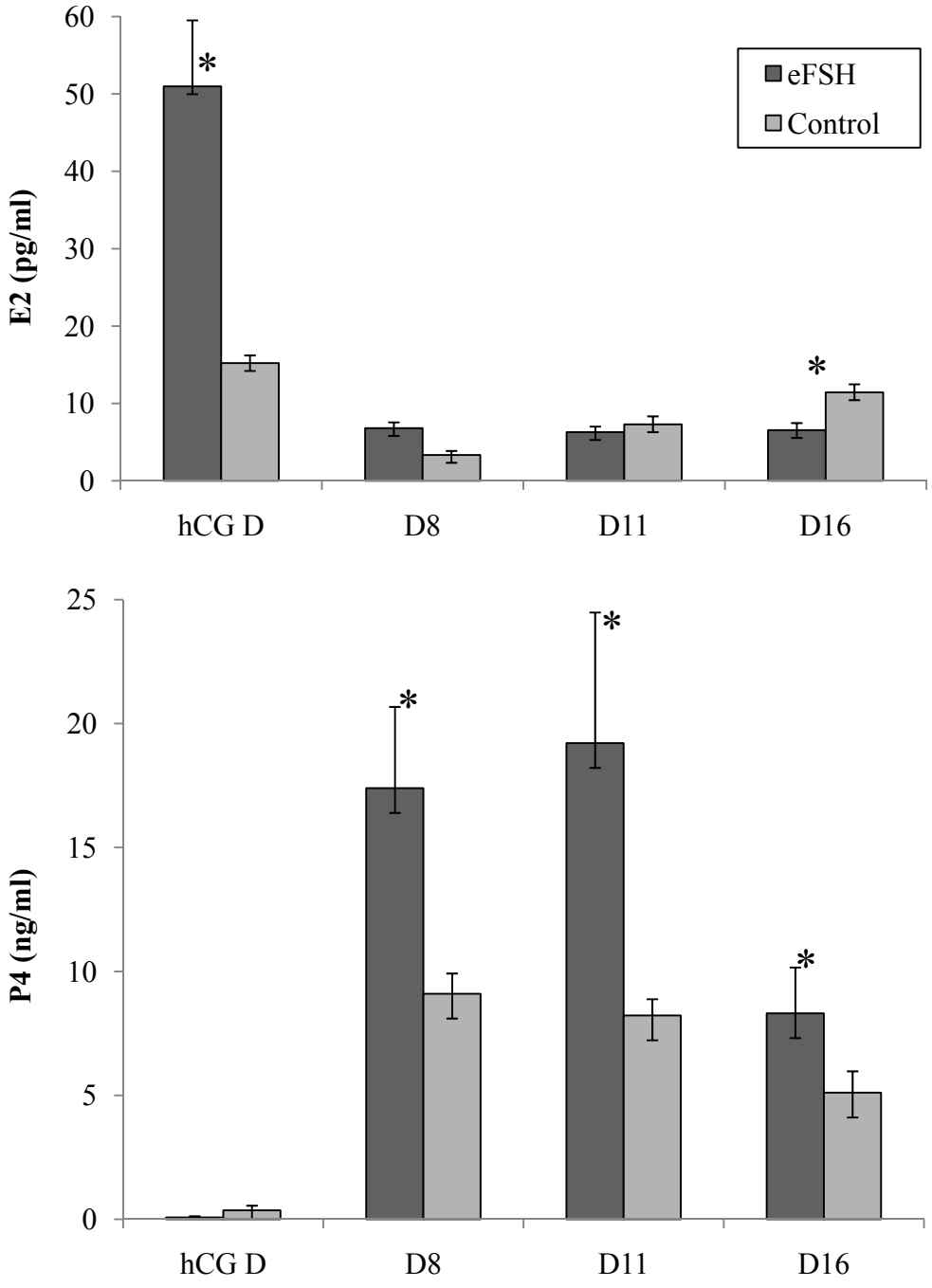


Figure 7.2 Serum estradiol (E2) and progesterone (P4) concentrations (mean ± SEM) on the day of hCG administration, and on days 8, 11 and 16 post-ovulation in eFSH-treated and control mares. Asterisks indicate values that are significantly different ($P < 0.05$) between groups on a specific day.



7.5. Discussion

To our knowledge, this is the first study that examined folliculogenesis and pregnancy rate, together with early embryonic development, reproductive tract variables, and serum P4 and E2 concentrations in eFSH-treated mares. The rationale of the current eFSH treatment regimen was to 'rescue' subordinate follicles in the follicular wave and promote their continued development and ovulation; and consequently to increase the number of ovulations and the probability of establishing a pregnancy. The dose and frequency of eFSH treatment used in the present study were according to the manufacture's recommended protocol, and as previously reported (McCue et al., 2007a). Administration of hCG was included in the treatment regimens of both groups for ovulation induction, as it is commonly used in the equine industry. In the eFSH group, hCG was administered in a 36 h delayed period after the last eFSH treatment, a period that is termed 'coasting', a regimen that is beneficial when estrous cycling mares were treated with eFSH (Welch et al., 2006). The rationale for the 'coasting' period is to allow the FSH concentration to decrease during the final maturation phase of the follicles. In the control group, a similar 'coasting' period was not utilized, as the endogenous FSH concentrations are expected to be suitable for follicular maturation (Ginther, 1992a). The design for the present study consisted of uneven number of estrous cycles in the two treatment groups due to the greater cost of eFSH and because it was anticipated that there would be a greater number of ovulations and embryos following eFSH treatment.

In accordance with previous reports, eFSH treatment stimulated the ovaries and increased the number of ovulations (McCue et al., 2007a). The number of preovulatory follicles and the number of ovulations in the eFSH and control groups were consistent with previous reports (Ginther, 1992a; Niswender et al., 2003; Niswender et al., 2004; Raz et al., 2005; Hudson et al., 2006; Raz et al., 2006b; Welch et al., 2006; Logan et al., 2007b). Interestingly, there was a greater incidence of large non-ovulatory follicles following the eFSH treatment; these follicles continued to grow and some became static in growth pattern after the eFSH treatment ceased, and later regressed or appeared luteinized. There are reports of a proportion of eFSH-treated mares that completely failed to have ovulations, or mares that experienced large non-ovulatory follicles accompanying normal appearing ovulations (Niswender et al., 2003; Raz et al., 2005; Raz et al., 2006b; Logan et al., 2007b; Niswender et al., 2007). In addition, in the current study, as in most other studies, the

number of ovulations was less than the number of the preceding preovulatory follicles in eFSH-treated mares, but not in controls (Niswender et al., 2003; Raz et al., 2005; Raz et al., 2006b; Logan et al., 2007b; Niswender et al., 2007).

The phenomenon of ovulation failure in eFSH-treated mares does not appear to be related to insufficient LH-like stimulatory agent as in this and other reports non-ovulatory large follicles are more commonly observed along with other ovulations. One possible explanation is the unique structure of the mare ovary in which follicles develop in the interior cortex of the ovary and with ovulations only occurring through the limited area of the ovulation fossa may create an anatomical barrier which restricts number of possible ovulations. Other explanations may be related to possible alteration of hormonal environment, alteration of the number of LH receptors, or changes in the affinity or hormone receptors in preovulatory large follicles in gonadotropin-superstimulated mares (Raz et al., 2006b). In the current study, the mean serum E2 concentration in eFSH-treated mares as compared with control mares was much greater on the day of hCG, probably due to the greater number of preovulatory follicles. In another study (Raz and Card, 2009), E2 concentrations were more than three times greater in eFSH-treated mares compared with control mares on the day the first ovulation was detected (Day 0). The E2 concentration pattern in non-eFSH-treated mares has been described (Ginther, 1992a), with a significant decrease in concentrations before ovulation. Greater and prolonged preovulatory E2 concentrations have been associated with persistent follicles and reduced fertility in mares and other species (Moor et al., 1985; Breuel et al., 1993; Wehrman et al., 1993; Ahmad et al., 1995; Ginther et al., 2007a). The greater and prolonged E2 concentrations in eFSH-treated mares may disturb normal follicular maturation and ovulation.

Although eFSH treatment stimulated the ovary and increased number of ovulations, pregnancy rates and mean embryo number were not different between groups. Among pregnant mares, mean embryo number per pregnancy tended to be greater in eFSH-treated as compared to control mares suggesting eFSH treatment may increase incidence of twin pregnancy in some individual mares. Niswender at al. (2003) evaluated pregnancies on days 14 to 16 post ovulation in five mares treated with eFSH followed by hCG, and reported 1.8 ± 0.7 embryos/mare; however, in 15 mares treated with eFSH followed by deslorelin, mean embryo number was not different from controls (0.6 embryos/mare).

The rhythmic changes in the reproductive tract, including gross, histologic, contractile and secretory changes, are under the control of ovarian steroids (Ginther, 1986; 1992a), and are in physiologic harmony with the stage of the estrous cycle and developmental transitions of oocytes and embryos. Therefore, in the present study uterine and cervical variables were evaluated, along with serum concentrations of E2 and P4 on selective days, as indicators for reproductive tract function in eFSH-treated as compared with control mares. As expected, changes in uterine and cervical variables paralleled serum concentrations of E2 and P4. The eFSH treatment modified the hormonal and reproductive tract milieu on the days that oocyte maturation, fertilization, and early embryonic development occurred in the present study as expected. Serum E2 concentrations in the eFSH-treated mares were greater on the day of hCG administration. At the time of this treatment and up to 2 days post ovulation, lesser scores of uterine and cervical tone, and increased endometrial edema were recorded for eFSH treated as compared to control mares. Prolonged and/or greater preovulatory secretion of E2 has been reported in animals of other species that were induced to superovulate (Moor et al., 1985; Kelley et al., 2006), and was associated with lower cleavage rates, early embryonic losses, abnormal embryonic development, and lesser conception rates (Lewinthal et al., 1987; Breuel et al., 1993; Johnson and Lewis, 1993; Wehrman et al., 1993; Ahmad et al., 1995); this has been attributed to the negative effect on oocyte maturation and alterations in the uterine environment (Roberson and Baker, 1969; McGaughey, 1977; Butcher and Pope, 1979; Moor et al., 1985; Ahmad et al., 1995). Considering these previous findings, it is reasonable to presume that mares treated with eFSH have altered oocyte maturation, modified secretions and contractility of the oviduct and uterus that may have a negative effect on fertilization, transfer of gametes, and early embryonic development.

The greater serum P4 concentrations observed in the eFSH-treated mares in the present study was an expected finding as more corpora lutea were present. There is limited information on the effects of progestin treatment on embryonic development in horses; however, results of some studies indicate progesterone treatment during early pregnancy stimulates expression of genes associated with embryonic development possibly resulting in an increased pregnancy rate in mares and females of other species (McKinnon et al., 1988; Ginther, 1992a; Mann et al., 1999; Budik et al., 2006). Therefore, the greater concentrations of P4 during diestrus in eFSH-treated mares seem to be advantageous, and may be beneficial in some mares with a history of luteal dysfunction.

Interestingly, mares treated with eFSH had significantly lower E2 concentrations on Day 16 as compared with mares of the control group. To elucidate the cause, records were re-examined, and number of follicles with a diameter ≥20mm on Day 16 was found to not be different between groups, however, diameter of the largest follicle on Day 16 was less in mares treated with eFSH (24.8 ± 2.2 compared with 31.2 ± 2.0 mm; $P < 0.05$). Although the present study was not designed to evaluate this phenomenon, it is hypothesized that early and prolonged E2 concentration before and around ovulation had transient suppressive effects on endogenous FSH secretion, which potentially resulted in a delay in subsequent follicular wave emergence and smaller follicle diameter on Day 16. The specific effect of alteration in P4 to E2 ratio on embryonic development in eFSH treated mares could not be determined in the present study, however, it may be related to the greater variability in growth rate of embryos in FSH-treated mares.

Pregnancy rate and mean embryo number in the eFSH group in the present study were less than was anticipated. It is a possibility that if there had been recovery attempts for embryos on day 6 to 8 post ovulation, more embryos may have been found. There are a considerable number of reports outlining the increased embryo recovery rate 6 to 8 days post ovulation in donor mares treated with eFSH or other superovulatory treatments, showing that embryos reach the uterus. Nevertheless, in some studies superovulatory treatments of mares were associated with lower than expected embryos per ovulation, reduced embryo quality, and lesser pregnancy rate in recipients that received an embryo from superstimulated donors (Woods and Ginther, 1983b; Woods, 1984; Alvarenga et al., 2001; Niswender et al., 2003; Peres et al., 2005; Raz et al., 2005; Carmo et al., 2006; Raz et al., 2006a; Raz et al., 2006b; Logan et al., 2007b). Similarly, in other species, superovulatory treatments impact viability of some proportion of embryos recovered (Akira et al., 1993; Youngs, 2001; Mapletoft et al., 2002; Cognie et al., 2003; Kelley et al., 2006). Considering this information and the current findings, it is likely that some of the day 6 to 8 embryos from mares with induced multiple ovulations are developmentally impaired, and although these embryos reach the uterus, development is abnormal, and therefore embryos do not survive or grow to a stage and size that can be diagnosed by transrectal ultrasonography examination.

The present study design was not sensitive enough to detect significant differences in mean daily embryonic growth rate on Days 11 to 16; however, significantly greater variability in growth rate of embryos developed in mares treated with eFSH may indicate some of these embryos were

compromised. In mares with spontaneous double ovulations embryo reduction before day 11 is unlikely, however, in mares where superovulatory treatments are used with equine pituitary extract, embryo reduction or retarded growth of some conceptuses before day 11 has been reported (Woods and Ginther, 1983b; Woods, 1984; Squires et al., 1987a; Ginther and Bergfelt, 1988). The present study provides important information on alterations caused by eFSH treatment which may explain differences between viability of embryos produced by mares stimulated to superovulate as compared to untreated mares.

In summary, the eFSH treatment stimulated the ovary, and increased the number of preovulatory size follicles and number of ovulations, however, the probability of establishing a pregnancy was not increased. Administration of eFSH modified the reproductive tract variables and serum concentrations of progesterone and estraiol-17β on days that oocyte maturation, fertilization, and early embryonic development are expected to occur. These alterations may be related to the greater incidence of non-ovulatory follicles, lower embryo per ovulation rate, and the lesser than expected pregnancy rates in the eFSH-treated mares.

8. EFFICIENCY OF SUPEROVULATION AND *IN-VIVO* EMBRYO PRODUCTION IN eFSH-TREATED DONOR MARES FOLLOWING ESTRUS SYNCHRONIZATION WITH PROGESTERONE AND ESTRADIOL-17B

This study was accepted for publication in Theriogenology
(Theriogenology 2009, 72 169-178. PMID: 19344941)

8.1. Abstract

Reliable methods of regulating estrus and stimulating superovulations in equine embryo transfer programs are desirable. Our objectives were to investigate the efficacy of a progesterone and estradiol-17β estrus synchronization regimen (P&E) in mares with and without subsequent eFSH treatment, and to examine the effects of eFSH on folliculogenesis and embryo production. Cycling mares were treated with P&E daily for 10 d. On the final P&E treatment day, PGF2α was administered and mares were randomly assigned to one of two treatment groups (n = 20 mares/group). In both groups mares were examined daily by transrectal ultrasonography. In the eFSH group, twice daily eFSH treatments were initiated at follicle size ≥25 mm and ceased at follicle size ≥35 mm; hCG was administered 36 h after cessation of eFSH treatment. In the control group, eFSH treatments were not given, but hCG was administered at follicle size ≥35 mm. Mares were inseminated with fresh semen, and embryo recovery attempts were performed 8 d post-ovulation. Synchrony of ovulations within each group appeared to be similar. Six mares in the eFSH group failed to ovulate. The eFSH treatment resulted in significantly ($P < 0.05$) higher numbers of preovulatory follicles and ovulations; however, embryo recovery rate did not increase (eFSH: 1.0 ± 0.4 vs. control: 0.95 ± 0.1 embryos/recovery attempt), and embryo per ovulation rate was significantly lower (36% vs. 73%). The eFSH-treated mares had significantly higher frequency of non-ovulatory follicles (28% vs. 0%), and higher periovulatory serum concentrations of estradiol-17β. Based on our findings, combined P&E and eFSH regimens cannot be recommended for cycling donor mares.

8.2. Introduction

In the last few decades embryo transfer technologies became more attractive to horse owners as more breed registries approved the registration of multiple embryo transfer foals. Nevertheless, the commercial expansion of embryo transfer in the equine industry is lagging behind the cattle industry, mainly due to limited success rate and high cost of superovulation and embryo cryopreservation methods (Dobrinsky, 2002; Stout, 2006; Scherzer et al., 2008). In addition, adequate synchronization of estrous cycles of donor and recipient mares can be labor intensive and difficult because of the great variation in estrous length among mares (Ginther, 1992b). Therefore, a demand for a reliable means of predicting or regulating estrus and multiple ovulations in the mare has increased, particularly for situations when embryo transfer, artificial insemination, and other forms of appointment breeding are to be performed.

Currently, it appears that most embryos are collected from single ovulating mares on Day 7 or 8 after ovulation, and transferred as fresh or after a short cooling period (12-24 h) to the uterus of a synchronized recipient (Squires et al., 2003a; Stout, 2006). Recipients ovulating between one day before and 3 d after the donor are equally likely to become pregnant after receiving an embryo; however, outside this window of acceptable synchrony, pregnancy rates may drop significantly (Allen and Rowson, 1975; Squires et al., 1986; Stout, 2006). Treatment regimens that can be used to synchronize estrus and ovulation in the mare include follicular ablation (Bergfelt et al., 2007), administration of prostaglandins (Douglas and Ginther, 1975b), and administration of progestagens alone or together with estradiol (Blanchard and Varner, 1995). These treatments are all usually combined with administration of ovulation inducing agents such as hCG or GnRH analogue (Samper, 2008). The administration of progesterone and estradiol-17β (P&E), daily, for 10-12 d, followed by administration of prostaglandin on the last day of the P&E treatment, and hCG when a large preovulatory follicle is subsequently detected, is one of the most predictable hormonal regimens to synchronize ovulation in mares used in embryo transfer programs, particularly in situations when only one or two recipients per donor are to be used (Loy et al., 1981; Taylor et al., 1982b; Bristol et al., 1983).

The recent availability of a commercial equine FSH preparation (eFSH, Bioniche Animal Health Inc., Athens, GA, USA) has created the potential for successful superovulatory treatment in mares (Niswender et al., 2003). In many studies eFSH treatment has been shown to increase the

number of ovulations and embryo recovery rates in transitional and cycling mares used for embryo transfer (reviewed by Squires and McCue, 2007). However, in some studies, eFSH treatments of cycling mares have been associated with variability in the superovulatory response among mares (Squires et al., 2006), as well as with unwanted hyperstimulatory effects such as ovulation failure, non-ovulatory follicles, and lower than expected embryo per ovulation rates (Niswender et al., 2003; Briant et al., 2004; Raz et al., 2005; Raz et al., 2006a; Raz et al., 2006b; Squires, 2006a; Logan et al., 2007b; Niswender et al., 2007; Alvarenga et al., 2008).

The synchrony of ovulations among mares after P&E regimen, with and without subsequent eFSH treatment apparently has never been compared. In addition, there is very limited information on the use of eFSH subsequent to a P&E estrus synchronization regimen (Raz et al., 2005; Logan et al., 2007b). Potentially, P&E treatment may result in a uniform population of follicles (Loy et al., 1981; Taylor et al., 1982b; Bristol et al., 1983) and a more consistent superovulatory response to eFSH; however, there is a risk that the P&E treatment would alter the ovarian sensitivity to eFSH, or that the eFSH treatment would stimulate follicles that are in an advanced degree of atresia, resulting from the endogenous gonadotropins reduction caused by the P&E (Evans et al., 1982; Plata-Madrid et al., 1992).

In the present study donor mares were first allocated to a P&E estrus synchronization regimen, and subsequently were assigned to either eFSH treatment or control group. The objectives of this study were 1) to investigate the efficacy of the P&E protocol as an estrus synchronization method in mares with and without subsequent eFSH treatment, and 2) to investigate the effects of eFSH on folliculogenesis, ovulation, and embryo production subsequent to P&E estrus synchronization method. We hypothesized that: 1) daily P&E treatments for 10 d would suppress follicular growth, and would effectively synchronize ovulation in mares that were subsequently treated with eFSH, and in controls; 2) Following the P&E treatments, ovulation synchrony within each group would be similar, however, the mean interval to ovulation would be different; 3) the eFSH treatment would result in higher numbers of preovulatory follicles, ovulations, and embryos, as compared to control; 4) endogenous serum estradiol-17β and progesterone concentrations would be different between mares treated with eFSH as compared to controls; and 5) embryo per ovulation rate would be different between the groups.

8.3. Materials and methods

8.3.1. Animals and reproductive tract examinations

Non-lactating Quarter Horse-Percheron cross mares (n = 40), aged 3 to 10 years, with a body condition score of at least 5 (range 5 - 7) on a 9 point assessment system (Henneke et al., 1983), were used during the physiologic breeding season (June to August 2006 in the Northern Hemisphere). Mares were kept in outdoor paddocks with sheds, and were maintained on alfalfa and grass hay with access to water and trace-mineralized salt, in accordance with the University of Saskatchewan's Institutional Animal Care and Use Committee. Mares had no signs of systemic disease, and were of good perineal conformation. Examinations of the reproductive tract were performed using a transrectal approach and included manual palpation followed by an ultrasonographic examination. A B-mode ultrasound scanner (GE Ausonics, Universal Ultrasound, Bedford Hills, NY, USA) equipped with a 5.0 MHz linear-array transducer was used to measure the maximum diameter of all follicles ≥20 mm, and to detect ovulation(s). Before the study was initiated, mares were examined every 1 to 3 d for at least one estrous cycle, to determine health of the reproductive tract and that the ovarian activity was consistent with the breeding season.

8.3.2. Estrus synchronization with progesterone and estradiol-17β (P&E)

All mares (n = 40) were first administrated a P&E compounded product (WCVM Veterinary Pharmacy, University of Saskatchewan, Saskatoon, SK, Canada) containing 150 mg of progesterone and 10 mg of estradiol-17β in sesame oil, intramuscularly, once daily for 10 d. Prostaglandin F2α (PGF2α, 5 mg s.c., Lutalyse®, Pharmacia Animal Health, Orangeville, ON, Canada) was administered on the final day of P&E regimen (Blanchard and Varner, 1995). The P&E treatments were initiated in predetermined dates, regardless of the physiologic status of the mare (estrus/diestrus). To spread the amount of work and to avoid exhausting our stallion, the P&E treatments were not initiated on the same date for all mares; instead, the mares were randomly divided into 4 synchronization clusters, in which the P&E treatments were initiated 4 d apart (the first cluster of the P&E treatments was initiated on June 21st). The mares were examined per-rectum, as described, on days 1, 3, 5, 7, 9, and 10 of the P&E treatment (PE1, PE3, PE5…, and PE10, respectively), and then daily until three days post ovulation, and on the day that embryo recovery attempt was performed.

8.3.3. Treatment groups

Mares were randomly assigned into either eFSH or control groups (20 mares/group) on the last day of P&E regimen. The number of mares assigned to each treatment group was balanced within each synchronization cluster. In the eFSH group mares were treated with twice daily eFSH (12.5 mg i.m., eFSH®, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) initiated when a follicle ≥25 mm in diameter was detected (Squires and McCue, 2007). When a follicle ≥35 mm in diameter was detected the last eFSH treatment was given, and approximately 36 h later hCG (2000 IU i.m., Chorulon®, Intervet Canada Ltd, Whitby, ON, Canada) was administered to induce ovulation (Welch et al., 2006). In the control group mares were examined daily by transrectal ultrasonography, and when a follicle ≥35 mm in diameter was detected, hCG was administered.

In both treatment groups, mares were artificially inseminated 24 h after hCG was administrated, and again every 48 h until ovulation (Day 0 = day of the first ovulation), with fresh semen collected from a 6 yr old Quarter-Horse stallion using a Missouri-type artificial vagina. The stallion was of proven past fertility and successfully passed a breeding soundness evaluation test (Kenney et al.; Varner) before the study was initiated. A dose of 5 to 6 x 10^8 progressively motile spermatozoa extended 1:1 with Kenney extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA, USA) was used for each insemination.

8.3.4. Embryo recovery attempts

Embryo recovery attempts were performed on Day 8 using a routine nonsurgical trans-cervical technique as described elsewhere (McKinnon and Squires, 1988a). A total of 4 L of embryo flush medium (ViGro Complete Flush Solution®, Bioniche Animal Health Canada Inc.) was used per mare, and mares were administered oxytocin (40 IU i.v., Oxytocin®, Vêtoquinol N.-A. Inc., Lavaltire, QC, Canada) before the procedure was completed to facilitate recovery. Embryos were identified using a stereomicroscope, enumerated, and rinsed 3 times in Holding Medium (Vigro Holding Plus®, Bioniche Animal Health Canada Inc.). Embryos were scored for quality (1-excellent, 2- good, 3-fair, 4-poor) according to their morphology as described by McKinnon and Squires (McKinnon and Squires, 1988b), and were subjectively assessed for age according to their developmental stage (morula/ early blastocyst/ expanded blastocyst) and size.

8.3.5. Measurement of serum estradiol-17β and progesterone concentrations

Jugular blood samples were collected into sterile plain Vacutainer tubes just prior to hCG administration (hCG-Day), on the day of ovulation (Day 0), and on day 8 post ovulation (Day 8), just prior to the embryo recovery attempt. The blood samples were centrifuged, and the sera were separated and stored frozen (-20°C) until progesterone (P4) and estradiol-17β (E2) radioimmunoassays were performed. For P4, all samples were analyzed in duplicates in one assay, using the Coat-A-Count® Progesterone In-vitro Diagnostic Test Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) (Blight and White, 1983); the intra-assay coefficient of variation was <3.4%. For E2, samples were analyzed in duplicates in a total of six assays using a radioimmunoassay developed and validated by the Western College of Veterinary Medicine Endocrinology Laboratory, University of Saskatchewan (Joseph et al., 1992; Bragg Wever et al., 2002); standards (17β-Estradiol, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) were prepared in charcoal stripped equine serum, and ranged from 1 to 100 pg/mL. Samples from each mare were analyzed in the same assay, and the numbers of samples from the two treatment groups were balanced within an assay. For E2, the intra- and inter-assay coefficients of variation were < 6.3% and < 8.9%, respectively.

8.3.6. Statistical analyses

Statistical analyses were performed with analytic computerized software (Statistix 8 Student Edition, Analytical Software, Tallahassee, FL, USA). Pearson chi-square test analysis was used to compare proportional data such as the proportion of mares that ovulated, proportion of mares with multiple ovulations, proportion of mares with a large non-ovulatory follicle, and proportion of successful embryo recoveries. The effect of the synchronization cluster on the main outcomes was analyzed by a General Analysis of Variance test; it was not significant, and therefore was pooled within the residual. Continuous data were evaluated for normality of distribution and for equality of variances using the Shapiro-Wilk Test and the Bartlett's Test, respectively. Accordingly, comparisons between groups were performed with either Student's *t*-test or Kruskal–Wallis non-parametric one-way ANOVA; Student's *t*-test was used to analyze normally distributed data that had equal variances between groups (largest follicle diameter at the last day of P&E treatment, interval to preovulatory follicle, interval to hCG administration, interval to ovulation); Kruskal–

Wallis non-parametric one-way ANOVA was used to analyze data which were not normally distributed and/or had unequal variance between groups (number of preovulatory follicles, number of ovulations, number of preovulatory follicles that failed to ovulate, number of embryos). Categorical data, such as embryo morphological grade and embryo age, were compared with Kruskal–Wallis non-parametric one-way ANOVA. The general effects of the treatment, the day, and the day by treatment interaction, on the largest follicle diameter, and on the serum steroid hormone concentrations, were analyzed in a Repeated Measures ANOVA, followed by Tukey HSD All-Pairwise Comparisons Test. Pearson's Correlation test was used to determine the correlation between the number of preovulatory follicles and serum E2 concentrations, and the correlation between the number of ovulations and serum P4 concentrations. Differences were considered significant at $P < 0.05$.

