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The Effects of Equine Somatotropin (eST) on Reproductive Function in the Domestic Mare.

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THE EFFECTS OF EQUINE SOMATOTROPIN (eST) ON REPRODUCTIVE FUNCTION IN THE DOMESTIC MARE

A Dissertation

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The effects of somatotropin on ovarian reproductive function has become a keen research area in mammalian species over the past decade. However, to date, very little information is available as to the effects of somatotropin administration on reproduction in the domestic mare. Therefore, the purpose of these experiments was (1) to determine the potential effects (if any) of administration of exogenous equine somatotropin on ovarian follicular development and plasma and follicular fluid hormone concentrations in cyclic and noncyclic mares, (2) to determine the mechanism(s) of action of somatotropin treatment on these parameters in the mare, (3) to develop an efficient protocol for stimulating ovarian follicular development using somatotropin treatment to mares and (4) to develop a repeatable method for the induction of ovulation in seasonally anestrous mares treated with somatotropin. It was determined that somatotropin treatment increased the number of ovarian follicles in both cyclic and noncyclic mares. In addition, plasma and follicular fluid levels of IGF-I were increased in mares treated with somatotropin. In seasonally anestrous mares, somatotropin treatment in conjunction with daily administration of a GnRH agonist was effective in inducing a 5-fold increase in the number of mares ovulating when compared with treatment with the GnRH agonist alone. In equine granulose cells cultured *in vitro*, both somatotropin and IGF-I were successful in enhancing cellular proliferation and steroidal output, providing evidence that these compounds act directly at the ovarian level to affect follicular development. In addition, it was determined that treatment to cyclic mares with somatotropin caused a significant delay in the process of follicular atresia, suggesting that it may be through this mechanism that somatotropin increases the

number of ovarian follicles in the mare. Further studies are needed in this area of equine reproduction so that the exact mechanism(s) through which somatotropin affects follicular development can be determined.

INTRODUCTION

Recently, the role that somatotropin (ST) plays on ovarian follicular dynamics and normal reproductive function has come under intense investigation in various mammalian species. It has been well documented that administration of exogenous ST increases follicle numbers on the ovaries of pigs (Echternkamp *et al.*, 1994), cattle (Gong *et al.*, 1991; Lucy *et al.*, 1993) and women (Hugues *et al.*, 1991; European and Australian Multicenter Study, 1995). For example, Spicer *et al.* (1992) demonstrated that female pigs given exogenous pST had more follicles (1 to 4 mm in diameter) present on their ovaries after treatment than did contemporary control gilts. This response seems to be the most common, since most studies report increases in follicles in the small- to medium-size range (1 to 9 mm), with little to no difference in the numbers of larger follicles (Gong *et al.*, 1991). The exact mechanism through which ST affects ovarian follicular populations is not currently known, however, its ability to stimulate IGF-I production from the liver, thereby increasing systemic IGF-I concentrations, may play a crucial role in this process (Homburg *et al.*, 1988). It should be noted, though, that a direct effect of ST at the ovarian level cannot be ruled out, as ST receptors have been detected in ovarian tissue of cattle (Gong *et al.*, 1991).

Since most studies dealing with the effects of ST on reproductive function have used animal models in which circulating concentrations of ST were brought to supraphysiological levels as a result of exogenous administration, the question of whether ST at normal physiologic levels was critical to proper reproductive performance was raised. One study using hypophysectomized ewes demonstrated that, in the absence of ST, gonadotropin administration alone was insufficient in returning

these animals to a normal reproductive state (Eckery *et al.*, 1997). However, when ST was administered in addition to LH and FSH, the animals began showing normal ovarian follicular development (Eckery *et al.*, 1997). These researchers were able to isolate ST receptors located on the surface of the follicular granulosa cells, and presumed that ST acted locally within the follicle to enhance the ability of the follicular cells to respond to available gonadotropins, which is currently one of the most prevailing hypotheses concerning the effects of ST on reproduction. Also, in Ames dwarf mice, a condition that is brought about due to a genetic defect which causes the absence of the development of the somatotrophs within the adenohypophysis, a significant reduction in both gonadotropic output as well as ovarian follicular development was noted and the administration of bST reversed these conditions (Bartke *et al.*, 1996). These studies provide strong evidence that ST provides a physiologic, rather than a pharmacologic, effect on reproductive function in domestic species.

It has been proposed that one of the mechanisms of action for ST on ovarian follicle populations arises from its ability to stimulate IGF-I production and secretion, and that IGF-I then works synergistically with available gonadotropins to enhance their response at the ovarian level (European and Australian Multicenter Study, 1995; deMoura *et al.*, 1997). In one study using perifused rabbit ovaries, it was demonstrated that ST caused an approximate 3-fold increase in IGF-I mRNA within the granulosa cells of the ovarian follicles, showing that ST can indeed cause effects at the ovarian level (Yoshimura *et al.*, 1996a), and that these effects were directly correlated with follicular IGF-I concentrations (Yoshimura *et al.*, 1994). Also, it is known that IGF-I stimulates the formation of LH receptors in rat granulosa cells (Liu *et al.*, 1998), which

would account for its ability to enhance the follicular response to LH. Also, IGF-I increases FSH-stimulated progesterone production in rat granulosa cells cultured *in vitro*, further demonstrating an effect of IGF-I at the level of the ovary (deMoura *et al.*, 1997). It is therefore likely that many of the effects of ST are due to increased IGF-I concentrations, which increase the follicular response to available gonadotropins.

A second hypothesis involves an ability of ST and/or IGF-I to increase the number of actively growing follicles present within a follicular wave. Since the discovery of cyclical, wave-like growth patterns of follicular growth in most mammalian species, the mechanism for selection of small antral follicles to become responsive to growth and development within the follicular wave has been a major research focus (for review see Ginther, 1992). It is not currently known what factors are involved with follicular selection and recruitment to begin the growth process, but increased blood flow to the ovary may play a role (Staigmiller, 1982; Driancourt *et al.*, 1991; Bao and Garverick, 1998). It is known, however, that these early-antral follicles are gonadotropin insensitive, so it is possible that various proteins and growth factors are involved in follicular selection (Britt, 1988). Therefore, it is possible that ST, through its ability to stimulate IGF-I, could play a role in early follicular activation into a follicular wave.

Another possibility is that the increased IGF-I concentrations could prolong the lifespan of the follicle by delaying the process of atresia, during which a follicle ceases to grow and begins to regress until it is no longer detectable on the ovary. The atretic response is thought to be brought about primarily through spontaneous apoptosis, or cellular death, of the follicular granulosa cells (Guthrie *et al.*, 1998). It has been

demonstrated that FSH is the most potent inhibitor of apoptosis in rat granulosa cells cultured *in vitro*, and it is well known that the mechanism for the ability of FSH to "superstimulate" follicular growth in mammals is by rescuing these follicles from the process of atresia (Chun *et al.*, 1996). Since IGF-I has also been shown to have potent inhibitory actions on granulosa cell apoptosis (Chun *et al.*, 1996), this hypothesis should not be overlooked.

Another possibly important aspect of this facet of follicular dynamics is the ability of both ST and IGF-I to increase steroidogenesis within the follicle. Administration of ST increases levels of intrafollicular 3β-hydroxysteroid dehydrogenase and aromatase mRNA, allowing for more progesterone and estradiol to be produce by the follicular cells (Tapanainen *et al.*, 1992). Although IGF-I causes higher granulosa cell production of both progesterone (Xia *et al.*, 1994) and estradiol (Echternkamp *et al.*, 1994; Howard and Ford, 1994), it is the ability of IGF-I to enhance estradiol output that gives greater insight to this hormonal system and its effects on reproduction. It is known that large, pre-ovulatory follicular development and estradiol output are highly correlated in women (Thierry Van Dessel *et al.*, 1996), and it has been recently shown that intrafollicular IGF-I concentrations are greatest in pre-ovulatory follicles in cattle (Yuan *et al.*, 1998). Thus, it is possible that another mechanism of action for IGF-I on follicle development is through stimulation of steroidogenesis within the follicle, allowing for an increased likelyhood for follicular dominance to occur (Yuan *et al.*, 1998).

During the winter months, which correspond to periods of shortened daylength, most mares undergo a period of reproductive inactivity termed "seasonal anestrous".

During this anovulatory period, GnRH production and output from the hypothalamus is very low, and normal ovarian reproductive activity ceases (Hart *et al.*, 1984). For many years, scientists have attempted to stimulate these mares to begin normal follicular growth and ovulation using GnRH therapy, with very limited success (Hyland *et al.*, 1987; Mumford *et al.*, 1994). Given that one of the mechanisms of action for ST/IGF-I is to enhance the follicular response to available gonadotropins, It is possible that the addition of daily administration of ST in conjunction with GnRH treatment could improve the response of seasonally anovulatory mares to GnRH therapy.

In addition, the development of an efficient, repeatable method to collect large numbers of viable oocytes from mares, for the purpose of *in vitro* fertilization (IVF) techniques, would benefit the scientific as well as the commercial horse industry. Since IVF has become a routine method of treating clinical infertility in cattle and women, the development of this technique in horses has been under intense study (Meintjes *et al.*, 1995). One of the largest obstacles to overcome is the lack of response seen when traditional stimulation protocols involving gonadotropins are used. It is well known that the administration of exogenous gonadotropins collected from ovine, bovine and equine sources to mares has little to no effect on stimulating ovarian follicular development, even when extremely large doses are given (Ginther, 1992). Therefore, it is difficult to obtain the numbers of oocytes necessary to develop a method of producing equine embryos *in vitro*.

Also, conventional methods of IVF have yielded limited success in the horse, with only one foal being reported to date (Palmer *et al.*, 1991). However, in one study using pregnant mares, Meintjes *et al.* (1995) demonstrated that acceptable numbers of

oocytes could be collected and that fertilization of these oocytes could be achieved using a pre-fertilization technique of drilling a small hole in the zona pellucida of the oocytes to allow the sperm access to the oocyte. Unfortunately, no pregnancies were obtained using this technique. More recently, intracytoplasmic sperm injection (ICSI) has been used to produce equine embryos *in vitro* with success (Meintjes *et al.*, 1996; Dell'Aquila *et al.*, 1997), and two pregnancies have been reported using this technique (Meintjes *et al.*, 1996; Squires *et al.*, 1996). Since it has been shown that treatment with ST increases the ovarian response in women undergoing controlled ovarian hyper-stimulation procedures (Owen *et al.*, 1991b; Schoolcraft *et al.*, 1997), and that the fertilization rate of the oocytes from ST-stimulated women are greater than those from contemporary control subjects (Owen *et al.*, 1991a), it is possible that the same may hold true for the domestic mare. Therefore, the purpose of the experiments reported herein was to (1) determine the effects of ST administration on cyclic and seasonally anovulatory mares, (2) determine the mechanism(s) of action of ST on reproductive function in cyclic and noncyclic mares and (3) develop an efficient, repeatable protocol for the production of equine embryos *in vitro* using ST for ovarian stimulation and ICSI for fertilization of the collected oocytes.

CHAPTER I

LITERATURE REVIEW

THE FOLLICULAR WAVE PHENOMENON

Cyclic Animal Model

In recent years, the study of the pattern of growth and development of ovarian follicles has been a key research interest in domestic animals. It has been shown that, in most mammals, a group of follicles, termed a "cohort", grows and regresses in a cyclical, wave-like pattern, hence the term "follicular wave" (Sirois and Fortune, 1988; Adams *et al.*, 1992b; Lucy *et al.*, 1993; Pursley *et al.*, 1993). The one exception is the domestic pig, as there is no evidence to date that suggests the follicular wave phenomenon occurs (Ryan *et al.*, 1994b). Currently, the exact mechanisms involved in early follicle activation and introduction into the cohort are not fully understood. It is believed, however, that various factors such as increased blood flow, changes in local growth factor concentrations and dietary energy intake are involved, since these pre-antral and early-antral follicles are not gonadotropin-dependent at this time (Staigmiller, 1982; Britt, 1988; Bao and Garverick, 1998). It is known that once these small, antral follicles are recruited into the cohort a surge of FSH secretion by the adenohypophysis preceeds the visible (via transrectal ultrasonography) initiation of the new follicular wave in both cattle and mares (Evans and Irvine, 1975; Adams *et al.*, 1992b; Badinga *et al.*, 1992; Ginther and Bergfelt, 1993; Pursley *et al.*, 1993; Beam and Butler, 1997).

The number of follicular waves per estrous cycle varies between individual animals and species, but in general cattle demonstrate 2 to 3 waves of follicular growth during each estrous cycle (Adams *et al.*, 1992a,b), while mares exhibit 1 to 2 follicular

waves per cycle (Ginther, 1990; Pursley *et al.*, 1993; Buratini *et al.*, 1997). Likewise, the duration of growth for each wave varies, with cattle showing ~7 days of growth per follicular wave while mares exhibit ~10 days of follicular growth per wave. In general, the cohort of follicles begins to grow synchronously, but during this growth phase oneor more follicles are "selected" for dominance, at which time these follicles begin to grow at a more rapid pace, while simultaneously deterring the further growth of the remaining subordinate follicles within the cohort. Depending upon which stage of the estrous cycle this cohort begins to grow, the dominant follicle can either ovulate and release its oocyte to be made available for fertilization, or it can regress at the time a new wave of follicular growth is initiated (for review see Ginther, 1992).

It has been proposed that the surge of FSH detected just prior to wave emergence is necessary for the promotion of normal growth and development of the new follicular wave, however, definitive evidence for this hypothesis is currently lacking, as it is generally accepted that these small antral follicles are gonadotropin insensitive (Britt, 1988). It is believed, however, that changes in FSH secretion are primarily responsible for the divergence in growth of the dominant follicle, which then decreases the growth of the remaining subordiate follicles within the current follicular wave (Mihm *et al.*, 1997). For example, Mihm *et al.*, (1997) demonstrated that in cyclic beef cows, a decline in circulating FSH concentrations by 2 days post-wave emergence could cause a change in available growth factors within the cohort of growing follicles that would promote follicular divergence of the dominant follicle and atresia of the subordinate follicles. It has also been shown that treatment with a bolus injection of FSH to heifers caused a delay in the divergence of the dominant follicle

from its subordinates, further suggesting the importance of the decline in FSH concentrations on follicular divergence and follicular dominance (Adams *et al.*, 1993).

Another important facet of follicular wave growth and development revolves around the fate of the dominant follicle. Once selected, this follicle will usually either ovulate or regress, depending upon the stage of the estrous cycle. It has been demonstrated that the decline in FSH is related to subsequent increases in intrafollicular content of estradiol and IGF-I within the dominant follicle, as well as with a decrease in intrafollicular IGF-I binding proteins (IGFBP) that allows for an overall increase in the bioavailable IGF-I within the follicle, which has important roles in follicular growth and ovulation (Mihm *et al.*, 1997). Also, it has been demonstrated that, in cattle, a decline in LH pulse frequency from the adenohypophysis results in atresia of the dominant follicle, and therefore no ovulation (Lucy *et al.*, 1993). Therefore, for ovulation to occur, the animal must be in the proper stage of the estrous cycle where progesterone concentrations are low so that an increase in LH pulse frequency can take place propagating the final growth and maturation of the dominant follicle for ovulation to occur (Lucy *et al.*, 1993).

As previously stated, the exact mechanisms allowing for follicular recruitment into a follicular wave, as well as some of the mechanisms involved in follicle growth and selection and continued growth of the dominant follicle, in addition to ovulation of the selected follicle(s), are not fully understood. It has been proposed that ST as well as IGF-I and, to a lesser extent insulin, are all involved in this dynamic process of follicular growth and development, but the exact mechanisms of action for these growth factors are as yet unclear (Bartke *et al.*, 1996; Cox, 1997). It is known that increases in

intrafollicular IGF-I and insulin can enhance the ability of the follicle to respond to available gonadotropins in the pig, but whether or not these growth promotors can influence early follicle growth and/or the selection process by which one or more follicles becomes dominant over the other follicles within a follicular wave remains to be determined (Cox, 1997).

Follicular Waves During Pregnancy

Interestingly, both cows and mares continue to exhibit waves of follicular growth and development throughout early pregnancy (Ginther *et al.*, 1989; Ginther and Bergfelt, 1992). In cattle, follicular wave growth has been demonstrated to continue during pregnancy as well as during treatment with exogenous progesterone up to 100 days (Bergfelt *et al.*, 1991). Also, pregnant cows usually exhibit follicular growth patterns similar to that of cyclic cows up to the last 22 days of gestation, when follicular activity seems to temporarily shut down, possibly due to high levels of estrogens being produced by the developing fetus (Ginther *et al.*, 1996). Of interest is the fact that, during pregnancy, follicles rarely ovulate in cattle, and the diameter of the largest follicle has been shown to be smaller in pregnant compared with nonpregnant cows (Ginther *et al.*, 1989).

In mares, the ovaries are extremely active during the first 30 to 50 days of gestation, with large numbers of follicles growing and regressing in wave-like patterns (Ginther and Bergfelt, 1992). Unlike cattle, mares have developed a mechanism to produce addition corpora lutea during pregnancy as a method to ensure that adequate concentrations of progesterone are maintained throughout early pregnancy so as to support continued growth of the fetus (Ginther, 1992). It has been demonstrated that, as

is the case with cyclic animal models, surges of FSH secretion are associated with initiation of new follicular waves during pregnancy for mares (Ginther and Bergfelt, 1992). However, individual follicular growth characteristics are extremely variable between mares, with some mares exhibiting few waves of follicular growth while others show a great deal more follicles per wave, more total number of waves, and a higher number of secondary corpora lutea during early pregnancy (Ginther and Bergfelt, 1992). In general, however, unlike the cow model, the ovaries of pregnant mares tend to cease new follicular growth by day 70 to 100 of pregnancy, at which time the ovary becomes quiescient for the remainder of pregnancy (Ginther, 1992). This decrease in follicular activity is thought to be caused primarily by elevated circulating concentrations of estrogens present at this time (McKinnon and Voss, 1993). Currently, the reason for continued growth and development of ovarian follicles during pregnancy is not fully understood in cattle, since these follicles rarely ovulate and therefore would not serve the same function as in the mare, where supplemental corpora lutea formation is a necessary event to ensure the continued survival of the early conceptus. However, one possible explanation for continued follicular wave emergence in early pregnancy in cattle may be to provide a source of oocytes in the event of abortion.