8.4. Results

8.4.1. Folliculogenesis and ovulation

On the day of P&E treatment initiation (PE1) 29/40 of the mares had a corpus luteum (CL) detected via transrectal ultrasonography; the number of mares with a CL was not different ($P > 0.05$) for mares that subsequently were assigned to the eFSH (15/20, 75%) or to the control group (14/20, 70%). The P&E treatment regimen was completed for all mares. The mean diameter of the largest follicle during the P&E regimen was significantly affected by the day, but was not different for mares subsequently assigned to the eFSH or control groups. The largest follicle diameter (Mean ± SEM) gradually decreased during the P&E regimen (PE1 26.9 ± 1.7; PE3 25.0 ± 1.6; PE5 21.8 ±1.5; PE7 20.4 ± 1.2; PE9 17.4± 1.0; PE10 16.1 ±0.9 mm). In most mares all follicles were ≤ 25 mm in diameter on last day of the P&E regimen; however, in one mare assigned to the eFSH group, a follicle of 26 mm was detected, and the eFSH treatment was initiated on that day; in two mares assigned to the control group, 28 and 30 mm follicles were detected.

The eFSH treatment was initiated 4.4 ± 0.5 d (range 0-7 d) after the P&E regimen was completed, and lasted for 5.4 ± 0.4 d (range 4-10 d; Table 8.1). All mares, in both groups, developed at least one follicle ≥35mm and received hCG. The mean interval from PE10 to a follicle ≥35 mm did not differ ($P > 0.05$) between the eFSH group (8.9 ± 0.3 d), and the control group (9.5 ± 0.4 d). The mean interval from PE10 to hCG administration was significantly longer

in mares treated with eFSH as compared to controls (10.4 ± 0.2 vs. 9.5 ± 0.4 d, respectively). During the four days before a follicle ≥35 mm was detected, the mean daily increasing rate of the diameter of the largest follicle was higher in mares treated with eFSH (4.5 ± 0.3 vs. 3.1 ± 0.2 mm/d; $P < 0.05$). The number of preovulatory follicles (≥30 mm) at the time of hCG administration was higher in the eFSH group (4.7 ± 0.5 vs. 1.3 ± 0.1 follicles; $P < 0.05$).

In the control group all mares ovulated within 72 h after hCG was administered, however, in the eFSH group 6/20 (30%) mares failed to ovulate ($P < 0.05$). In ovulating mares, the number of preovulatory follicles (≥30 mm) and mean ovulation number were significantly higher in mares treated with eFSH (4.1 ± 0.5 follicles; 2.8 ± 0.5 ovulations) as compared to controls (1.3 ± 0.1 follicles; 1.3 ± 0.1 ovulations). Furthermore, a higher ($P < 0.05$) proportion of mares had multiple ovulations in the eFSH group (11/14, 79%) as compared to controls (6/20, 30%). Within the 11 mares with multiple ovulations in the eFSH group, all ovulations were detected on the same day (3 mares), within 24 h (5 mares), or within 48 h (3 mares). In the 6 mares with multiple ovulations in the control group, all ovulations were detected on the same day (3 mares), or within 24 h (3 mares). For each mare, the interval to ovulation was calculated according to the first detected ovulation.

For ovulating mares, the mean interval from PE10 to ovulation tended ($P < 0.07$) to be longer in mares treated with eFSH (13.1 ± 0.5 d) as compared to controls (11.8 ± 0.45 d); however, the synchrony of ovulation within each group appeared to be similar ($P > 0.05$), with a 95% Confidence Interval (95% CI) gap of approximately two days in each of the groups (95% CI eFSH 12.0-14.3 d vs. control 10.8-12.8 d). Data regarding the time of ovulation of individual mares, in relation to PE10, are presented in Figure 8.1.

Within the eFSH group, the number of preovulatory follicles tended ($P < 0.08$) to be higher in the six mares that failed to ovulate within 72 h after hCG administration (5.8 ± 0.9 follicles), when compared to mares that successfully ovulated (4.1 ± 0.5 follicles). In the six mares that failed to ovulate, hCG was administered 8.6 ± 0.5 d after the P&E regimen was completed; however, we observed that their follicles either continued to grow, remained static, appeared as hemorrhagic or luteinized follicles, and later regressed. A single spontaneous ovulation was subsequently detected 21.0 ± 1.1 d (range 18-26 d) after hCG was administrated, which was 30.8 ± 0.9 d (range 29-35 d) after the P&E regimen was completed.

Among some of the mares that successfully ovulated in the eFSH group, a large non-ovulatory follicle was observed in addition to the CL(s). A large non-ovulatory follicle was defined as a preovulatory follicle (≥30 mm) that was observed on the day of hCG, but failed to ovulate within 3 d of the first ovulation. In the eFSH group, in 8/14 (57%) of the mares at least one non-ovulatory follicle (2.0 ± 0.3, range 1-3 non-ovulatory follicles) was observed, whereas in the control group we did not observe any large non-ovulatory follicles in any of the mares ($P < 0.05$). In the overall per-follicle comparison among ovulating mares, significantly more preovulatory follicles failed to ovulate in the eFSH group than in the control group (Table 8.1).

8.4.2. Embryo production

Embryo production data are summarized in Table 8.2. A total of 14 embryos were recovered from 14 ovulating eFSH-treated mares, and a total of 19 embryos were recovered from 20 control mares ($P > 0.05$). Therefore, the mean embryo recovery was similar between the groups. Embryo recovery attempts were successful (≥1 embryo recovered) in 8/14 (57%) eFSH-treated mares, and in 16/20 (80%) control mares ($P > 0.1$). Embryo morphology grades and assessed ages were not significantly different between the groups.

8.4.3. Serum progesterone and estradiol-17β concentrations

Serum P4 and E2 concentration data are presented in Figure 8.2. The number of preovulatory follicles was positively correlated ($P < 0.05$) with serum E2 concentrations on the hCG-Day ($r = 0.9$), and on Day 0 ($r = 0.7$). The number of ovulations was positively correlated ($P < 0.05$) with serum P4 concentrations on Day 8 ($r = 0.7$). The treatment, day, and treatment by day interaction significantly affected both serum E2 and P4 concentrations. Comparisons by day revealed that serum E2 concentrations were significantly higher on the hCG-Day, and on Day 0, in the eFSH group than in the control group. Within the eFSH group, serum concentrations of E2 were significantly higher on Day 0 when compared to Day 8; however, there was no such difference ($P > 0.05$) in the control group. Serum concentrations of P4 on Day 8 were significantly higher in the eFSH group.

Among the eFSH treated mares, mares that failed to ovulate tended ($P < 0.09$) to have higher E2 concentration on the day of hCG administration, as compared to mares that successfully

ovulated. For the six mares that failed to ovulate, blood samples were also taken every 3 - 5 d after hCG administration, until spontaneous ovulation was detected (data not presented). Generally, in these mares, serum E2 concentrations first decreased, and then later gradually increased, but to levels that were still markedly lower than measured initially, when hCG was administered. In 5/6 failing mares, serum P4 concentrations were < 0.3 ng/mL until spontaneous ovulation was detected. In one mare, follicles appeared to luteinize about a week after hCG administrations, which corresponded to a gradual increase in P4 concentrations.

Table 8.1 Ovarian parameters in mares after an estrus synchronization regimen with progesterone and estradiol-17β (P&E), with or without subsequent twice daily eFSH treatments. In both groups hCG was administered to estrous mares to induce ovulation. Results are presented as mean (±SEM) or percentages (%).

	eFSH (n = 20)	Control (n = 20)
Duration of eFSH treatment (d)	5.4 ± 0.4	---
Proportion of mares that ovulated within 72 h of hCG administration	70% (14/20) [a]	100% (20/20) [b]
[1]No. of preovulatory follicles (≥30 mm)	4.1 ± 0.5 [a]	1.3 ± 0.1 [b]
[1]No. of ovulations	2.8 ± 0.5 [a]	1.3 ± 0.1 [b]
[1]Proportion of preovulatory follicles failing to ovulate	28% (16/57) [a]	0% (0/26) [b]

[ab] Values within a row with different superscripts are significantly different ($P < 0.05$)

[1] Values were calculated for ovulating mares only (control group n = 20 mares; eFSH group n = 14 mares).

Figure 8.1 Time of first ovulation in mares after estrus synchronization regimen with progesterone and estradiol-17β (P&E), with or without subsequent eFSH treatments. All mares were given P&E, daily for 10 d, and PGF2α was given with the last P&E treatment. In the eFSH group, twice daily eFSH treatments were initiated at follicle size ≥25 mm and ceased at follicle size ≥35 mm; hCG was administered after 36 h coasting. In the control group, hCG was administered when a follicle ≥35 mm was detected. Each dot represents ovulation of an individual mare in relation to the last P&E treatment. Vertical lines represent the median of the group.

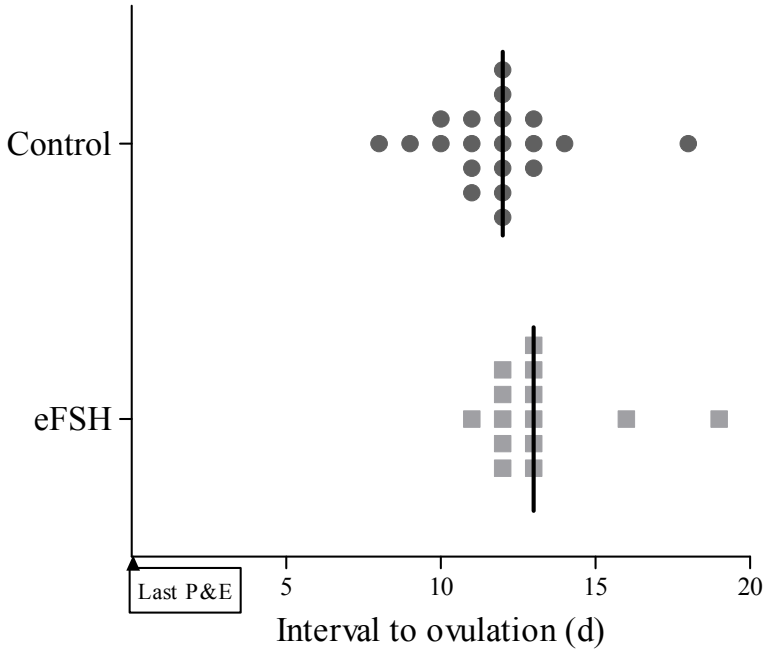
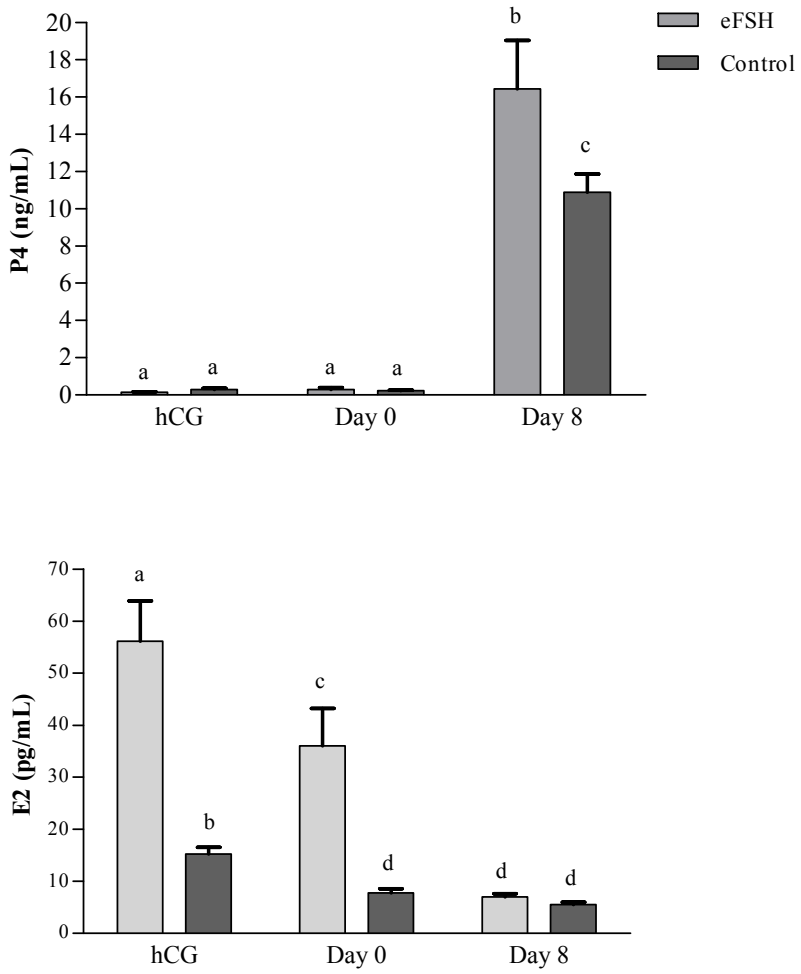


Table 8.2 Embryo production in mares that ovulated after an estrus synchronization regimen with progesterone and estradiol-17β (P&E), with or without subsequent twice daily eFSH treatments. In both groups, estrous mares were given hCG to induce ovulation and artificially inseminated with fresh semen; embryo recovery attempts were performed 8 d after ovulation. Values are presented as mean ± SEM or presentages (%)

	eFSH (n = 14)	Control (n = 20)
Embryo number	1.0 ± 0.4	0.95 ± 0.1
Successful embryo recovery attempts	57% (8/14)	80% (16/20)
Embryo per ovulation rate	36% (14/39) [a]	73% (19/26) [b]
Embryo morphology grade (1-excellent; 4-poor)	2.4 ± 0.2	1.8 ± 0.1
Embryo assessed age (d)	7.6 ± 0.2	7.7 ± 0.1

[ab]Values with different superscripts within a row are significantly different ($P < 0.05$)

Figure 8.2 Mean (± SEM) serum progesterone (P4) and E2 estradiol-17β (E2) concentrations in mares after estrus synchronization with progesterone and estradiol-17β (P&E), with or without subsequent eFSH treatments. In the eFSH group, twice daily eFSH treatments were initiated at follicle size ≥25 mm and ceased at follicle size ≥35 mm; hCG was administered after 36 h coasting. In the control group, hCG was administered when a follicle ≥35 mm was detected. Values are presented for the day of hCG administration (hCG), day of ovulation (Day 0), and 8 days post ovulation (Day 8). Different letters above bars represent significant differences ($P < 0.05$).



8.5. Discussion

This is the first study that critically compared the efficacy of P&E protocol as an estrus synchronization method in mares with and without subsequent eFSH treatment. The P&E treatment was initiated at random stages of the estrous cycle. The number of mares with a CL (73%) at that time reflected the expectation that most mares would be in diestrus and fewer in estrus according to a 21 - 22 d interovulatory interval with approximately a 16 d luteal phase and a 5 - 6 d follicular phase (Ginther, 1992b). As anticipated, mean diameter of the largest follicle significantly decreased during the P&E treatment regimen, and in most mares all follicles were ≤ 25 mm in diameter on last day of the regimen. These results are with concurrence with previous studies (Loy et al., 1981; Taylor et al., 1982b; Bristol et al., 1983), which attributed the suppression of folliculogenesis to the negative influence of P&E treatment on endogenous gonadotropin concentrations (Evans et al., 1982; Plata-Madrid et al., 1992).

The synchrony of ovulations among the 14/20 mares in the eFSH group that successfully ovulated, and among control mares, were similar, as most mares ovulated within a three-day period. These extents of ovulation synchrony following P&E regimen are with agreement with previous reports (Loy et al., 1981; Taylor et al., 1982b; Bristol et al., 1983; Plata-Madrid et al., 1992; Raz et al., 2005). However, on average, the interval from the last P&E treatment to ovulation tended to be longer in ovulating mares in the eFSH group. This can be attributed, at least in part, to the 36 h delay period in the administration of hCG in mares treated with eFSH. The delayed period in the hCG administration is termed “coasting”, and it is believed to be beneficial when cycling mares are treated with eFSH (Welch et al., 2006); the rationale for the coasting period is to allow the high FSH concentration to decrease during the final maturation phase of the follicles. A similar coasting period was not implemented in the control group, since the endogenous FSH concentrations were not expected to be as high, and since a coasting period is not a recommended practice in the equine breeding industry when hCG is used alone. Indeed, the interval from the last P&E treatment to a follicle ≥35 mm was not significantly different between the groups; however, the interval to hCG administration was significantly longer in mares treated with eFSH. These differences in the interval to hCG administration and to ovulation should be taken in consideration in situations when a donor and recipient mares are synchronized with P&E, and eFSH regimen is planned for the donor mare.

Treatment with eFSH was reported previously to increase the number of preovulatory follicles and the number of ovulations (reviewed by Squires and McCue, 2007). As expected, treatment with eFSH in the current study resulted in a significantly greater degree of ovarian stimulation. This was apparent in the significantly higher daily increasing rate of the largest follicle diameter before a preovulatory follicle was detected, higher number of preovulatory follicles, and higher mean number of ovulations in ovulating mares. Consistent with the greater number of ovulations in eFSH-treated mares, serum P4 concentrations were significantly higher on Day 8. In addition, the higher degree of ovarian stimulation in mares treated with eFSH was also reflected by the differences in serum E2 concentrations between the groups; on the hCG-Day and on Day 0, serum concentrations of E2 were correlated with the number of preovulatory follicles, and were significantly higher in mares treated with eFSH. However, six mares in the eFSH group completely failed to ovulate, and among mares that did ovulate, we observed a significant high number of non-ovulatory follicles. The mares that failed to ovulate appeared to be highly responsive to stimulation with eFSH, and tended to have higher number of preovulatory follicles and higher serum concentrations of E2 than the other mares; however, they failed to respond to hCG.

Ovulation failure and increased frequency of large non-ovulatory follicles have been reported previously in cycling mares following eFSH treatment (Niswender et al., 2003; Raz et al., 2005; Raz et al., 2006a; Raz et al., 2006b; Logan et al., 2007b; Niswender et al., 2007). In addition, in many studies that examined the use of eFSH treatment during the breeding season, as in the current study, the number of ovulations was commonly lower than the number of the preceding preovulatory follicles (Niswender et al., 2003; Raz et al., 2005; Raz et al., 2006b; Logan et al., 2007b; Niswender et al., 2007). These are not surprising findings; non-ovulatory follicles and reduced oocyte competence were reported following superovulatory treatments in other species, and are considered to be hyperstimulatory effects (Armstrong et al., 1983; Laurincik et al., 1993; Chao et al., 2005; Colazo et al., 2005; Barati et al., 2006; Lee et al., 2006; Bartlewski et al., 2008). In mares, ovulation failure following eFSH treatment could be related to the unique structure of the ovary, in which follicles develop in the interior cortex of the ovary and ovulate only through the limited area of the ovulation fossa; this anatomical barrier may restrict the number of ovulations and may be disturbed in superstimulated mares (Carmo et al., 2006; Alvarenga et al., 2008). Other explanations may be related to possible alterations of the hormonal environment,

alterations in the number of LH receptors, insufficient LH-like stimulation, or perhaps changes in the affinity or hormone receptors in preovulatory follicles in superstimulated mares (Raz et al., 2009c).

In the current study, higher mean serum E2 concentrations in eFSH-treated mares allowed us to conclude that these mares experienced significantly higher and prolonged periovulatory E2 levels. The E2 concentration pattern in single ovulating mares has been described previously, with a marked decrease in levels before ovulation (Ginther, 1992b; Ginther et al., 2007c). The concentration of plasma E2 was reported to decrease immediately when an hCG injection was given at a follicle of approximately 35 mm (Gastal et al., 2006). Furthermore, lower E2 concentrations in the follicular fluid of preovulatory follicles were associated with maturation of the oocyte (Ginther et al., 2007b). In the control group, E2 concentrations were similar on the day of ovulation to those measured in diestrus (Day 8); conversely, within the eFSH group, E2 concentrations on Day 0 were still significantly higher than on Day 8. Higher and prolonged E2 levels before and around the time of ovulation have been associated with persistent follicles and reduced fertility in the mare, and in other species (Moor et al., 1985; Breuel et al., 1993; Wehrman et al., 1993; Ahmad et al., 1995; Ginther et al., 2007a). Therefore, it is likely that the higher and prolonged periovulatory E2 levels in the eFSH-treated mares may have disturbed normal follicular maturation and ovulation.

Mean embryo number per recovery attempt was not different between the groups despite significantly higher number of ovulations in the eFSH group. The mean embryo number was comparable to those reported previously in mares that were treated with eFSH after a P&E treatment regimen (Raz et al., 2005; Logan et al., 2007b). In the control group all preovulatory follicles ovulated, and the mean embryo recovery per ovulation rate (73%) was significantly higher than obtained in the eFSH group (36%). Inconsistent and lower than expected embryo per ovulation rates have been reported previously in superstimulated cycling mares and have been proposed to be associated with high ovarian response (Alvarenga et al., 2001; Niswender et al., 2003; Logan et al., 2007b; Alvarenga et al., 2008). The lower embryo per ovulation rate in our study could have resulted from the eFSH-related hyperstimulatory effects, as was discussed above, possible alterations in oocyte maturation and transport, as well as possible alterations in reproductive tract parameters mediated by the modified steroidogenic hormonal environment

(Carmo et al., 2006; Alvarenga et al., 2008; Raz et al., 2009c). However, other possible explanations could be the quality of the follicles that were present in the ovaries at the time that the eFSH treatment was initiated, or possible alteration in the sensitivity of the ovary to eFSH caused by the pre-treatment with P&E (Alvarenga et al., 2008). Prolonged gonadotropic reduction probably resulted from the P&E treatment regimen and may have led to populations of static or atretic follicles; however, some of these follicles may have been "rescued" by the eFSH treatment. It is our speculation that these could have been factors that contributed to ovulation disturbance, or to oocytes that were not competent for normal fertilization, normal oviductal transport, or normal embryonic development. In the control group, however, it appeared that only competent follicles continued to develop and ovulated.

In summary, the synchrony of ovulations among mares after P&E regimen, with and without subsequent eFSH treatment, appeared to be similar; but the interval to ovulation tended to be 1-2 d longer, on average, in mares treated with eFSH. The eFSH treatment resulted in a higher number of preovulatory follicles and a higher number of ovulations; however, embryo recovery rate did not increase, and embryo per ovulation rate was significantly lower than in the control. In addition, a significantly higher proportion of mares failed to ovulate, and a significantly higher frequency of non-ovulatory follicles was observed in ovulating mares following the eFSH treatment. These negative hyperstimulatory effects in mares treated with eFSH were associated with significantly higher and prolonged periovulatory E2 concentrations. Based on the findings in this study, we cannot recommend the combined use of P&E estrus synchronization regimen and eFSH superovulatory treatment in cycling donor mares.

9. REPRODUCTIVE PERFORMANCE OF DONOR MARES SUBSEQUENT TO eFSH TREATMENT IN EARLY VERNAL TRANSITION; COMPARISON BETWEEN THE FIRST, SECOND, AND MID-SEASON ESTROUS CYCLES OF THE BREEDING SEASON

This study was accepted for publication in Animal Reproduction Sciences (Anim Reprod Sci 2009, 116 107-118. PMID: 19171444)

9.1. Abstract

The objective was to compare the reproductive performances associated with the first (Cycle-1), second (Cycle-2), and mid-season (MS-Cycle) ovulations of the breeding season in donor mares that were treated with equine FSH (eFSH) in the early vernal transition. Mares (n = 15) kept under ambient light were examined ultrasonographically per-rectum starting January 30. When an ovarian follicle ≥25mm in diameter was detected, twice daily eFSH treatments were initiated. The eFSH treatments ceased when a follicle ≥35mm was detected, and hCG was administered 36 h later. Thereafter, mares were artificially inseminated every 48 h until ovulation (Day 0). Trans-cervical embryo recovery attempts were performed on Day 8, and PGF2α was subsequently administered. Equine FSH was not administered in the subsequent estrous cycles. In Cycle-2 and in the MS-Cycle, hCG was administered when a follicle ≥35mm was detected; breeding, embryo recovery, and PGF2α administration were similar to Cycle-1. Mares had an untreated estrous cycle (no treatment or breeding) between Cycle-2 and the MS-Cycle. All mares developed follicle(s) ≥35mm after 4.9 ± 0.6 days of eFSH treatment, and subsequently ovulations occurred; mean (95% CI) interval from treatment initiation to ovulation was 7.9 (6.5-9.3) days. The number of preovulatory follicles (≥30mm) at the time of hCG administration (Cycle-1: 2.2 ± 0.3 compared with Cycle-2: 1.0 ± 0 compared with MS-Cycle: 1.1 ± 0.1 follicles), and the number of ovulations (2.5 ± 0.4 compared with 1.0 ± 0 compared with 1.1 ± 0.1 ovulations), were greater ($P < 0.05$) in Cycle-1. Nevertheless, mean embryo numbers did not differ among cycles (0.8 ± 0.2 compared with 0.5 ± 0.1 compared with 0.5 ± 0.1 embryo/mare). On average, embryo morphology grade was less ($P < 0.05$) in Cycle-1 as compared to non-eFSH cycles (combined Cycle-2 and MS-Cycle).

This impaired embryo quality could be due to a seasonal effect, or negative effect of the eFSH treatment, which was possibly related to alterations in the hormonal environment (estradiol-17β and progesterone). A prolonged inter-ovulatory interval (> 21 d) was recorded in 7 of 15 mares following the Cycle-1 ovulation, but not subsequently. In conclusion, eFSH treatment of vernal transitional donor mares stimulated ovulation within only a few days of treatment, and the following embryo recovery rate was at least as good as in the subsequent estrous cycles; however, on average, embryos were morphologically impaired. In subsequent estrous cycles in the breeding season, ovulations, embryo recovery rates, and embryo variables did not appear to be negatively affected; however, the first inter-ovulatory interval of the breeding season was prolonged in approximately half of the mares.

9.2. Introduction

Mares are seasonal, polyestrous, long day breeders. Following winter solstice, as daylight length increases, anestrous mares gradually obtain ovulatory competence during a prolonged phase called vernal transition. The vernal transition phase is characterized by increased GnRH and gonadotropin secretion, resurgence of follicular development, estrous behavior and, ovulation (Sharp, 1980b; Nagy et al., 2000). Prior to the first ovulation of the breeding season systemic estrogen concentrations are similar, serum LH concentrations do not increase to extent that occurs later in the breeding season, and the ovulatory follicle grows slower, but to a larger size, than in preovulatory periods later of the breeding season (Freedman et al., 1979b; Fitzgerald et al., 1987b; Ginther, 1992b; Bogh et al., 2000; Gastal et al., 2007). However, there appears to be no difference in the pregnancy and foaling rates following ovulations that occur at various times during the breeding season, or among the first, second and third ovulation of the season (Butterfield et al., 1964; Ginther, 1992b; Carnevale et al., 2000; Morris and Allen, 2002). Mares in which the first ovulation occurred late in the year may, however, have a lesser period over which pregnancy can occur, or may have fewer embryo recovery attempts completed in a breeding season.