MECHANISMS OF FOLLICULAR GROWTH AND DEVELOPMENT

Follicle Activation

Early follicular growth, from the pre-antral to early-antral stages, is a complex and puzzling series of events that is not well understood at present. The exact mechanisms involved in the process of follicular activation, when a quiescent pre-antral follicle suddenly begins to grow and develop, as well as the mechanisms involved in

recruitment of early-antral follicles into a newly emerging follicular wave, are not currently known, although it is known that these follicles are gonadotropin-insensitive at this time (Britt, 1988; Findlay, 1994; Gong *et al.*, 1996b). One possibility is that increased blood flow into local areas containing pre-antral and early-antral follicles is involved in the processes of follicular activation and recruitment, as evidenced by the fact that, in dairy heifers, it has been demonstrated that the ovary bearing the corpuus luteum has significantly more visible ovarian follicles than the ovary without a corpus luteum within the same animal (Staigmiller, 1982; Driancourt *et al.*, 1991; Bao and Garverick, 1998).

This theory is inadequate, however, since follicular waves can occur on opposite ovaries simultaneously, even though one ovary may not have a corpus luteum present at the time of wave emergence (Ginther, 1992; McKinnon and Voss, 1993). Recent evidence suggests that estradiol, ST, IGF-I and other growth factors may play a role in follicular activation and recruitment (Bergh *et al.*, 1994; Liu *et al.*, 1998). For example, it has been suggested that higher concentrations of ST may be involved in early follicle growth, and that protocols involving ST administration to increase the number of follicles should be given for relatively long durations, since it is estimated that it takes 30 to 60 days for a follicle to develop from a pre-antral state until the time it becomes visible via ultrasound (Bergh *et al.*, 1994). Also, in *in vitro*-cultured ovarian follicles collected from mice, it has been demonstrated that ST as well as activin can enhance pre-antral and early-antral growth even though these follicles are gonadotropin-independent (Liu *et al.*, 1998). It is clear, however, that further research is needed in

this area of follicular dynamics to better understand the underlying mechanisms involved in early follicle activation and recruitment.

Gonadotropin Input

Once the growing follicles have become part of a new follicular wave, they reach a stage in which they become gonadotropin-dependent, that is, gonadotropic input is required for further growth and development. This input starts at the level of the hypothalamus, with the production and secretion of gonadotropin-releasing hormone (GnRH) into the median eminence. This in turn supplies the adenohypophysis with blood flow so that the GnRH, along with other releasing factors, can stimulate the production and secretion of various hormones by the adenohypophysis that are necessary for normal function of the entire body. GnRH from the hypothalamus stimulates gonadotropes, which are the cells responsible for producing gonadotropins, within the adenohypophysis to produce and secrete LH and FSH (Evans and Irvine, 1976; Alexander and Irvine, 1987; Johnson *et al.*, 1988; Pantke *et al.*, 1991; Irvine and Alexander, 1993; Porter *et al.*, 1997), both of which are vital to the continued growth and development of ovarian follicles in the horse.

GnRH secretion is regulated through neuronal input, and the primary substances that are believed to be responsible for the regulation of GnRH secretion are nitric oxide, PGE_2 and leptin. Using an immortalized cell line derived from rats, it has been shown that leptin has both direct and indirect effects on GnRH secretion, primarily through OB-R neuronal receptors (Magni *et al.*, 1999). Nitric oxide is a necessary component of this pathway, as it has been demonstrated that nitric oxide inhibitors can block leptin-induced GnRH and LH secretion (Yu *et al.*, 1997). Adequate feed intake is essential for

normal leptin production, as fasting and feed restriction both decrease basal leptin concentrations in pigs (Mao *et al.*, 1999) and rats (Nagatani *et al.*, 1998). Therefore, in order to ensure normal GnRH production, and hence normal LH and FSH secretion, proper nutrition should be maintained at all times.

Normal secretion of GnRH is crucial to the reproductive cycle in most mammalian species. In the domestic mare, this is shown by the fact that during times of decreased daylength (winter), GnRH secretion by the hypothalamus is severly depressed or absent, causing the animal to undergo a period of acyclicity termed "seasonal anestrous" (Ginther, 1992), during which time the mare ceases to have a normal cycle, and becomes anovuiatory. In the cyclic mare, it has been demonstrated that up to 98% of all GnRH pulses secreted by the hypothalamus were associated with a subsequent pulse of LH secretion by the adenohypophysis, and 83% of these GnRH pulses caused a pulse of FSH secretion (Alexander and Irvine, 1987). Also, Irvine and Alexander (1993) demonstrated that secretion of FSH and LH usually occurred within 5 minutes after a GnRH pulse, showing that GnRH is the primary hormone controlling gonadotropin secretion. However, in mares immunized against GnRH, basal LH secretion is more severly affected than that of FSH, and when these mares are given a GnRH agonist, FSH secretion appears normal relative to control animals, while LH response to the agonist is minimal, suggesting a differential regulatory mechanism for the two hormones (Garza *et al.*, 1986).

Conversely, it has been shown that basal FSH is not affected by GnRH administration in mares, while basal LH increases dramatically in response to GnRH therapy (Nett *et al.*, 1989). However, other studies have shown that there is a greater

response of FSH secretion to GnRH administration in mares when compared with LH secretion (Evans and Irvine, 1976). It is believed that increasing the pulsatile frequency of GnRH release, along with increasing estradiol levels in the blood, is the major cause for the "surge" of LH secretion noted during behavioral estrus in the mare, and it is this LH surge that is ultimately involved in ovulation of the dominant follicle (Irvine and Alexander, 1994). Therefore, it is clear that GnRH is the primary secretagogue for both LH and FSH, and therefore critical for normal reproductive cyclicity in the domestic mare (Irvine and Alexander, 1994).

Administration of GnRH and/or one of its agonists is usually carried out to achieve ovulation, and this type of approach has been very successful in the horse (Meinert *et al.*, 1993; Swinker *et al.*, 1993; Skidmore *et al.*, 1996; Meyers *et al.*, 1997). Since GnRH is secreted in a pulsatile manner, it should come as no surprise that this is the preferred manner of administration of GnRH for the purpose of inducing ovulation in the horse (Becker and Johnson, 1992). Also, this route of administration yields superior results for inducing ovulation in cattle (Gong *et al.*, 1996b). This is primarily due to the fact that the ultimate cause of ovulation is the rise in cirulating levels of LH, and that pulsatile GnRH input is more effective in inducing LH secretion in treated animals (Vivrette and Irvine, 1979). Also, while large doses of GnRH can cause a substantial rise in plasma LH, the biological activity of this LH is reduced, and it is therefore not as effective in inducing ovulation in the mare (Pantke *et al.*, 1991).

Both FSH and LH are necessary hormones for normal reproductive cyclicity in most mammalian species. FSH is required for follicular growth and maturation, and increasing the amount of available FSH can increase the growth of small- and medium-

sized follicles by up to 7-fold (Gibbons *et al.*, 1997). Unfortunately, there has been no clear establishment of a relationship between the amount of FSH secreted and ovulation rate in the mare, and mares are much less sensitive to ovarian stimulation by administration of exogenous FSH than other animals (Urwin and Allen, 1983). In the mare, FSH is low during the anovulatory season, but during vernal transition, which is the period of time that the animal goes from an acyclic to a cyclic state, FSH production and secretion increases, with larger and larger peak levels of FSH secretion occurring until the first ovulation of the breeding season (Hines *et al.*, 1991). Once cyclicity has resumed, lower baseline concentrations of FSH are detected, possibly due to the secretion of inhibitory substances by the growing follicles (Hines *et al.*, 1991). Also, it has been demonstrated that certain steroids, such as testosterone, may affect the amount of FSH accumulation within the adenohypophysis, further causing a decrease in basal FSH secretion (Thompson *et al.*, 1984). Although different isoforms of FSH do exist, there is no evidence of a difference in sensitivity to these different isoforms (Cooke *et al.*, 1997).

LH is the hormone primarily responsible for the final growth and maturation of the pre-ovulatory follicle, with serum concentrations being low during diestrus, high during behavioral estrus, and reaching peak levels 1 to 2 days post-ovulation (Evans and Irvine, 1975). The key events seem to be (1) an increase in pulsatile amplitude, i.e. higher amounts of LH secreted per secretion episode, which is important in inducing final growth and maturation of the pre-ovulatory follicle (Evans *et al.*, 1997) and (2) an increase in LH pulse frequency just prior to ovulation, which appears to be a triggering mechanism for ovulation to occur (Stagg *et al.*, 1998). As stated earlier, GnRH is the

primary regulatory hormone for LH release in the mare (Garza *et al.*, 1986), so it is apparent that pulsatile secretion of GnRH must follow a similar pattern throughout the estrous cycle. That LH is the primary hormone responsible for the induction of ovulation in the mare is evidenced by the fact that human chorionic gonadotropin (hCG), a compound secreted by the live human conceptus, has strick LH-like capabilities in the mare and is extremely useful for inducing ovulation in this species (Duchamp *et al.*, 1987), even though its administration causes a decrease in the release of both GnRH and LH (Mores *et al.*, 1996).

Hormonal Regulation of LH and FSH

The female reproductive system is composed of a highly complex, interactive set of checks and balances, which serve to ensure normal reproductive cyclicity. The production and secretion of both FSH and LH are governed not only by GnRH input, but also by several other steroid and protein hormones that allow for the process of follicle growth, ovulation and corpus luteum formation to proceed at a normal pace. For instance, estradiol, a steroid hormone produced by growing ovarian follicles, has many implications in the production and secretion of gonadotropins (Vivrette and Irvine, 1979; Ireland, 1987; Pinaud *et al.*, 1991). In the granulosa cells, estradiol is important in the amplification of the actions of FSH, specifically the "priming" action FSH has on granulosa cells to render them more susceptible to the further stimulatory actions of LH (Ireland, 1987), and it is well known that, within the follicle, only the granulosa cells are capable of producing estradiol (Watson and Thomson, 1996). It has been suggested that estradiol production by the ovarian follicles is an indicator that the follicles are in a healthy, growing state, with much higher production of estradiol in

largest, dominant follicle within a follicular wave in cattle (Ireland and Roche, 1983; Badinga *et al.*, 1992).

Unfortunately, this difference in estradiol secretory rates between the dominant and subordinate follicles does not manifest itself until after follicular divergence has occurred, which rules out the notion that one of the factors influencing the divergence of the dominant follicle from its subordinates is an innate ability to produce higher amounts of estradiol (Ginther *et al.*, 1997). In addition, although the tendency for atretic follicles is to decrease estradiol output while increasing the production of follicular androgens, this is not a sure sign of follicular atresia, since growing follicles produce androgens in addition to estradiol (Kenney *et al.*, 1979; Davis and Sharp, 1991). Interestingly, it has been demonstrated that androgens have a positive effect on FSH, as well as LH, secretion during diestrus by allowing for an accumulation of FSH within the adenohypophysis prior to its release, causing larger amounts to be secreted per secretion episode (Thompson *et al.*, 1983b; Reville-Moroz *et al.*, 1984; Thompson *et al.*, 1987).

However, other studies have shown that androgens have either a negative effect on FSH secretion (Evans *et al.*, 1997), or no effect at all (Thompson *et al.*, 1987). It is known, though, that the increased production of estradiol by the dominant follicle inhibits FSH secretion by the adenohypophysis, decreasing the amount of FSH available for stimulation of the subordinate follicles (Ginther, 1992; Evans *et al.*, 1997). This is thought to be one of the primary mechanisms through which the dominant follicle suppresses the growth of the other subordinate follicles within the follicular wave, thereby ensuring that it will remain the follicle destined to ovulate (Evans *et al.*, 1997).

Estradiol also affects the production and secretion of LH, by both direct and indirect pathways (Pinaud *et al.*, 1991). First, estradiol directly stimulates production of LH in the adenohypophysis while inhibiting its secretion to allow for an accumulation of LH so that greater than normal amounts are available for release (Vivrette and Irvine, 1979). This increase in pituitary stores of LH can be detected within 24 hours after estradiol exposure (Vivrette and Irvine, 1979). Estradiol can then increase GnRH output by the hypothalamus to cause the release of the LH stored within the pituitary, creating a net increase in pituitary production of LH (Vivrette and Irvine, 1979). It is primarily through this mechanism that estradiol causes the pre-ovulatory surge of LH noted in most domestic species (McKinnon and Voss, 1993).

Once ovulation of the pre-ovulatory follicle has occurred, the formation of the corpus luteum begins and progesterone production by this gland increases (Ginther, 1992). Progesterone is the steroid hormone most important for the maintenance of pregnancy, hence its name ("pro" meaning for, "gesterone" meaning gestation). It is also intricately involved in the regulation of LH production and secretion (Adams *et al.*, 1992a). It has been shown that progesterone can inhibit LH secretion, probably through decreasing GnRH pulse frequency (Evans *et al.*, 1982; Adams *et al.*, 1992a). In one study using *Bos indicus* cattle, administration of progesterone during the follicular phase of the estrous cycle delayed ovulation and the timing of the LH peak, demonstrating its ability to suppress LH secretion (Cavalieri *et al.*, 1997). This should be expected since, in cyclic animals, there is normally no need for ovulation to occur at the time that functional corpora lutea are present. Finally, although progesterone is very important in the regulation of LH secretion, most evidence to date suggests a minimal

role for this steroid in the regulation of FSH synthesis and release (Evans *et al.*, 1982; Adams *et al.*, 1992a).

In addition to steroidal influence, there are also protein hormones produced by the developing follicles that aid in the regulation of pituitary FSH (Miller *et al.*, 1981). Most notable of these is inhibin, a protein produced primarily by the dominant follicle, which regulates FSH secretion (Britt, 1988). Inhibin functions to directly inhibit FSH secretion by the pituitary, as another mechanism of reducing the amount of FSH available to the subordinate follicles within a follicular wave (Britt, 1988; Roser *et al.*, 1994). It has been demonstrated in mares that peripheral inhibin concentrations increase beginning on day 8 post-ovulation and continue rising until the day of ovulation, closely following the emergence and growth of the dominant follicle (Roser *et al.*, 1994). These levels of inhibin then decline rapidly until day 7 post-ovulation (Roser *et al.*, 1994). Interestingly, the circulating concentrations of FSH show the opposite pattern, with higher levels during the first 7 days post-ovulation and then a decrease as inhibin levels rise (Roser *et al.*, 1994). In mares, inhibin antiserum has been shown to increase both plasma FSH as well as the number of ovulations in treated mares, with no effect on circulating LH concentrations (Nambo *et al.*, 1998). This demonstrates the ability of inhibin to negatively regulate FSH release from the adenohypophysis.

Finally, a second protein hormone produced by growing follicles, activin, aids in the regulation of pituitary FSH. Activin directly stimulates the release of pituitary FSH to aid in follicular growth and development, and may actually cause some of the somatotropes within the adenohypophysis, which produce ST, to undergo a

conformational change into gonadotropes capable of secreting FSH in the rat (Childs and Unabia, 1997). Further study into the mechanisms of action of these protein hormones in gonadotropin regulation and follicular development is needed.

The Ovulatory Response

The final response of the growing follicle, after follicular activation, recruitment, growth and selection of the dominant follicle has occurred, is the process of ovulation. Ovulation has been described as an inflammatory process (Watson *et al.*, 1991), which is thought to be ultimately mediated by prostaglandins (Jochle *et al.*, 1987; Yamauchi *et al.*, 1997). After the ovulatory surge of LH has been released, a complex pathway of events involving prostaglandin production and release is set in motion, which culminates in the ovulation of the pre-ovulatory follicle (Yamauchi *et al.*, 1997). It is believed that nitric oxide is intricately involved in this pathway, and it has been shown that nitric oxide inhibitors can block ovulation primarily through the blockage of prostaglandin formation (Yamauchi *et al.*, 1997). Once ovulation has occurred, the ovum is released and made available for fertilization, and the resultant ruptured follicle begins the process of forming a corpus luteum, which is responsible for progesterone production for the maintenance of pregnancy.

Nutritional Effects on Follicular Development

The body condition of the animal, as well as the overall nutritional plane on which the animal is maintained, can have profound effects on reproductive performance. Cows undergoing feed restriction and those that have a low body condition score, which reflects the nutritional status of the animal, have lower numbers of ovarian follicles than those with a high body condition score or those fed adequate

energy intake (Rutter and Manns, 1991; Ryan *et al.*, 1994a). Also, increasing the dietary energy has been shown to be beneficial in increasing the number of follicles (Lammoglia *et al.*, 1997), without affecting gonadotropin concentrations (Gutierrez *et al.*, 1997b). It appears that the effects of dietary energy on follicular development occur during the time of early follicular activation and subsequent recruitment into a follicular wave, since the process seems to be gonadotropin-independent (Gutierrez *et al.*, 1997b). In addition, adequate dietary energy is crucial in maintaining normal reproductive cyclicity, since restricting the dietary energy intake lowers basal estradiol and progesterone production by the follicles and corpora lutea in Brahman cattle, respectively (Lammoglia *et al.*, 1997).

One mechanism for the stimulation of ovarian follicular development may be through dietary effects on circulating ST and IGF-I, two growth promoters that also have effects on the reproductive axis (Rhind and Schanbacher, 1991). It is known that, during periods of feed restriction, serum ST rises, presumably due to its repartitioning abilities so that available nutrients are routed away from fat deposition (Rhind and Schanbacher, 1991), but also in part due to the ability of blood glucose to suppress ST secretion (Barb *et al.*, 1995). In contrast, IGF-I is higher in pigs that are on diets consisting of adequate energy intake, presumably due to its growth promoting abilities in many cell-types throughout the body (Ryan *et al.*, 1994a). In addition, it has been demonstrated that the circulating levels of high-density lipoproteins can influence hepatic IGF-I production by increasing the amount of IGF-I produced (Bao *et al.*, 1995). Finally, circulating insulin levels are affected by nutritional status, and insulin can also have effects in reproduction (Conway *et al.*, 1990). For example, insulin

treatment to pigs increases the number of ovarian follicles, but this stimulation is primarily through the IGF-I type-I receptor, essentially making the effects of insulin virtually identical to those of IGF-I (Edwards *et al.*, 1996).