Efficacious regimens to stimulate cycles of ovarian follicular development in mares to overcome winter anestrus and/or the prolonged transitional phase are of interest to the horse breeding industry, as economic pressures exist to produce foals early in the year. This economic pressure has become even more significant in the recent decades, as embryo transfer technologies

improved and more breed registries approved the registration of foals resulting from embryo transfer. Many horse owners would like to increase the number of embryos produced per mare in a given year; others would like to have mares conceive early in the year, after she has already produced embryos. Various therapeutic strategies to advance first ovulation of the year have been investigated. In addition to artificial photoperiod (McCue et al., 2007c), hormonal strategies employed include the use of GnRH and GnRH analogues (Ginther and Bergfelt, 1990; Harrison et al., 1990; McCue et al., 1991; Fitzgerald et al., 1993; Hyland, 1993; Johnson and Becker, 1993), progesterone and progestins (Webel and Squires, 1982; Newcombe et al., 2002), hCG (Carnevale et al., 1989), prolactin (Thompson et al., 1997a), dopamine antagonists (Besognet et al., 1997), prostaglandins (Jochle et al., 1987), and equine pituitary extract (Douglas et al., 1974; Lapin and Ginther, 1977).

More recently, treatment with a purified equine-FSH (eFSH®, Bioniche Animal Health Inc.) has advanced first ovulation of the breeding season (Niswender et al., 2004), and resulted in successful embryo recoveries from donor mares on day 7 or 8 post ovulation (Peres et al., 2007). However, to date, there is very limited information whether mares will continue having estrous cycles in a typical manner after their first ovulation of the breeding season is induced by eFSH, and there is no information regarding possible alterations in reproductive performance in subsequent estrous cycles. In addition, in some studies, but not others, superovulatory treatments of mares having typical estrous cycles have been associated with less than expected embryos per ovulation rate and alteration of embryo quality (Woods and Ginther, 1984; Alvarenga et al., 2001; Niswender et al., 2003; Peres et al., 2005; Raz et al., 2005; Carmo et al., 2006; Raz et al., 2006b; Logan et al., 2007b). To date, however, the quality of embryos produced subsequent to eFSH treatment of transitional mares has not been reported, nor compared to those produced by non-treated mares having typical patterns of estrous cyclicity.

The objective of the present study was to examine and compare reproductive variables associated with the first (Cycle-1), second (Cycle-2), and mid-season (MS-Cycle) ovulations of the breeding season in donor mares that were treated with eFSH in the early vernal transitional period to hasten first ovulation. In these estrous cycles folliculogenesis, time to ovulation, inter-ovulatory intervals (IOI), ovulation number, embryo recovery rate, and embryo quality, were investigated and compared. The hypothesis was that 1) an eFSH treatment regimen would induce ovulation in

early transitional mares within a few days of treatment initiation; 2) the eFSH-induced estrous cycle (Cycle-1) would result in greater numbers of preovulatory follicles, ovulations, and embryos, as compared to Cycle-2, and to the MS-Cycle; 3) the first inter-ovulatory interval (IOI) of the breeding season would be prolonged (> 21 days) in some mares; 4) serum estradiol-17β and progesterone concentrations would be different in Cycle-1, as compared to Cycle-2, and to the MS-Cycle, and 5) embryo morphology grade might be different among estrous cycles, or between the eFSH-induced cycle (Cycle-1) to the non-eFSH cycles (combined Cycle-2 and MS-Cycle).

9.3. Materials and methods

9.3.1. Animals

This study was performed between January and July 2005, in the research facility of the University of Saskatchewan in Canada, which is located at 52°07′ latitude in the Northern Hemisphere. Percheron cross type mares (n = 15), ages 3 to 6 years, with a body condition score of at least 5 out of 9, were used. Mares had no signs of systemic disease or lameness, and had acceptable perineal conformation. Mares were kept under ambient light in outdoor paddocks with sheds, and were maintained on alfalfa and grass hay with access to water and trace-mineralized salt, in accordance with the University of Saskatchewan's Institutional Animal Care and Use Committee.

9.3.2. Experimental design and treatments

9.3.2.1. *First, eFSH-cycle (Cycle-1)*

Trans-rectal palpation and ultrasonographic examinations of the reproductive tracts were performed starting January 30th. A B-mode ultrasonographic scanner (GE Ausonics, Universal Ultrasound, Bedford Hills, NY, USA) equipped with a 5.0 MHz linear-array transducer was used to monitor follicular growth and ovulation. At the beginning of the study, the reproductive status of all mares was defined as winter anestrus due to the absence of luteal tissue and follicles >15 mm in diameter on repeated examinations. Mares were examined every 2 to 3 d until an ovarian follicle ≥18 mm in diameter was detected and daily thereafter. When a follicle ≥25 mm in diameter was first detected in a mare, the mare was categorized as being transitional, and twice

daily eFSH treatment (12.5 mg i.m., eFSH®, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) was initiated. When a follicle ≥35 mm in diameter was detected the last eFSH treatment was given, and approximately 36 h later hCG (2000 IU i.m., Chorulon®, Intervet Canada Ltd, ON, Canada) was administered. Mares were artificially inseminated 24 h after hCG was administered, and again every 48 h until ovulation (Day 0 = day of ovulation) with fresh semen collected from a stallion of proven fertility. A minimum dose of 5 x 10^8 progressively motile spermatozoa extended 1:1 with Kenney extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA, USA) was used to inseminate the mares.

Embryo recovery attempts were performed 8 d after ovulation using a routine nonsurgical trans-cervical technique as described elsewhere (McKinnon and Squires, 1988a). A total of 4 L of embryo flush medium (ViGro Complete Flush Solution®, Bioniche Animal Health Canada Inc.) was used per mare, and mares were administrated oxytocin (40 IU i.v., Oxytocin®, Vêtoquinol N.-A. Inc., QC, Canada) before the procedure was completed to facilitate recovery. Embryos were identified using a stereomicroscope, enumerated, and rinsed three times in Holding Medium (Vigro Holding Plus®, Bioniche Animal Health Canada Inc.). Embryos were scored for quality (1-excellent, 2- good, 3-fair, 4-poor) according to their morphology as described by McKinnon and Squires (McKinnon and Squires, 1988b), and were subjectively assessed for age according to developmental stage (morula/ early blastocyst/ expanded blastocyst) and size.

9.3.2.2. *Second cycle (Cycle-2)*

Mares received PGF2α (5 mg s.c., Lutalyse®, Pharmacia Animal Health, Orangeville, ON, Canada) after the first embryo recovery attempt was completed, and they were examined every 1-3 d thereafter to evaluate whether they continued to have estrous cycles. When a follicle ≥35 mm in diameter was detected, hCG was administered to induce ovulation. Subsequently, the mares were bred and embryo recovery attempts were performed as was described for Cycle-1.

9.3.2.3. *Mid-season cycle (MS-Cycle)*

Following the embryo recovery attempt of Cycle-2, the mares received PGF2α, and thereafter were examined every 1 to 3 d until spontaneous ovulation was detected. The mares were not treated or bred, as this estrous cycle was considered as a “rest cycle”. Eight days after the rest

cycle ovulation occurred, PGF2α was administered, the mares were monitored for estrus and when a follicle ≥35 mm in diameter was detected, hCG was administered to induce ovulation. Subsequently, mares were bred and embryo recovery attempts were performed as for Cycle-1 and Cycle-2.

To impartially compare the IOI following each of the estrous cycles, PGF2α was administered after the embryo recovery attempt (Day 8) of the MS-Cycle, and mares were monitored for estrus. When a follicle ≥35 mm in diameter was detected, hCG was administered and mares were examined daily until ovulation was detected.

9.3.3. Measurement of serum estradiol-17β and progesterone concentrations

Jugular blood samples were collected into sterile plain Vacutainer tubes before the eFSH treatment was initiated and for each estrous cycle prior to hCG administration (hCG-Day) and on Day 8 (just prior to the embryo recovery attempt). Blood samples were centrifuged, and sera separated and stored frozen (-20° C) until hormone assays were performed. Serum concentrations of progesterone (P4) and estradiol-17β (E2) were determined using radioimmunoassays. For P4, all samples were analyzed in duplicates in one assay, using the Coat-A-Count® Progesterone In-vitro Diagnostic Test Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) (Blight and White, 1983); the intra-assay coefficient of variation was <5.8%. For E2, samples were analyzed in five assays using a radioimmunoassay developed and validated by the WCVM Endocrinology Laboratory, University of Saskatchewan (Joseph et al., 1992; Bragg Wever et al., 2002); standards (17β-Estradiol, E8875, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) were prepared in charcoal stripped equine serum, and ranged from 1 to 100 pg/ml. Samples from each mare (all estrous cycles) were analyzed in duplicates in the same assay. For E2, the intra- and inter-assay coefficients of variation were < 8.9% and < 12.7%, respectively.

9.3.4. Statistical analyses

Statistical analyses were performed with analytic computerized software (Statistix 8 Student Edition, Analytical Software, Tallahassee, FL, USA). Pearson chi-square test analysis was used to compare proportional data such as the proportion of mares which ovulated, proportion of mares with multiple ovulations, proportion of successful embryo recoveries, and proportion of mares

with prolonged IOI. Continuous data, such as number of preovulatory follicles, largest follicle diameter prior to ovulation, number of ovulations, number of embryos, and IOI, were compared between estrous cycles using paired-t test analysis. Categorical data, such as embryo morphological grade and embryo age, were compared with Kruskall–Wallis non-parametric one-way ANOVA. The general effects of the Estrous Cycle, the Day, and the Cycle by Day interaction, on serum steroid hormone concentrations, were analyzed in a Repeated Measures ANOVA, followed by Tukey's HSD All-Pairwise Comparisons Test. Pearson's Correlation test was used to determine the correlation between the number of preovulatory follicles and serum E2 concentrations, and the correlation between the number of ovulations and serum P4 concentrations. Differences were considered significant at $P < 0.05$. Results are presented as mean ± S.E.M.

9.4. Results

9.4.1. Folliculogenesis and ovulation

Treatment protocols were completed for all mares, and data was collected for all estrous cycles. Following the eFSH treatment, all mares (15 of 15, 100%) developed at least one ovarian follicle ≥35 mm in diameter, and subsequently ovulated. Mean duration of eFSH treatment was 4.9 ± 0.6 d, and the interval from eFSH treatment initiation to ovulation was 7.9 ± 0.6 d. The mean date of the first ovulation of the breeding season was March 13th ± 3 d.

Mean diameter of the largest preovulatory follicle was greater prior to the first ovulation of breeding season, (46.2 ± 1.4 mm) as compared to the follicle diameter prior to the ovulations of Cycle-2 (41.0 ± 1.0 mm) and MS-Cycle (41.5 ± 1.5 mm). The number of preovulatory follicles (≥30 mm) at the time of hCG administration, and the number of ovulations, were greater in Cycle-1 (2.2 ± 0.3 follicles, 2.5 ± 0.4 ovulations) as compared to Cycle-2 (1.0 ± 0 follicles, 1.0 ± 0 ovulations) and to the MS-Cycle (1.1 ± 0.1 follicles, 1.1 ± 0.1 ovulations) (Table 9.1). Greater proportions of mares had multiple ovulations in Cycle-1 (11 of 15, 73%) as compared to Cycle-2 (0 of 15, 0%) or to the MS-Cycle (2 of 15, 13%). Ovulations were asynchronous in 7 of 11 (64%) mares that had multiple ovulations following the eFSH treatment. In three mares all ovulations were detected within 24 h (examinations on two consecutive days); and in four mares all ovulations were detected within 48 h (examinations on three consecutive days). In the two mares

that had double ovulations in the MS-Cycle, ovulations were synchronous. In the rest cycle, and in the estrous cycle that followed the MS-Cycle, each mare had an ovulation from a single follicle.

A prolonged IOI (> 21 d) was observed in 7 of 15 (47%) mares following the first eFSH-induced ovulation, however, subsequent to Cycle-2 ovulation none of the mares (0/15, 0%) had a prolonged IOI in any of the estrous cycles ($P < 0.05$). The mean IOI following the first ovulation of the breeding season (24.0 ± 2.4 d, range 16 to 50 d) was longer, as compared to the second (18.3 ± 0.4 d, range 16 to 21 d), third (16.5 ± 0.7 d, range 11 to 21 d), and fourth (16.8 ± 0.5 d, range 14 to 20 d) IOI. However, it was not significantly different among the IOI following the second, third, and fourth ovulations of the breeding season. Data regarding IOI of individual mares are presented in Figure 9.1.

9.4.2. Embryo production

Embryo recovery rates were not different among the estrous cycles (Table 9.1). In Cycle-1 a total of 12 embryos were recovered, and in each of the other two cycles a total of seven embryos were recovered. Mean embryo ages were not different among estrous cycles or between embryos that were recovered following the eFSH cycle (7.2 ± 0.2 d) compared with non-eFSH estrous cycles (7.6 ± 0.2 d). However, mean embryo morphology grade was inferior in the eFSH-cycle (2.7 ± 0.3) as compared to Cycle-2, or as compared to embryos from non-eFSH estrous cycles (Cycle-2 and MS-Cycle combined; 1.6 ± 0.2).

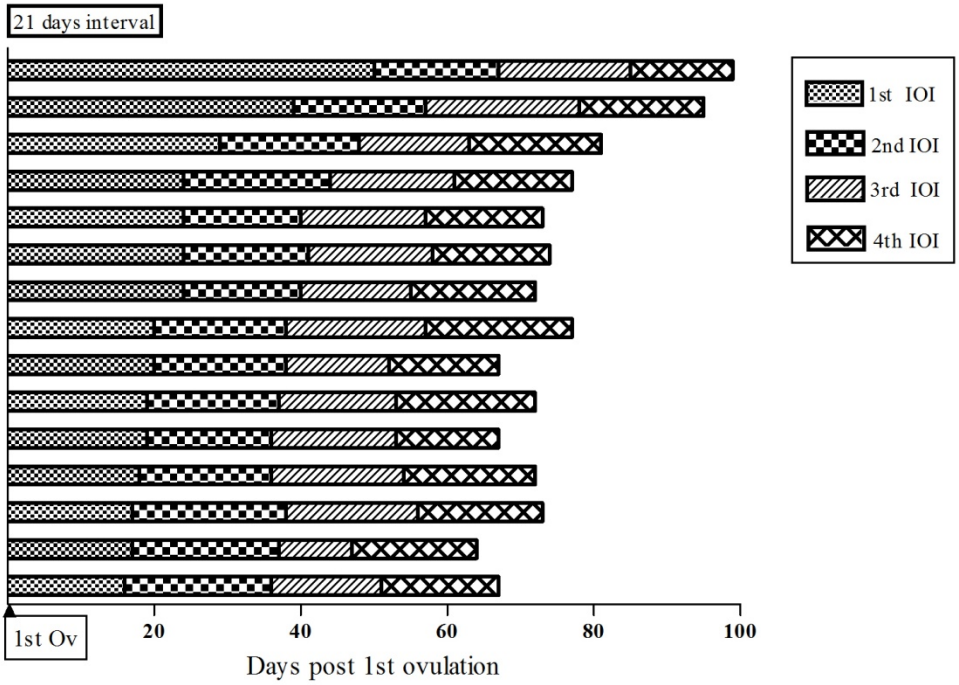
Table 9.1 Reproductive variables associated with the first (Cycle-1), second (Cycle-2), and mid-season (MS-Cycle) ovulations of the breeding season in donor mares treated with twice daily eFSH in the early vernal transitional period to hasten first ovulation. Results are presented as mean (± S.E.M) or percentages (%).

	Cycle-1*	Cycle-2*	MS-Cycle*
Duration of eFSH treatment (d)	4.9 ± 0.6	---	---
Proportion of mares having ovulations	100% (15/15)	100% (15/15)	100% (15/15)
No. of preovulatory follicles (≥30 mm)	2.2 ± 0.3 [a]	1.0 ± 0 [b]	1.1 ± 0.1 [b]
No. of ovulations	2.5 ± 0.4 [a]	1.0 ± 0 [b]	1.1 ± 0.1 [b]
Proportion of mares with ≥2 ovulations	73% (11/15) [a]	0% (0/15) [b]	13% (2/15) [b]
No. of embryos	0.8 ± 0.2	0.5 ± 0.1	0.5 ± 0.1
Embryo morphology grade (1-excellent; 4-poor)	2.7 ± 0.3 [a]	1.5 ± 0.2 [b]	1.7 ± 0.2 [ab]

[ab] Values with different superscripts within a row are significanlly different ($P < 0.05$)

*For the three estrous cycles, hCG was administered to induce ovulation, the mares were bred, and embryo recovery attempts were perform 8 days after ovulation

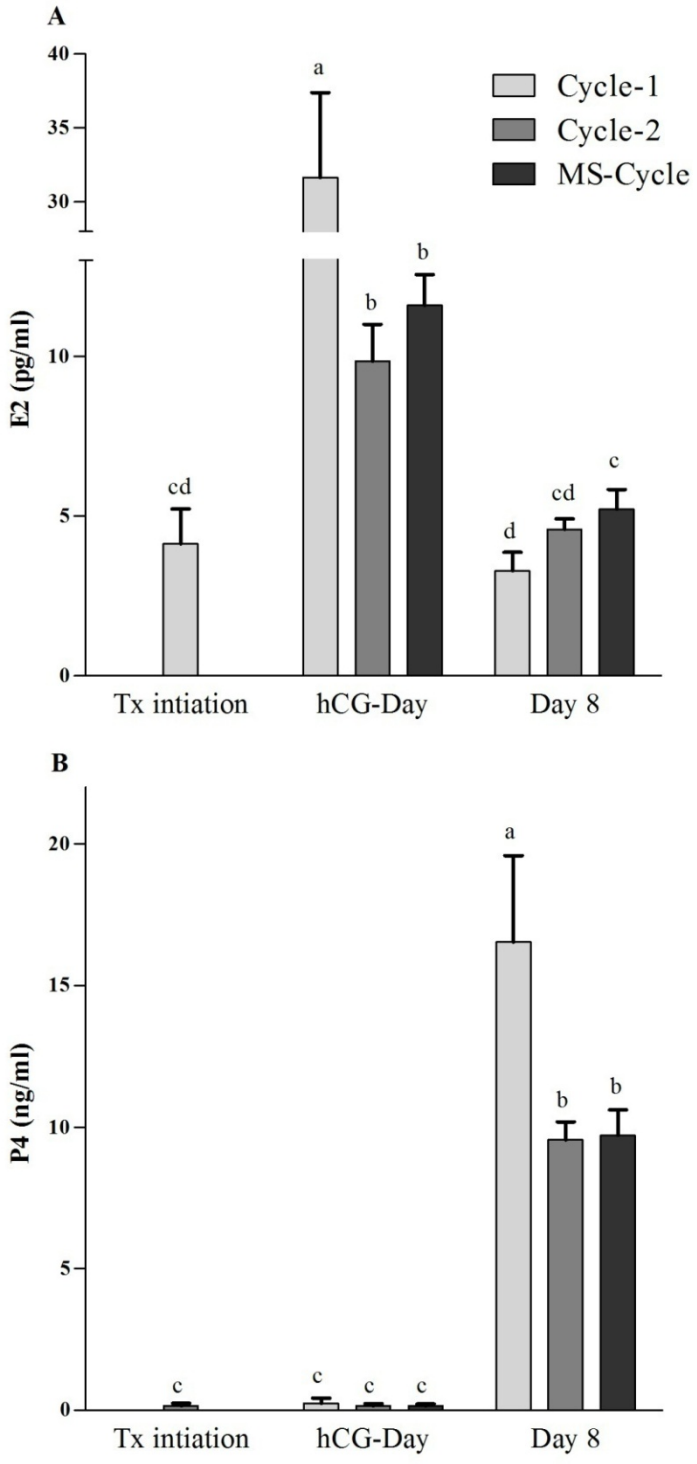
Figure 9.1 The first four inter-ovulatory intervals (IOI) of the breeding season in individual mares (n = 15). The first ovulation of the breeding season (1st OV) was subsequent to an eFSH treatment regimen which was initiated in the early vernal transitional period. Prostaglandin F2α was given 8 days post ovulation (all estrous cycles), and hCG was administered after a follicle ≥35mm was detected (all estrous cycles, except prior to the third ovulation of the breeding season). Each horizontal column represents data from an individual mare.



9.4.3. Serum progesterone and estradiol-17β concentrations

Serum E2 and P4 concentrations were affected by the Cycle, the Day, and the Cycle by Day interaction (Figure 9.2). At the time of eFSH treatment initiation all mares had serum P4 concentration < 0.1 ng/mL, and mean serum E2 concentration was 4.1 ± 1.1 pg/ml. However, subsequent to the eFSH treatment, as the first follicle ≥35 mm in diameter was detected (first hCG-Day), serum E2 concentrations increased. On the days of hCG administration serum E2 concentrations were positively correlated with the number of preovulatory follicles ($r = 0.8$, $P < 0.05$), and were greater in Cycle-1, as compared to Cycle-2 and the MS-Cycle. However, on the days of embryo recovery attempts (Day 8), serum E2 concentrations were less following Cycle-1, as compared to the MS-Cycle, but were not different from Cycle-2. Serum P4 concentrations were not different among the cycles on the days of hCG administration. However, on Day 8, serum P4 concentrations were positively correlated with the number of ovulations ($r = 0.7$, $P < 0.05$), and were greater following Cycle-1, as compared to Cycle-2 and to the MS-Cycle.

Figure 9.2 (next page) Serum E2 (A) and P4 (B) concentrations (mean ± S.E.M) in mares in the first (Cycle-1), second (Cycle-2), and mid-season (MS-Cycle) estrous cycles of the breeding season. The first ovulation of the breeding season (Cycle-1) was subsequent to an eFSH treatment regimen which was initiated in the early vernal transitional period (Tx initiation). In the three estrous cycles depicted, hCG was administered after a follicle ≥35mm was detected (hCG-Day); the mares were bred and embryo recovery attempts were perform 8 days post ovulation (Day 8). Different letters above bars represent significant differences ($P < 0.05$).


A
40
35
30
10
5
0
E2 (pg/ml)
Cycle-1
Cycle-2
MS-Cycle
a
b
b
cd
d
cd
c
Tx intiation
hCG-Day
Day 8
B
20
15
10
5
0
P4 (ng/ml)
a
b
b
c
c
c
c
Tx intiation
hCG-Day
Day 8

9.5. Discussion

To our knowledge, this is the first study that compared the reproductive performance among the first, second, and mid-season estrous cycles in the breeding season in donor mares that were treated with eFSH in the early vernal transitional period. Treatment protocols and management over these estrous cycles were selected to be as similar as possible to that commonly used in the horse breeding industry. Mares were monitored from winter anestrus to the transitional phase to initiate eFSH treatment as early as possible before the breeding season, but at a time that mares become more likely to be responsive to exogenous hormonal stimulation. Serial examinations of the ovaries, and the hormonal analyses indicated that mares were in the early transitional period at time of eFSH treatment initiation (Oxender et al., 1977b; Ginther, 1992a).

The eFSH treatment in early transitional mares was aimed to promote development of preovulatory follicle(s), responsive to ovulation-induction, by bypassing the hypothalamic-pituitary axis and stimulating the ovary directly. Administration of hCG was included in the treatment protocols of all estrous cycles (excluding the rest cycle) to shorten time to ovulation, and increase likelihood of successful ovulation, regardless of ability of the hypothalamic-pituitary-ovarian axis to generate an endogenous LH surge. In Cycle-1, the hCG was administered after a delayed period of 36 h following the last eFSH treatment, a period that is termed “coasting” and has been reported to be beneficial when mares having typical estrous cycles were treated with eFSH (Welch et al., 2006). The rationale for the “coasting” period is to allow the increased FSH concentration to decrease during the final maturation phase of the follicles. A similar “coasting” period was not utilized in the other estrous cycles, because the endogenous FSH concentrations were not expected to be as great, and because a “coasting” period is not a recommended practice in the equine breeding industry when hCG is used alone.

The University of Saskatchewan is located in the Canadian Northwest, which has a naturally long and cold winter. Clinical experience over the years indicates a majority of mares under ambient light at this location commonly have a first spontaneous ovulation of the breeding season in May. In the current study, eFSH treatment was effective and stimulated first ovulation of the season in all mares after only a few days of treatment. Mean date of first ovulation of the season was March 13 ± 3 d. In the current study design, there was not a group of untreated mares to evaluate extent in which the eFSH treatments advanced the breeding season, as this was not one of

the objectives of the current study. However, in the teaching facility at the University of Saskatchewan, none of 10 untreated light mares that were monitored during the same time period had ovulations during the period that eFSH-treated mares completed the first estrous cycle. Results in the current study are consistent with previous studies which reported that eFSH hastened time of first ovulation of the breeding season (Niswender et al., 2004; Peres et al., 2007).

Reproductive efficiency at the onset of the breeding season may be affected by irregularity in expression of estrus, such as split, prolonged, or covert estrus, and by irregularities in the interval from the end of estrus to ovulation (Hughes et al., 1975; Allen, 1979; Asa, 1986; Ginther, 1992b). In the current study, first ovulation of the breeding season occurred within a short period of the time after the eFSH treatment was initiated (95% CI: 6.5 to 9.3 d). Therefore, it appears that eFSH treatment might increase reproductive efficiency by inducing the first ovulation of the breeding season within a predictable time period from treatment initiation. As anticipated, twice daily eFSH treatment followed by hCG in early transitional mares resulted in greater ovarian stimulation. Greater numbers of preovulatory sized follicles, and a greater number of ovulations, as compared with other subsequent cycles in the breeding season, are evidence for this finding. The greater ovarian stimulation was also reflected by differences in serum E2 and P4 concentrations among estrous cycles. On the day of hCG treatment, serum concentrations of E2 were correlated with number of preovulatory follicles, and were greater following the eFSH treatment. Consistent with the greater number of ovulations in the first estrous cycle, serum P4 reached greater concentrations on Day 8. The corpora lutea that developed following ovulation during all estrous cycles appeared ultrasonographically normal, and resulted in Day 8 serum P4 concentrations which presumably were capable of supporting embryonic development at least until embryo recovery attempts were performed.

Prior to the first spontaneous ovulation of the breeding season E2 is similar, but the ovulatory follicle grows to a larger size, than later in the ovulatory season (Freedman et al., 1979b; Fitzgerald et al., 1987b; Ginther, 1992b; Bogh et al., 2000; Gastal et al., 2007). In the current study, mean E2 concentrations and mean diameter of largest follicles were both greater prior to the first ovulation of breeding season, as compared to the subsequent estrous cycles. The differences in the diameter of the largest follicle prior to ovulation could be associated to a seasonal effect;

however, it is more reasonable that supplementary exogenous FSH treatment as well as the "coasting" period resulted in larger follicles prior to first ovulation of the breeding season.