SOMATOTROPIN AND IGF-I IN REPRODUCTION

Somatotropin is secreted by the adenohypophysis in response to growth hormone releasing-hormone (GHRH) secreted by the hypothalamus (Dubreuil *et al.*, 1990; Hugues *et al.*, 1991). Its primary role is to promote growth in most cell-types within the body, however it appears to be very important in reproductive function as well. It has been demonstrated that immunization of prepubertal heifers against GHRH significantly delays the onset of puberty and reduces the number of ovarian follicles (Cohick *et al.*, 1996). Furthermore, administration of GHRH significantly raises plasma ST as well as follicular steroids, and increases follicle growth in cattle (Spicer and Enright, 1991) and women (Hugues *et al.*, 1991). It has been proposed that ST plays a permissive role in reproduction, and that only in cases of ST deficiency should one expect ST treatment to show a benefit (Piaditis *et al.*, 1995). However, this may not be entirely true, as numerous studies have demonstrated positive effects on ovarian follicular dynamics with ST administration to normally cycling cattle (Gong *et al.*, 1991; Rieger *et al.*, 1991; Spicer *et al.*, 1992; Lucy *et al.*, 1993; Echternkamp *et al.*, 1994b; Gong *et al.*, 1996a). It has also been reported that cows deficient in ST receptors have lower numbers of follicles on their ovaries at any given time, shorter waves of follicular growth and depressed growth of the dominant follicle within each follicular wave compared with normal cattle (Chase *et al.*, 1998). It is therefore evident

that ST plays some role in ensuring normal reproductive function in mammalian species.

The administration of exogenous ST has been shown to be beneficial in increasing follicular numbers in cattle (Lucy *et al.*, 1993), pigs (Echternkamp *et al.*, 1994a) and women (Owen *et al.*, 1991a). It is thought that these effects are brought about, at least in part, through the ability of ST to stimulate hepatic as well as locally produced IGF-I (Homburg *et al.*, 1988), although a direct effect of ST at the level of the ovary has been suggested (Gong *et al.*, 1991). Early studies have shown that exogenous ST increases ovarian follicle numbers in the small- to medium-size categories, and this effect seems to be highly correlated to circulating IGF-I concentrations (Owen *et al.*, 1991a). Since daily ST secretion patterns are variable and pulsatile in nature, with frequent sampling times needed in order to establish baseline patterns for individual animals (Thompson *et al.*, 1992), it may be important to establish baseline IGF-I values, since ST stimulates IGF-I production and baseline IGF-I concentrations tend to be less variable within individual animals (Stewart *et al.*, 1993). However, it is also likely that ST exhibits direct effects at the ovarian level as well, since some studies have demonstrated positive effects of ST adimnistration on the number of follicles without an effect on IGF-I concentrations (Tapanainen *et al.*, 1992). In light of these findings, it is likely that ST has both direct effects at the level of the ovary, as well as indirect effects through the stimulation of systemic and intrafollicular of IGF-I production.

Many reports have demonstrated that ST administration increases ovarian follicle numbers, and also that the administration of ST affects follicular and systemic steroid concentrations (Spicer *et al.*, 1992; Tapanainen *et al.*, 1992; Echternkamp *et al.*,

1994b; Gong *et al.*, 1996a). However, other studies in women have shown that ST administration has no effect on ovarian follicular dynamics, since exogenous treatment with ST resulted in no noticeable effect on follicular growth and development (Hofferer *et al.*, 1991; Owen *et al.*, 1991b; Shaker *et al.*, 1992; Eckery *et al.*, 1994; Driancourt and Disenhaus, 1997). However, it is important to note that most of these studies involved normally cycling experimental subjects, in which additional ST may not be beneficial to reproductive function. In addition, several factors including the time of the cycle, the amount of ST given, as well as the duration of treatment could all have affected the outcome of these studies. It is evident, in any case, that ST is important in normal reproductive function as evidenced in animals with little to no endogenous ST input (Bartke *et al.*, 1996; Eckery *et al.*, 1997).

In Ames dwarf mice, a genetic condition in which no somatotropes develop within the adenohypophysis, resulting in no ST production, normal patterns of ovarian follicular development do not occur, but treatment with exogenous ST can completely reverse this phenomenon (Bartke *et al.*, 1996). In addition, in hypophysectomized ewes, gonadotropin administration alone is insuffucient in restoring normal reproductive cyclicity (Eckery *et al.*, 1997). However, once ST is given in addition to gonadotropins, the animals will begin to cycle and ovulate in a normal fashion once again. (Eckery *et al.*, 1997). These findings demonstrate the importance of ST in normal reproductive function.

It is well documented that treatment with exogenous ST causes plasma IGF-I concentrations to rise within 2 to 3 days of the initiation of treatment, and it is believed that this growth factor is responsible for many of the ovarian effects demonstrated with

ST administration in cows (Gong *et al.*, 1991; De La Sota *et al.*, 1993; Eckery *et al.*, 1994; Lucy *et al.*, 1994; Samaras *et al.*, 1994; Gong *et al.*, 1997). For example, ST administration to cyclic gilts resulted in an increase in the number of small ovarian follicles, which was highly correlated to rises in both plasma and intrafollicular IGF-I concentrations (Spicer *et al.*, 1992). Interestingly, most studies in domestic species have only reported increases in small- to medium-sized follicles, with no difference seen in large follicle numbers per animal in animals treated with ST (Gong *et al.*, 1991; Spicer *et al.*, 1992; Lucy *et al.*, 1993). However, in one study using gonadotropin-stimulated Holstein heifers, ST administration significantly increased both large follicle numbers as well as the number of ovulations per heifer when compared with those heifers receiving gonadotropin treatment only (Rieger *et al.*, 1991).

Similar findings have been reported in clinically infertile women, especially those with polycystic ovarian disease, in which ST treatment in conjunction with gonadotropin administration significantly increased the number of large follicles available for transvaginal oocyte collection procedures for use in *in vitro* fertilization protocols (Homburg *et al.*, 1988; Owen *et al.*, 1991a,b). It is proposed that elevated levels of ST as well as IGF-I act synergistically with available gonadotropins, causing an enhancement of the follicular response to gonadotropin stimulation, which could explain the apparent lack of large follicle stimulation in experimental subjects receiving no gonadotropin stimulation (European and Australian Multicenter Study, 1995). This could be important in the treatment of clinical infertility in not only cattle and humans, where gonadotropin treatments alone are often successful, but also in the domestic mare, since various protocols involving treatment with gonadotropins have shown little

to no real success (Rosas *et al.*, 1998). In a recent study in cyclic mares, it was shown that treatment with both porcine ST as well as bovine ST resulted in significantly higher plasma IGF-I concentrations when compared with untreated control mares, demonstrating that ST from other species has biological activity in the domestic mare (Buonomo *et al.*, 1996). However, in this study, no reproductive parameters were monitored, and the study was discontinued due to severe local reactions with the commercial preparation of these compounds.

Further studies showing the synergistic effects of ST/IGF-I on gonadotropic action in follicular development involve a reduction in the amount of gonadotropins necessary to elicit the desired response (Homburg *et al.*, 1988; Owen *et al.*, 1991a; Yoshimura *et al.*, 1994). In women undergoing controlled ovarian hyperstimulation treatments for use with *in vitro* fertilization protocols, ST administration significantly reduced the total amount of gonadotropins needed to successfully stimulate the desired number of follicles when compared with women receiving no ST treatment (Homburg *et al.*, 1988; Owen *et al.*, 1991a; Bergh *et al.*, 1994; European and Australian Multicenter Study, 1995). In addition, using perifused rabbit ovaries, Yoshimura *et al.* (1994) demonstrated that ST enhanced the follicular response to available gonadotropins by increasing both follicular growth as well as oocyte maturation. Furthermore, it was demonstrated that these effects were highly correlated to increased intrafollicular IGF-I production, providing further evidence that many of the effects of ST treatment are IGF-I mediated (Yoshimura *et al.*, 1994). From a practical sense, it should be noted that treatment with ST in no way impairs the fertility of the resultant oocytes, which

would obviously limit its use in ovarian stimulation protocols (Homburg *et al.*, 1988; Owen *et al.*, 1991a,b; Bilby *et al.*, 1998).

Finally, treatment with exogenous ST can also affect follicular wave patterns. In lactating dairy cattle, ST administration has been shown to decrease the interval between follicular waves (Kirby *et al.*, 1997b). That is, when cows were given exogenous ST, the emergence of the subsequent follicular wave was hastened by ~48 hours (Kirby *et al.*, 1997a). This same phenomenon has been reported in young dairy heifers, where treatment with both ST and placental lactogen hastened follicular wave emergence (Lucy *et al.*, 1994). Since serum IGF-I was increased in all treated animals, an IGF-I-mediated response cannot be ruled out (Lucy *et al.*, 1994). That ST/IGF-I seems to have the ability to affect follicular wave patterns in this fashion lends further evidence that these growth factors may be involved in early follicular activation and recruitment into a follicular wave.

Effects of ST on Hormone Production

Conflicting results have been reported in relation to the effects of ST on gonadotropin as well as steroid production. In both cattle (Gong *et al.*, 1991) and women (Tapanainen *et al.*, 1992), it has been demonstrated that ST treatment had no effect on plasma LH or FSH concentrations. Also, it was also reported that ST administration has no effects in cattle on estradiol or progesterone production in cyclic animals (Gong *et al.*, 1991). However, women treated with ST exhibited a decrease in serum estradiol as well as serum progesterone levels, with an increase in intrafollicular steroid production (Tapanainen *et al.*, 1992). This in contrast to a study by Lanzone *et al.* (1996), in which it was demonstrated that ST administration to subfertile women

caused a rise in serum estradiol concentrations throughout the duration of treatment. In addition, pigs treated with ST exhibited no differences in serum concentrations of LH, FSH, estradiol or progesterone, however, intrafollicular estradiol production was affected, with a decrease in estradiol production noted in medium-sized follicles in pigs (Spicer *et al.*, 1992).

Interestingly, in rats treated with equine chorionic gonadotropin (eCG), it was shown that, while ST treatment had no effect on absolute serum values of LH, administration of ST did cause slightly different (pleiomorphic) forms of LH to be secreted, which appear to have a lower biological activity than LH secreted in the absence of ST treatment (Wilson *et al.*, 1985). In any case, it currently remains unclear what effects, if any, ST administration may have on the production and secretory patterns of gonadotropins and steroid hormones in mammalian species.

Insulin-like Growth Factor-I

Since treatment with either GHRH and/or ST causes significant rises in both plasma and intrafollicular IGF-I concentrations, it is important to gain a better understanding of the exact mechanisms through which IGF-I may affect mammalian reproductive function (Owen *et al.*, 1991b; Kirby *et al.*, 1993; Stanko *et al.*, 1994; Gong *et al.*, 1997; Sirotkin *et al.*, 1998a). It has been demonstrated that IGF-I increases the growth and development of ovarian follicles in the rat (Yoshimura *et al.*, 1996a,b), as well as stimulating resumption of meiosis in the oocyte (Zhou and Bondy, 1993; Yoshimura *et al.*, 1996a), and that blocking the IGF-I Type-I receptor reverses these effects (Yoshimura *et al.*, 1996a). In addition, treatment of granulosa cells collected from growing ovarian follicles with IGF-I increases the responsiveness of the granulosa

cells to the stimulatory effects of FSH, possibly through increasing FSH receptor numbers (Owen *et al.*, 1991a). This seems to be the most likely mechanism of action for the synergistic effect detected between IGF-I and the gonadotropins, since treatment with IGF-I also increases LH receptor numbers in cultured rat granulosa cells (Liu *et al.*, 1998). This synergistic effect can be detected in lactating dairy cows during the first 14 days postpartum, as it was demonstrated that all cattle that ovulate within this time frame have 40 to 50% higher basal IGF-I concentrations, with no differences in plasma steroid or gonadotropin levels (Beam and Butler, 1997). This clearly shows that higher IGF-I concentrations in the plasma can be important for the resumption of reproductive cyclicity in cattle, and that IGF-I is involved in reproductive function. Since there was no difference in circulating plasma levels of LH or FSH in this study, it is probable that the elevated IGF-I allowed for an enhancement of the response to the available gonadotropins in these postpartum cattle (Beam and Butler, 1997). Also, it has been demonstrated in subfertile women that the circulating concentration of IGF-I is inversely related to the amount of gonadotropins needed to achieve an adequate stimulatory response when undergoing treatment for use in *in vitro* fertilization protocols (Rabinovici *et al.*, 1990).

It has been suggested that intrafollicular IGF-I concentrations may play a role in the selection and continued growth of the dominant follicle within a follicular wave (Yuan *et al.*, 1998). This is due to the fact that, in some reports, intrafollicular concentrations of IGF-I are higher in dominant compared with subordinate follicles (Rabinovici *et al.*, 1990; Monget and Monniaux, 1995; Yuan *et al.*, 1998). Also, it has been shown that IGF-I receptor numbers are higher in dominant follicles when

compared to those of subordinate follicles (Stewart *et al.*, 1996; Zhou *et al.*, 1996; Yuan *et al.*, 1998). However, others have reported no difference in intrafollicular concentrations of IGF-I between dominant and subordinate follicles (De La Sota *et al.*, 1996; Thierry Van Dessel *et al.*, 1996). Aside from total concentrations of IGF-I within the follicular fluid of dominant and subordinate follicles, the bioavailability of the IGF-I that is present may play an even greater role in dominant follicle growth and development. It is known that, in the dominant follicle, intrafollicular concentrations of IGF-I binding proteins change over time, allowing for an overall increase in the IGF-I available for cellular stimulation (Monget *et al.*, 1993; Monget and Monniaux, 1995; Yuan *et al.*, 1998). It is therefore possible that this increase in bioavailable IGF-I, through its interactions with gonadotropins, allows for the stimulation of growth of the dominant follicle(s) over that of the subordinate follicles within a follicular wave. Finally, it has been demonstrated in mares (Malinowski *et al.*, 1996) as well as women (Klein *et al.*, 1996) that basal IGF-I concentrations decrease as age increases, which may partially explain the age related decrease in fertility noted in these species.

Other Factors Influencing IGF-I

Aside from ST stimulation, IGF-I production can be influenced by various other compounds within the body. It has long been known that estradiol effects both IGF-I production as well as IGF-I receptor formation (Monget and Monniaux, 1995; Johnson *et al.*, 1996; Johnson *et al.*, 1998; Wilson, 1998a,b). However, there seems to be an age-related uncoupling of the estradiol-IGF-I axis, since prepubertal and adolescent females respond differently than adults (Wilson, 1998a,b). In adolescent female Rhesus monkeys, estradiol increases systemic IGF-I production, but in adults the opposite

effect occurs (Wilson, 1998b). In addition, in these adult monkeys, estradiol increases IGF-I binding protein concentrations within the follicle, which would further limit the amount of IGF-I available for use by the granulosa cells (Wilson, 1998a).

In addition to estradiol, progesterone has also been shown to effect IGF-I production by decreasing systemic concentrations of IGF-I (Manikkam and Rajamahendran, 1997). Also, treatment of both sheep (Khalid *et al.*, 1997) as well as pigs (Hammond *et al.*, 1988) with gonadotropins or GnRH has been shown to increase intrafollicular IGF-I concentrations, however, no effects were detected in systemic IGF-I levels. In addition, glucocorticoids have been shown to negatively affect follicular growth (Asa and Ginther, 1982; Asa *et al.*, 1983), and this is correlated to a reduction in intrafollicular IGF-I levels (Peter and Asem, 1996). Finally, other factors, such as tumor necrosis factor alpha (TNFα) have been reported to inhibit IGF-I binding to granulosa cells, however, no effects were detected in actual IGF-I production (Spicer, 1998b).

Intrafollicular Effects of ST and IGF-I

Both ST and IGF-I have potent stimulatory effects at the level of the granulosa cells within ovarian follicles. ST increases the FSH-stimulated differentiation of cultured rat granulosa cells (Gong *et al.*, 1991), and it synergizes with FSH and LH to enhance the effects of both of these gonadotropic hormones within the granulosa cells (Homburg and Ostergaard, 1995). Also, granulosa cells of antral follicles do possess ST receptors, however, those of pre-antal follicles do not, suggesting that the increases in small follicle numbers noticed during ST treatment is not a result of an increase in early follicle activation (Sharara and Nieman, 1994). Also, since ST administration to

granulosa cells collected from women has been shown to have no effect on subsequent steroidal output by these granulosa cells, it is assumed that most of the effects noted with ST treatment are, in fact, mediated through IGF-I (Foster *et al.*, 1995).

The effects of IGF-I on granulosa cells function are similar to those of ST, but perhaps more broad in nature. It is well documented that IGF-I synergizes with available gonadotropins within the follicle, and it is through this mechanism that IGF-I causes most of its ovarian effects (Homburg and Ostergaard, 1995; De La Sota *et al.*, 1996; deMoura *et al.*, 1997; Singh and Armstrong, 1997). It has been shown that granulosa cells from most domestic species have the capacity to produce IGF-I, and also that these granulosa cells possess IGF-I receptors, which would suggest that IGF-I has both paracrine and autocrine functions within the granulosa cells (Xia *et al.*, 1994; Stewart *et al.*, 1997; Sirotkin *et al.*, 1998b).