The greater E2 serum concentration prior to first ovulation of the breeding season as compared to later in the breeding season is probably due to the stimulatory effect of the eFSH treatment and the E2 contribution of multiple preovulatory large follicles. In another study (Raz et al., 2009b), concentrations of serum E2 were approximately three times greater in transitional eFSH-treated mares compared to control mares on the day the first ovulation was detected (Day 0). In mares having typical estrous cycles treated with eFSH as compared to controls, there were similar results in the current study; greater E2 prior to and at time of ovulation. The systemic E2 concentrations pattern in non-eFSH-treated mares has been described (Ginther, 1992a), with decreased concentrations of E2 prior to ovulation. Prolonged and/or greater preovulatory secretion of E2 also occurs in superovulating animals of other species (Moor et al., 1985; Kelley et al., 2006), and was associated with lesser cleavage rates, early embryonic losses, abnormal embryonic development, and a lesser conception rate (Lewinthal et al., 1987; Breuel et al., 1993; Johnson and Lewis, 1993; Wehrman et al., 1993; Ahmad et al., 1995). This has been attributed to the negative effect on oocyte maturation and alterations in the uterine environment (Roberson and Baker, 1969; McGaughey, 1977; Butcher and Pope, 1979; Moor et al., 1985; Ahmad et al., 1995).

In the current study, there was a lesser embryo quality following the first ovulation(s) of the breeding season, which can be related to a seasonal effect. However, it is more likely to be related to a negative effect of eFSH on embryo quality, possibly mediated by greater than typical preovulatory E2. Interestingly, in three mares treated with eFSH (Cycle-1) progesterone concentrations were increased (> 0.6 ng/ml) on the day of hCG administration. Increased preovulatory progesterone concentrations before ovulation occurs in some mares and cows following superovulation treatment; this type of endocrine pattern has been associated with abnormal follicular/oocyte maturation, and loss of transferable embryos (Callesen et al., 1986; Roberge et al., 1995; Greve and Callesen, 2001; Briant et al., 2004). In many studies, eFSH treatment increases number ovulations and embryo recovery rates in mares having typical estrous cycles that are used for embryo transfer (McCue et al., 2007a; Squires and McCue, 2007). However, in some studies, superovulatory treatments of mares having typical estrous cyclic patterns have been associated with less than expected embryos per ovulation rate, alteration of

embryo quality, and less than expected pregnancy rates after embryos were transferred to recipients (Woods and Ginther, 1984; Alvarenga et al., 2001; Niswender et al., 2003; Peres et al., 2005; Raz et al., 2005; Carmo et al., 2006; Raz et al., 2006b; Logan et al., 2007b). Nevertheless, the current study is the first that examined embryo morphology following eFSH treatment in transitional mares, and compared findings in non-eFSH-treated mares that had been undergoing typical estrous cyclic patterns.

In Cycle-2 and in the MS-Cycle number of ovulations, embryo recovery rate, and embryo quality were similar to those expected in cycling non-superstimulated donor mares (Ginther, 1992b; Squires et al., 1999; Squires et al., 2003a). Treatments of transitional mares with twice daily 12.5 mg of eFSH have been reported previously in studies conducted in Colorado, USA (latitude 40°585' N) (Niswender et al., 2004), and Sao-Paulo, Brazil (latitude 22°52' S) (Peres et al., 2007). Duration of eFSH treatment, proportion of mares that had ovulations, and proportion of mares with ≥2 ovulations following the eFSH treatment in the current study were comparable with those reported in previous studies. In the study conducted in Colorado, mares were monitored until ovulation, but were not bred; mean number of ovulations was similar to that reported in the current study. However, in the study conducted in Sao-Paulo, mean number of ovulations and embryo recovery rate (5.6 ± 4.5 ovulations; 2.0 ± 1.8 embryos) were superior to that reported in the current study. These differences may be related to the disparity in the body weight of the mares in the two studies, as mares in the current study were Percheron cross type, but the eFSH dose was not adjusted for the greater body weight. Nevertheless, embryos per ovulation rate were similar between the studies (32% and 36%). A considerable variation in ovulation rate and embryo recovery among mares having typical estrous cyclic patterns that were treated with eFSH was noted by Squires et al. (2006); this variation may be due to the influence of different management, treatment protocol, environmental factor, and genetic makeup of the animals used. Furthermore, variation in ovulation rate and embryo recovery following ovarian superstimulation treatments have been observed in cows, goats, and ewes (Mapletoft et al., 2002; Cognie et al., 2003). Interestingly, in cattle embryo transfer programs, approximately 70% of embryos are collected from 30% of the ovarian superstimulated cows, and only 50 to 60% of the embryos are of transferable quality (grade 1 or 2 out of 4) (Mapletoft et al., 2002; Hasler, 2003; Bo et al., 2008).

The mean IOI from the first to the second ovulations of the breeding season was longer than the subsequent IOIs. Inter-ovulatory interval during the breeding season, when PGF2α and hCG are used to shorten the estrous cycle and hasten ovulation is expected to be < 21 d (Lofstedt, 1988; Ginther, 1992a). In the current study, first IOI was prolonged in about half of the mares; however, subsequent to second ovulation of the breeding season, none of the mares had a prolonged IOI in any of the estrous cycles. The prolonged first IOI may reflect lack of full seasonal maturity of the hypothalamic-pituitary-ovarian axis in some mares, even after these mares had ovulations and produced embryos.

Palmer et al. (1978) concluded that length of interval from luteolysis to ovulation is dependent on the extent of follicular development, and is negatively correlated to estrogen concentrations at that time. In the current study, on Day 8, day PGF2α was administered, serum E2 concentrations were less following Cycle-1, as compared to the MS-Cycle. Follicular records were re-examined on these days and following Cycle-1, number of medium size follicles (≥20 mm diameter), and mean diameter of largest follicle were less than in the MS-Cycle. Nevertheless, although the prolonged first IOI may reflect a seasonal effect, the possibility that early and possibly prolonged E2 concentration before and around eFSH-induced ovulation had a transient suppressive effect on the endogenous gonadotropins secretion from the pituitary cannot be exclued; this potentially resulted in a delay in the subsequent follicular wave emergence and smaller follicle diameter on Day 8, which may have contributed to the longer IOI.

In summary, the current study compared reproductive variables among first, second, and mid-season estrous cycles in the breeding season in donor mares treated with eFSH in the early vernal transitional period. Follicular development and ovulation were induced in early transitional donor mares within a few days of eFSH treatment. Most of these mares had ≥2 ovulations, and embryo recovery rate was similar or greater than during the breeding season. However, on average, the embryos recovered in the first, eFSH induced estrous cycle, were morphology impaired as compared to embryos recovered during other estrous cycles of the breeding season. This impaired embryo quality could be related to a seasonal effect, or negative effect of the eFSH treatment. There were no significant differences in number of ovulations, and in number and quality of embryos recovered in the subsequent estrous cycles of the breeding season. However, the first

inter-ovulatory interval of the breeding season was prolonged (> 21 d) in about half of the mares, yet, subsequent intervals in the breeding season were not.

10. COMPARISON OF THE EFFECTS OF eFSH AND DESLORELIN TREATMENT REGIMENS ON OVARIAN STIMULATION AND EMBRYO PRODUCTION OF DONOR MARES IN EARLY VERNAL TRANSITION

This study was accepted for publication in Theriogenology
(Theriogenology 2009, 71(9):1358-1366. PMID: 19329174)

10.1. Abstract

The objective of this study was to compare the effects of eFSH and deslorelin treatment regimens on ovarian stimulation and embryo production of donor mares in early spring transition. Mares kept under ambient light were examined ultrasonographically per-rectum, starting January 30th, and were assigned to one of two treatment groups using a sequential alternating treatment design when a follicle ≥25 mm was detected. In the eFSH group, mares (n = 18) were treated twice daily with eFSH (12.5 mg i.m.) until a follicle ≥35 mm was detected, and hCG was administered 36 h later. In the deslorelin group, mares (n = 18) were treated twice daily with deslorelin (63 μg i.m.) until a follicle ≥35 mm was detected, and were then given hCG. Estrous mares were inseminated with fresh semen. Embryo recovery attempts were performed eight days post-ovulation. In each group, 14/18 (78%) mares ovulated following the eFSH or deslorelin treatment regimens. The mean (95% CI) interval from treatment initiation to ovulation was 8.2 (7.3, 8.9) and 7.2 (6.2, 8.1) days in the eFSH and deslorelin groups, respectively. In the eFSH group, the number of ovulations was significantly higher (mean ± SEM; 3.4 ± 0.4 vs. 1.1 ± 0.1 ovulations), and more embryos were recovered (2.6 ± 0.5 vs. 0.4 ± 0.2 embryos/recovery attempt). We concluded that eFSH and deslorelin treatment regimens were equally effective in inducing ovulation in early transitional mares within a predictable time of treatment; however, the eFSH regimen increased the number of ovulations and embryos recovered per mare.

10.2. Introduction

Mares are seasonal, polyestrous, long day breeders. During the winter months the function of the mare's hypothalamic–pituitary-ovarian axis changes, as GnRH secretion is minimal, and follicular waves cease in the majority of mares (Johnson and Becker, 1993; Nagy et al., 2000). Following winter solstice, anestrous mares gradually obtain ovulatory competence during a prolonged phase called vernal transition. This transition phase is characterized by a series of stages or events characterized by increased GnRH and gonadotropin secretion, resurgence of follicular development, estrous behavior and, finally ovulation (Sharp, 1980a; Nagy et al., 2000). During the early transitional phase the number of follicles with a diameter ≥20 mm in the mare's ovaries increases, and ovaries usually contain several developing and atretic follicles (Turner et al., 1979a; Ginther, 1992b). During the late transition phase most mares develop 1 - 3 anovulatory follicular waves each characterized by a large dominant follicle (≥35 mm); follicles continue to emerge and regress until one is ultimately recruited to be the ovulatory follicle (Turner et al., 1979b; Ginther, 1992b). Increasing daylight length plays the major role in the resurgence of ovulatory competence, and the mean month that mares experience the first ovulation of the breeding season depends on the geographical latitude at which they live, genetics, nutrition, climate, and other environmental factors (Sharp, 1980b; Ginther, 1992b; Guerin and Wang, 1994; Niekerk and Niekerk, 1997; Nagy et al., 2000).

Successful regimens to stimulate ovarian cyclicity in mares to overcome winter anestrus and/or the prolonged transitional phase is of interest to the horse breeding industry, as economic pressures exist to produce foals early in the year. This has become even more pronounced in the last 3 decades since the use of embryo transfer and other technologies to improve embryo production has grown and become more attractive. Many horse owners would like to have embryos recovered from their performance mare early in the year, before the show season; others would like to have their mare carry a pregnancy to term early in the year, after she has already produced a few embryos. Various therapeutic strategies to advance the first ovulation of the year have been investigated. In addition to artificial photoperiod (McCue et al., 2007c), hormonal strategies employed include the use of GnRH and GnRH analogs (Ginther and Bergfelt, 1990; Harrison et al., 1990; McCue et al., 1991; Fitzgerald et al., 1993; Hyland, 1993; Johnson and Becker, 1993), progesterone and progestins (Webel and Squires, 1982; Newcombe et al., 2002),

hCG (Carnevale et al., 1989), prostaglandins (Jochle et al., 1987), prolactin (Thompson et al., 1997a), dopamine antagonists (Besognet et al., 1997), equine pituitary extract (Douglas et al., 1974; Lapin and Ginther, 1977), and more recently, equine FSH (eFSH) (Niswender et al., 2004; Peres et al., 2007). However, information on efficacious and practical hormonal treatment regimens for early transitional mares used for embryo transfer, with a predictable interval to ovulation, embryo production, and continuation of cyclicity, is still limited.

The objective of this study was to determine and compare the efficacy of deslorelin (GnRH agonist) and eFSH treatment regimens in donor mares in early spring transition. We investigated the effects of two treatment protocols on folliculogenesis, interval to first ovulation, ovulation rate, ovulation synchrony, embryo recovery rate, and continuation of cyclicity. We hypothesized that both treatment regimens would stimulate follicular growth and ovulation in early transitional mares, within only a few days of treatment; however, we anticipated that the eFSH treatment would result in higher number of ovulations and higher embryo recovery rate.

10.3. Materials and methods

10.3.1. Animals and reproductive tract examinations

This study was performed between January and June 2006, in the research facility of the University of Saskatchewan in Canada, which is located at 52°07′ latitude in the Northern Hemisphere. Thirty-six mares, Quarter Horse / Percheron cross type, ages 3 to 10 years, with a body condition score of at least 5 out of 9, were used for this study. They had no signs of systemic disease or lameness, and had good perineal conformation. Mares were kept under ambient light in outdoor paddocks with sheds, and were maintained on alfalfa and grass hay with access to water and trace-mineralized salt, in accordance with the University of Saskatchewan's Institutional Animal Care and Use Committee.

Transrectal palpation and ultrasonographic examinations of the reproductive tracts were performed starting on January 30th, 2006. Uterine and cervical tone was assessed and separately scored from 1 to 4 (1- soft, 2- moderately soft, 3- moderately toned, 4- toned). A B-mode ultrasound scanner (GE Ausonics, Universal Ultrasound, Bedford Hills, NY, USA) equipped with a 5.0 MHz linear-array transducer was used to monitor ovarian activity and to subjectively score

endometrial edema from 0 (no edema, homogeneous grey) to 4 (marked edema with a distinct black and white pattern) (Samper, 1997). At the beginning of the study, the reproductive status of all mares was defined as winter anestrus due to the absence of luteal tissue and follicles <15 mm in diameter on repeated examinations. Mares were examined every 2 - 3 d until a follicle ≥18 mm in diameter was detected and daily thereafter until 3 d post ovulation(s), and on day 8 after ovulation (day of embryo recovery attempt).

10.3.2. Experimental design and treatment groups

Mares were assigned to one of two treatment groups when a follicle ≥25 mm in diameter was detected using a sequential alternating treatment design. Thus, the first mare that had a follicle ≥25 mm in diameter was randomly assigned to a treatment group. Thereafter, mares were assigned to a treatment group by alternate sequence in order to balance the date of treatment initiation and reduce the effect of mare variability between the groups.

In the eFSH group, mares (n = 18) were treated with eFSH (12.5 mg i.m., eFSH®, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) twice daily until a follicle ≥35 mm in diameter was detected, and approximately 36 h later hCG (2000 IU i.m., Chorulon®, Intervet Canada Ltd, Whitby, ON, Canada) was administered. In the deslorelin group, mares (n = 18) were treated with deslorelin (63 μg, i.m., Bet Pharm, Lexington, KY, USA) twice daily until a follicle ≥35 mm in diameter was detected, at which time hCG was administered.

In both treatment groups, mares were artificially inseminated 24 h after a follicle ≥35 mm in diameter was detected, and again every 48 h until ovulation (Day 0 = day of the first ovulation) with fresh semen collected from a stallion of proven fertility. A minimum dose of 5 x 10^8 progressively motile spermatozoa extended 1:1 with Kenney extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA, USA) was used to inseminate the mares.

A treatment failure was defined as a mare that did not develop a follicle ≥35 mm in diameter within 12 d of eFSH/deslorelin treatment, or if a mare developed a follicle ≥35 mm but failed to ovulate within 72 h of hCG administration. Mares that were deemed treatment failures were followed until their first ovulation was detected.

10.3.3. Embryo recovery attempts

In mares that ovulated following eFSH or deslorelin treatment, embryo recovery attempts were performed 8 d after ovulation using a routine nonsurgical transcervical technique as described elsewhere (McKinnon and Squires, 1988a). A total of 4 L of embryo flush medium (ViGro Complete Flush Solution®, Bioniche Animal Health Canada Inc.) was used per mare, and mares were administrated oxytocin (40 IU i.v., Oxytocin®, Vêtoquinol N.-A. Inc., Lavaltire, QC, Canada) before the procedure was completed to facilitate recovery. Embryos were identified using a stereomicroscope, enumerated, and rinsed 3 times in Holding Medium (Vigro Holding Plus®, Bioniche Animal Health Canada Inc.). Embryos were scored for quality (1-excellent, 2- good, 3-fair, 4-poor) according to their morphology as described by McKinnon and Squires (McKinnon and Squires, 1988b), and were subjectively assessed for age according to their developmental stage (morula/ early blastocyst/ expanded blastocyst) and size.

10.3.4. Evaluation of subsequent cyclicity

Mares received PGF2α (5 mg s.c., Lutalyse®, Pharmacia Animal Health, Orangeville, ON, Canada) after an embryo recovery attempt was completed. Mares were examined every 1 -3 d to evaluate whether they continued to cycle. When a follicle ≥35 mm in diameter was detected hCG was administered to induce ovulation. The mares were not bred in this second cycle, but they were monitored as in the first cycle until 8 d after their second ovulation.

10.3.5. Measurement of serum estradiol-17β and progesterone concentrations

During the first cycle, jugular blood samples were collected into sterile plain vacutainer tubes at the time of treatment assignment, just prior to hCG administration (hCG-Day), on the day of ovulation (Day 0), and on day 8 post ovulation (Day 8) just prior to the embryo recovery attempt. For the second cycle, jugular blood samples were collected on hCG-Day, Day 0, and Day 8. The blood samples were centrifuged, and the sera were separated and stored frozen (-20°C) until hormone assays were performed. Serum concentrations of progesterone (P4) and estradiol-17β (E2) were determined using RIA validated for use in horses. For P4, all samples were analyzed in duplicates in one assay, using the Coat-A-Count® Progesterone In-vitro Diagnostic Test Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) (Blight and White, 1983); the

intra-assay coefficient of variation was <4.9%. For E2, samples were analyzed in nine runs using an assay developed and validated by the Western College of Veterinary Medicine endocrinology laboratory, University of Saskatchewan (Joseph et al., 1992; Bragg Wever et al., 2002); standards (17β-Estradiol, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) were prepared in charcoal stripped equine serum, and ranged from 1 to 100 pg/mL. Samples from each mare were analyzed in duplicates in the same run, and the numbers of samples from the two treatment groups were balanced within a run. For E2, the intra- and inter-assay coefficients of variation were <6.1% and <11.8%, respectively.

10.3.6 Statistical analyses

Statistical analyses were performed with analytic computerized software (Statistix 8 Student Edition, Analytical Software, Tallahassee, FL, USA). Continuous data were evaluated for normality of distribution and for equality of variances using the Shapiro-Wilk Test and the Bartlett's Test, respectively. Accordingly, comparisons between groups were performed with either Student t-test or Kruskall–Wallis non-parametric one-way ANOVA; Student *t*-test was used to analyze normally distributed data that had equal variances between groups (the number of days of treatment, interval to 1st ovulation, interval between 1st and 2nd ovulations) ; Kruskall–Wallis non-parametric one-way ANOVA was used to analyze data which were not normally distributed and/or had unequal variance between groups (number of preovulatory follicles, number of ovulations, number of embryos). The general effects of the treatment, the day, and the day by treatment interaction, on serum steroid hormone concentrations were analyzed in a General Analysis of Variance test, followed by Tukey HSD All-Pairwise Comparisons Test. Pearson's Correlation test was used to determine the correlation between the number of preovulatory follicles and serum E2 concentrations, and the correlation between the number of ovulations and serum P4 concentrations. Pearson chi-square test analysis was used to compare proportional data such as the proportion of mares whom ovulated, proportion of mares with multiple ovulations, and proportion of successful embryo recoveries. Categorical data (such as embryo morphological grade and embryo age) were compared with Kruskall–Wallis non-parametric one-way ANOVA. Differences were considered significant at $P < 0.05$.

10.4. Results

10.4.1. Folliculogenesis and ovarian stimulation

Folliculogenesis and ovarian stimulation data are summarized in Table 10.1. In the eFSH group 18/18 (100%) mares developed at least one follicle ≥35 mm in diameter, and 14/18 (78%) ovulated and had embryo recovery attempts. In the deslorelin group, 16/18 (89%) developed at least one follicle ≥35 mm in diameter, and 14/18 (78%) ovulated and had embryo recovery attempts. Mean date (range) of the first ovulation of the season was March 30th (March 13th to April 27th) for mares treated with eFSH, and April 6th (March 14th to April 24th) for mares treated with deslorelin ($P > 0.10$). The 4 mares that failed to ovulate following the eFSH treatment protocol, ovulated on April 26th, April 29th, May 2nd, and May 15th. The 4 mares that failed to ovulate following the deslorelin treatment protocol, ovulated on April 25th, April 29th, May 6th, and May 9th.

Unless specified differently, subsequent results are presented as mean ± SEM for ovulating mares in each group. Duration of treatment was not significantly different between the eFSH group (4.5 ± 0.4 d) and the deslorelin group (5.3 ± 0.5 d). Mares treated with eFSH had higher ($P < 0.05$) number of follicles ≥30 mm in diameter at the time of hCG administration (eFSH: 3.1 ± 0.4 vs. deslorelin: 1.1 ± 0.1 preovulatory follicles) and higher ($P < 0.05$) number of ovulations (3.4 ± 0.4 vs. 1.1 ± 0.1 ovulations). Higher ($P < 0.05$) proportions of mares had multiple ovulations in the eFSH group (13/14, 93%) as compared to the deslorelin group (1/14, 7%). The mean (95% CI) interval from treatment initiation to ovulation was 8.2 (7.3, 8.9) d in mares treated with eFSH, and 7.2 (6.2, 8.1) d in mares treated with deslorelin ($P > 0.10$). Data regarding the time of the first ovulation of individual mares, in relation to the day of treatment initiation, are presented in Figure 10.1.

10.4.2. Embryo production

Embryo production data are summarized in Table 10.2. A total of 37 embryos were recovered from 14 ovulating eFSH-treated mares, and a total of 6 embryos were recovered from 14 ovulating deslorelin-treated mares. Therefore, mean embryo recovery was higher ($P < 0.05$) in the eFSH group (2.6 ± 0.5 embryos/recovery attempt) as compared to the deslorelin group (0.4 ± 0.2

embryos/recovery attempt). Embryo recovery attempts were successful (≥1 embryo recovered) in 13/14 (93%) eFSH-treated mares, and in 5/14 (36%) deslorelin-treated mares ($P < 0.05$). Mean embryo morphology grades were not significantly different between the groups, and a morphology grade of good or excellent was given to 25/37 (68%) embryos in the eFSH group, and to 4/6 (67%) embryos in the deslorelin group.

10.4.3. Continuation of cyclicity

In the second cycle each mare ovulated a single follicle. The mean interval from the first to second ovulation was not significantly different between mares in the eFSH group (22.7 ± 2.1 d) and the mares in the deslorelin group (20.6 ± 1.7 d). However, the proportion of mares which ovulated within a 21 d interval tended ($P = 0.10$) to be lower in the eFSH group (7/14, 50%) as compared to the deslorelin group (12/14, 86%). The inter-ovulatory intervals of individual mares are presented in Figure 10.2.

10.4.4. Serum progesterone and estradiol-17β concentrations

Serum P4 and E2 concentration data are presented in Figure 10.3. The number of preovulatory follicles was positively correlated ($P < 0.05$) with the serum E2 concentrations on the hCG-Day ($r = 0.9$), and on Day 0 ($r = 0.6$). The number of ovulations was positively correlated ($P < 0.05$) with the serum P4 concentrations on Day 0 ($r = 0.8$), and on the Day 8 ($r = 0.6$). The treatment, day, and treatment by day interaction significantly affected both serum P4 and E2 concentrations. At the time of treatment assignment all mares had serum P4 concentration <0.1 ng/mL. Comparisons by day revealed that serum P4 concentrations were higher ($P < 0.05$) on Day 0 and on Day 8 of the first cycle in mares treated with eFSH as compared to mares treated with deslorelin. In all mares that ovulated (both groups, both cycles) serum P4 concentrations on Day 8 were higher than 3.5 ng/mL. Serum E2 concentrations were higher ($P < 0.05$), in the first cycle, on the hCG-day and Day 0 in mares treated with eFSH as compared to mares treated with deslorelin. In the second cycle there were no significant differences between groups in serum P4 and E2 concentrations when analysed by day, but there was a significant day effect.

Table 10.1 Ovarian response in spring transitional mares treated with twice daily eFSH or deslorelin followed by hCG administration. Results are presented as mean (± SEM) or percentages (%).

	eFSH (n = 18)	Deslorelin (n = 18)
Duration of treatment (d)	4.5 ± 0.4	5.3 ± 0.5
Proportion of mares that ovulated	78% (14/18)	78% (14/18)
[1]No. of preovulatory follicles (≥30 mm)	3.1 ± 0.4 [a]	1.1 ± 0.1 [b]
[1]No. of ovulations	3.4 ± 0.4 [a]	1.1 ± 0.1 [b]

[ab] Values with different superscripts within a row are significantly different ($P < 0.05$)

[1] Values were calculated for ovulating mares only

Table 10.2 Embryo production of spring transitional mares ovulating subsequent to treatment with twice daily eFSH or deslorelin followed by hCG administration; estrous mares were artificially inseminated with fresh semen, and embryo recovery attempts were performed 8 d after ovulation.

	eFSH (n = 14)	Deslorelin (n = 14)
[1]Embryo number	2.6 ± 0.5 [a]	0.4 ± 0.2 [b]
Successful embryo recovery attempts	93% (13/14) [a]	36% (5/14) [b]
Embryo per ovulation rate	77% [a]	38% [b]
[2]Embryo morphology grade (1-excellent; 4-poor)	2 (1, 3.5)	1 (1, 4)
[2]Embryo assessed age (d)	8 (7, 8)	8 (6, 8)

[ab] Values with different superscripts within a row are significantly different ($P < 0.05$)

[1]Values are presented as mean ± SEM

[2]Values are presented as median (1st and 3rd quartiles)

Figure 10.1 Time of first ovulation of the breeding season in mares treated with twice daily eFSH or deslorelin, followed by hCG. Each dot represents time of ovulation of an individual mare, in relation to the day of treatment initiation (1st Tx). Vertical lines represent the mean time of ovulation of the group.

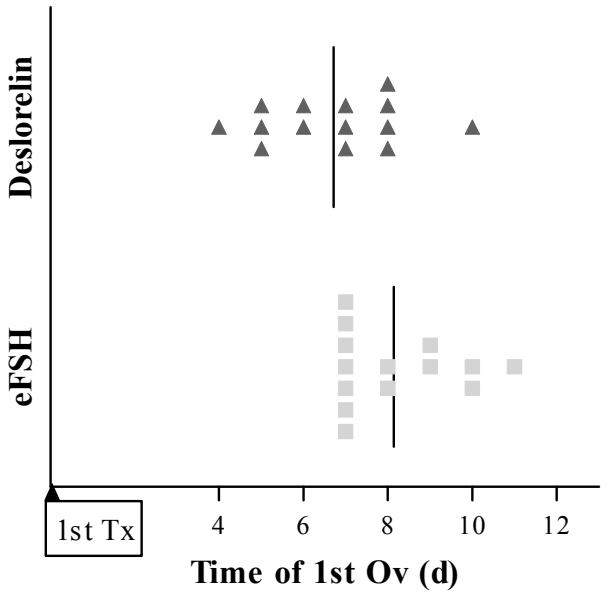


Figure 10.2 Inter-ovulatory interval between the 1st and 2nd ovulation of the breeding season. First ovulation was following eFSH or deslorelin treatment of spring transitional mares; PGF2a was administered 8 d after the 1st ovulation, and hCG was administered when a follicle ≥35 mm in diameter was detected to induce the 2nd ovulation. Each bar represents the inter-ovulatory interval of one mare.