Since FSH seems to be required for optimal IGF-I production, and since FSH increases IGF-I receptor numbers within the granulosa cells, the interrelationship between FSH and IGF-I is very important in the growth and development of the follicular granulosa cells (Hammond *et al.*, 1988; Samaras *et al.*, 1996; Beam and Butler, 1997; Pawshe *et al.*, 1998). In addition, IGF-I has been shown to increase the number of LH receptors in cultured rat granulosa cells, and this, along with the ability of IGF-I to increase inhibin secretion (De La Sota *et al.*, 1996) may be an important mechanism in the selection of the dominant follicle within a follicular wave (Beam and Butler, 1997). In most domestic species, only the dominant follicle exhibits LH receptors within the granulosa cells (England *et al.*, 1981; Staigmiller, 1982), and since IGF-I and IGF-I receptors are higher in the follicular fluid collected from dominant

follicles, the hypothesis that intrafollicular IGF-I is necessary for the selection of the dominant follicle is supported (Beam and Butler, 1997). However, unlike most domestic species, ovarian follicles in mares have been shown to possess LH receptors from sizes of 5 mm in diameter upwards (Goudet *et al.*, 1999). Since the ovarian follicles of mares grow to very large sizes (>40 mm in diameter) when compared with most other species evaluated, this phenomenon of only dominant follicles possessing LH receptors does not hold true. However, even in mares, those granulosa cells from subordinate follicles have less total number of LH receptors than do those from dominant follicles, which leaves the possibility that intrafollicular IGF-I could play a role in dominant follicle selection in the mare as well (Goudet *et al.*, 1999). Finally, unlike those of LH, FSH receptors are found in all follicles from 0.5 mm in diameter upwards, so the potential effect of IGF-I on dominant follicle selection may be limited to increasing LH receptor numbers rather than an overall increase in both FSH and LH receptors (Findlay, 1994).

Both IGF-I and insulin have mitogenic effects on the growth and development of granulosa cells in mammalian species. IGF-I has been shown to increase DNA synthesis in cultured bovine granulosa cells (Khamsi and Armstrong, 1997), which in turn leads to an increase in cell proliferation. Numerous studies have clearly demonstrated that IGF-I greatly increases granulosa cells proliferation *in vitro* in virtually every farm animal species studied (Xia *et al.*, 1994; Spicer and Echternkamp, 1995; Armstrong *et al.*, 1996b; Campbell *et al.*, 1996; Gutierrez *et al.*, 1997a; Mariana *et al.*, 1998). Furthermore, while insulin has similar effects (Matamoros *et al.*, 1991; Simpson *et al.*, 1994; Whitley *et al.*, 1998), insulin is not as potent an ovarian stimulator

as is IGF-I, probably due to the fact that it appears that both factors work through the type-I IGF-I receptor, which has a greater affinity for IGF-I (Monget and Monniaux, 1995; Gutierrez *et al.*, 1997a; Willis *et al.*, 1998). In addition, when FSH is administered to these *in vitro* cultured granulosa cells, the cellular response is increased by 2- to 3-fold (Xia *et al.*, 1994; Armstrong *et al.*, 1996b; Campbell *et al.*, 1996). Finally, while cumulus cell expansion in mature follicles is generally thought to be FSH-dependent, there is growing evidence that this process is also IGF-I mediated, and therefore, IGF-I is required for proper maturation of the ovarian follicle (Lorenzo *et al.*, 1994; Singh and Armstrong, 1997).

In addition, IGF-I has potent stimulatory actions on steroid production by the granulosa cells, which are, again, augmented by the presence of FSH (Gong *et al.*, 1991; Spicer and Echternkamp, 1995; Armstrong *et al.*, 1996b; deMoura *et al.*, 1997). It has been demonstrated that the addition of IGF-I to the culture medium of caprine (Behl and Pandey, 1999), bovine (Schams *et al.*, 1988; Armstrong *et al.*, 1996b), ovine (Mariana *et al.*, 1998), human (Mason *et al.*, 1993; Foster *et al.*, 1995) and porcine (Howard and Ford, 1994; Xia *et al.*, 1994; Sirotkin *et al.*, 1998a) granulosa cells increases the steroidal output of these cells. In general, both progesterone and estradiol production is enhanced through IGF-I treatment, and insulin can cause similar effects (Spicer, 1998a). It appears that IGF-I increases aromatase enzyme activity within the granulosa cells, and thereby increases estradiol production (De La Sota *et al.*, 1996). In addition, it has been shown that IGF-I increases the production of steroid acute regulatory protein (StAR protein), and in this way augments progesterone production by the granulosa cells (Bao *et al.*, 1998; LaVoie *et al.*, 1999). It is therefore evident that

IGF-I is crucial to maintaining normal granulosa cells proliferation and steroid production to ensure that follicular growth and development proceed in a normal manner.

IGF-I Binding Proteins

In addition to stimulating IGF-I production, ST administration has been shown to increase the production of IGF-I binding proteins (IGFBP) within the follicle (Echternkamp *et al.*, 1994a; Rabinovici *et al.*, 1997). Other factors, including epidermal growth factor (stimulatory) and FSH (inhibitory), affect IGFBP production within the follicle (Mondschein *et al.*, 1990; Yap *et al.*, 1998). These binding proteins are responsible for regulating the amount of available IGF-I in the plasma and follicular fluid, since without them, the IGF-I found in plasma and follicular fluid would be utilized by the various cells throughout the body in a short period of time. IGFBP are therefore used to increase the half-life of circulating IGF-I so that it will be available for longer periods of time. Incidentally, an ST binding protein has also been isolated (Amit *et al.*, 1993). IGFBP are produced within the follicle by both theca and granulosa cells (San Roman and Magoffin, 1992), and they act primarily by binding IGF-I and making it less available for cellular utilization (Samaras and Hammond, 1995), which would block the stimulatory effects of IGF-I (Spicer and Chamberlain, 1999). Other factors involved in regulating IGFBP production are LH and FSH, which seem to increase intrafollicular IGFBP concentrations (Armstrong *et al.*, 1996a). In addition, both IGF-I as well as insulin are potent inhibitors of IGFBP production, with IGF-I having stronger inhibitory actions on IGFBP production than insulin (Poretsky *et al.*, 1996).

Several IGFBP have been identified to date, and they can be classed as follows: (1) small molecular weight IGFBP and (2) large molecular weight IGFBP (Gerard and Monget, 1998). The small molecular weight IGFBP include IGFBP-2, -4 and -5, whereas the large molecular weight IGFBP are limited to IGFBP-3 (Funston *et al.*, 1996). Typically, growing follicles have less amounts of intrafollicular IGFBP than do atretic follicles in both pigs and cattle (Samaras *et al.*, 1993; Stanko *et al.*, 1994; Funston *et al.*, 1996; Stewart *et al.*, 1996). In addition, it appears that the types of IGFBP present are also important, since follicular fluid from dominant follicles usually contain IGFBP-3, with little to no small molecular weight IGFBP present (San Roman and Magoffin, 1993; Echternkamp *et al.*, 1994a; Funston *et al.*, 1996; Gerard and Monget, 1998). This is probably due to the fact that IGFBP-3 tends to bind IGF-I less tightly than the smaller molecular weight IGFBP, and therefore more IGF-I would be bioavailable in large, dominant follicles for the stimulation of the granulosa cells (Mondschein *et al.*, 1991; Echternkamp *et al.*, 1994b; Thierry Van Dessel *et al.*, 1996).

It has been suggested that decreasing concentrations of IGFBP, which in turn increases bioavailable IGF-I, thereby increasing the number of LH receptors within the granulosa cells, is necessary for the establishment of follicular dominance (Stewart *et al.*, 1996; Zhou *et al.*, 1996). In addition, IGFBP appear to play a prominant role in follicular atresia (De La Sota *et al.*, 1996). It has been hypothesized that the onset of folliclular atresia may be determined more by changes in intrafollicular IGFBP concentrations than by absolute levels of IGF-I (Monget *et al.*, 1993). This is supported by the finding that, during follicular atresia, the ratio of IGFBP to IGF-I changes, and that less IGF-I is available for granulosa cells stimulation (De La Sota *et al.*, 1996).

Also, it is known that FSH stimulates proteases designed to break down IGFBP, which may be one mechanism of avoiding follicular atresia (Erickson *et al.*, 1994). Further studies are needed to more clearly define the precise role of IGFBP in ovarian follicular dynamics.

IMPLICATIONS OF SOMATOTROPIN TREATMENT IN THE MARE

Seasonality

The reproductive cycle in the domestic mare is seasonal in nature, with peak breeding activity coinciding with periods of increasing daylength (summer), while during reduced daylength conditions, mares typically undergo a period of reproductive inactivity termed "seasonal anestrous" (Ginther, 1992). The term "anestrous" is not entirely accurate, since sporatic exhibitions of behavioral estrus are often detected in these anovulatory mares (Thompson *et al.*, 1983a). However, this is currently the most widely accepted term used to describe this part of the mare's seasonal cycle. Interestingly, although the majority of mares do undergo this anovulatory state, up to 25% of mares actually continue reproductive cyclicity complete with an ovulatory response virtually year-round (Hughes *et al.*, 1972). The current theory for this winter inactivity in the mare is that, when daylength is decreased, melatonin production from the pineal increases, and this increase in melatonin production decreases GnRH production and secretion from the hypothalamus (McKinnon and Voss, 1993).

However, it should be noted that, to date there has been no direct evidence linking melatonin to GnRH production or secretion (McKinnon and Voss, 1993). It is known, though, that hypothalamic GnRH is severely decreased or absent in the mare during the winter, and this is the direct cause of the anovulatory season (Hart *et al.*,

1984). It has been shown that ovarian follicular development and ovarian size is dramatically decreased in seasonally anovulatory mares, and this can be directly correlated to decreased GnRH output from the hypothalamus (Hart *et al.*, 1984). That actual daylength is involved in this process is evidenced by the fact that exposure of seasonally anovulatory mares to artificial lighting regimes (~16 hours of light per day) can hasten the onset of reproductive cyclicity within a relatively short period of time (Kooistra and Ginther, 1975; Sharp *et al.*, 1975; Oxender *et al.*, 1977; Nequin *et al.*, 1989). This occurs presumably through increased hypothalamic GnRH, which then stimulates both LH and FSH production, and this aids in restoring reproductive function (Cleaver *et al.*, 1991).

Since most of the major breed registries require a mandatory birthdate of January 1 for foals born within a year in the United States, along with the fact that the gestation length in the domestic mare is ~11 months, it is of importance to the commercial breeder to have mares cycling in February, which is during the seasonal anovulatory state for most mares in North America. Artificial lighting regimens are the most repeatable method of inducing seasonally anovulatory mares to begin reproductive cyclicity (Ginther, 1992). However, this method of inducing cyclicity in the mare is labor intensive, expensive and time consuming, since it takes 60 to 70 days from the onset of treatment with increasing light exposure until ovulation is achieved (Ginther, 1992).

For this reason, GnRH treatment to these anovulatory mares has become an intense area of study in recent years. It has been demonstrated that continuous infusion with GnRH can induce ovulation in up to 50% of seasonally anovulatory mares within 4

weeks (Hyland *et al.*, 1987). However, in all studies to date, the ovulatory response in seasonally anovulatory mares treated with GnRH has been shown to be highly variable, and that either continuous infusion, multiple daily injections or long-term subcutaneous depots are required for any response at all (Johnson, 1986; Hyland *et al.*, 1987; Johnson, 1987; Ginther and Bergfelt, 1990; Fitzgerald *et al.*, 1993). The variability exhibited in the ovulatory response to these different GnRH therapy regimes is probably primarily reflected by the state of anestrous that the mares in question are actually in, since mares in a deeper state of anestrous, as determined by plasma progesterone concentrations as well as ovarian follicular activity, tend to be less responsive to GnRH treatment (Fitzgerald *et al.*, 1993; Mumford *et al.*, 1994; Hyland *et al.*, 1987).

The ability of GnRH to stimulate both LH and FSH production is critical to its ability to promote ovulation in some seasonally anovulatory mares (McKinnon and Voss, 1993). It is well known that pituitary and serum concentrations of LH (Hart *et al.*, 1984; Thompson *et al.*, 1986b), as well as serum levels of FSH (Alexander and Irvine, 1991), are reduced during the winter months in mares, and that GnRH can increase both LH and FSH output by the pituitary (Thompson *et al.*, 1986a ; Johnson, 1987). It is this action of GnRH that allows for the resumption of reproductive cyclicity in seasonally anovulatory mares treated with GnRH or one of its more potent agonists.

It is known that prolactin is also decreased during the winter months (Thompson *et al.*, 1986b), and that treatment of anovulatory mares with prolactin can hasten the date of the first ovulation in these mares, possibly through an increase in LH receptors among the granulosa cells (Thompson *et al.*, 1997). It is possible that, through a similar mechanism, treatment of anovulatory mares with ST could enhance the response of

these mares to GnRH therapy by increasing intrafollicular IGF-I concentrations, which could in turn increase LH and FSH receptor production on the granulosa cells (deMoura *et al.*, 1997). Since it is well documented that ST administration significantly increases the response of both cattle and women to available gonadotropins, this could prove to be a viable method of reducing the variability noticed with traditional GnRH treatment to seasonally anovulatory mares, and it could make this type of treatment regimen economically viable for the commercial horse breeder.

OBJECTIVES

The objectives of the present study were, therefore, to: (1) determine the effects of ST administration to both cyclic as well as seasonally anovulatory mares, (2) determine if ST administration to seasonally anovulatory mares receiving GnRH treatment would enhance the ovulatory response over that of GnRH treatment alone, (3) determine if a repeatable protocol, including ST administration, could be developed for the stimulation and subsequent collection of viable oocytes from the ovaries of cyclic mares for use in *in vitro* fertilization treatments and (4) determine the mechanism of action of ST administration to stimulate ovarian follicular development.

CHAPTER II

THE EFFECTS OF EQUINE SOMATOTROPIN (eST) ON OVARIAN STIMULATION AND SYSTEMIC HORMONE CONCENTRATIONS IN CYCLIC MARES TREATED AT TWO STAGES OF THE ESTROUS CYCLE

INTRODUCTION

In recent years, the role of somatotropin (ST) and its effects on ovarian function has become a major area of interest to reproductive physiologists studying follicular development. However, little research has been reported defining the function of ST in reproduction in the cyclic mare. Short-term daily ST secretion in mares is pulsatile in nature, much like that of other species (Thompson *et al.*, 1994). With a large variation exhibited in the quantity and in the number of daily pulses of ST secreted among individual mares, frequent blood sampling over an extended period of time is needed to establish baseline secretion patterns for an individual animal (Thompson *et al.*, 1992;1994). Several studies in the human and the cow suggest that ST may have direct effects on ovarian activity based on the identification of ST receptors and binding proteins (BP) in the ovary (Spicer and Enright, 1991; Amit *et al.*, 1993; Cohick *et al.*, 1996). Also, it has been demonstrated that ST administration increases circulating levels of IGF-I in cattle, pigs, horses and women (Owen *et al.*, 1991a; Spicer *et al.*, 1992; European and Australian Multicenter Study, 1995; Buonomo *et al.*, 1996). Furthermore, treatment with ST has been shown to affect follicular dynamics and estradiol and progesterone secretion patterns in cattle, pigs and humans (Owen *et al.*, 1991a; Rieger *et al.*, 1991; Spicer *et al.*, 1992; 1993; Lucy *et al.*, 1994).

It is well known that ST mediates many of its effects through insulin-like growth factor-I (IGF-I), and apparently it is through this mechanism that growth hormone alters

ovarian function (Spicer and Echternkamp, 1995). Since IGF-I levels can be determined with much less frequent blood sampling, it may be more feasible to monitor circulating IGF-I rather than ST in mares. Also, follicular fluid from larger follicles tends to have higher concentrations of IGF-I, and this IGF-I could play an important role in the selection of dominant follicles, as has been suggested in humans and in cattle (Rabinovici *et al.*, 1990; Spicer and Enright, 1991).

The purpose of this experiment was to (1) determine if treatment with exogenous equine somatotropin (eST) would increase the number of small- and/or medium-size follicles on the ovaries of cyclic mares and (2) to determine the effects of eST treatment on circulating levels of LH, estradiol, progesterone and IGF-I in cyclic mares.

MATERIALS AND METHODS

Experimental Design

A group of mixed-breed horse and pony mares (n=20), maintained on bermudagrass pasture and in good body condition (body condition scores ranged from 5 to 8 on a scale of 1 to 9), was selected for this experiment. Experimental animals were randomly allocated to one of four treatment groups during the breeding season of 1996 (June through July). Since mares were to be monitored over parts of two successive estrous cycles, the estrous cycles were designated as "T" for the treated cycle and "P" for the post-treated cycle. Treatment A consisted of mares (n=8) each receiving an intramuscular (i.m.) injection of 25 μg/kg of body weight of equine somatotropin (eST, BresaGen Ltd., Thebarton, South Australia) daily beginning on day T6 and ending on day T12. The time of treatment for this group was determined so that elevated plasma

ST and/or IGF-I levels would be present at the time of follicular wave emergence, which is at approximately day 9 post-ovulation. The ovaries of these mares were monitored daily by ultrasound to determine the day of ovulation (day of ovulation = day T0) and then were monitored every other day beginning on the first day of treatment (day T6) and ending on the subsequent day of ovulation (day P0) using an Aloka 500-V ultrasound unit with a 5 MHz rectal probe (Corometrics Medical Systems, Wallingford, Connecticut). A blood sample was collected from each of the mares in this group at 24-hour intervals during the same time period. The blood samples were collected on ice using sterile glass collection tubes (Monoject Vacutainers®, Sherwood Medical, St. Louis, Missouri). Treatment B consisted of mares (n=8) each similarly receiving an i.m. injection of eST daily beginning on day T13 and ending on day T19. This time period was used so as to coincide with divergence of the dominant follicle from the subordinate within the ovulatory follicular wave, which occurs at approximately day 14 post-ovulation. Blood sample collection and ultrasonographic monitoring of ovarian structures were performed on these mares beginning on day T13 and ending on day P5.

Mares randomly assigned to Treatments C and D (n=2/treatment) were used as controls, and were administered an i.m. injection of a sodium borate vehicle equal in volume to that containing the eST given to mares in Treatments A and B. Follicle growth and ovulation in control mares were monitored by ultrasonography and blood sample collection was performed in the same manner as the mares allotted to Treatments A and B, respectively.

The crystalline eST was reconstituted in sterile water at a concentration of 2.5 mg/ml. Injection sites were rotated daily on opposite sides of the neck region. The volume of each injection ranged from 2.8 to 5.7 ml, depending upon the body weight of the mare (25 μg/kg of body weight).

Ultrasound prints (Sony®, Model No. UP850 thermal printer, Tokyo, Japan) of the ovaries of each mare were collected to monitor follicular development. Each ovary was scanned from dorsal to ventral and from lateral to medial sides, and still prints were taken each time a follicle was present at its maximum diameter. Each follicle was then identified, measured and placed into one of five follicle classification categories, based on its diameter (Category I = ≤7 mm, II = 8 to 16 mm, III = 17 to 24 mm, IV = 25 to 32 mm and V = >32 mm).

Hormone Assays

Blood samples were centrifuged at 300 x g for 10 minutes, and the plasma was then stored in individually-labeled 7 ml plastic tubes (Curtin Matheson Scientific, Inc., Houston, Texas) and frozen within 30 minutes of collection. Validated radio-immunoassay (RIA) (Sticker *et al.*, 1995) was performed on the plasma samples collected from days 1, 2, 3, 5 and 7 of treatment, the day of the onset of standing estrus and the day of ovulation to determine circulating IGF-I levels for each mare. Also, LH (Thompson *et al.*, 1983) and estradiol-17β (Diagnostic Systems Laboratories, Webster, Texas) levels were determined from samples collected on day P0 and for the 8 days prior to day P0 (days –8 through P0) for Treatments A and C, or day -8 to day P5 for Treatments B and D.

In addition, progesterone (Diagnostic Systems Laboratories) concentrations were assayed from plasma samples collected every other day beginning on day T6 and ending on the first day of standing estrus (Treatments A and C) or on days P1 to P5 (Treatments B and D). The selection of these treatment days was determined based on cyclic patterns of endogenous secretion of these hormones by mares previously monitored at this laboratory, and to determine if exogenous eST treatment would affect the circulating plasma levels of progesterone, estradiol, LH and IGF-I in cyclic mares.

Statistical Analyses

Total number of follicles present in each follicle size category for each mare was obtained from the ultrasound prints and analyzed across treatment groups using analysis of variance (ANOVA). The mean follicle diameter within each size category for each mare and the number of ovulations per mare were also analyzed using the same ANOVA procedure. Hormone levels obtained for each mare from RIA were analyzed across treatment groups (Treatments A *vs.* C and Treatments B *vs.* D, respectively) using a split-plot ANOVA procedure.

RESULTS

Effect on Follicle Populations

The results of treatment with exogenous eST on follicle populations in cyclic mares are shown in Tables 2.1 through 2.3. In this study, eST administration did not significantly alter the mean number of follicles ≥8 mm per mare in cyclic mares treated daily either on days T6 through T12 (Treatment A) or on days T13 through T19 (Treatment B) when compared with corresponding control mares (Treatments C and D) on day 7 of treatment, days 3 and 7 post-treatment, or on the first day of standing estrus.

However, mares administered daily eST early in the estrous cycle (Treatment A) had a significantly greater number of follicles ≤7 mm in diameter ($P<0.05$) when compared with control mares (Treatment C) on days 3 and 7 post-treatment and at the onset of behavioral estrus (mean number of follicles per mare [± SEM] of 12.9, 12.8, 13.0 [± 1.68] *vs.* 5.5, 5.5, 4.0 [± 3.36], respectively).

Correspondingly, daily treatment with eST to mares on days T13 through (Treatment B) did not result in increased numbers of follicles within follicle size categories over that of control mares (Treatment D). In addition, the mean number of follicles per size category per mare ≥8 mm in diameter was not different among treatment and control groups across all days evaluated in this study. Furthermore, neither the mean follicular diameter for follicles within each size category nor the number of ovulations per mare was affected by treatment with eST or the carrier vehicle.

Hormone Profiles

Plasma hormone profiles from this study are presented in Figures 2.1 through 2.8. In summary, eST treatment did not affect plasma progesterone concentrations in either eST treatment group (Treatments A and B) compared with control mares (Treatments C and D) for any of the time periods monitored in this study (a total of 6 days for Treatments A and C and 5 days for Treatments B and D). Also, circulating estradiol levels were not significantly different from day -8 to day P0 in Treatments A and B when compared with respective control groups (Treatments C and D). However, on days P1 through P5 mares in Treatment B had significantly lower plasma estradiol concentrations ($P<0.05$) than control mares in Treatment D (0.21, 0.24, 0.07, 0.13 and

0.31 [± 0.33] pg/ml per mare compared with 4.6, 2.6, 2.3, 4.1 and 1.9 [± 0.61] pg/ml per mare, respectively). While the number of follicles per mare was not significantly different between these two groups, it can be speculated that secondary follicles present in the emerging follicular wave may have contributed to this increase in estradiol concentrations post-ovulation in the control group. Mares in Treatments A and C were not monitored past day P0, and therefore plasma estradiol concentrations during this time for these mares are not known.

In addition, plasma LH concentrations were significantly lower on day -7 through day -1, but not on day -8 (pre-ovulation of the treated cycle) or day P0 for mares in Treatment A compared with control mares in Treatment C (0.36, 0.40, 0.34, 0.67, 1.6, 3.6 and 6.0 [± 0.9] ng/ml per mare *vs.* 5.3, 7.5, 8.1, 7.7, 9.6, 9.5 and 10.5 [± 1.8] ng/ml per mare, respectively). However, this may have been due to the fact that one of the control mares had unusually high plasma LH concentrations during this time peroid. Also, there was no detectable difference in circulating LH levels in mares from Treatment B when compared with mares from Treatment D on day -8 pre-ovulation through day P5.

Finally, there was a significant increase in plasma IGF-I concentrations for mares treated with eST by day 3 of treatment when compared with those for control mares (144.99 [± 12.77] ng/ml *vs.* 64.19 [± 25.55] ng/ml for Treatments A and C, respectively and 179.02 [± 16.02] ng/ml *vs.* 101.08 [± 32.03] ng/ml for Treatments B and D, respectively). Increased IGF-I levels were not evident by the first day of standing estrus in mares in Treatment A compared with mares in Treatment C. However, eST treated mares in Treatment B, unlike control mares in Treatment D, continued to have greater circulating IGF-I levels until the time of ovulation.

Table 2.1. Mean number of follicles within size category per mare per treatment group on day 3 post-treatment

Treatment	≤7 mm	8 to 16 mm	17 to 24 mm	25 to 32 mm	≥32 mm
A. eST	12.9[a] (±1.7)	8.0 (±1.7)	2.8 (±1.7)	0.9 (±1.9)	0.6 (±2.1)
C. Control	5.5[b] (±3.4)	6.5 (±3.4)	3.0 (±3.4)	0.5 (±4.8)	0.5 (±4.8)
B. eST	10.3 (±1.7)	7.8 (±1.7)	2.5 (±1.7)	0.6 (±2.8)	0.1 (±4.8)
D. Control	11.0 (±3.4)	4.5 (±3.4)	1.5 (±3.4)	0.0	1.0 (±3.4)

[a,b]Means within columns with different superscripts are different ($P < 0.05$).

Table 2.2. Mean number of follicles within size category per mare per treatment group on day 7 post-treatment

Treatment	≤7 mm	8 to 16 mm	17 to 24 mm	25 to 32 mm	≥32 mm
A. eST	12.8[a] (±1.7)	8.5 (±1.7)	1.4 (±1.9)	0.6 (±2.8)	0.4 (±2.8)
C. Control	5.5[b] (±3.4)	8.0 (±3.4)	3.5 (±3.4)	0.5 (±4.8)	1.0 (±3.4)
B. eST	11.8 (±1.7)	8.6 (±1.7)	1.1 (±2.4)	0.1 (±4.8)	0.3 (±3.4)
D. Control	12.5 (±3.4)	3.0 (±3.4)	0.0	0.0	1.0 (±3.4)

[a,b]Means within columns with different superscripts are different ($P < 0.05$).

Table 2.3. Mean number of follicles within size category per mare per treatment group on the day of the onset of standing estrus

Treatment	≤7 mm	8 to 16 mm	17 to 24 mm	25 to 32 mm	≥32 mm
A. eST	13.0[a] (±1.7)	7.6 (±1.7)	3.3 (±1.8)	0.9 (±1.9)	0.5 (±2.4)
C. Control	4.0[b] (±3.4)	7.0 (±3.4)	3.5 (±3.4)	1.0 (±3.4)	0.5 (±4.8)
B. eST	7.6 (±1.9)	7.0 (±1.9)	3.6 (±2.1)	0.7 (±2.4)	0.7 (±2.1)
D. Control	8.0 (±3.4)	5.5 (±3.4)	1.5 (±3.4)	0.0	1.0 (±3.4)

[a,b]Means within columns with different superscripts are different ($P < 0.05$).

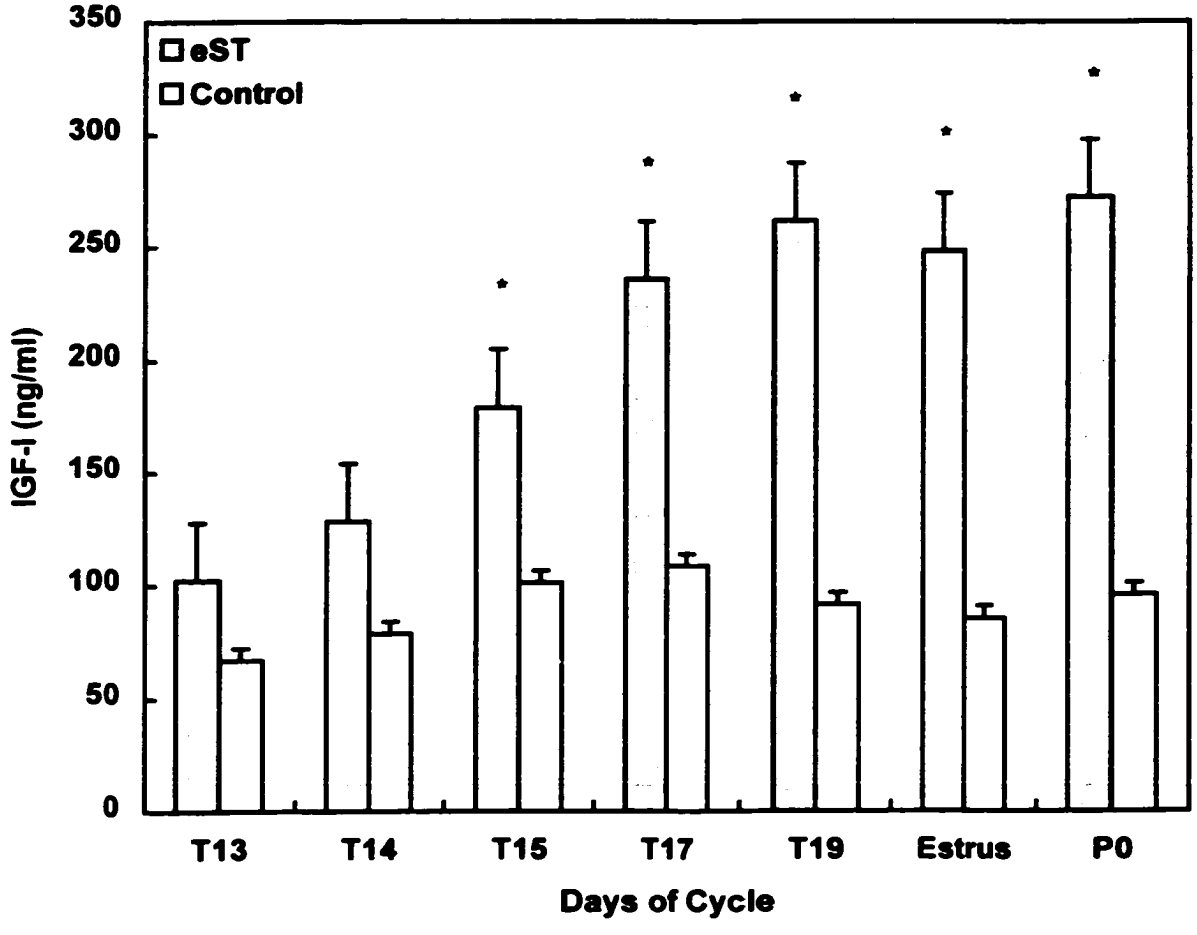

Figure 2.1. Plasma progesterone concentrations (±SEM) for mares treated with eST early in the estrous cycle (Treatments A and C). T = day of treatment cycle.

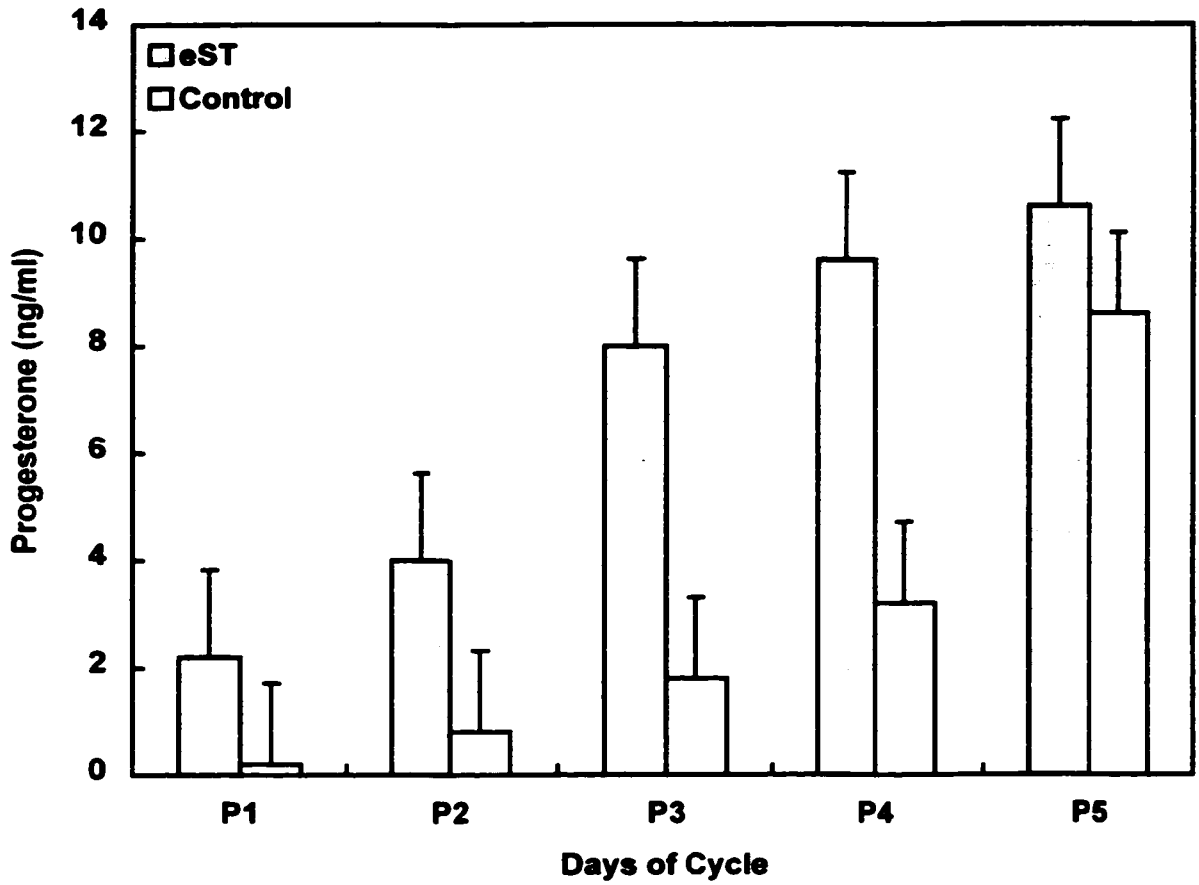

Figure 2.2. Plasma progesterone concentrations (±SEM) for mares treated with eST late in the estrous cycle (Treatments B and D). P = day of post-treatment cycle and P1 = day 1 of post-treatment cycle.