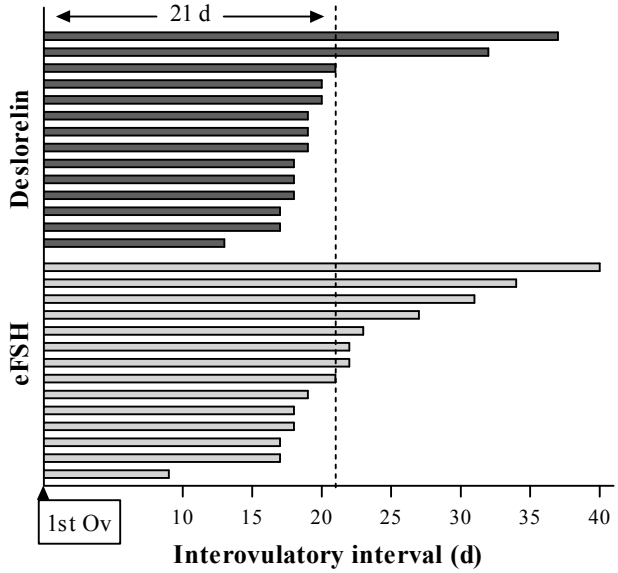
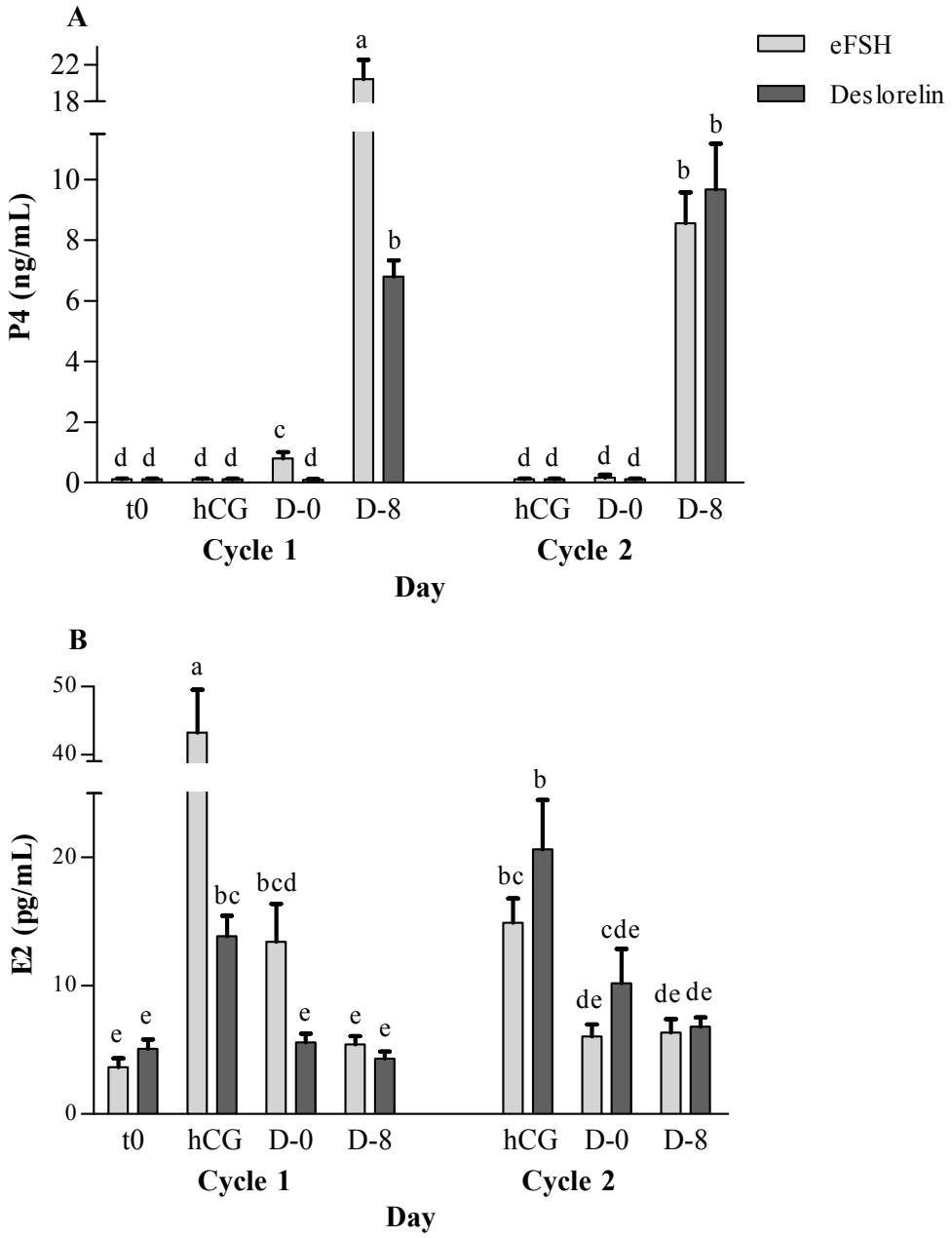


Figure 10.3 Serum P4 (A) and E2 (B) concentrations in spring transitional mares treated with eFSH or deslorelin. For the eFSH or deslorelin treatment cycle (Cycle 1), values are presented for the day of treatment assignment (t0), day of hCG administration (hCG), day of ovulation (D-0), and 8 days post ovulation (D-8). For the subsequent cycle, values are presented for hCG, D-0 and D-8. Error bars represent SEM. Different letters above bars represent significant differences ($P < 0.05$).



10.5. Discussion

This is the first study that has critically compared the efficacy of eFSH and deslorelin treatment protocols in early transitional mares. Both treatment protocols intend to promote the development of preovulatory follicle(s), responsive to ovulation induction. The deslorelin treatment protocol works through stimulation of gonadotropin secretion from the pituitary, whereas the eFSH treatment protocol bypasses the hypothalamic-pituitary axis and stimulates the ovary directly. The dose and frequency of the eFSH treatment used in the study were selected according to the manufacturer's recommended protocol, and as previously reported (McCue et al., 2007a). The dose and frequency of the deslorelin treatment were selected according to preliminary results and information provided to us by Bet-Pharm, the compounding pharmacy supplying the deslorelin. Administration of hCG was included in both treatment protocols in order to increase the likelihood of successful ovulation, regardless of the ability of the hypothalamic-pituitary-ovarian axis to generate an endogenous LH surge. In the eFSH group, hCG was administered after a delayed period of 36 h following the last eFSH treatment, a period that is termed "coasting" and has been reported to be beneficial when cycling mares were treated with eFSH (Welch et al., 2006); the rationale for the coasting period is to allow the high FSH concentration to decrease during the final maturation phase of the follicles. A similar coasting period was not utilized in the deslorelin group, as we believed that the endogenous FSH concentrations were not expected to be as high, and since a coasting period was not a part of the protocol recommended by Bet-Pharm, or in other previously reported GnRH-analogue treatment regimes.

Treatment assignment was performed in a sequential alternating design to balance the date of treatment initiation and reduce the effect of mare variability between the groups. We followed the mares from winter anestrus to the transitional phase in order to initiate the treatment as early as possible before the breeding season, and as mares become more likely to be responsive to exogenous hormonal stimulation. We concluded from the serial examination of the ovarian activity, and the hormonal analysis, that mares were indeed in the early transitional period at the time of treatment initiation (Oxender et al., 1977b; Ginther, 1992b).

The University of Saskatchewan is located in the Canadian Northwest, which has a naturally long and cold winter. Our clinical experience over the years is that the majority of mares under ambient light at our location commonly have their first spontaneous ovulation of the breeding

season in May. In the current study both eFSH and deslorelin treatment protocols were effective and stimulated the first ovulation of the season in 78% of mares after only a few days of treatment. Mean date of the first ovulation of the season was March 30th for mares treated with eFSH, and April 6th for mares treated with deslorelin. We did not have a group of untreated mares to evaluate the extent in which the treatments advanced the breeding season, as this was not one of the objectives of this study. However, our results may be consistent with such an effect, based on our clinical data, and in concurrence with previous reports (Johnson, 1987; Johnson and Becker, 1993; McKinnon et al., 1997; Niswender et al., 2004; Peres et al., 2007).

Equal proportions of mares successfully ovulated in both groups; however, treatment with eFSH resulted in a significantly greater degree of ovarian stimulation, which was apparent in higher number of preovulatory sized follicles, and higher number of ovulations. This was also reflected by the differences in serum E2 and P4 concentrations between the groups; on the hCG-Day and on Day 0, serum concentrations of E2 were correlated with the number of preovulatory follicles, and were significantly higher in mares treated with eFSH; consistent with the greater number of ovulations in eFSH-treated mares, serum P4 concentrations increased faster and reached higher concentrations, on Day 0 and Day 8, respectively. These differences in the magnitude of ovarian response between the two groups may be related to the physiologic differences in the routes of ovarian stimulation, and to the subsequent amount of gonadotropins that were available to stimulate the ovary. Bioavailability, potency, and the doses of eFSH or deslorelin used in the study, may have also led to such differences.

The corpora lutea that developed following the eFSH or deslorelin treatments appeared ultrasonographically normal, and resulted in Day 8 serum P4 concentrations capable of supporting embryonic development and pregnancy. Embryo recovery results were superior in mares treated with eFSH due to the higher number of ovulations, and higher embryo per ovulation rate. Embryos were recovered from mares in both groups, and most of them were graded as good to excellent in quality; this may indicate normal release of cumulus-oocyte-complexes, fertilization, and early embryo development following at least some of the eFSH- and deslorelin-induced ovulations in transitional mares. However, some of the recovered embryos were of lower quality (grade 3 or 4). This was in accordance with previous studies which suggested that in many species superovulation treatments for ovarian stimulation may impact the viability of a proportion of embryos recovered;

nevertheless, this is a controversial issue in the mare (Woods, 1984; Akira et al., 1993; Youngs, 2001; Mapletoft et al., 2002; Cognie et al., 2003; Raz et al., 2005; Kelley et al., 2006; Raz et al., 2006b).

Treatment of transitional mares with eFSH have been reported previously following studies conducted in Colorado, USA (latitude 40°585' N) (Niswender et al., 2004), and Sao-Paulo, Brazil (latitude 22°52' S)(Peres et al., 2007). The eFSH treatment protocol reported here is different as mares were maintained under ambient light before and during the study, and an hCG coasting period was utilized. The duration of eFSH treatment, the proportion of mares that successfully ovulated, and the number of ovulations obtained following the eFSH treatment in the current study were comparable with those reported in the studies conducted in Colorado and Sao-Paulo. Using this eFSH treatment protocol, we obtained multiple ovulations in the vast majority of ovulating mares, and a high embryo recovery rate. Embryo per ovulation rate in cycling superstimulated mares was reported to be equal or lower than expected in cycling control mares (Alvarenga et al., 2001; Niswender et al., 2003; Raz et al., 2005; Carmo et al., 2006; Logan et al., 2006; Welch et al., 2006; Logan et al., 2007b); interestingly, the embryo per ovulation rate in the current study was notably high. It is possible that eFSH treatment is more valuable for transitional mares than for cycling mares, however, this has not been critically examined to date.

Several studies utilized GnRH, or GnRH analogues, in a combination of doses, routes, and patterns of administration for the induction of ovulation in seasonally anovulatory mares, which makes it difficult to compare them with this study (Evans and Irvine, 1976; 1977; 1979; Allen and Alexeev, 1980; Allen et al., 1987; Fitzgerald et al., 1987a; Fitzgerald BP, 1987; Hyland et al., 1987; Johnson, 1987; Minoia and Mastronardi, 1987; Hyland and Jeffcott, 1988; Johnson and Becker, 1988; Ginther and Bergfelt, 1990; Harrison et al., 1990; Ainsworth and Hyland, 1991; McCue et al., 1991; McCue et al., 1992b; Fitzgerald et al., 1993; Hyland, 1993; Johnson and Becker, 1993; McKinnon et al., 1997; Nagy et al., 2000; Johnson et al., 2002a). Nevertheless, the current study utilized a practical treatment protocol that combined injectable deslorelin and hCG, which are commercially available. Ovulation rate (78% of mares) was superior to most GnRH treatment regimens previously reported, and mean ovulation number (1.1± 0.1) was similar to that accepted from cycling mares spontaneously ovulating (Ginther, 1992b). Higher incidence of multiple ovulations following GnRH or GnRH analog regimens have been reported by few

investigators (Johnson and Becker, 1988; Ginther and Bergfelt, 1990); others, like us, have failed to obtained similar results (Palmer and Quellier, 1988; Harrison et al., 1990; McCue et al., 1992b; McKinnon et al., 1997). The embryo recovery rate obtained in the current study (0.4 ± 0.2 embryos/recovery attempt) was lower than what is expected in single-ovulating mares (0.5 embryos/recovery attempt), but still acceptable. This was anticipated, considering the normal pregnancy rate previously reported following ovulations induced by GnRH in seasonally anestrous mares (Allen et al., 1987; Hyland et al., 1987; Minoia and Mastronardi, 1987; Ginther and Bergfelt, 1990; McCue et al., 1992b).

The mean inter-ovulatory interval from the first, eFSH- or deslorelin- induced ovulation, to the second ovulation was longer than the expected interval during the breeding season, particularly when PGF2α and hCG are used to shorten the cycle and hasten ovulation (Lofstedt, 1988; Ginther, 1992b). This may reflect lack of full seasonal maturity of the hypothalamic-pituitary-ovarian axis in a few of the treated mares, even after they successfully ovulated and produced embryos. Anovulatory mares induced to ovulate with GnRH have been reported to have prolonged intervals to their next ovulation, particularly when ovulation was induced early before the onset of the natural breeding season (Ginther and Bergfelt, 1990; McKinnon et al., 1997; McCue et al., 2007b). Prolonged inter-ovulatory interval was also anecdotally reported following eFSH treatment (McCue et al., 2007b; Peres et al., 2007). We found a tendency for more eFSH-treated mares than deslorelin-treated mares to have a delayed return to normal estrus in a reasonable time; these findings may be due to chance, the date of ovulation of each individual mare, the potential effect of each hormone on the hypothalamic-pituitary-ovarian axis, or the possible suppression of the high levels of P4 and E2 found in eFSH-treated mares.

In summary, twice daily administration of eFSH or deslorelin for a short treatment period, followed by hCG, were equally effective in inducing ovulation in transitional mares. However, treatment with eFSH resulted in a significantly higher number of preovulatory size follicles, a greater number of ovulations, and a higher embryo recovery rate than obtained following the deslorelin treatment. Corpora lutea developed following both treatment protocols produced adequate serum P4 concentrations. Prolonged intervals between the first and second ovulation of the season tended to be more common in mares treated with eFSH than in mares treated with deslorelin.

11. OVULATION, PREGNANCY RATE, AND EARLY EMBRYONIC DEVELOPMENT IN VERNAL TRANSITIONAL MARES TREATED WITH EQUINE OR PORCINE FSH

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11.1. Abstract

The objective of this study was to compare the efficacy of purified equine- and porcine- FSH treatment regimens in mares in early vernal transition. Mares (n = 22) kept under ambient light were examined ultrasonographically per-rectum, starting January 30th. They were assigned to one of two treatment groups using a sequential alternating treatment design when a follicle ≥25 mm was detected. In the eFSH group, mares were treated twice daily with equine-FSH, and in the pFSH group mares were treated twice daily with porcine-FSH; treatments were continued until follicle(s) ≥35 mm were detected, and hCG was administered 24 h later. Estrous mares were inseminated with fresh semen, and examined for pregnancy on days 11 - 20 post ovulation. In the eFSH group 11/11 (100%) mares developed follicle(s) ≥35 mm, 8/11 (73%) ovulated, and 6/8 (75%) ovulating mares conceived. In the pFSH group, 5/11 (45%) developed follicle(s) ≥35 mm, 4/11 (36%) ovulated, and 3/4 (75%) ovulating mares conceived. Treatment with eFSH resulted in a greater ovarian stimulation; higher number of preovulatory-sized follicles, higher number of ovulations, and higher number of embryos ($P < 0.05$). Following ovulation, serum progesterone concentrations were correlated with the number of CLs, and supported early embryonic development; maternal recognition of pregnancy occurred in all pregnant mares. We concluded that eFSH can be used to effectively induce follicular growth and ovulation in vernal transitional mares; however, if bred, diagnosis and management of twin pregnancies would be required prior to Day 16 due to the increased risk of multiple embryos per pregnancy. Conversely, the current pFSH treatment regimen cannot be recommended.

11.2. Introduction

Mares are seasonal, polyestrous, long day breeders. Following winter solstice, anestrous mares gradually obtain ovulatory competence during a prolonged phase called vernal transition. This transitional phase is characterized by a series of stages or events characterized by increased GnRH and gonadotropin secretion, resurgence of follicular development, estrous behavior and, finally ovulation (Sharp, 1980b; Nagy et al., 2000). During the early transitional phase the number of follicles with a diameter ≥20 mm in the mare's ovaries increases, and ovaries usually contain several developing and atretic follicles (Turner et al., 1979b; Ginther, 1992a). During the late transitional phase most mares develop 1 - 3 anovulatory follicular waves each characterized by a large dominant follicle (≥35 mm); follicles continue to emerge and regress until one is ultimately recruited to be the ovulatory follicle (Turner et al., 1979b; Ginther, 1992a). Increasing daylight length plays the major role in the resurgence of ovulatory competence, and the mean month that mares experience the first ovulation of the breeding season depends on the geographical latitude at which they live, genetics, nutrition, climate, and other environmental factors (Sharp, 1980b; Ginther, 1992b; Guerin and Wang, 1994; Niekerk and Niekerk, 1997; Nagy et al., 2000).

Successful regimens to stimulate ovarian cyclicity in mares to overcome winter anestrus and/or the prolonged transitional phase is of interest to the horse breeding industry, as economic pressures exist to produce foals early in the year. Various therapeutic strategies to advance the first ovulation of the year have been investigated. In addition to artificial photoperiod (McCue et al., 2007c), hormonal strategies employed include the use of GnRH and GnRH analogs (Ginther and Bergfelt, 1990; Harrison et al., 1990; McCue et al., 1991; Fitzgerald et al., 1993; Hyland, 1993; Johnson and Becker, 1993), progesterone and progestins (Webel and Squires, 1982; Newcombe et al., 2002), hCG (Carnevale et al., 1989), prostaglandins (Jochle et al., 1987), prolactin (Thompson et al., 1997a), dopamine antagonists (Besognet et al., 1997), and equine pituitary extract (Douglas et al., 1974; Lapin and Ginther, 1977). More recently, a purified equine FSH (eFSH®, Bioniche Animal Health Inc.) has been shown to advanced the first ovulation of the breeding season, and to result in successful embryo recoveries from donor mares on day 7 or 8 post ovulation (Niswender et al., 2004; Peres et al., 2007). However, to date, there is no information regarding the potential benefit of purified equine-FSH treatment in transitional mares that are intended to carry their own pregnancy.

Purified porcine FSH (Folltropin®-V, Bioniche Animal Health Inc.) is an affordable commercially available form of FSH, which is widely used to induce superovulation in donor cows (Mapletoft et al., 2002). In mares, different forms of porcine FSH have been reported to increase the number of ovulations (Irvine, 1981; Squires et al., 1986; Fortune and Kimmich, 1993) and the rates of embryo recovery in cycling donor mares (Veselinovic et al., 1994; Krekeler et al., 2006), as compared to non-treated control; however, it was not widely accepted as a superovulatory treatment in the equine industry since the results were inferior to those obtained with gonadotropins of equine origin (Squires et al., 1986; Veselinovic et al., 1994; Squires and McCue, 2007). Nevertheless, the potential benefits of purified porcine FSH in transitional mares have never been examined. Potentially, porcine FSH may stimulate follicular growth and ovulation in transitional mares, and might result in a pregnancy early in the year.

The objective of this study was to determine and compare the efficacy of purified equine and porcine FSH treatment regimens in mares in early vernal transition, which were intended to carry their own pregnancy. We investigated the effects of the two treatment protocols on folliculogenesis, interval to first ovulation, ovulation rate, number of ovulations, pregnancy rate, embryo number, early embryonic development, and serum concentrations of progesterone and estradiol-17β. We hypothesized that both treatment regimens would stimulate follicular growth and ovulation in early transitional mares within only a few days of treatment; however, we anticipated that the equine-FSH treatment would result in greater ovarian stimulation and a higher pregnancy rate.

11.3. Materials and methods

11.3.1 Animals and reproductive tract examinations

This study was performed between January and May 2007, in the research facility of the University of Saskatchewan in Canada, which is located at 52°07′ latitude in the Northern Hemisphere. Twenty-two mares, Quarter Horse / Percheron cross type, ages 3 to 6 years, with a body condition score of at least 5 out of 9, were used for this study. They had no signs of systemic disease or lameness, and had good perineal conformation. Mares were kept under ambient light in outdoor paddocks with sheds, and were maintained on alfalfa and grass hay with access to water

and trace-mineralized salt, in accordance with the guidelines of the University of Saskatchewan's Institutional Animal Care and Use Committee.

Transrectal palpation and ultrasonographic examinations of the reproductive tracts were performed starting on January 30th, 2007. Uterine and cervical tone was assessed and separately scored from 1 to 4 (1- soft, 2- moderately soft, 3- moderately toned, 4- toned) (Samper, 1997). A B-mode ultrasound scanner (GE Ausonics, Universal Ultrasound, Bedford Hills, NY, USA) equipped with a 5.0 MHz linear-array transducer was used to monitor ovarian activity and to subjectively score endometrial edema from 0 (no edema, homogeneous grey) to 4 (marked edema with a distinct black and white pattern) (Samper, 1997). At the beginning of the study, the reproductive status of all mares was defined as winter anestrus due to the absence of luteal tissue and follicles <15 mm in diameter on repeated examinations. Mares were examined every 2-3 d until a follicle ≥18 mm in diameter was detected and daily thereafter until 3 d post ovulation (Day 0 = day of ovulation), and on Days 5, 8, and 11-20.

11.3.2. Experimental design and treatment groups

Mares were assigned to one of two treatment groups when a follicle ≥25 mm in diameter was detected using a randomized sequential alternating treatment design. Thus, the first mare that had a follicle ≥25 mm in diameter was randomly assigned to a treatment group; thereafter, mares were assigned to a treatment group by alternate sequence in order to balance the date of treatment initiation and reduce the effect of mare variability between the groups.

In the eFSH group, mares (n = 11) were treated twice daily with equine-FSH (12.5 mg i.m., eFSH®, Belleville, ON, Canada), and in the pFSH group, mares (n = 11) were treated twice daily with porcine-FSH (25 mg i.m., Folltropin®-V, Bioniche Animal Health Canada Inc.). The twice daily FSH treatments were continued until a follicle ≥35 mm in diameter was detected, and approximately 24 h later hCG (2000 IU i.m., Chorulon®, Intervet Canada Ltd, ON, Canada) was administered. A treatment failure was defined as a mare that did not develop a follicle ≥30 mm in diameter within 7 d of FSH treatment, or if a mare developed a follicle ≥35 mm but failed to ovulate within 72 h of hCG administration. Mares that were deemed treatment failures were followed until their first spontaneous ovulation was detected.

In both treatment groups, estrous mares were artificially inseminated 24 h after hCG was administered, and again every 48 h until ovulation, with fresh semen collected from a stallion of proven fertility. A minimum dose of 5 x 10^8 progressively motile spermatozoa extended 1:1 with Kenney extender (EZ-Mixin, Animal Reproduction Systems, Chino, CA, USA) was used to inseminate the mares. Mares that were deemed treatment failures were not bred.

11.3.3. Pregnancy diagnosis

On Days 11 - 20, daily transrectal ultrasonographic examinations were performed to evaluate the mares' pregnancy status (pregnant yes/no) and the number of embryos present. In addition, on Days 11 - 16 ultrasound images of each embryonic vesicle were frozen 3 times at the largest subjective image of the vesicle, and the average diameter for each vesicle was recorded. Mean daily growth rate was calculated for each embryonic vesicle on Days 11 – 16. In singleton pregnancies, daily growth rate was determine by calculating the differences of the vesicle's diameters measured on two subsequent days; however, in some of our mares two or three embryonic vesicles were detected in a pregnancy, all of which were ultrasonographically visible on Day 11. Since we could not follow each of these embryonic vesicles individually, the daily growth rate was averaged for the embryos in a pregnancy. Following the examination on Day 20 the mares received PGF2α (5 mg subcutaneously, Lutalyse®, Pharmacia Animal Health, Orangeville, ON, Canada) to terminate their pregnancies.

11.3.4. Measurement of serum estradiol-17β and progesterone concentrations

Jugular blood samples were collected into sterile plain vacutainer tubes on selected days during the study. The blood samples were centrifuged, and the sera were separated and stored frozen (-20°C) until progesterone (P4) and estradiol-17β (E2) radioimmunoassays were performed. Serum concentrations of P4 were evaluated for the day of treatment assignment, day of hCG administration (hCG-Day), and Days 0 - 3, 5, 8, 11, 14, 17 and 20. Serum concentrations of E2 were evaluated for the day of treatment assignment, hCG-Day, and Days 0 - 3, 5, and 20. In mares that failed to develop a follicle ≥35 mm in diameter, the P4 and E2 hormonal analysis included the day of treatment assignment, and the day of the last treatment. In mares that developed a follicle

≥35 mm in diameter, but failed to ovulate within 72 h of hCG administration, the hormonal analysis included the day of treatment assignment and the hCG-Day.

For P4, samples were analyzed in duplicates in a total of three assays using the Coat-A-Count® Progesterone In-vitro Diagnostic Test Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) (Blight and White, 1983); the intra- and inter-assay coefficients of variation were <3.6% and <8.8%, respectively. For E2, samples were analyzed in duplicates in a total of six assays using a radioimmunology developed and validated by the Western College of Veterinary Medicine Endocrinology Laboratory, University of Saskatchewan (Joseph et al., 1992; Bragg Wever et al., 2002); standards (17β-Estradiol, E8875, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) were prepared in charcoal stripped equine serum, and ranged from 1 to 100 pg/ml. For E2, the intra- and inter-assay coefficients of variation were <8.9% and <13.8%, respectively.

11.3.5. Statistical analyses

Statistical analyses were performed with analytic computerized software (Statistix 8 Student Edition, Analytical Software, Tallahassee, FL, USA; and SAS Version 9.1, SAS Institute Inc., Cary, NC, USA). Pearson chi-square test analysis was used to compare proportional data such as the proportion of mares that developed a follicle ≥35 mm, proportion of mares that ovulated, proportion of mares with multiple ovulations, and proportion of pregnant mares. Kruskall–Wallis non-parametric one-way ANOVA was used to analyze continuous end points such as size of the largest follicle diameter at treatment initiation, number of days of treatment, interval to first ovulation, number of preovulatory large follicles (≥30 mm), number of ovulations, and number of embryos. The general effects of the treatment, the day, and the day by treatment interaction, on serum steroid hormone concentrations, and on uterine and cervical tone and edema, were analyzed in a Repeated Measures ANOVA, followed by Tukey HSD All-Pairwise Comparisons Test. Pearson's Correlation test was used to determine the correlation between the number of preovulatory follicles and serum E2 concentrations, and the correlation between the number of ovulations and serum P4 concentrations. Between groups, embryonic vesicle diameters and growth rates (Days 11 to 16) were compared with Kruskall–Wallis non-parametric one-way ANOVA analysis. Differences were considered significant at $P < 0.05$. Results are presented as mean ± SEM.

11.4. Results

11.4.1. Folliculogenesis and ovarian stimulation

Folliculogenesis and ovarian stimulation data are summarized in Table 11.1 (for all mares) and Table 11.2 (for ovulating mares). At the time of treatment initiation, the diameter of the largest follicle was not significantly different between the groups. In the eFSH group 11/11 (100%) mares developed at least one follicle ≥35 mm in diameter, and 8/11 (73%) ovulated. In the pFSH group, 5/11 (45%) developed at least one follicle ≥35 mm in diameter, and 4/11 (36%) ovulated. The duration of treatments in mares that developed a follicle ≥35 mm were 4.8 ± 0.6 d and 6.5 ± 1.8 d for the eFSH and pFSH groups, respectively ($P > 0.10$). Mares treated with eFSH developed a higher ($P < 0.05$) number of preovulatory follicles (≥30 mm), and had a higher ($P < 0.05$) number of ovulations. In mares that had ≥2 ovulations, all ovulations were detected on the same day, except for 2 eFSH-treated mares in which ovulations were detected within a 48 h period.

The mean date of treatment initiation was not significantly different between the groups (eFSH: March 10th ± 6 vs. pFSH: March 13th ± 7 d). In mares in which the treatment was successful, the mean date of the first ovulation of the season was March 17th ± 7 d for mares treated with eFSH, and March 26th ± 10 d for mares treated with pFSH ($P > 0.10$). The mares that failed to ovulate following the eFSH- and pFSH- treatment regimes, ovulated on average on April 9th ± 10 d, and April 22nd ± 6 d, respectively ($P > 0.10$); each of these mares had a single ovulation. Overall, the first ovulation of the breeding season was significantly earlier in mares that developed a follicle ≥35 mm and ovulated following the FSH treatment regimens (March 21st ± 6 d) as compared to mares that were deemed treatment failures (April 19th ± 5 d). Between groups, when all mares in the study were evaluated, the first ovulation of the season was significantly earlier in mares in the eFSH group (March 24th ± 6 d) as compared to mares in the pFSH group (April 12th ± 6 d).

11.4.2. Pregnancy rates and early embryonic development

In the eFSH group a pregnancy was diagnosed in 6/11 mares; two mares with a singleton pregnancy (one embryo), three mares with a twin pregnancy (two embryos), and one mare with a triplet pregnancy (three embryos). In the pFSH group, a pregnancy was diagnosed in 3/11 mares;

all of which had a singleton pregnancy. Therefore, pregnancy rate in ovulating mares was not significantly different between the groups (6/8, 75% vs. 3/4, 75%). However, mean embryo number tended ($P < 0.08$) to be higher in pregnant mares in the eFSH group (1.8 ± 0.3 vs. 1.0 ± 0 embryos/pregnancy). Overall, there were significantly more embryos per eFSH-treated mare (1.0 ± 0.3 embryo/mare) than per pFSH-treated mare (0.3 ± 0.1 embryo/mare).

In all pregnant mares, pregnancy was diagnosed on Day 11, and all embryonic vesicles were identified on that day. On Days 11 to 16, mean diameter (Figure 11.1) and mean daily growth rates of embryonic vesicles (3.6 ± 0.2 vs. 3.8 ± 0.4 mm/day) were not significantly different between the eFSH and pFSH groups. In the eFSH group, in 5/6 pregnant mares, embryonic vesicles appeared to develop normally until Day 20. However, we observed early embryonic death in the mare that had a triplet pregnancy. In that mare, embryos appeared to develop normally until Day 15; however, on Day 16 only one embryonic vesicle was detected, and on Day 18 no embryonic vesicles were detected. In the pFSH group, all embryos appeared to develop normally until Day 20.

11.4.3. Uterine and cervical parameters

At the time of treatment initiation, the mean scores for uterine tone and edema, and mean score for cervical tone, were not significantly different between the groups, and were not significantly different between mares that subsequently were deemed treatment failure and those who were not. Overall, the scores of uterine and cervical tones were significantly affected by Day, but they were not different between groups. Generally, uterine and cervical tones were scored lower (softer), with higher endometrial edema scores just before and after ovulation (uterine tone 1.5 ± 0.1; cervical tone 1.4 ± 0.1; uterine edema 2.4 ± 0.3), as compared to Days 5 - 20 (uterine tone 3.1 ± 0.1; cervical tone 3.1 ± 0.1; uterine edema 0.1 ± 0.1). Uterine and cervical tone increased and endometrial edema decreased from the day of hCG administration to two days after ovulation ($P < 0.01$). On the day of hCG administration, mares treated with eFSH had a significantly higher endometrial edema, as compared to mares treated with pFSH (2.9 ± 0.4 vs. 1.5 ± 0.3).

Table 11.1 Ovarian parameters in early vernal transitional mares treated twice daily with equine or porcine FSH followed by hCG administration. Results are presented as mean (± SEM) or percentages (%).

	eFSH (n = 11)	pFSH (n = 11)
Largest follicle size at treatment initiation (mm)	25.6 ± 0.4	25.7 ± 0.3
Proportion of mares that developed a follicle ≥35 mm and received hCG	100% (11/11) [a]	45% (5/11) [b]
Proportion of mares that ovulated following the FSH treatment regime	73% (8/11)	36% (4/11)
No. of preovulatory follicles (≥30 mm) per treated mare	2.6 ± 0.4 [a]	0.6 ± 0.2 [b]
No. of ovulations per treated mare	1.9 ± 0.5 [a]	0.5 ± 0.2 [b]
Proportion of mares with ≥2 ovulations	64% (7/11) [a]	9% (1/11) [b]

[ab] Values with different superscripts within a row are significantly different ($P < 0.05$)

Table 11.2 Ovarian and pregnancy parameters in mares that ovulated following equine or porcine FSH treatment regimens during early vernal transition. Results are presented as mean (± SEM) or percentages (%).

	eFSH (n = 8)	pFSH (n = 4)
Duration of FSH treatment (d)	4.8 ± 0.6	6.5 ± 1.8
Interval from treatment initiation to ovulation (d)	6.7 ± 0.5 [a]	10.5 ± 1.8 [b]
No. of preovulatory follicles (≥30 mm)	2.3 ± 0.4 [a]	1.2 ± 0.3 [b]
No. of ovulations	2.6 ± 0.4 [a]	1.2 ± 0.3 [b]
Pregnancy rate	75% (6/8)	75% (3/4)
[1]Embryo number per pregnancy	1.8 ± 0.3	1.0 ± 0
Daily growth rate of embryonic vesicles on Days 11-16 (mm/day)	3.6 ± 0.2	3.8 ± 0.4

[ab] Values with different superscripts within a row are significantly different ($P < 0.05$)

[1] $P < 0.08$

11.4.4. Serum progesterone and estradiol-17β concentrations

There was a significant effect of Day and Treatment on serum P4 and E2 concentrations. At the time of treatment assignment all mares had serum P4 concentration < 0.1 ng/ml, and serum E2 concentrations were not significantly different between the groups (4.6 ± 0.8 vs. 5.5 ± 1.2 pg/ml). In both groups, in mares that developed follicle(s) ≥35 mm, serum E2 concentrations were significantly higher on the hCG-Day as compared to the day of treatment assignment. In mares that did not develop a follicle ≥35 mm, there were no significant differences in serum E2 concentrations between the day that the treatment was stopped (3.9 ± 0.4 pg/ml) and the day of treatment assignment (4.1 ± 0.6 pg/ml). On the hCG-Day, serum E2 was positively correlated ($r=0.6$; $P < 0.05$) with the number of preovulatory large follicles, and it was significantly higher in mares treated with eFSH (38.2 ± 7.1 vs 13.8 ± 4.1 pg/ml). Mean serum E2 concentrations on Days 0 - 3, 5, and 20, were not different between the groups.

Following ovulation, serum P4 concentrations increased in both groups, and reached its peak concentrations on Day 11 and 8 in the eFSH and pFSH groups, respectively (Figure 11.2). Following that, there was a decrease in mean serum P4 concentrations in both groups; however, in all pregnant mares, serum P4 concentrations were > 5 ng/ml until Day 20. In mares which were not pregnant, serum P4 concentrations decreased to < 0.2 ng/ml on Days 17 and 20. The overall serum P4 concentrations were significantly higher in mares treated with eFSH; and the number of ovulations was positively correlated ($r=0.6$; $P < 0.05$) with the overall serum P4 concentrations (area under the curve) on Days 0 to 20.

Figure 11.1 Embryonic vesicle diameters (mean ± SEM) on Days 11 to 16 of gestation as detected by transrectal ultrasonographic examination. Mares were induced to ovulate during early vernal transition by twice daily treatment with equine or porcine FSH, followed by hCG administration.

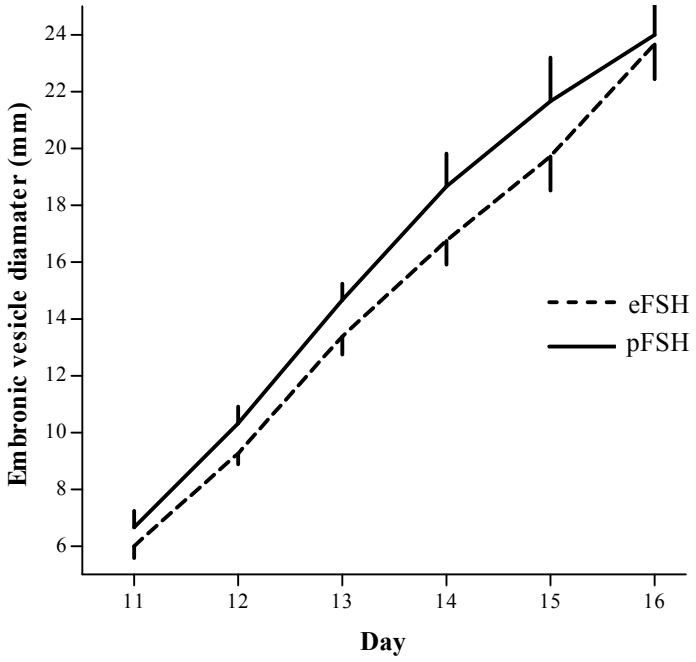
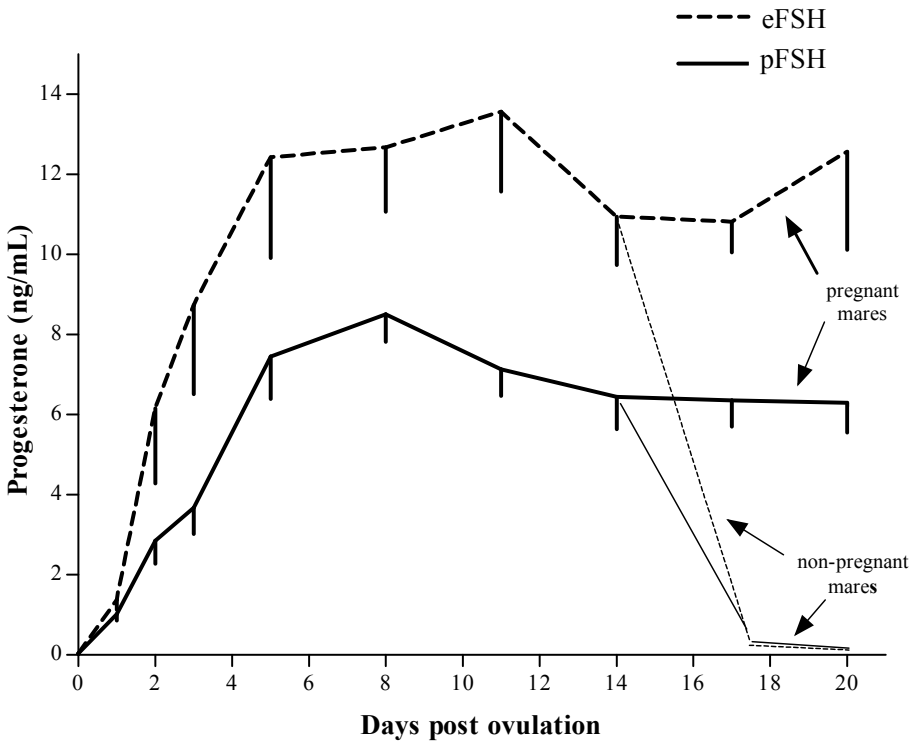


Figure 11.2 Serum progesterone concentrations (mean ± SEM) following ovulation (Day 0) in vernal transitional mares treated twice daily with equine or porcine FSH, followed by hCG administration. All mares were artificially inseminated with fresh semen; pregnancy was detected by transrectal ultrasonographic examination in 75% of the ovulating mares in each group. Serum progesterone concentrations were significantly affected by the day and by the treatment ($P < 0.05$).



11.5. Discussion

This is the first study that has critically compared the efficacy of purified equine- and porcine-FSH treatment regimens in vernal transitional mares. Treatment assignment was performed in a sequential alternating design to balance the date of treatment initiation and reduce the effect of mare variability between the groups. We followed the mares from winter anestrus to the transitional phase in order to initiate the treatment as early as possible before the breeding season, and as mares become more likely to be responsive to exogenous hormonal stimulation. We concluded from the serial examination of the ovarian activity, and the hormonal analysis, that mares were indeed in the early transitional period at the time of treatment initiation (Oxender et al., 1977b; Ginther, 1992a).

The eFSH and pFSH treatment protocols intend to promote the development of preovulatory follicle(s), responsive to ovulation-induction, by bypassing the hypothalamic-pituitary axis and stimulating the ovary directly. Administration of hCG was included in both treatment protocols in order to increase the likelihood of successful ovulation, regardless of the ability of the hypothalamic-pituitary-ovarian axis to generate an endogenous LH surge. The hCG was administered after a delayed period of 24 h following the last FSH treatment, a period that is termed “coasting” and has been reported to be beneficial when cycling mares were treated with eFSH (Welch et al., 2006); the rationale for the coasting period is to allow the high FSH concentration to decrease during the final maturation phase of the follicles.

Treatment with eFSH resulted in a significantly greater degree of ovarian stimulation, which was apparent in higher number of preovulatory sized follicles, higher number of ovulations, higher proportion of mares with multiple ovulations, and higher number of embryos. In addition, more mares in the eFSH group developed a follicle ≥35 mm and experienced their first ovulation following the treatment; however, the difference in the proportions of mares that ovulated was not statistically significant, probably due to small number of horses in each group. Overall, mares in the eFSH group had their first ovulation of the season approximately 3 weeks earlier than mares in the pFSH group. However, we did not have a group of untreated mares to evaluate the extent in which the treatments advanced the breeding season, as this was not one of the objectives of this study.

The higher degree of ovarian stimulation in mares treated with eFSH was also reflected by the differences in serum E2 and P4 concentrations between the groups; on the hCG-Day, serum concentrations of E2 were correlated with the number of preovulatory follicles, and were significantly higher in mares treated with eFSH; accordingly on that day, mares treated with eFSH had a significantly higher endometrial edema, as compared to mares treated with pFSH. Consistent with the greater number of ovulations in eFSH-treated mares, an larger amount of luteal tissue, serum P4 concentrations increased at a faster rate and reached higher concentrations in those mares that ovulated following the eFSH treatment as compared to those that ovulated following the pFSH treatment.

The eFSH and pFSH used in this study are highly purified extracts from equine- and porcine-pituitary glands, respectively; according to the manufacturer, the extraction and purification processes are the same for the two products. The differences in the magnitude of ovarian response following equine- and porcine- FSH treatments may be related to several factors, such as: the dosages used, bioavailability, and potency. We selected the amount and frequencies of the FSH administrations according to previous reports describing treatments of cycling mares with eFSH® (McCue et al., 2007a) and with Folltropin®-V (Fortune and Kimmich, 1993; Krekeler et al., 2006). However, the doses of the two hormones cannot be compared; whereas the amount of FSH in the Folltropin®-V is given in NIH-FSH-P1 units (National Institutes of Health USA Reference Standard of FSH), the amount of FSH in the eFSH® is given in B-FSH-ReF-001units (Bioniche Animal Health Standard of FSH); yet, the mode of conversion of these units have not been determined by the manufacturer (personal communication with Dr. Duncan K. Hockley, Bioniche Animal Health Ltd.).

To the best of our knowledge, the bioavailability and potency of eFSH® and Folltropin®-V in horses have never been critically compared; still, it has been suggested that porcine-FSH does not bind to the equine FSH receptor with the same affinity as equine-FSH (Chopineau et al., 1997; Squires and McCue, 2007). A comparison of porcine gonadotropins with equine gonadotropins shows that equine gonadotropins have a higher carbohydrate and sialic acid content, which may play an important role in their biological activity and may extend their half life (Whitley et al., 1981; Ginther, 1992a; van Dijk and Ward, 1992; Rose et al., 2000). In a recent study, ultrastructural analysis of equine oocytes matured *in-vitro* showed that the use of a homologous

source of gonadotropins (eFSH or crude Equine Pituitary Extract) in the maturation media provided better cytoplasmic maturation than if the maturation media was supplemented with porcine-FSH. Oocytes that matured in the presence of pFSH showed morphological characteristics of immaturity such as heterogeneity of the cortical granules and the presence of junctions between cumulus cells and oocytes, as well as indications of the precocious release of cortical granules; while in contrast, oocytes cultured in the presence of eFSH and Equine Pituitary Extract showed cytoplasmic characteristics similar to oocytes that were matured *in vivo* (Landim-Alvarenga and Alvarenga, 2006).

The duration of treatment, the proportion of mares that successfully ovulated, and the mean ovulation number obtained in the eFSH group were comparable with those reported in other studies which utilized eFSH treatment for transitional donor mares (Niswender et al., 2004; Peres et al., 2007). The corpora lutea that developed following the eFSH treatments appeared ultrasonographically normal, and resulted in serum P4 concentrations capable of supporting embryonic development and pregnancy. In all pregnant mares, serum P4 concentration were high (>5 ng/ml) up to Day 20, which allowed us to conclude that maternal recognition of pregnancy occurred as expected, since luteolysis did not occur. In the two mares that were not pregnant serum P4 concentration decreased to < 0.2 ng/ml on Days 17 and 20. In the one mare in which embryonic losses occurred, serum P4 concentrations were high up to Day 20, and we could not determine the cause for the loss; in this mare serum E2 concentration before and after ovulation were the highest in the group. In a recent study (Raz et al., 2009c), we examined the effects of eFSH in cycling mares carrying their own pregnancy for 16 days, as compared to non-treated control, and found that although the number of ovulations was increased (2.3 ± 0.5 vs 1.1± 0.3 ovulations/mare), the embryo per ovulation rate was significantly lower in the eFSH treated mares (0.3 ± 0.1 vs 0.6 ± 0.1 embryo/ovulation); this was associated with significant alterations of the reproductive tract parameters (tone and edema) and serum concentrations of progesterone and estradiol-17β on the days that oocyte maturation, fertilization, and early embryonic development are expected to occur. In the current study, pregnancy rate, embryo per ovulation rate, and daily embryonic growth rate were similar to what has been reported for single-ovulating mares during the season (Ginther, 1992b). It is possible that eFSH treatment is more valuable for vernal transitional mares than for cycling mares; however, this has not been critically examined to date.

The results obtained in the pFSH group were poor. In two previous studies, the same purified pFSH product was used in cycling mares and resulted in 1.5-2.2 ovulations per mare. However, we found very limited value to the current pFSH treatment regimen in vernal transitional mares. The difference in the efficacy of FSH treatments in cycling versus transitional mares is probably related to the differences in their physiologic state, which may affect the sensitivity of the ovary to stimulation, steroidogenic capacity of the ovary, and the consequent degree of stimulation or hyperstimulation.

A drawback for the use of superovulatory treatments, such as eFSH, in mares intended to carry their own pregnancy would be the increased risk of multiple embryos pregnancy, which may lead to pregnancy loss. Therefore, early transrectal ultrasonographic detection of twins would be required in mares detected to have multiple ovulations. Twin pregnancies detected prior to Day 16 of gestation, when the embryos are still mobile, are best managed by crushing one embryonic vesicle manually, with expected survival rate for the remaining twin exceeding 90% (Bowman, 1986; Pascoe et al., 1987; Macpherson and Reimer, 2000). Therefore, efficacious superovulatory treatment would still potentially increase the likelihood of a successful pregnancy in mares that are managed appropriately. Nevertheless, the mares in our study were allowed to carry their pregnancy only to Day 20, and manual reduction of embryos in twin pregnancies was not performed. Therefore, further studies are indicated to evaluate if there is a difference in pregnancy loss rate later in the pregnancy in transitional mares initially treated with eFSH, and also in mares treated with eFSH that undergo embryo reduction procedures versus controls.

In summary, as compared to treatment with pFSH, treatment with eFSH was significantly more efficacious, and resulted in a significantly higher extent of ovarian stimulation in mares in early vernal transition. Mares treated with eFSH had their first ovulation of the season significantly earlier, and most of them ovulated multiple follicles, and within few days of treatment; the subsequent serum progesterone concentrations were high and supported early embryonic development. In addition, normal maternal recognition of pregnancy occurred in all pregnant mares. We concluded that eFSH can be used to effectively induce follicular growth and ovulation in early vernal transitional mares intended to carry their own pregnancy; however, pregnancy diagnosis and appropriate management of twins would be required prior to Day 16 of

gestation due to the increased risk of multiple embryos in a pregnancy. In contrast, the current pFSH treatment regimen cannot be recommended.

12. GENERAL DISCUSSION

Effective and reliable treatments to induce multiple ovulations or superovulation are of great interest to the equine industry because they may potentially increase embryo recovery and the success rates of embryo transfer programs. In addition, these treatments may have the ability to increase the per cycle pregnancy rate in normal or subfertile mares and to stimulate follicular growth and fertile ovulations in seasonal anovulatory mares, thereby hastening and extending the annual period of reproductive production. Since 2003, the availability of eFSH, a purified equine pituitary extract product with a high FSH to LH ratio, has raised the potential for a successful and practical superovulatory treatment for mares. This took place after more than 35 years, in which different treatments were found to be of limited success or were not practical. The overall objective of the experiments reported here was to examine reproductive parameters and fertility in cycling and vernal transitional mares following eFSH treatments. The following sections are intended to review the reported studies, and to discuss factors that were found to affect fertility in superstimulated mares and that should be considered in future studies.

12.1. eFSH treatment protocol

In the studies reported here, eFSH treatment of cycling and vernal transitional mares resulted in significant ovarian stimulation. The eFSH treatment protocol included administration of 12.5 mg of eFSH twice daily initiated at approximately the time of follicle diameter deviation (largest follicle ≥20 or 25 mm); these treatments were given in order to stimulate, or "save", or "rescue" subordinate follicles to achieve ovulatory competency and result in multilple ovulations. In mares, follicle deviation occurs during the decline in the wave-stimulating endogenous FSH surge, at a time when the subordinate follicles of the wave do not reach an appropriate preparatory stage, and therefore are negatively affected by the changing gonadotropin concentrations (Ginther et al., 2003; Ginther et al., 2004a; Ginther et al., 2005a; Checura et al., 2008). Despite the rapid designation of follicle status, a subordinate follicle may maintain adequate viability for at least one day after the beginning of deviation so that it can acquire dominant status if the dominant follicle fails or is ablated (Ginther et al., 2003; Ginther et al., 2004a). Based on several previous studies, FSH stimulates the production of several factors which have intra-follicular roles in the

development of dominant follicle status (Khalid et al., 2000; Campbell and Baird, 2001; Glister et al., 2001; Ginther et al., 2003; Beg and Ginther, 2006).

In cattle, initiating gonadotropin treatments at the time of follicular wave emergence is most beneficial, as it increases the number of follicles in a wave and the number of ovulations (Bergfelt et al., 1997; Bo et al., 2002; Mapletoft et al., 2002; Bo et al., 2006). This approach is practical in the cattle industry since the follicular growth pattern in the cow is quite predictable, and since several methods for synchronizing follicular wave emergence have been developed. However, in mares, there is no efficient hormonal treatment for the induction of a new follicular wave. Therefore, the superovulatory approach in mares is to initiate treatment with gonadotropins at the early stage of the follicular wave when all, or most, of the antral follicles of the wave still maintain adequate viability to obtain a preovulatory status. Many of the initial studies with EPE were conducted prior to the knowledge regarding ovarian follicular waves in the mare, and superovulatory treatments were initiated according to the day in the estrous cycle. However, the length of the estrous cycle as well as the pattern and timing of follicular waves vary significantly among mares (reviewed by Ginther, 1992b); this, in part, could be responsible for the variability in superovulatory results. Pierson and Ginther (1990) reported that initiation of EPE treatment when the largest follicle had reached a diameter of 15, 20 or 25 mm, but not 30 mm, gave a superstimulatory response. In the first study which utilized eFSH (Niswender et al., 2003), the eFSH treatment was initiated on Day 5 or 6 post ovulation. Later on, eFSH treatment in vernal transitional mares was initiated according to follicle diameter (≥25 mm) and resulted in ≥2 ovulations in most mares (Niswender et al., 2004).

In the studies reported in this thesis, the timing of initiation of eFSH treatment was selected based on follicular diameter, regardless of seasonal reproductive status of mares (cycling or vernal transition). In cycling mares we used a criterion of follicle size ≥20 mm (Chapters 6 and 7) or follicle size ≥25 mm (Chapters 5 and 8), and in vernal transitional mares a criterion of follicle size ≥25 mm. The rationale was that monitoring follicular activity before the initiation of eFSH treatment could reduce the variability in the superovulatory outcomes and could result in fewer days of treatment. Reducing the days of treatment is important because of the high expense of gonadotropin therapy (approximately $100 USD per day) and limited availability of equine pituitaries. Results of our studies allowed us to conclude that eFSH treatment initiated according to

follicle diameter deviation increases the development of more preovulatory follicles and increases the number of ovulations compared to non-treated control mares. We did not critically compare superovulatory results between eFSH treatment initiated at follicle size ≥20 mm and treatment initiated at follicle size ≥25 mm; however, both were effective. Furthermore, in cycling mares, mean duration of eFSH treatment in the studies in which the treatment was initiated at follicle size ≥25 mm were 5.3 ± 0.8 d (Chapter 5) and 5.4 ±0.4 d (Chapter 8), whereas mean duration of eFSH treatment in the studies in which the treatment was initiated at follicle size ≥20 mm were 8.4 ± 1.1 d (Chapter 6, PG group), 7.2 ± 1.5 d (Chapter 6, P&E group) and 8.9 ± 0.6 d (Chapter 7). In the studies we conducted in vernal transitional mares, mean duration of eFSH treatment were 4.9 ± 0.6 (Chapter 9), 4.5 ± 0.4 d (Chapter 10), and 4.8 ± 0.6 d (Chapter 11). In accordance with our findings, Welch et al. (2006) reported a similar number of ovulations when the size of the largest follicle at the onset of eFSH treatment was ≤ 25 mm as compared to at least one follicle having a size between 25 and 29 mm when treatment began. Moreover, the duration of treatment was shorter for mares in which treatment was initiated at a follicle size ≥25 mm.