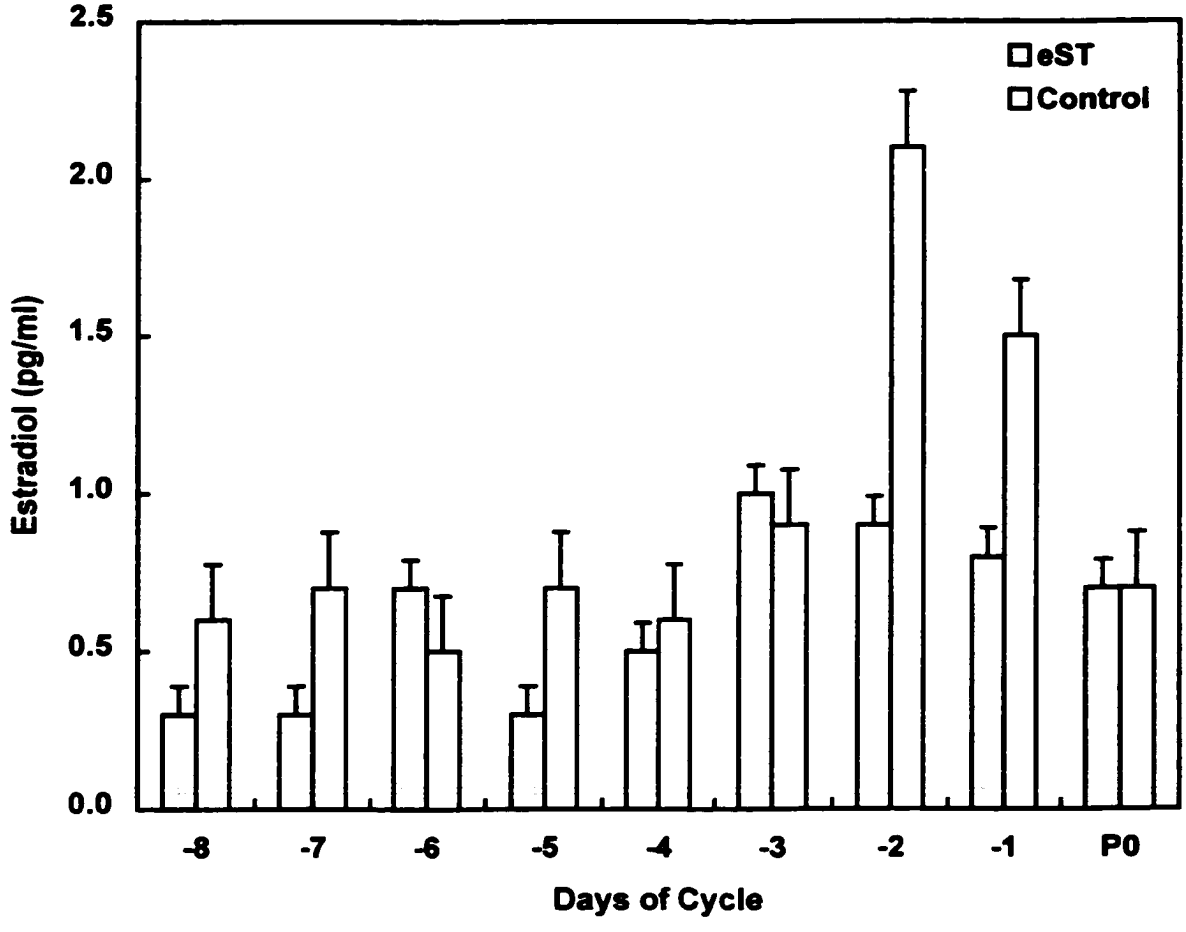

Figure 2.3. Plasma estradiol concentrations (±SEM) for mares treated with eST early in the estrous cycle (Treatments A and C). P0 = day of ovulation of the post-treatment cycle.

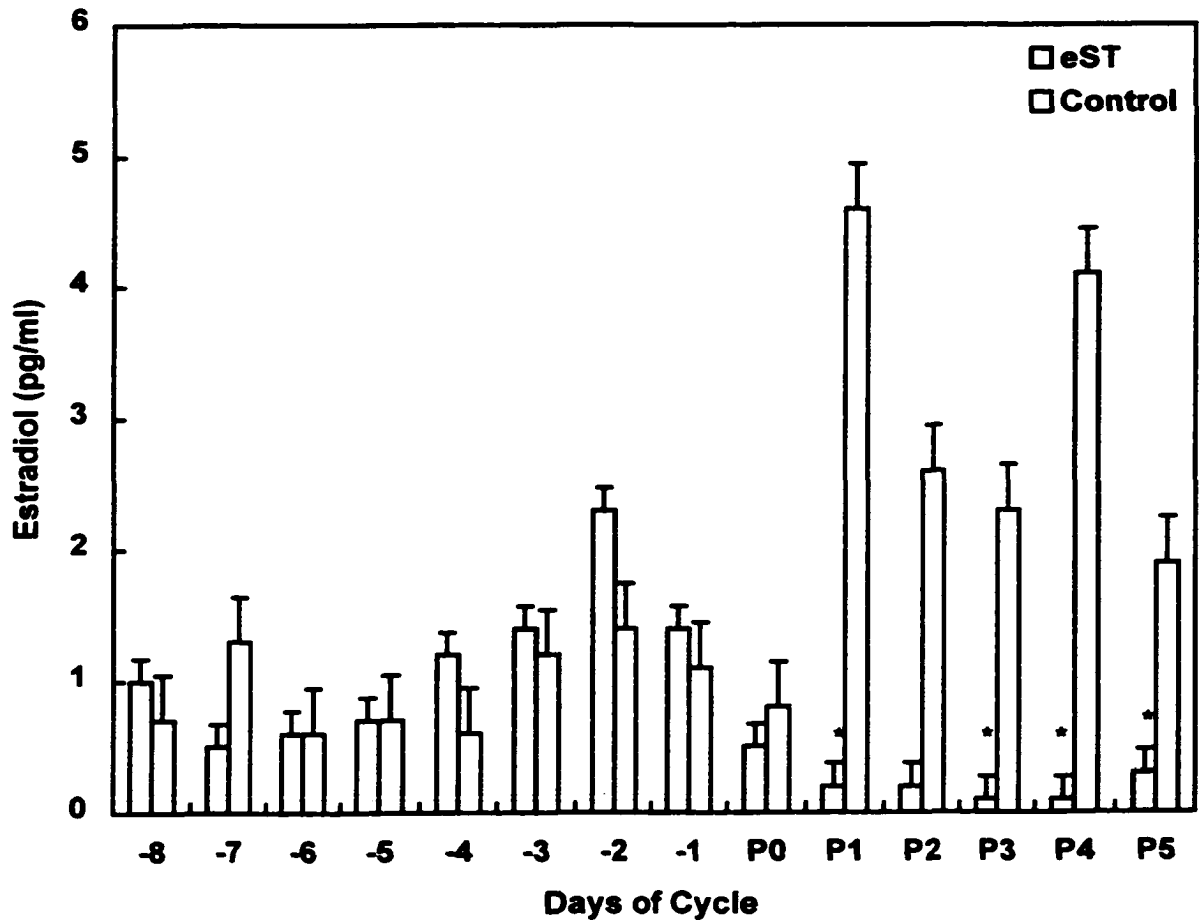

Figure 2.4. Plasma estradiol concentrations (±SEM) for mares treated with eST late in the estrous cycle (Treatments B and D). P = day of post-treatment cycle and P0 = day of ovulation of the post-treatment cycle. (*) Denotes significant difference within days.

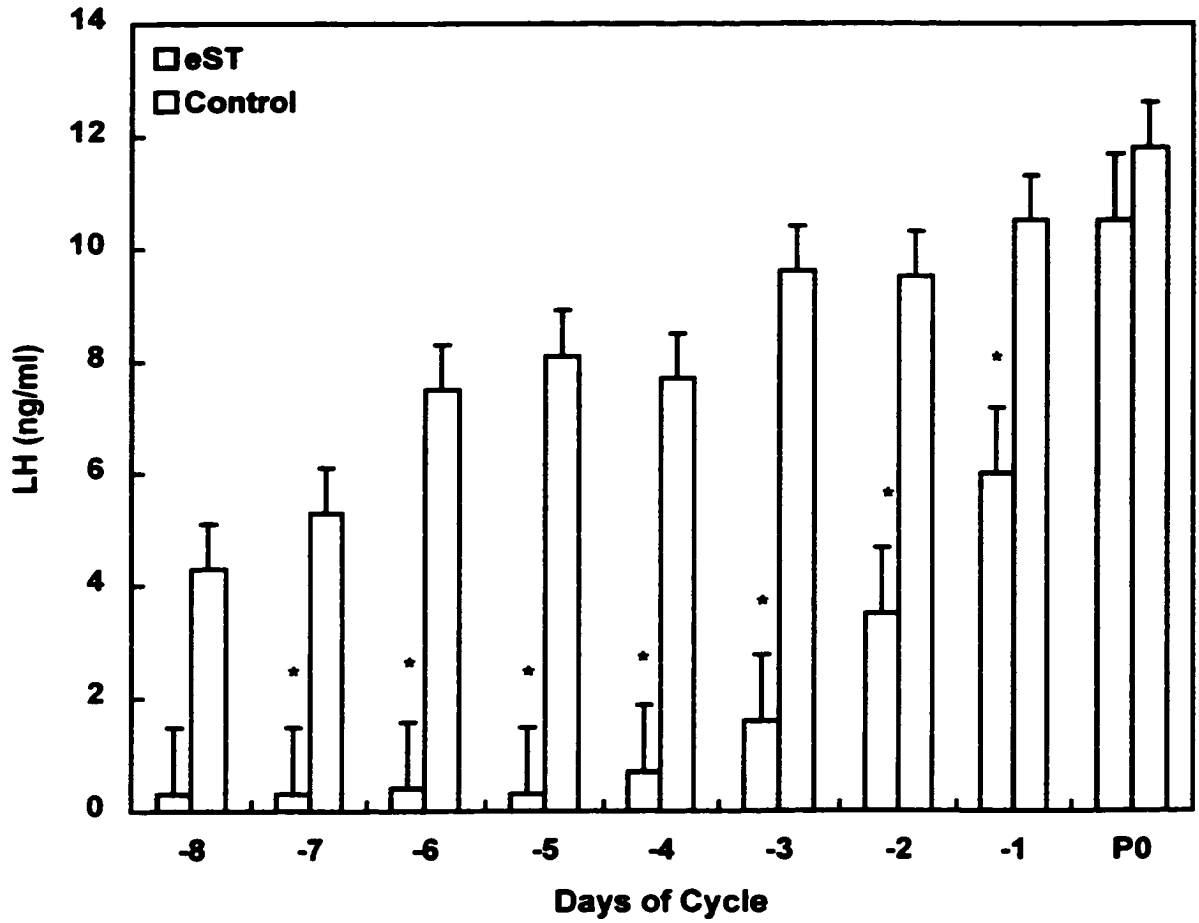

Figure 2.5. Plasma LH concentrations (±SEM) for mares treated with eST early in the estrous cycle (Treatments A and C). P0 = day of ovulation of the post-treatment cycle. (*) Denotes significant difference within days prior to ovulation.

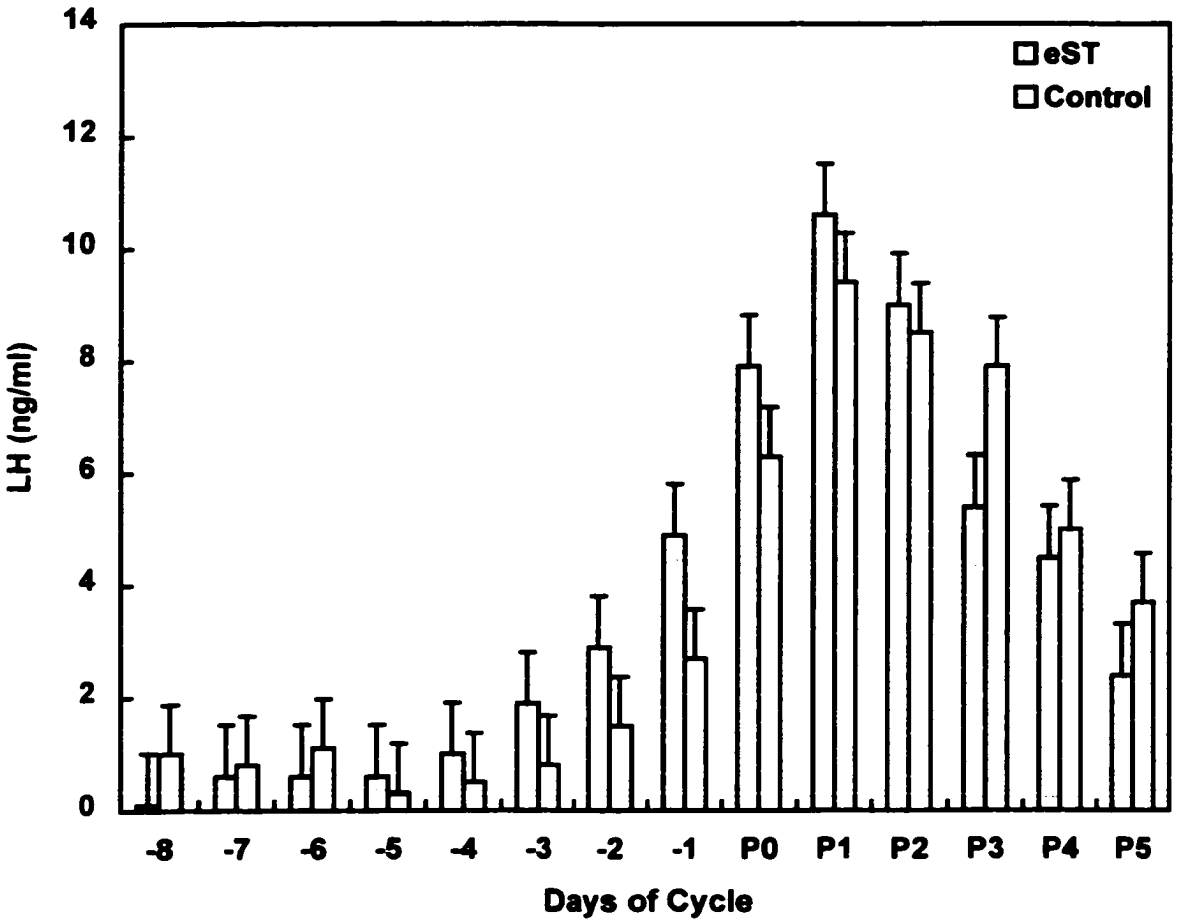

Figure 2.6. Plasma LH concentrations (±SEM) for mares treated with eST late in the estrous cycle (Treatments B and D). P = day of post-treatment cycle and P0 = day of ovulation of the post-treatment cycle.

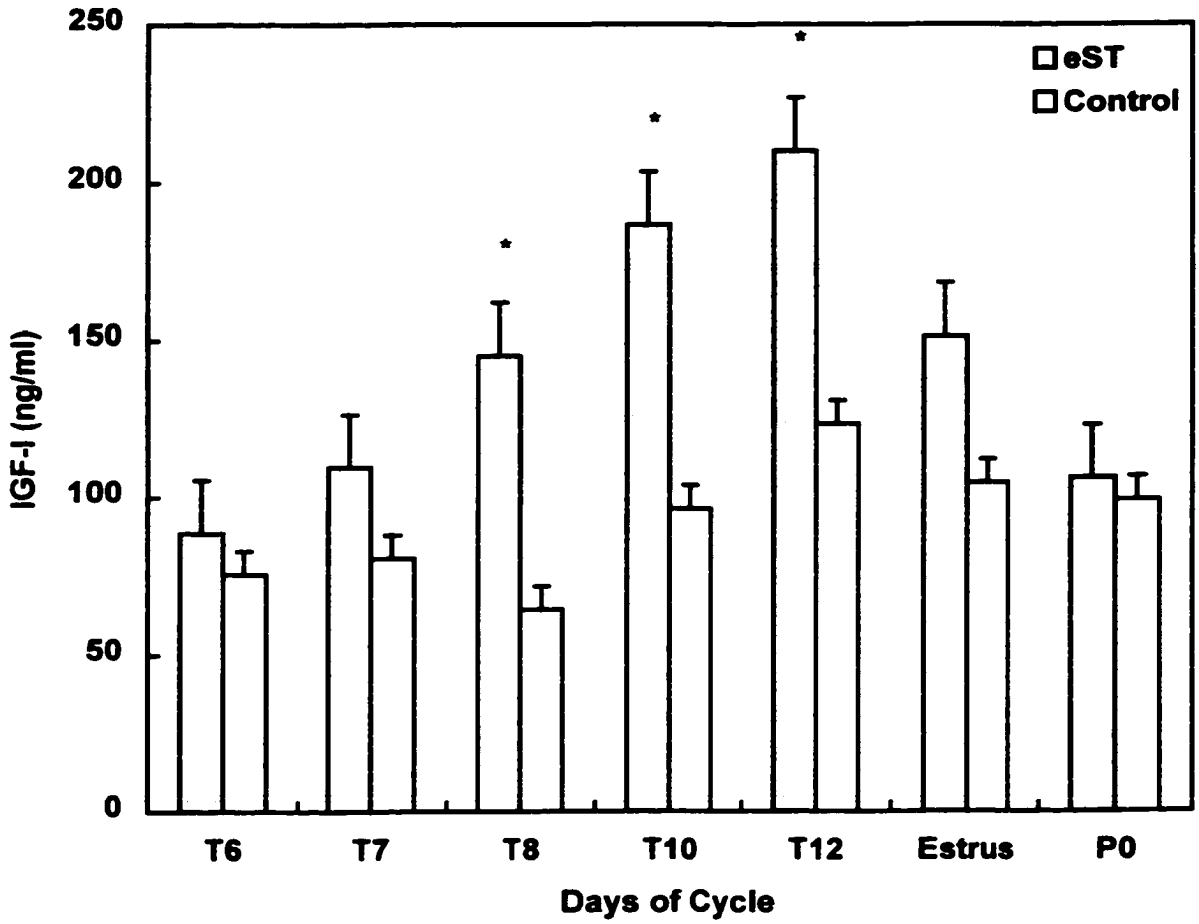

Figure 2.7. Plasma IGF-I concentrations (±SEM) for mares treated with eST early in the estrous cycle (Treatments A and C). T = day of treatment cycle and P0 = day of ovulation of post-treatment cycle. (*) Denotes significant difference within days.