The dose and frequency of eFSH treatment used by us were according to the manufacturer's recommended protocol at the time the studies were conducted, and according to other reports that had employed EPE or eFSH treatments. It has been shown that frequency of injections is a factor that affects the response to EPE, and superior results in terms of ovulations and embryo recovery have been achieved with twice daily as compared to once daily administration (Alvarenga et al., 2001; Scoggin et al., 2002). Similarly, studies that examined once versus twice daily administration of eFSH reported a similar trend as with EPE, with the administration of 12.5 mg twice daily being superior to other eFSH protocols (Niswender et al., 2003; Welch et al., 2006). Logan et al. (2007b) examined the effect of eFSH dose on the superovulatory response in donor cycling mares and reported higher numbers of preovulatory follicles and ovulations in mares treated twice daily with 12.5 mg as compared to 6.25 mg eFSH. Furthermore, McCue et al. (2008) compared a standard eFSH protocol (12.5 mg, twice daily until 35 mm follicle), to a 3-day eFSH treatment protocol (12.5 mg, twice daily), and to a declining eFSH treatment protocol (12.5 mg twice daily day 1, 8.0 mg twice daily day 2, 4.0 mg twice daily day 3), and reported superior results for the standard 12.5 mg twice daily eFSH protocol.

Several studies in mares and in other species concluded that inclusion of an ovulation inducing agent in the superovulatory protocols, given at the time preovulatory sized follicles are present, is beneficial (Woods and Ginther, 1983a; Bo et al., 2002; Mapletoft et al., 2002; Niswender et al., 2003; Bo et al., 2006; Scherzer et al., 2008). In mares, hCG was reported to be beneficial following EPE treatment (Woods and Ginther, 1983a; Alvarenga et al., 2001), and was superior to deslorelin administration in mares treated with eFSH (Niswender et al., 2003; Logan et al., 2007b). Endogenous LH secretion has been shown to be suppressed in mares treated with purified EPE containing a high FSH:LH ratio (Briant et al., 2004). Similarly, in cattle, treatments with unpurified (Roberge et al., 1995) or purified porcine FSH (Gosselin et al., 2000), eCG (Gosselin et al., 2000), or rhFSH (Takagi et al., 2001), were associated with a suppression of endogenous LH secretion Therefore, it is likely that the administration of hCG in vernal transitional and cycling mares treated with eFSH may not only shorten the interval to ovulation, but also increase the likelihood of successful ovulations, regardless of the ability of the hypothalamic-pituitary-ovarian axis to generate an endogenous LH surge.

In the studies reported here, eFSH treatments ceased when at least one follicle ≥35 mm in size was detected, and hCG was administered with the last eFSH treatment (Chapters 5 and 6), or after a delayed period of 24 to 36 h (Chapters 7 - 11) which is termed “coasting”. The rationale for the “coasting” period is to allow the increased FSH concentration to decrease during the final maturation phase of the follicles. Follicle coasting has been used in women to prevent hyperstimulation of the ovaries, which is characterized by an excessive rise in E2 levels or numerous incompletely mature follicles (Fluker et al., 1999; Mathur et al., 2007); however, the benefit of follicular coasting in women is controversial (Aboulghar and Mansour, 2003). Follicle coasting has also been employed in cattle superovulatory treatment protocols (Sirard et al., 1999; Bo et al., 2002; Bo et al., 2006). In mares, Welch et al. (2006) reported that the highest number of ovulations following twice daily 12.5 mg of eFSH was obtained when the follicles were allowed to “coast” before hCG administration; however, this result was not significantly different when compared to a protocol without the coasting period. Therefore, although quite commonly employed, there is no strong evidence to support the view that coasting is actually beneficial for superstimulated mares. Our studies were not designed to examine the effect of follicle coasting in the eFSH treatment protocol; nevertheless, we observed signs of hyperstimulation of the ovaries

(non-ovulatory follicles, and high peri-ovulatory E2 concentrations) even when a coasting period was employed in the treatment protocol.

12.2. eFSH treatment in cycling mares

We examined the effect of eFSH on cycling mares in four different studies. In all of these studies eFSH treatment resulted in significant ovarian stimulation, as it increased the number of preovulatory size follicles, and the number of ovulations as compared to non-treated mares (Table 12.1). The eFSH treatment also appeared to enhance follicular growth rate; on the days before a preovulatory follicle was detected, the daily growth rate (mm/day) of the largest follicle was significantly higher in mares treated with eFSH as compared to control. Furthermore, our work demonstrates the potential of eFSH to increase embryo recovery in cycling donor mares. Higher numbers of preovulatory follicles, ovulations, and recovered embryos in eFSH-treated mares have also been reported in other studies published prior to (Niswender et al., 2003) and during (Welch et al., 2006; Logan et al., 2007b; Araujo et al., 2008; McCue et al., 2008) our research. We found that estrus synchronization method plays an important role in the superovulatory outcomes, with eFSH treatment after PGF2α administration resulting in higher numbers of ovulations as compared to eFSH treatment following P&E regimen. We also found that administration of eFSH modified reproductive tract variables (tone and edema) and serum concentrations of progesterone (P4) and estradiol-17β on the days that oocyte maturation, fertilization, and early embryonic development are expected to occur. The eFSH treatment was associated with a significantly higher incidence of non-ovulatory follicles, a greater variation in embryo per ovulation rate, lower quality of some proportions of embryos recovered, and lower than expected pregnancy rate in recipients. Moreover, we found that eFSH treatment did not increase pregnancy rate per estrous cycle in mares intended to carry their own pregnancy; however, the incidence of twin pregnancy increased.

12.3. Estrus synchronization in eFSH-treated cycling mares

In an embryo transfer program, donor and recipient mares' ovulations need to be closely aligned, as synchrony is a critical factor affecting pregnancy rates (Carnevale et al., 2000). Therefore, timing of ovulations in eFSH-treated donor mares has practical importance. We found that the extents of ovulation synchrony following a P&E regimen and a PGF2α administration in

eFSH-treated mares were similar to those reported previously for non-superstimulated mares (Loy et al., 1981; Bristol et al., 1983; Blanchard and Varner, 1995). The P&E regimen was more efficient than PGF2α administration for ovulation synchrony among eFSH-treated mares, with ≥80% of ovulations occurring within a 3 day period. However, the highest superovulatory results were obtained when mares were treated with PGF2α as compared to P&E regimen.

When PGF2α was used, all eFSH-treated mares developed at least one preovulatory-sized follicle, approximately 90% ovulated successfully, and about 75% of the mares had ≥2 ovulations; the mean number of ovulations ranged from 2.3 to 2.8 per mare (Chapters 5, 6, and 7). However, when mares were pre-treated with the P&E regimen, results of the subsequent superovulatory treatment were disappointing. Although all mares pre-treated with P&E developed preovulatory-sized follicles after a few days of eFSH treatment, we observed up to 30% of mares that completely failed to ovulate following hCG administration. In addition, a lower proportion of mares had ≥2 ovulations (36% in one study, and 55% in another), and the mean number of ovulations per P&E-eFSH treated mare ranged from 1.6 to 1.9 ovulations (Chapters 6 and 8).

Embryo recoveries following P&E treatment in eFSH-treated mares that indeed ovulated were also disappointing; with averages of 0.9 and 1.0 embryos per recovery attempt (Chapter 6 and 8, respectively). Embryo per ovulation rate was 56% in one study (Chapter 6) and 36% in another (Chapter 8). Similar to our findings, Logan et al. (2007b) reported lower than expected ovulation and embryo recovery rates in eFSH-treated cycling mares pre-treated with the P&E regimen. The rationale for the P&E regimen was to use a treatment to initiate a new wave of follicle growth arising from small young follicles with fresh oocytes. It is possible that pre-treatment with P&E had the opposite effects and altered the quality of follicles that were present in the ovaries at the time the eFSH treatment was initiated, altered the sensitivity of the ovary to eFSH, or decreased the size of the available pool of follicles. In addition a prolonged reduction in gonadotropin levels, which was expected to arise from the P&E treatment regimen, may have led to populations of static or atretic follicles; however, some of these aged follicles may have been "rescued" by the eFSH treatment. We speculate that these could have disturbed follicular growth, ovulation, oocytes transfer, fertilization, or embryonic development.

Therefore, although the P&E estrus synchronization regimen is more efficient than PGF2α administration for synchrony of ovulation among eFSH-treated mares, the poor superovulatory

results eliminates any benefit for its use. Future studies should examine other alternatives for estrus and ovulation synchrony in eFSH-treated mares. In cattle, aspiration of follicles prior to FSH treatment as a means of increasing the superovulatory response has been used successfully (Bergfelt et al., 1994; Bergfelt et al., 1997; Baracaldo et al., 2000; Bo et al., 2002). However, in mares, a similar approach to estrus synchronization combined with superovulation has not been attempted to date.

Table 12.1 Summary of ovarian parameters following eFSH treatment of cycling mares in four different studie

Chapter	Treatment group	n	Follicle criterion for eFSH treatment initiation	Days of eFSH treatment (mean±SEM)	Mares developing preovulatory follicles	Mares ovulating	Mares with ≥2 ovulations	Number of preovulatory follicles* (mean±SEM)	No. of Ovulations* (mean±SEM)	Range. of Ovulations per treated mare
5	eFSH	12	F≥25mm	5.3 ± 0.8	100% (12/12)	83% (10/12)	75% (9/12)	3.8 ± 0.6	2.8 ± 0.5	0 - 6
	Control	12	---	---	100% (12/12)	100% (12/12)	42% (5/12)	1.4 ± 0.1	1.4 ± 0.1	1 - 2
6	PG+eFSH	12	F≥20mm	8.4 ± 1.1	100% (12/12)	92% (11/12)	75% (9/12)	2.6 ± 0.5	2.5 ± 0.4	0 - 5
	P&E+eFSH	11	F≥20mm	7.2 ± 1.5	100% (11/12)	91% (10/11)	36% (4/11)	1.8 ± 0.3	1.6 ± 0.3	0 - 3
7	eFSH	16	F≥20mm	8.9 ± 0.6	100% (16/16)	100% (16/16)	69% (11/16)	2.6 ± 0.4	2.3 ± 0.5	1 - 6
	Control	26	---	---	100% (26/26)	100% (26/26)	12% (3/26)	1.1 ± 0.1	1.1 ± 0.3	1 - 2
8	P&E+eFSH	20	F≥25mm	5.4 ± 0.4	100% (20/20)	70% (14/20)	55% (11/20)	4.1 ± 0.5	2.8 ± 0.5	0 - 8
	P&E+Control	20	---	---	100% (20/20)	100% (20/20)	30% (6/20)	1.3 ± 0.1	1.3 ± 0.1	1 - 2

* Marked values (*) were calculated for ovulating mares only

Table 12.2 Summary of ovarian parameters following eFSH treatment of vernal transitional mares in three different studies. Data shown include ovarian parameters in only hCG-treated cycling mares (Chapter 9), and in vernal transitional mares treated with eFSH (Chapter 9), deslorelin (Chapter 10) or pFSH (Chapter 11).

Chapter	Treatment group	n	Follicle criterion for hormonal treatment initiation	Days of hormonal treatment* (mean±SEM)	Mares developed preovulatory follicles	Mares ovulated	Mares with ≥2 ovulations	Number of preovulatory follicles* (mean±SEM)	No. of Ovulations* (mean±SEM)	Range No. of ovulations per treated mare
9	Cycle-1 eFSH	15	F≥25mm	4.9 ± 0.6	100% (15/15)	100% (15/15)	73% (11/15)	2.2 ± 0.3	2.5 ± 0.4	1 - 7
	Cycle-2	15	---	---	100% (15/15)	100% (15/15)	0% (0/15)	1.0 ± 0.0	1.0 ± 0.0	1 - 2
	Mid-season Cycle	15	---	---	100% (15/15)	100% (15/15)	13% (2/15)	1.1 ± 0.1	1.1 ± 0.1	1 - 2
10	eFSH	18	F≥25mm	4.5 ± 0.4	100% (18/18)	78% (14/18)	72% (13/18)	3.1 ± 0.4	3.4 ± 0.4	0 - 7
	Deslorelin	18	F≥25mm	5.3 ± 0.5	89% (16/18)	78% (14/18)	6% (1/18)	1.1 ± 0.1	1.1 ± 0.1	0 - 2
11	eFSH	11	F≥25mm	4.8 ± 0.6	100% (11/11)	73% (8/11)	64% (7/11)	2.3 ± 0.4	2.6 ± 0.5	0 - 4
	pFSH	21	F≥25mm	6.5 ± 1.8	45% (5/11)	36% (4/11)	9% (1/11)	1.2 ± 0.3	1.2 ± 0.3	0 - 2

* Values were calculated for ovulating mares only

12.4. eFSH treatment in vernal transitional mares

The effects of eFSH in vernal transitional mares were examined in three studies reported herein. In these studies mares maintained under ambient light were monitored from winter anestrus to the vernal transitional phase and eFSH treatment was initiated as soon as the first follicle ≥25mm in size was detected. We aimed to initiate the treatment as early as possible before the breeding season, but still at a time when mares are more likely to respond to exogenous gonadotrpin stimulation. In all of these studies, eFSH treatment stimulated the development of at least one preovulatory follicle in all mares within a few days of treatment (Table 12.2). Following hCG administration, most of these mares successfully ovulated (100%, 78%, and 73%, Chapters 9, 10, and 11, respectively). Results from our studies are in agreement with other studies which reported that eFSH is effective in inducing and advancing the first ovulation of the breeding season (Niswender et al., 2004; Peres et al., 2007). The extent to which eFSH treatment advanced the breeding season as compared to untreated mares was not tested in our studies; however, due to 1) the synchronized follicular growth and the short interval to ovulation among eFSH-treated mares, 2) our clinical data regarding the date of first ovulation in untreated mares in our geographical location, and 3) significantly less successful ovulation results in transitional mares treated with porcine-FSH (Chapter 11), it appears reasonable to assume that eFSH treatment in early vernal transitional mares will advance the first ovulation of the breeding season by 3 to 6 weeks.

The eFSH treatment in vernal transitional mares resulted in a higher number of preovulatory-sized follicles and a greater number of ovulations, as compared to vernal transitional mares treated with deslorelin (Chapter 10) or pFSH (Chapter 11), or as compared to non-superstimulated cycling, control mares (Chapter 9). Following eFSH treatment in the vernal transition, multiple ovulations (≥2) were detected in most ovulating mares (73%, 93% and 64%; Chapter 9, 10, and 11, respectively), and the mean number of ovulations ranged from 2.5 to 3.4. Corpora lutea that developed following these ovulations appeared ultrasonographically normal, and resulted in high progesterone concentrations in diestrus, supporting early embryonic development at least until embryo recovery attempts were performed (Chapters 9 and 10), or until Day 20 of pregnancy in mares allowed to carry their own pregnancy (Chapter 11).

In vernal transitional mares intended to carry their own pregnancy, treatment with eFSH was significantly more efficacious as compared to treatment with pFSH, and resulted in pregnancies that were significantly earlier in the breeding season. It appeared that maternal recognition of these pregnancies occurred as we did not detect any signs of luteolysis in any of the pregnant mares, at least until Day 20 post-ovulation. However, the eFSH treatment appeared to increase the risk for multiple embryos in a pregnancy. Therefore, pregnancy diagnosis and appropriate management of twins should be performed prior to Day 16 of gestation if eFSH treatment is used in vernal transitional mares intended to carry their own pregnancy.

Embryo recovery rate in vernal transitional donor mares treated with eFSH was similar or greater than that expected from single ovulating cycling mares, or that of vernal transitional mares treated with deslorelin. However, we observed that a proportion of these embryos had a low morphology grade. Following eFSH treatment in the vernal transition, the first inter-ovulatory interval of the breeding season was prolonged (>21 d) in about half of the mares, yet, subsequent intervals in the breeding season were not affected; there appeared to be no signs of any abnormalities in the subsequent estrous cycles of the breeding season in terms estrous cycle length, the number of ovulations, or embryo recovery.

12.5. Variability in the superovulatory response in eFSH-treated mares

In all studies reported here, eFSH treatment increased the number of preovulatiory follicles (≥30 mm in diameter) and the number of ovulations, which is in agreement with previous reports utilizing crude or purified equine pituitary extract gonadotropin stimulation (Hofferer et al., 1993; Alvarenga et al., 2001; Niswender et al., 2003; Logan et al., 2007b; McCue et al., 2008). However, it is clear that there is significant variation in the response to eFSH among mares in the same study (0 to more than 8 ovulations, Chapter 8), among different reports (means ranged from 1.4 to 5.5 ovulations) (Niswender et al., 2003; Niswender et al., 2004; Raz et al., 2005; Raz et al., 2006b; Welch et al., 2006; Logan et al., 2007b; Peres et al., 2007; McCue et al., 2008; Raz et al., 2009a; Raz and Card, 2009; Raz et al., 2009b; Raz et al., 2009c; Raz et al., 2009d), and even within subsequent estrous cycles in the same mare (Squires et al., 2006).

Squires et al. (2006) were the first to note considerable variation in ovulation and embryo recovery rates among mares treated with eFSH. Furthermore, variations in ovulation and embryo recovery rates following superstimulation treatments have been observed in cows, goats, and ewes (Mapletoft et al., 2002; Cognie et al., 2003). Interestingly, in bovine embryo transfer programs, approximately 70% of embryos are collected from 30% of the superstimulated cows and approximately 25% of cows produce no transferable embryos (Mapletoft et al., 2002), not very different than eFSH-treated mares.

Variability in ovarian responsiveness in mares may be related to differences in superovulatory treatments, such as gonadotropin preparation; batch and total dose; duration and timing of treatment; and the use of additional hormones in the superovulatory regimen (e.g. for estrus synchronization or ovulation induction as discussed above). However, as shown in our studies, results may vary significantly even when the same standard eFSH treatment protocol is used. Therefore, additional factors inherent to the animal and its environment may play a crucial role in the observed variability. These factors may include nutritional status, reproductive history, age, season, genetics, breed, and ovarian status at the time of treatment. It is our speculation that eFSH treatment is more cost-effective in vernal transitional mares as compared to cycling mares. This is because the currently available alternative management options (e.g. no treatment; GnRH treatment regimen; pFSH treatment regimen) are not as effective. Furthermore, the highest number of ovulations (3.4 ± 0.4) and highest number of embryos (2.6 ± 0.5) were obtained in the study in which eFSH treatment was administered to vernal transitional mares (Chapter 10). In another study (Chapter 9) embryo recovery in vernal transitional mares treated with eFSH was only 0.8 ± 0.2 embryos per mare; however, mares in that study were of higher body weights and the eFSH dose was not adjusted accordingly. Another interesting finding is that although we observed mares that completely failed to ovulate following eFSH treatment in both vernal transitional and cycling mares, we observed non-ovulatory follicles accompanying normal ovulations mostly in cycling mares. In vernal transitional mares the response to hCG administration was uniform in the vast majority of preovulatory follicles within a mare; either all of them ovulated (most mares), or all of them failed to ovulate. Non-ovulatory follicles in eFSH-treated mares will be discussed in the next section. Future studies should focus on the factors affecting variability and success rate of superovulatory treatments in mares.

12.6. Non-ovulatory follicles in eFSH-treated mares

In studies reported here, all mares (cycling and in vernal transition) treated with eFSH developed preovulatory follicles following a few days of eFSH treatment. However, we observed that some mares did not ovulate (0 ovulations) following hCG administration, and some mares experienced non-ovulatory follicles accompanying normal appearing ovulations. Some of those follicles continued to grow and some became static after the eFSH treatment ceased, and later regressed or appeared luteinized. As mentioned before, the response to hCG administration in vernal transitional mares was uniform in the vast majority of preovulatory follicles within a mare (either all ovulated or all failed to ovulate), whereas non-ovulatory large follicles accompanying normal appearing ovulations were observed in some cycling mares. Hence, the number of preovulatory follicles following eFSH treatment of cycling mares was consistently higher than the subsequent number of ovulations. Similar trends have been reported in other studies which utilized eFSH treatment in cycling mares (Niswender et al., 2003; Logan et al., 2007b; McCue et al., 2008). Among vernal transitional mares that ovulated following the eFSH-hCG treatment regimen, the mean number of ovulations was similar or even slightly higher than the mean number of preovulatory follicles recorded at the time of hCG administration.

In studies reported here, the incidences of ovulation failure and non-ovulatory follicles were higher in eFSH-treated mares as compared to controls. It appeared that up to approximately 40% of the preovulatory follicles that develop in eFSH treated mares may not ovulate. Furthermore, our results suggest that about 10 to 20% of cycling mares can be expected to completely fail to ovulate following an eFSH treatment regimen; the incidence of ovulation failure may be as high as 30% if a P&E estrus synchronization regimen is used prior to the superovulatory treatment. The incidences of vernal transitional mares that failed to ovulate (0 ovulations) following the eFSH treatment regimen were 0% (Chapter 9), 22% (Chapter 10) and 27% (Chapter 11). The previously reported incidence of ovulation failure in non-eFSH-treated mares during the physiologic breeding season has ranged from 3.1% (Hughes et al., 1972) to 8.2% (McCue and Squires, 2002), and has been attributed to insufficient pituitary gonadotropin stimulation, insufficient estrogen production from the follicle itself or hemorrhage into the lumen of the preovulatory follicle (Ginther, 1992a). In eFSH treated mares it is clear that both FSH stimulation and estrogen production are very high. Still, it is possible that some of the

mechanisms involved in ovulation failure in non-stimulated mares are also involved in the development of non-ovulatory follicles in eFSH-treated mares. However, the exact cause of the significantly lower ovulation rate per follicle ≥30 mm in eFSH-treated mares has not been elucidated.

Non-ovulatory follicles following superovulatory treatments have been reported in mares (Alvarenga et al., 2001; Scoggin et al., 2002; Briant et al., 2004) and in other species (Armstrong et al., 1983; Boland et al., 1991; Laurincik et al., 1993; Aboulghar and Mansour, 2003; Boyle et al., 2004; Chao et al., 2005; Colazo et al., 2005; Barati et al., 2006; Lee et al., 2006; Veiga-Lopez et al., 2006; Bartlewski et al., 2008), and are considered to be a result of ovarian hyperstimulation. Ovulation failure has been suggested to be related to the high gonadotropin dose, FSH to LH ratio in the hormone preparation, alteration in endogenous hormones concentrations, down-regulation of LH receptors on theca and/or granulosa cells, increased vascular permeability of the ovarian tissue, as well as individual sensitivity to hormonal stimulation among treated animals. In mares (Hofferer et al., 1992; 1993; Rosas et al., 1998) and in other species (Murphy et al., 1984; Wollen et al., 1985; Veiga-Lopez et al., 2006), source and purity of the gonadotropin preparation was identified as a causal factor for ovulation failure, with the presence of LH in the FSH preparation being the major cause. However, even the use of purified FSH in mares and other species may not avoid the presence of non-ovulatory follicles completely. In mares, ovulation failure following eFSH treatment could also be related to the unique structure of the ovary, in which follicles develop in the interior cortex of the ovary and ovulate only through the limited area of the ovulation fossa; this anatomical barrier may restrict the number of ovulations and may be disturbed in superstimulated mares (Carmo et al., 2006; Alvarenga et al., 2008).

Detection of serum E2 concentrations in eFSH-treated mares in our studies allowed us to conclude that these mares experienced significantly higher and prolonged periovulatory E2 levels. Our results suggest that this alteration in E2 concentrations does occur in most mares treated with eFSH, but there might be some variation related to the estrus synchronization method employed (PGF2α or P&E regime), and to seasonal status (cycling vs. vernal transitional). In single-ovulating mares, E2 concentrations increase as follicle diameter increases, but there is a marked decrease in levels before ovulation (Ginther, 1992b; Ginther et al., 2007c).

Similarly, in non-superstimulated mares the concentration of plasma E2 was reported to decrease immediately following hCG treatment at a follicle size of approximately 35 mm (Gastal et al., 2006). Higher and prolonged E2 concentrations before and around the time of ovulation have been associated with persistent follicles and reduced fertility in the mare (Ginther et al., 2007a) and in other species (Moor et al., 1985; Breuel et al., 1993; Wehrman et al., 1993; Ahmad et al., 1995; Veiga-Lopez et al., 2006). In our studies, eFSH-treated mares that failed to ovulate tended to have a higher number of preovulatory follicles and higher E2 concentration on the day of hCG administration as compared to mares that ovulated. It is therefore likely that higher and prolonged periovulatory E2 levels in the eFSH-treated mares may have disturbed normal follicular maturation and ovulation. The cause of the differences in the number of ovulation failures between cycling mares and vernal transitional mares was not detected in our studies. Hence, it is clear that ovulation failure in superstimulated mares requires further study.

12.7. Embryo production in eFSH-treated donor mares

Embryo production parameters following eFSH treatment in cycling and in vernal transitional donor mares are summarized in Tables 12.3. and 12.4. In the studies reported here, treatment with eFSH increased donor embryo recovery yield. At least one embryo was obtained in 71.1% (32/45), and in 79.3% (23/29) of the recovery attempts performed in eFSH-treated cycling and vernal transitional donor mares, respectively. In all studies combined, 74% (55/74) of embryo recovery attempts following eFSH treatment were successful, whereas in unstimulated cycling mares (Chapters 5, 6, and 8, and 9) embryo recovery was successful in only 56% (35/62) of the attempts ($P < 0.05$; Chi-squire analysis), as was expected in single-ovulating mares. This is in agreement with other studies which utilized eFSH or EPE in order to increase the probability of recovering one or more embryos from donor mares. Still, there are great variations in the mean number of embryos obtained among different studies, and within mares in the same study following superovulatory treatment. In our studies, eFSH treatment resulted in mean embryo numbers that ranged from 0.9 to 2.6 embryos per recovery attempt; embryo recovery in mares within the same study ranged from 0 to 7 embryos per attempt. The variation in embryo yield among different studies are likely related to the variation in superovulatory response and the number of ovulations, estrus synchronization method employed prior to eFSH treatment initiation, as well as variation in embryo per ovulation rates.

Table 12.3 Summary of embryo production parameters in donor cycling and vernal transitional mares treated with eFSH in five different studies.