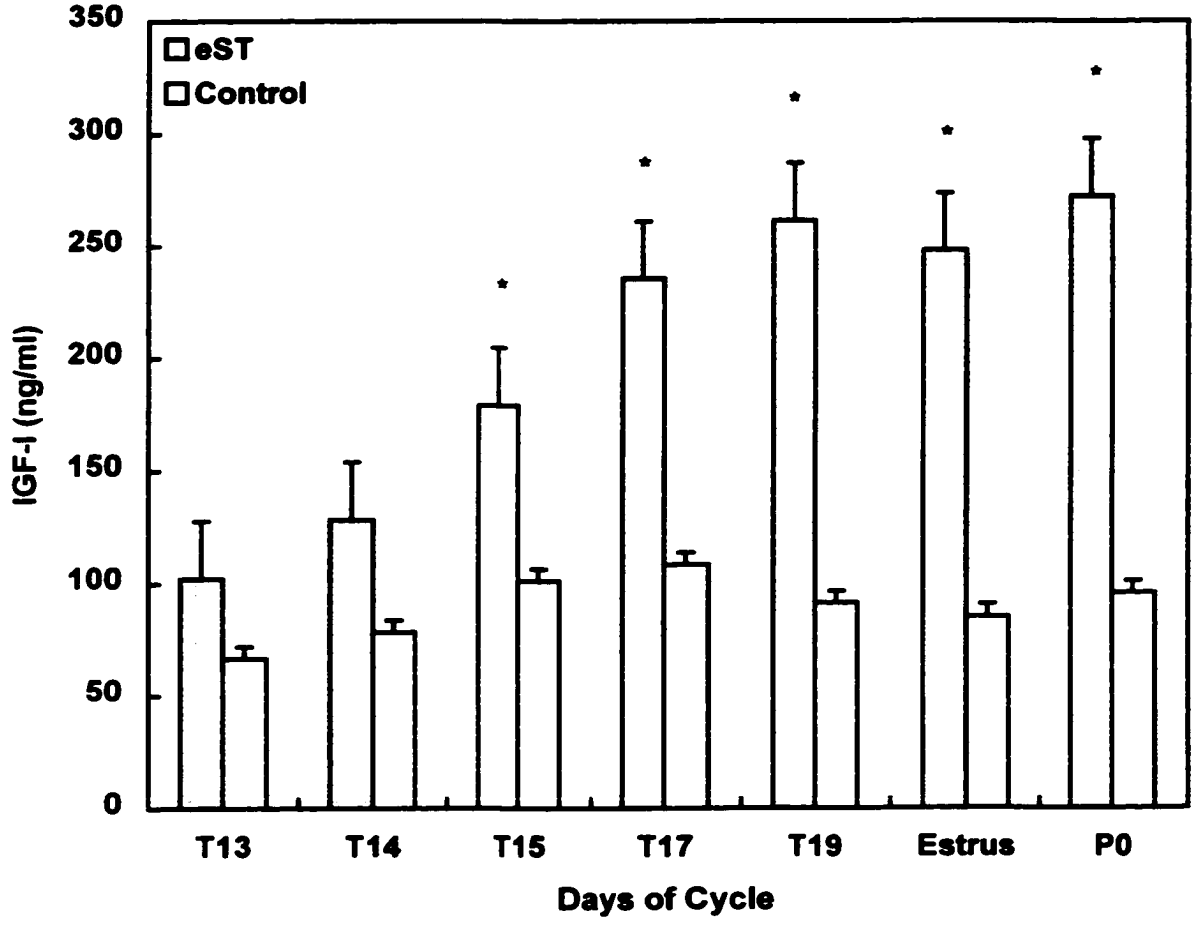

Figure 2.8. Plasma IGF-I concentrations (±SEM) for mares treated with eST late in the estrous cycle (Treatments B and D). T = day of treatment cycle and P0 = day of ovulation of the post-treatment cycle. (*) Denotes significant difference within days.

DISCUSSION

Early follicular dynamics in the equine species is not well understood when compared with other domestics species. Currently, it is believed that follicles ≤15 mm are growing and/or regressing in a continuous fashion, in order to provide a reserve for the selection of the larger follicles. The phenomenon of follicular waves has been documented in the mare, with mares exhibiting either 1 or 2 waves per interovulatory interval. In mares with 2 follicular waves, the first wave emerges at or around day –1 (day 0 = day of ovulation) and peaks at day 10. The second follicular wave (as well as the first follicular wave in mares with only 1 wave per interovulatory interval) emerges at approximately day 10 and ends with ovulation of the dominant follicle of that wave (for review see Ginther, 1992). Further studies are needed in order to develop a better understanding of early follicular dynamics in the horse.

In the present study, we have shown that treating cyclic mares with eST increases the number of small follicles present on the ovaries on days 3 and 7 post-treatment and at the onset of standing estrus (post-treatment). This finding is similar to the ovarian response of swine, in which ST administration has been shown to increase either small- (Buonomo *et al.*, 1996) or medium-sized follicles (Echternkamp *et al.*, 1994a). Also, Hereford x Friesian crossbred heifers treated with recombinant bovine somatotropin (rbST) responded by increasing the number of small, but not medium or large follicles (Gong *et al.*, 1991). This suggests that ST, either through IGF-I or directly, may play a more important role in follicle recruitment than in follicle growth (Buonomo *et al.*, 1996). It should be noted that Spicer and Enright (1991) have shown that treatment of Hereford x Friesian heifers with ST releasing factor increased the mean diameter of large follicles. In the present study, however, there was no increase in the mean diameter of follicles within any of the follicle size categories evaluated.

A multitude of studies have evaluated the effects of ST on both systemic and intraovarian IGF-I production and the possibility of direct effects of ST on ovarian

function. It has been well established that ST administration increases both circulating and follicular fluid levels of IGF-I in cattle, pigs and humans (Mason *et al.*, 1990; Rieger *et al.*, 1991; European and Australian Multicenter Study, 1995), and ST has also been shown to increase plasma IGF-I in horses (Buonomo *et al.*, 1996). Also, a positive correlation between serum and IGF-I levels in follicular fluid has been established, with the higher circulating IGF-I levels serving as one source of ovarian IGF-I, by diffusing from circulation into the follicles (Amit *et al.*, 1993).

In the present study, plasma IGF-I levels for mares treated with eST did not rise above that of control mares until the third day of treatment with eST. Correspondingly, Owen *et al.* (1991b) noted in women that serum IGF-I levels did not rise until 2 days after the onset of ST administration, and it was not until 3 to 4 days after the final injection of ST that these IGF-I levels began to return to normal baseline levels, which is in agreement with our findings on circulating IGF-I levels in cyclic mares.

It has also been proposed that the effects of ST on ovarian activity are not a result of higher IGF-I levels, but are, instead, indicative of a direct effect of ST at the level of the ovary (Gong *et al.*, 1991; Spicer *et al.*, 1992; Cohick *et al.*, 1996). This hypothesis is supported by the data of Cohick *et al.* (1996), who detected ST receptors within the ovary in prepubertal beef heifers. Cohick *et al.* (1996) have also reported that immunizing these heifers against ST releasing factor resulted in a change in follicular development between 3 and 6 months of age, which caused the heifers to reach puberty at an older age (>14 months). Also, human pre-ovulatory follicles have been found to contain ST binding proteins, lending more evidence to a direct effect of ST on ovarian function (Owen *et al.*, 1991a). Spicer and Enright (1991) found that administration of ST releasing factor to Hereford-Friesian crossbred heifers did not increase follicular fluid IGF-I, providing further evidence that ST may be the mediator of the increase in follicular activity noted with ST administration. Based on these

findings, it is most probable that ST acts both directly on the ovary and indirectly through IGF-I.

One very important mode of action of ST on reproduction is its ability to act synergistically with gonadotropic hormones to stimulate ovarian activity. Studies in women undergoing ovarian stimulation protocols have shown that administration of ST in concert with gonadotropins can either increase the ovarian response and/or lower the dose of gonadotropins needed to stimulate the ovaries in some women (Homburg *et al.*, 1988; Gong *et al.*, 1991; Hugues *et al.*, 1991; Owen *et al.*, 1991b; Bergh *et al.*, 1994). For example, two studies have reported that treatment with ST increased the number of fertilized oocytes recovered from superovulated women (Owen *et al.*, 1991b; Bergh *et al.*, 1994), while one study indicated that ST did not increase the overall number of oocytes collected from superovulated women, but did increase the number of oocytes recovered from women with polycystic ovaries (European and Australian Multicenter Study, 1995). In contrast, another study indicated no difference in the amount of gonadotropins needed to stimulate ovarian activity in women treated with ST compared with those not receiving ST (Tapanainen *et al.*, 1992).

While systemic IGF-I is not affected by treatment with gonadotropins, it is interesting to note that steroidogenic activity within the ovary can be modified by exogenous ST and/or IGF-I (Adashi *et al.*, 1985). It has been shown that IGF-I can enhance FSH activity on bovine and porcine granulosa cells in culture (Spicer *et al.*, 1992). Furthermore, it has been hypothesized that ST/IGF-I acts in concert with FSH and possibly insulin to induce its stimulatory effects on the ovary (Gong *et al.*, 1991). This could be one mechanism for selection of follicle dominance, since the higher levels of IGF-I in large follicles could serve to stimulate their response to available FSH and thereby enable the follicles to develop at a greater rate than subordinate follicles.

The effects of ST and IGF-I on blood levels of estradiol, progesterone, LH and FSH have been reported for cattle and humans (Gong *et al.*, 1991; Tapanainen *et al.*,

1992; Lucy *et al.*, 1994). In dairy heifers and in women, ST treatment has been shown to decrease serum levels of estradiol and while increasing serum IGF-I levels (Rabinovici *et al.*, 1990; Tapanainen *et al.*, 1992). This is similar to our finding in cyclic mares, where ST-treated mares also had lower circulating estradiol levels on days 1 to 5 post-ovulation. The finding that the control mares had higher circulating estradiol levels post-ovulation than pre-ovulation was unexpected, and it can be speculated that this may be due, in part, to the presence of secondary follicles in the newly emerging follicular wave. Why exogenous ST decreases circulating estradiol concentrations is unclear, but it could involve a reduction in the ability of the ovarian follicles to convert progestins to androgens and subsequently to estrogens.

Treatment with bST has been shown to increase plasma progesterone levels in Holstein heifers, and IGF-I increases progesterone production in both cattle and pigs (Schams *et al.*, 1988; McArdle *et al.*, 1991; Lucy *et al.*, 1994; Spicer *et al.*, 1995). This is in contrast to the present study in cyclic mares, where we found no differences in the amount of circulating progesterone levels between animals treated with ST and those receiving the carrier vehicle. One possibility for this difference could be that the mares were treated for only 7 days, while animals in most other studies were treated with sustained release subcutaneous injections or implants, which generally last much longer, or they were treated for longer intervals. Gong *et al.* (1991) reported no difference in estradiol, progesterone, FSH or LH levels in bST-treated heifers compared with control heifers. In our study, mares treated with ST early in the estrous cycle had significantly lower levels of plasma LH, but this was likely due to the fact that one of our control mares had abnormally high plasma LH and not a result of the effects of ST treatment *per se*.

In summary, exogenous eST treatment increased both the plasma levels of IGF-I and the number of small follicles present on the ovaries of cyclic mares. Exogenous equine somatotropin treatment did not result in larger follicle diameters, as has been

shown in other species, possibly due to the timing of ST administration with respect to the stage of the estrous cycle, or perhaps insufficient quantities and/or number of doses of ST administered. It is evident, however, that ST treatment does have physiological effects on ovarian function in the horse and pony mare.

CHAPTER III

EFFECTS OF ADMINISTRATION OF EXOGENOUS eST TO SEASONALLY ANOVULATORY MARES

INTRODUCTION

Recently, the role of ST and insulin-like growth factor-I (IGF-I) in reproduction have come under intense investigation in domestic species and women. It is known that treatment with ST causes an increase in circulating IGF-I levels in horses (Buonomo *et al.*, 1996; Cochran *et al.*, 1999). In addition, treatment with ST has been reported to cause an increase in the number of small follicles in horses (Cochran *et al.*, 1999), pigs (Echternkamp *et al.*, 1994a) and cattle (Gong *et al.*, 1991). It has been proposed that ST/IGF-I causes its effects on ovarian function by acting synergistically with gonadotropins, allowing an increased response of the follicular cells to available gonadotropins (European and Australian Multicenter Study, 1995).

During times of decreased day length, mares undergo a period of acyclicity, termed the anovulatory or anestrous season. At this time, GnRH secretion from the hypothalamus is severely decreased or absent (Hart *et al.*, 1984), causing virtually no LH to be secreted from the anterior pituitary of these mares. During this period, the ovaries of the mare become small and relatively inactive, with little or no follicular development taking place.

The purpose of the present experiment was to determine if eST administration would increase the ovarian activity of seasonally anovulatory mares.

MATERIALS AND METHODS

Experimental Design

Anovulatory mares (n=10), all in good body condition, were randomly allocated to one of two treatment groups. Treatment A consisted of anovulatory mares (n=5), each treated with 25 μg of eST (EquiGen™, BresaGen Ltd., Thebarton, South Australia) per kg of body weight (i.m.) once daily for 28 days. Treatment B also consisted of anovulatory mares (n=5) from the same experimental herd, similarly treated with the eST vehicle. Daily blood sample collections were performed on each mare beginning on day 1 (first day of treatment) and continuing until day 28 (last day of treatment). In addition, follicular development was assessed at 72-hour intervals, beginning on day 1 of treatment and ending on day 28 of treatment.

Follicle Assessment and Hormone Analyses

The ovaries of each mare were examined at 72-hour intervals via transrectal ultrasonography using an Aloka 500-V ultrasound unit with a 5 MHz rectal probe (Corometrics, Wallingford, CT). Each ovary was scanned from dorsal to ventral and from lateral to medial sides, and the size of each follicle was recorded. Follicles were assigned to developmental categories based upon their diameter (Category I = <10 mm, Category II = 10 to 20 mm, Category III = 21 to 30 mm, Category IV = >30 mm). In addition, jugular blood samples were collected using sterile glass collection tubes (Vacutainers®, Sherwood Medical, St. Lcuis, MO) and centrifuged at 300 x g for 15 minutes. The plasma was then frozen within 30 minutes of collection. Validated radioimmunoassay (RIA) (Sticker *et al.*, 1995) was performed on the plasma samples collected every other day, beginning on day 1 of treatment and ending on day 28 of

treatment to determine circulating IGF-I concentrations for each mare. Similarly, LH and FSH (Thompson *et al.*, 1983a,b) concentrations were analyzed from these mares.

Experimental Conditions

This study was conducted during the calendar month of January, 1997 in south Louisiana, USA. The average daylength (sunrise to sunset) during the experimental period was 10.2 hours. In addition, the daily low temparature ranged from -5°C to 16°C and the daily high temperature ranged from 1°C to 26°C. All mares were selected from the same experimental herd and housed together in the same pasture during the experimental period. All mares were maintained on ryegrass pastures and bahia grass hay, free-choice, and supplemented with 2.2 kg of a commercial 8% protein feed per mare per day throughout the experimental period. Each mare used in the present study was determined to be seasonally anovulatory by monitoring plasma progesterone concentrations as well as ovarian follicular activity for at least 4 weeks prior to the initiation of treatment. Briefly, all mares had plasma progesterone levels of <0.5 ng/ml in addition to having no follicles >15 mm in diameter for the 4-week period prior to treatment.

Statistical Analyses

The number of follicles present in each follicle size category, as well as the total number of follicles present for each mare, were analyzed across time among treatment groups using analysis of variance (ANOVA). Hormone concentrations from mares were analyzed across time among treatment groups using a split-plot ANOVA procedure (Cochran *et al.*, 1999).

RESULTS

Overall, there was a positive effect of treatment of anovulatory mares with eST on the total number of follicles per mare ($P<0.05$) (Figure 3.1), with follicle numbers being significantly higher in treated mares by the seventh day of treatment. However, this observation was largely due to an increase in the number of small (<10 mm) follicles present on the ovaries of each mare ($P<0.05$) (Figure 3.2). There was no significant effect of eST administration on the number of follicles ≥10 mm (Categories II-IV) per mare throughout the experimental period (data not shown).

As expected, plasma IGF-I concentrations were elevated ($P<0.05$) in mares treated with eST over those of control mares by the third day of treatment (Figure 3.3). However, circulating plasma concentrations of LH and FSH in these mares were not affected by treatment with exogenous eST at any time throughout the experimental period.

DISCUSSION

In the present study, treatment with exogenous eST caused an increase in the number of follicles per mare in seasonally anovulatory mares by the seventh day of treatment (Figure 3.1). This was due mostly to an increase in the number of small (<10 mm) follicles (Figure 3.2), as there was no significant difference in the number of follicles ≥10 mm in diameter per mare for these mares throughout the experimental period. This is in agreement with a previous report from this laboratory, which demonstrated that treatment with eST increased the number of small follicles per mare in cyclic mares during the breeding season (Cochran *et al.*, 1999). In addition, similar

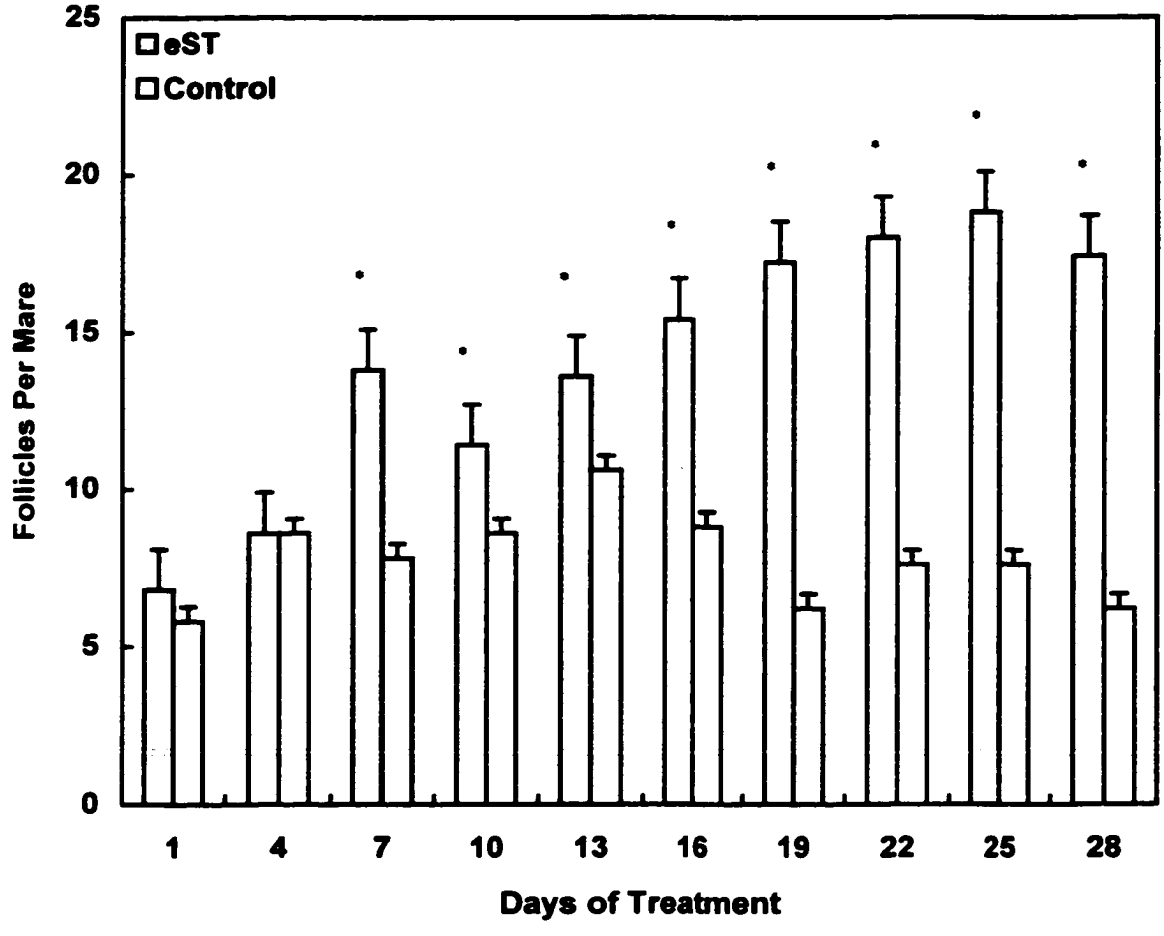

Figure 3.1. Total number of follicles per mare for seasonally anovulatory mares treated with eST. (*) Denotes significant differences within days.