Chapter	Seasonal status of mares at treatment initiation	Treatment group	Successful embryo recovery attempts (≥1 embryo)	No. of embryos (mean±SEM)	Range No. of embryos per mare	Embryo grade (mean±SEM)	Embryo per ovulation rate
5	Cycling	eFSH	90% (9/10)	1.5 ± 0.3	0 - 3	2.7 ± 0.2	54% (15/28)
	Cycling	Control	42% (5/12)	0.5 ± 0.2	0 - 2	1.8 ± 0.3	38% (6/16)
6	Cycling	PG+eFSH	8/11 (73%)	1.4 0.3	0 - 3	2.6 ± 0.2	50% (15/30)
	Cycling	P&E+eFSH	7/10 (70%)	0.9 ± 0.3	0 - 3	2.4 ± 0.3	56% (9/16)
8	Cycling	P&E+eFSH	57% (8/14)	1.0 ± 0.4	0 - 6	2.4 ± 0.2	36% (14/39)
	Cycling	P&E+control	80% (16/20)	0.95 ± 0.1	0 - 2	1.8 ± 0.1	80% (16/20)
9	Transition	Cycle-1 eFSH	67% (10/15)	0.8 ± 0.2	0 - 3	2.7 ± 0.3	32.4% (12/37)
	Cycling	Cycle-2	47% (7/15)	0.5 ± 0.1	0 - 1	1.5 ± 0.2	47% (7/15)
	Cycling	Mid-season Cycle	47% (7/15)	0.5 ± 0.1	0 - 1	1.7 ± 0.2	44% (7/16)
10	Transition	eFSH	93% (13/14)	2.6 ± 0.5	0 - 7	2.3 ± 0.2	77% (37/48)
	Transition	Deslorelin	36% (5/14)	0.4 ± 0.2	0 - 2	2.0 ± 0.6	38% (6/16)

In most of our studies, embryo per ovulation rate was similar to what is expected in single-ovulating mares, but there was a wide range from 31% to 77%. Inconsistent and lower than expected embryo per ovulation rates in superstimulated cycling mares have been reported previously and have been attributed to a high ovarian response (Alvarenga et al., 2001; Niswender et al., 2003; Logan et al., 2007b). Our data demonstrate that there is also variation among vernal transitional mares treated with eFSH. The lower embryo per ovulation rate in some of our studies could have resulted from the eFSH-related hyperstimulatory effects, possible alterations in oocyte maturation and transport, as well as possible alterations in reproductive tract parameters (tone and edema) mediated by the modified steroidogenic hormonal environment (Carmo et al., 2006; Alvarenga et al., 2008; Raz et al., 2009c). Furthermore, number of ovulations and embryo recovery per ovulation in studies that utilized superovulation treatment varied significantly, possibly due to a failure of fertilization or due to high embryonic loss in the uterine tube or uterus (Douglas et al., 1974; Woods and Ginther, 1982; Palmer et al., 1993; Alvarenga et al., 2001; Carmo et al., 2006; Squires and McCue, 2007). Other potential explanations include the quality of oocytes within follicles present at the time eFSH treatment was initiated, alteration in the sensitivity of the ovary to eFSH related to seasonal status, or pre-treatment effects from the estrus synchronization regimen.

A study conducted by Carmo et al. (2006) indicated that disruption of the transport of oocytes to the oviduct might be involved in the reduced embryo recovery rates per ovulation seen in superovulated mares. In that study, mares treated with EPE were slaughtered 12 to 24 hours after ovulation; the ovaries and oviducts were examined and oocytes were collected. The EPE treatment increased the number of recovered oocytes; however, the percentages of morphologically-normal oocytes recovered per ovulation tended to be lower ($P = 0.07$) than in control mares. In addition, the ovulatory fossa of most of the superovulated mares had an excessive accumulation of coagulated blood, which was not observed in the fossa of control mares. It is likely that this excessive haemorrhage would alter the macro- and micro- enviroment, and may disturb oocyte entrance to the oviduct, oocyte maturation, and early embryonic development.

In the studies reported here, mean embryo morphology grade of mares treated with eFSH was either not statistically different or was lower than that obtained in controls. Numerical mean

embryo morphology grade of embryos obtained from eFSH-treated mares was consistently lower than that of controls. Overall, when data from all studies are combined, 67% (68/102) of the embryos recovered from eFSH-treated donor mares were of good or excellent quality (grades 1 or 2), whereas 89% (35/39) of the embryos from non-stimulated mares were of good or excellent quality ($P < 0.05$; Chi-squire analysis). In cycling mares treated with eFSH, 64% (34/53) of the total embryos recovered were of good or excellent quality; and in vernal transitional mares treated with eFSH, 69.3% (34/49) of the total embryos recovered were of good or excellent quality.

There are conflicting previous results regarding the effect of superovulation treatment on equine embryo viability, with some studies suggesting that embryos collected from superovulated mares were less viable than those from single-ovulating mares (Woods and Ginther, 1982; 1983b; Woods, 1984). The variation among studies may be related, at least in part, to the variation in the FSH:LH ratio among gonadotropin preparations when EPE was utilized, and perhaps differences between batches of eFSH. The equine embryonic morphology grading system is modified from a similar bovine embryo morphology grading system. However, unlike the bovine embryo, the equine embryo develops an embryonic capsule. During our evaluations of the embryos recovered from eFSH-treated donor mares, a number of misshapen, wrinkled, or detached embryonic capsules were noted. It is possibile that the importance of the morphologic appearance of the equine embryonic capsule may have either been underestimated or not evaluated in other studies. Other influential factors may include the operators' experience and embryo evaluation skills, treatment protocols, breeding management, environmental factors, and the genetic makeup and inherent fertility of mares and stallions.

In two studies reported here (Chapters 5 and 6), a total of 39 embryos recovered from eFSH-treated donor mares were transferred to recipients; only 13 pregnancies (33%) resulted from these transfers. All pregnancies resulted from the transfer of grade 1 or 2 embryos. Embryo morphology assessment is considered a valuable tool to evaluate the likelihood of post-transfer pregnancy in the recipient (McKinnon and Squires, 1988b; a; Squires et al., 1999), and the incidence of subsequent pregnancy loss (Carnevale et al., 2000). It was reported that embryos graded 3 or 4 (fair to poor) are less likely to result in pregnancy than grade 1 or 2 (good to excellent) embryos (Squires et al., 1999; Carnevale et al., 2000). Therefore, indentifying the

factors that contribute to lower than expected embryo morphology grade following superovulatory treatment is of great importance.

The meeting between the maternal and paternal gametes is dependent upon a number of complicated processes. Furthermore, major demands are placed on oocytes and embryos with regards to developmental transitions, i.e., oocyte maturation, fertilization, compaction, and blastocyst formation; all of which need to be traversed successfully in a supportive reproductive tract environment, which is under the control of ovarian steroid hormones (Ginther, 1986; 1992a). Hence, in our studies, we evaluated uterine and cervical parameters, along with serum concentrations of E2 and P4 on selective days, as indicators for reproductive tract function in eFSH-treated mares. We found that administration of eFSH modified the reproductive tract variables and serum concentrations of P4 and E2 on the days that oocyte maturation, fertilization, and early embryonic development are expected to occur. As was anticipated, the changes in uterine and cervical parameters paralleled the serum concentrations of E2 and P4.

Before and around the time of ovulation, serum E2 concentrations in the eFSH-treated mares were significantly higher, and on those days we recorded softer uterine and cervical tone, and increased endometrial edema as compared to controls. In diestrus, mares treated with eFSH had significantly higher uterine tone and higher serum P4 concentrations. Prolonged and/or greater preovulatory secretion of E2 also occurs in superovulating animals of other species (Moor et al., 1985; Kelley et al., 2006), and was associated with reduced cleavage rates, higher early embryonic losses, abnormal embryonic development, and a decreased conception rate (Lewinthal et al., 1987; Breuel et al., 1993; Johnson and Lewis, 1993; Wehrman et al., 1993; Ahmad et al., 1995). This has been attributed to the negative effect on oocyte maturation and alterations in the uterine environment (Roberson and Baker, 1969; McGaughey, 1977; Butcher and Pope, 1979; Moor et al., 1985; Ahmad et al., 1995). In other species, as was shown in our studies in mares, the presence of non-ovulatory follicles at the time of ovulation, fertilization and early embryonic development was associated with high E2 concentration, reduced fertility and reduced final superovulatory yields (Armstrong et al., 1983; Moor et al., 1985; Callesen et al., 1986; Breuel et al., 1993; Wehrman et al., 1993; Ahmad et al., 1995; Veiga-Lopez et al., 2006; Ginther et al., 2007a; Bartlewski et al., 2008). In ruminants, the administration of exogenous estradiol around estrus, or immediately following ovulation, causes an impaired transport of sperm (Lightfoot et

al., 1967; Hawk and Conley, 1975; Hyttel et al., 1988) and oocytes (Hafez et al., 1963; Holst and Braden, 1972; Crisman et al., 1980). Prolonged high estradiol levels have also been associated with decreased fertility related to abnormal embryonic development and transport (Wehrman et al., 1993) and with a significant decrease in oocyte or embryo recovery (Breuel et al., 1993; Misra et al., 1998). Data presented here clarify some factors affecting the success rates of superovulatory treatment in mares. These factors should be considered in the cost-benefit analysis when eFSH treatment is considered.

12.8. eFSH treatment in mares intended to carry their own pregnancy

Superovulatory treatment has been suggested to increase the per-estrous cycle pregnancy rate in normal or subfertile mares, as more oocytes would be available for fertilization and more embryos would initially develop in the uterus. We conducted two studies in which the eFSH regimen was employed in mares not intended for embryo transfer; in one study (Chapter 7) we used cycling mares, and in the other study (Chapter 11) we used vernal transitional mares (Table 12.4). In the first study, cycling mares were treated with eFSH and bred, and pregnancy status was recorded by trans-rectal ultrasoundography on Days 11 to 16. The eFSH treatment stimulated the ovary, and increased the number of preovulatory-sized follicles and number of ovulations; however, the probability of establishing a pregnancy was not increased. Overall mean embryo numbers were not different between mares treated with eFSH and controls, and embryo per ovulation rate was less in the eFSH group (31%) compared to controls (59%). However, we found a tendency toward a higher incidence of multiple embryos in a pregnancy following eFSH treatment. This study was not designed to detect significant differences in the mean daily embryonic growth rate on Days 11 to 16; however, a significantly greater variability in growth rate of embryos in mares treated with eFSH may indicate that some of these embryos were compromised. In mares with spontaneous double ovulations, embryo reduction before Day 11 is unlikely; however, in cycling mares where EPE is used as a superovulatory treatment, embryo reduction or retarded growth of some conceptuses before Day 11 has been reported (Woods and Ginther, 1983b; Woods, 1984; Squires et al., 1987a; Ginther and Bergfelt, 1988). Our data provide important information on alterations caused by eFSH treatment which may explain differences between viability of embryos produced by mares stimulated to superovulate as compared to untreated mares.

Table 12.4 Pregnancy rates and number of embryos in eFSH-treated cycling and vernal transitional mares intended to carry their own pregnancy. Data are presented as compared to control cycling mares (chapter 7) or to vernal transitional mares treated with pFSH (Chapter 11).

Chapter	Seasonal status of mares at treatment initiation	Treatment group	n	Mares ovulated	Pregnancy rate per treated mare	Pregnancy rate per ovulating mare	No. of embryos per ovulating mare (mean±SEM)	No. of embryos per pregnancy (mean±SEM)	Overall embryo per ovulation rate
7	Cycling	eFSH	16	100% (16/16)	50% (8/16)	50% (8/16)	0.7 ± 0.2	1.4 ± 0.2	31% (11/36)
	Cycling	Control	26	100% (26/26)	62% (16/26)	62% (16/26)	0.7 ± 0.1	1.1 ± 0.2	59% (17/29)
11	Transition	eFSH	11	73% (8/11)	55% (6/11)	75% (6/8)	1.4 ± 0.4	1.8 ± 0.3	52% (11/21)
	Transition	pFSH	11	36% (4/11)	27% (3/11)	75% (3/4)	0.8 ± 0.3	1.0 ± 0.0	60% (3/5)

In our other study, vernal transitional mares were treated with either twice daily eFSH or pFSH; estrous mares were inseminated with fresh semen, and examined for pregnancy on Days 11-20 post ovulation. Treatment with eFSH resulted in a greater ovarian stimulation; higher number of preovulatory-sized follicles, higher number of ovulations, and higher number of embryos. Following ovulation, serum progesterone concentrations were correlated with the number of CLs and supported early embryonic development; maternal recognition of pregnancy occurred in all pregnant mares. Embryo per ovulation rate (52%) was as expected in single ovulating mares. We concluded that eFSH can be used to effectively induce follicular growth and ovulation in vernal transitional mares; however, similar to cycling mares, diagnosis and management of twin pregnancies in bred mares would be required prior to Day 16 due to the increased risk of multiple embryos per pregnancy. Twin pregnancies detected prior to Day 16 of gestation, when the embryos are mobile, are most effectively managed by manually crushing one embryonic vesicle, with expected survival rate for the remaining twin exceeding 90% (Bowman, 1986; Pascoe et al., 1987; Macpherson and Reimer, 2000). Mares in our studies were allowed to carry their pregnancy only for short time, and manual reduction of embryos in twin pregnancies was not performed. Future studies should evaluate pregnancy loss rate in eFSH-treated mares later in the pregnancy, and when those mares undergo embryo reduction procedures.

Data presented here elucidate several factors affecting the success rate of superovulatory treatment in mares treated with eFSH; these factors should be considered in the development of superovulation protocols in future studies in order to improve the number of ovulations and embryo yields, and to minimize possible negative effects on follicular development, ovulation, and embryo viability.

13. SUMMARY AND CONCLUSIONS

1. The administration of eFSH (12.5 mg twice daily), initiated according to follicle diameter (≥20 mm or ≥25 mm), stimulated the ovaries of cycling and vernal transitional mares. This resulted in the development of multiple preovulatory-sized follicles, and increased the number of ovulations.

2. Treatment with eFSH enhanced embryo recovery rate in most mares.

3. The superovulatory response to eFSH varied significantly among mares (cycling and vernal transitional) in terms of number of ovulations, number of embryos, and embryo per ovulation rates.

4. The extent of ovulation synchrony among eFSH-treated cycling mares following synchronization of follicle development with PGF2α administration or the P&E estrus synchronization regimen were similar to those reported for non-superstimulated mares. Hence, the P&E estrus synchronization regimen was more efficient than PGF2α administration in synchronizing ovulations among eFSH-treated mares, with ≥80% of ovulations occurring within a 3 day period.

5. The estrus synchronization regimen employed prior to initiation of eFSH treatment played an important role in the superovulatory outcomes (proportion of mares ovulating, number of ovulations and embryo recovery) in cycling mares; administration of PGF2α provided a better alternative than the P&E regimen.

6. Treatment with eFSH did not increase pregnancy rate per estrous-cycle in normal cycling mares.

7. In vernal transitional mares, eFSH treatment stimulated the development of at least one preovulatory follicle within only a few days of treatment. Most of these mares ovulated following hCG administration, and breeding resulted in pregnancy or successful embryo recovery in some mares.

8. Cycling and vernal transitional mares which conceived following eFSH treatment regimen showed a tendency toward (a higher incidence of) having multiple embryos per pregnancy.

9. eFSH (12.5 mg twice daily) and deslorelin (63 μg twice daily) treatment regimens were equally effective in inducing ovulation in early vernal transitional mares within a predictable interval from treatment; however, the eFSH regimen was more effective in increasing the number of ovulations and the number of embryos recovered per mare.

10. As compared to treatment with pFSH, treatment with eFSH was significantly more efficacious, and resulted in significantly more ovarian stimulation (number of preovulatory follicles and ovulations) in early vernal transitional mares. The first ovulation of mares treated with eFSH occured significantly earlier in the season, and most mares had multiple ovulations.

11. Following ovulations induced by an eFSH treatment regimen in early vernal transitional mares, the first inter-ovulatory interval of the breeding season was prolonged (>21 d) in about half of the mares; yet, subsequent inter-ovulatory intervals in the breeding season were not affected. Furthermore, there were no negative effects of eFSH in terms of ovulation or embryo recovery in the subsequent estrous cycles of the breeding season.

12. Corpora lutea that developed following eFSH treatment regimen in cycling and vernal transitional mares appeared ultrasonographically normal, and secreted high levels of progesterone during diestrus.

13. It appeared that maternal recognition of pregnancy occured in mares that conceived following eFSH treatment in the vernal transitional phase, as there were no signs of luteolysis in the pregnant mares until at least Day 20 post-ovulation. In non-pregnant mares, luteolysis occured as expected at approximately Day 16 post-ovulation.

14. Administration of eFSH modified serum concentrations of progesterone and estradiol-17β, and reproductive tract variables (palpable tone and edema score detected by ultrasonography) on the days that oocyte maturation and fertilization, and early embryonic development are expected to occur.

15. The incidence of ovulation failure (0 ovulations per mare) and non-ovulatory follicles were significantly higher in mares treated with eFSH as compared to controls. This was associated with a high number of preovulatory follicles and higher E2 concentrations in estrus before expected ovulation. The response to hCG administration following eFSH treatment in vernal transitional mares was uniform in the vast majority of preovulatory follicles within a mare (either all ovulated or all failed to ovulate), whereas non-ovulatory large follicles accompanying normal appearing ovulations were observed in some cycling mares. The incidence of ovulation failure appeared to be the highest in cycling mares which were pre-treated with P&E regimen.

16. A higher proportion of embryos recovered from eFSH-treated donor mares had lower morphology grades (grade 3 or 4 out of 4); this was associated with lower post-transfer pregnancy rates as compared to embryos recovered from non-superstimulated mares.

14. SUGGESTIONS FOR FUTURE RESEARCH

The studies reported in this thesis elucidate several aspects affecting the success rates of eFSH treatment in mares, and offer background for the formulation of new hypotheses and the design of future studies for superovulation.

Future research will have to focus on the development of alternative approaches for superovulation in mares, other than the use of equine pituitary extract products, like EPE or eFSH. The use of such animal products is problematic particularly because of biosecurity risks and the limited availability of horse pituitaries. Horse slaughter has been banned recently in most states in the USA; at present there is no active slaughterhouse in the USA that can supply horse pituitaries. In addition, Bioniche Animal Health has recently stopped producing eFSH, and the already-made lots are being distributed by BetPharm, Kentucky. Therefore, it is quite clear that such animal products will not be available in the near future. Future approaches to superovulation in mares should include the development of commercial recombinant equine FSH (reFSH) and the development of approaches other than gonadotropin or gonadotropin-like stimulation.

Single-chain recombinant equine FSH (reFSH) and recombinant LH (reLH) has recently been developed (Aspen Bio Pharma, Castle Rock, CO, USA). The reFSH was reported to increase the number of preovulatory sized follicles and ovulations (Niswender et al., 2007). However, the product is not commercially available, and probably will not be available at least until 2010 (Dr. Cory Niswender, personal communication). The information on the effects of reFSH is very limited, and further studies are needed to determine if and how it may be used to routinely enhance reproductive efficiency in mares. Using a reFSH or other bioengineered forms of FSH products to stimulate follicular development may offer the advantage of using an FSH product without the protein contaminants or LH activity of an equine pituitary extract. It may allow more accurate and repeatable control of stimulation using different protocols, alone and in combination with other hormones such as hCG, GnRH analogues, and reLH.

Passive (Nambo et al., 1998) and active (McCue et al., 1992a; McKinnon et al., 1992; Terhaar et al., 1997) immunization of mares against inhibin has been shown to induce multiple ovulations; this effect was attributed to the increase in FSH concentrations that followed the

reduction in circulating inhibin. However, the use of active immunization of mares against inhibin required multiple inoculations over several weeks; and adverse reactions, ranging from mild tissue swelling to abscessation occasionally occurred at the immunization site (Squires, 2006b). With the development of more effective and safer immunization techniques, the potential benefit of immunization against inhibin in mares should be further explored.

Upcoming research should examine methods to increase free IGF-I in several follicles simultaneously, which potentially may result in successful superovulation. Recent studies have shown that the IGF system has a key role in the initiation of the selection mechanism in mares, with free IGF-I elevated in the future dominant follicle. Free IGF-I stimulates granulosa cell proliferation and synergizes with gonadotropins to promote differentiation of follicle cells (Spicer and Echternkamp, 1995). *In vitro* effects of IGF-I in cattle include increased proliferation of granulosa cells and estradiol production (Glister et al., 2001), enhanced sensitivity of granulosa cells to FSH (Monget and Monniaux, 1995; Spicer and Echternkamp, 1995), increased secretion of inhibin-A, activin-A and follistatin from granulosa cells (Glister et al., 2001), and enhanced LH stimulation of androgen synthesis from theca cells (Stewart et al., 1995). A method to increase free IGF-I activity in multiple follicles may initially be by intra-follicular injections using trans-vaginal ultrasound-guided approaches (Ginther et al., 2004b); this can be used for intra-follicular treatment with minute quantities of free IGF-I, IGF-I analogues, or IGF-binding-protein proteases. If this approach proves to be successful with the result of multiple ovulations, other, less invasive alternatives should be explored in order to increase free IGF-I activity in multiple follicles of the wave by developing molecular approaches to activate IGF-I-related genes that are specific for the ovary.

Future studies should examine the role of other candidate factors that may be used to stimulate superovulation; such candidates could be activin and vascular endothelial growth factor. Activin induces granulosa cell proliferation; increases FSH receptor expression, granulosa cell steroidogenesis, basal and gonadotropin-stimulated aromatase activity and estradiol production; and also delays the onset of luteinization and atresia (Knight and Glister, 2001). Vascular endothelial growth factor (VEGF) stimulates mitosis of endothelial cells and increases vascular permeability and angiogenesis (Reynolds and Redmer, 1998; Tamanini and De Ambrogi, 2004). In the follicles of cattle and pigs, an increase in VEGF is correlated with an

increase in the follicle diameter (Barboni et al., 2000; Berisha et al., 2000). In horses, follicular fluid VEGF concentrations were reported to be higher in the largest follicle than in the second largest follicle on the day subsequent to the beginning of diameter deviation (Ginther et al., 2004a; Ginther et al., 2004d); however, the earlier temporal relationships before deviation have not been studied in horses. Increased vascularity would benefit the follicles in receiving preferential supply of growth factors, gonadotropins, steroid precursors and other nutrients required for continued development and ovulation. However, such an approach for superovulation may carry risk for negative effects, such as persistent anovulatory or hemorrhagic follicles; therefore, controlled methods to increase follicular vascularity should be studied with attention to such effects.

Information on other factors in the follicular fluid, hypothalamus and pituitary cells is still limited. An improved understanding of how these various local factors, steroids and gonadotropin secretions are integrated into a coordinated response in terms of follicular development to dominant status will improve our ability to control follicular growth and ovulation. Some of these factors have already been indentified, mostly in other species; these include the following: follistatin (Sugawara et al., 1999), fibroblast growth factor (Berisha et al., 2004; Buratini et al., 2005), epidermal growth factor (Driancourt and Thuel, 1998), keratinocyte growth factor (Osuga et al., 2001), gonadotropin surge-attenuating factor (Fowler and Spears, 2004), the transforming growth factor-β superfamily (Bilezikjian et al., 2006; Knight and Glister, 2006), and emerging new factors such as kisspeptins (Roa et al., 2008). Understanding the genetic regulations as well as the roles of these factors in the function of the hypothalamus-pituitary-ovarian axis would increase our knowledge on follicular development and ovulation and could potentially lead to the development of successful estrus synchronization and superovulation regimens in horses and in other species.

The objective of ovarian superstimulatory treatment is to obtain the maximum number of viable embryos by stimulating growth and subsequent ovulation of competent antral follicles. Therefore, whatever approach to superovulation in mares is used, it is clear that studies should not focus solely on the number of ovulations obtained following treatment. The studies presented here emphasize that the development of superovulatory treatment should improve the number of ovulations and embryo yield, but at the same time minimize possible negative effects on

follicular development, ovulations, and embryo viability. Future studies should be performed on larger sample size as the variability in the ovarian superovulatory response among treated mares is high. It is crucial that studies include the transfer of embryos recovered from superstimulated donor mares to recipient mares in order to evaluate their viability. Although this will add significant cost to studies, it is extremely important for the development of effective and reliable superovulatory treatment protocols. Other more objective and consistent methods than simple morphological assessment, should also be employed to evaluate embryo viability (e.g. electron microscopy, development in culture, metabolism of fluorescent substrates, molecular markers for apoptotic cells, and metabolic tests) (Vanderwall, 1996).

The data presented here show that superovulatory treatment with eFSH does not increase pregnancy rate in fertile mares intended to carry their own pregnancy, but increases the likelihood of multiple embryos per pregnancy. Future studies should examine the potential benefit of superovulatory treatment in subfertile mares. Such studies would be of further practical value if mares would be allowed to carry their pregnancy for longer time, optimally to term, and if manual reduction of embryos in twin pregnancies would be performed at an early stage (prior to Day 16 of gestation). Furthermore, specific superovulatory treatment protocols should be developed in order to increase pregnancy rate in mares intended to carry their own pregnancy, as other protocols used to increase donor embryo production might not be suitable.

Follicular fluid and oocyte analyses in superstimulated mares would further increase our knowledge of the possible effects of superovulatory treatment at the follicle level. These would be of significant value for the understanding and optimization of follicular and oocyte maturation and ovulation following such treatments. Furthermore, it would allow close inspection of the follicle and oocyte sub-populations in superstimulated mares, and perhaps would elucidate factors contributing to the variability in ovarian response and follicle and oocyte quality (e.g. breed, genetic, nutritional status, reproductive history, age, season, ovarian status at the time of treatment, etc.). Information obtained from such studies may have significant influence in optimizing donor selection, treatment protocols, and breeding management in superstimulated animals.

There is a need to further examine the alterations in the hormonal environment in superstimulated mares; specifically, the negative effects of such alterations should be studied and minimized. In the studies reported here hormonal analyses of E2 and P4 were performed only for specific days. It was demonstrated that serum E2 concentrations in superstimulated mares, both cycling and vernal transitional, are extremely high before and at the time of ovulation. However, serum E2 concentrations on the 4 days following the day of ovulation have not been compared to controls. Such information is crucial for the understanding of the hormonal environment during fertilization and early embryonic development, and the influence of non-synchronized ovulations or non-ovulatory follicles present at that time. Furthermore, the correlation between the hormonal environment in superstimulated mares, oviductal and uterine secretions, contractility, and gene expression, should be clarified. The potential effects of seasonal status (cycling vs. vernal transition) on those parameters should also be further explored.

Improved estrus synchronization and control of follicular wave emergence and development would significantly enhance reproductive efficiency in horses. This is important for appointment-breeding management of non-stimulated mares used for assisted reproductive techniques and even more essential when superovulatory treatments are employed. Our studies demonstrate that the progesterone and estradiol regimen (P&E) is not suitable for mares treated with eFSH; therefore, there is a necessity to develop alternatives for the synchronization of follicular wave growth. Other alternative methods to suppress follicular development might be more efficient than the P&E regimen for estrus synchronization and control of follicular wave development prior to superovlatory treatments. Interesting candidates that might be appealing to examine are testosterone or other anabolic steroids (Turner and Irvine, 1982; Thompson et al., 1983), inhibin (Ginther et al., 2005c), cortisol (Asa et al., 1983), GnRH agonists and antagonists (Watson et al., 2000; Evans et al., 2002; Checura et al., 2008), and GnRH vaccines (Imboden et al., 2006; Card et al., 2007). The merit of ultrasound-guided follicle aspiration or ablation, which at present is the only effective method to stimulate new wave emergence in mares, also requires further investigation. In cattle, aspiration of follicles before FSH treatment as a means of increasing the superovulatory response has been used successfully (Bergfelt et al., 1994; Bergfelt et al., 1997; Baracaldo et al., 2000; Shaw and Good, 2000; Bo et al., 2002). However, in mares, this approach to estrus synchronization combined with superovulation has not yet been examined.

Over the last few decades, many researchers have focused on the development of effective estrus synchronization and superovulatory regimens in order to improve reproduction success in horses. Conceivably, increased effectiveness and feasibility of other reproductive techniques, such as oocyte retrieval, *in-vitro* oocyte maturation, *in-vitro* fertilization, intracytoplasmic sperm injection, and *in-vitro* production of equine embryos, and cloning, would minimize the need for superovulatory treatments. Nevertheless, the combined effect of a short superovulatory pre-treatment prior to employment of such techniques, without allowing follicles to reach preovulatory status, should be further investigated.

In summary, future studies should seek alternatives to conventional superovulatory and estrus synchronization treatments for mares and address factors that affect oocyte and follicular recruitment, maturation and developmental, oviductal and uterine environment, and embryo viability and development. Identification of these factors will provide means for the development of strategies in which pregnancy rate and success rate of embryo transfer programs can be optimized in mares.

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