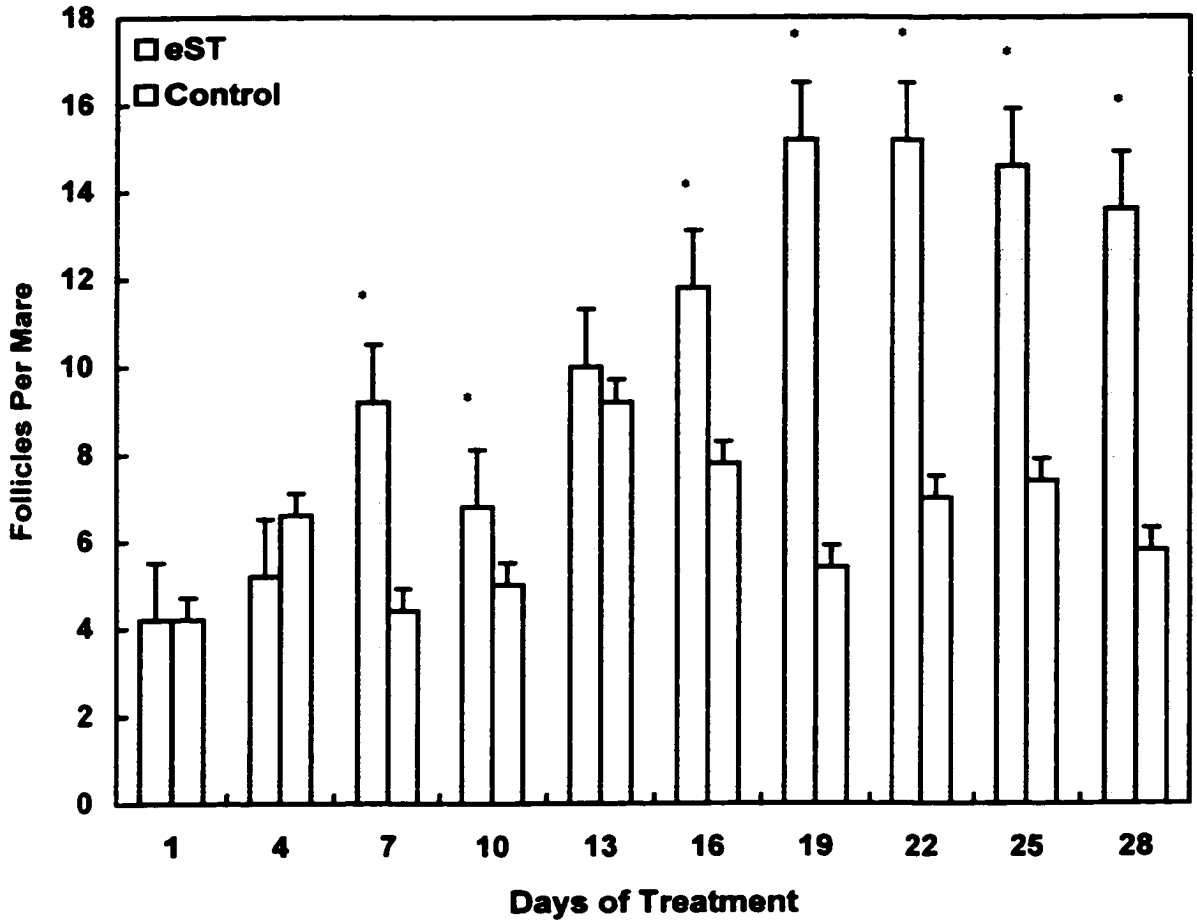

Figure 3.2. Number of small follicles (<10 mm) per mare for seasonally anovulatory mares treated with eST. (*) Denotes significant differences within days.

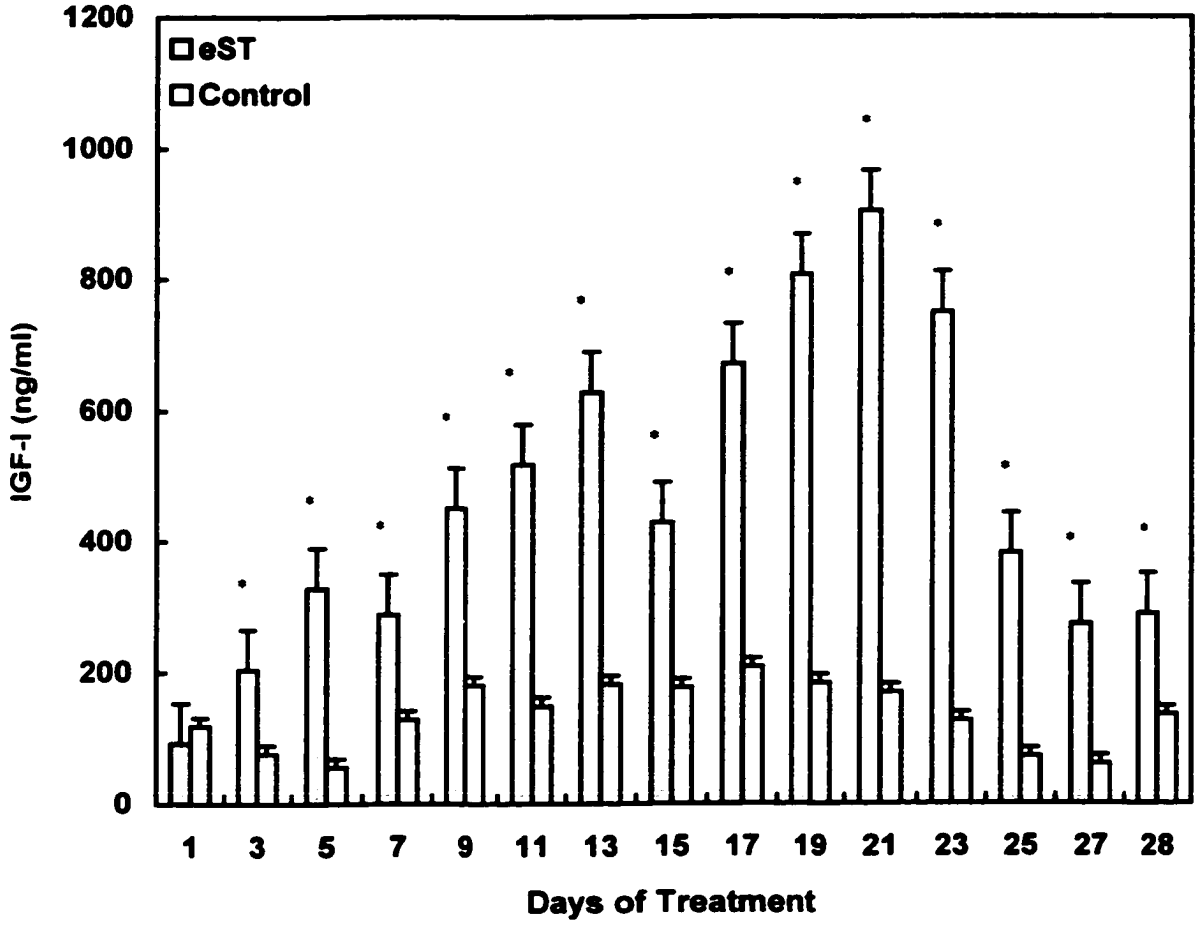

Figure 3.3. Plasma IGF-I concentrations for seasonally anovulatory mares treated with eST. (*) Denotes significant differences within days.

findings have been previously reported for pigs (Echternkamp *et al.*, 1994a) and cattle (Gong *et al.*, 1991).

In addition, plasma IGF-I was elevated in mares treated with eST over that of contemporary control mares by the third day of treatment (Figure 3.3). This is in agreement with previous reports in the horse (Cochran *et al.*, 1999) and in women (Owen *et al.*, 1991), where plasma IGF-I concentrations were not significantly elevated until 2 to 4 days of exogenous ST administration. This finding has been extensively demonstrated in various species, and is consistently found whenever exogenous ST is administered. Furthermore, there was no difference in circulating LH and FSH concentrations for seasonally anovulatory mares treated with exogenous eST. Gong *et al.* (1991) reported similar findings in a study with Holstein heifers treated with recombinant bovine ST (rbST), where they found no effect of treatment with rbST on plasma LH or FSH concentrations.

It is proposed that ST/IGF-I causes its effects on ovarian stimulation, at least in part, through its ability to work synergistically with available gonadotropins (European and Australian Multicenter Study, 1995). This has been shown *in vitro*, where administration of ST to porcine and bovine granulosa cells enhanced the action of FSH on these cells (Spicer *et al.*, 1993). This could be one explanation for the increase in small follicles in the present study, as it is currently believed that follicles in the 5 to 10 mm range are not responsive to LH, and would therefore be totally dependent on circulating FSH, or independent of gonadotropin control (Ginther, 1992). If these small follicles are at least partially responsive to FSH stimulation, then the ability of IGF-I to enhance the ovarian cells to available FSH could cause more follicles of this size

category to be present on the ovaries of mares treated with eST, as FSH, unlike LH, is detectable in jugular blood of seasonally anovulatory mares, although at highly reduced levels when compared with cyclic mares during the breeding season (Thompson *et al.*, 1986a).

Another possible explanation for the ability of ST, via elevated IGF-I, to increase the number of small follicles in treated animals could be through a prolongation of the time in which follicular atresia occurs. This hypothesis is supported by a study by Chun *et al.* (1996), where it was determined that in the granulosa cells of rats, FSH treatment decreased the rate of apoptosis by 60%, while IGF-I treatment decreased the rate of apoptosis by 45%. Therefore, one explanation for the findings reported in the present study could be that elevated plasma IGF-I levels, which may in turn cause elevated IGF-I concentrations in the follicular fluid, could act on the granulosa cells and reduce the rate of apoptosis, thereby, increasing the time of follicular regression and allowing the follicles to remain present on the ovaries for a longer period of time. This possible mechanism of action of IGF-I could, in effect, be implied to "rescue" early atretic follicles much the same way as FSH is currently used in cattle (Britt, 1988). Further studies are needed to clarify this hypothesis.

In summary, we have demonstrated that treatment of seasonally anovulatory mares with eST can increase the number of follicles present on their ovaries, with the greatest response being seen in the number of small follicles. In addition, plasma IGF-I was elevated in those mares treated with eST over that of contemporary control mares. Since there was no difference in circulating LH or FSH concentrations, the observed response in follicle numbers could be through the ability of IGF-I to enhance the

responsiveness of follicular cells to available gonadotropins, or through its apparent ability to prolong follicular atresia by decreasing the rate of cell death in the granulosa cells. However, further studies are needed in order to determine that exact mechanism(s) of action of ST and IGF-I on follicular development in the mare.

CHAPTER IV

THE EFFECTS OF ADMINISTRATION OF eST IN CONJUNCTION WITH A POTENT GnRH AGONIST ON REPRODUCTIVE FUNCTION IN SEASONALLY ANOVULATORY MARES

INTRODUCTION

It is well established that the reproductive cycle of the mare is seasonal in nature, with peak breeding activity coinciding with extended daylength. During times of decreasing daylength (winter), mares typically undergo a period of ovarian inactivity termed "seasonal anestrous" (Ginther, 1992). During this time, GnRH secretion from the hypothalamus is severely depressed, resulting in drastically reduced circulating levels of LH (Hart *et al.*, 1984). It has been demonstrated that increasing the photoperiod can increase GnRH ouput by the hypothalamus, thereby increasing plasma gonadotropin levels, resulting in cyclic recrudescence (Nequin *et al.*, 1989). From a practical standpoint, it would be beneficial to be able to find an efficient means of inducing reproductive cyclicity during the anovulatory season due to the fact that most major breed registries impose a mandatory birthdate of January 1 (in the USA) for all foals born the previous calendar year, regardless of when the foals were actually born. Given that the domestic mare has an ~11 month gestation length, mares would need to be mated in February in order for the foals born to fit in with their birthdate more closely. Unfortunately, this is a time that coincides with the anovulatory season, therefore, very few mares in this hemisphere would normally be in a cyclic state at this time.

It has been well demonstrated that increasing photoperiod will enable mares to begin the breeding season earlier in the year, however, this method can be costly and

labor intensive when dealing with large numbers of mares (for review see Ginther, 1992). Also, the administration of GnRH, along with some of its more potent agonists, has shown promise in inducing seasonally anovulatory mares to ovulate (Hyland *et al.*, 1987; Johnson, 1987; Ginther and Bergfelt, 1990; Fitzgerald *et al.*, 1993; Mumford *et al.*, 1994). However, this method has shown extreme variation in response, and therefore cannot be recommended for commercial purposes at this time (Mumford *et al.*, 1994). Also, most reports of successful induction of ovulation in seasonally anovulatory mares using any of the GnRH agonists (GnRHa) have come from either (1) continuous infusion, (2) multiple daily injections or (3) sustained-release formulations, which can also be costly and labor intensive from a practical standpoint (Hyland *et al.*, 1987; Johnson, 1987; Ginther and Bergfelt, 1990; Fitzgerald *et al.*, 1993; Mumford *et al.*, 1994).

Recently, the role of somatotropin (ST) and insulin-like growth factor-I (IGF-I) on reproduction has come under intense investigation in various species. It has been demonstrated that administration of ST can increase the number of follicles in mares (Cochran *et al.*, 1999), cattle (Gong *et al.*, 1996), pigs (Spicer *et al.*, 1992) and women (Owen *et al.*, 1991b). It has been proposed that this increase in follicle number is attributable, at least in part, to the ability of ST/IGF-I to enhance the granulosa cells within the ovarian follicle to the stimulatory effects of available gonadotropins, thus, enhancing follicular growth and maturation (Homburg *et al.*, 1988; Gong *et al.*, 1991; European and Australian Multicneter Study, 1995). This could be beneficial in inducing seasonally anovulatory mares to ovulate by enabling the follicles present to better respond to available gonadotropins secreted in response to GnRHa, thereby,

reducing the amount and number of injections of GnRHa, as well as reducing the variation in the treatment response.

Therefore, the purpose of these experiments was (1) to determine if administration of eST could enhance follicular growth, development and ovulation in seasonally anovulatory mares treated with once daily injections of a GnRHa and (2) to determine if ovulations obtained in this manner were fertile and could result in a viable pregnancy.

MATERIALS AND METHODS

Experiment 4.1

Experimental Design

Twenty seasonally anovulatory lighthorse mares of mixed breeds were randomly allocated into one of two treatment groups. Mares in Treatment A (n=10) were administered 25 μg eST (EquiGen™, BresaGen Ltd., Thebarton, South Australia) per kg body weight once daily, beginning on day 1 (first day of treatment) and continuing until day 35 or until ovulation was achieved. Mares in Treatment B (n=10) were similarly administered a sodium borate solution (vehicle) beginning on day 1 and continuing until day 35 or until ovulation was achieved. All mares (Treatments A and B) were administered 40 ng per kg body weight of a GnRHa (des-Gly^{10},[D-His(Bzl)^{6}]-Luteinizing Hormone Releasing Hormone Ethylamide, Sigma No. L-2761) once daily beginning on day 10 and continuing until day 35 or until ovulation was achieved. Also, all mares were administered a single dose of human chorionic gonadotropin (hCG) once a follicle ≥35 mm in diameter was detected to facilitate ovulation. Daily blood samples were collected for each mare throughout the experimental period via jugular veni-

puncture. In addition, ovarian follicular development was assessed at 72-hour intervals via transrectal ultrasonography throughout the experimental period.

Follicle Assessment and Hormone Analyses

Blood samples were collected via jugular venipuncture and immediately placed on ice using sterile glass collection tubes (Vacutainers®, Sherwood Medical, St. Louis, MO). They were then centrifuged at 300 x g for 15 minutes and the plasma stored in 7-ml plastic tubes (Curtin Matheson, Houston, TX) and frozen within 30 minutes of collection. A previously validated radioimmunoassay (RIA) (Sticker *et al.*, 1995) was performed on the plasma samples collected every other day, beginning on day 1 of treatment and ending on day 35 or until ovulation was achieved to determine circulating IGF-I concentrations for each mare within treatment group. In addition, plasma concentrations of FSH and LH (Thompson *et al.*, 1983) were analyzed from all mares.

The ovaries of each individual mare were examined at 72-hour intervals via transrectal ultrasonography using an Aloka 500-V ultrasound with a 5 MHz rectal probe (Corometrics, Wallingford, CT). Each ovary was scanned beginning from dorsal to ventral and then from lateral to medial sides, and the size of each follicle was recorded. Follicles were assigned to developmental categories based upon their diameter at the time of assessment (Category I = <10 mm, Category II = 10 to 20 mm, Category III = 21 to 30 mm, Category IV = >30 mm). Once a follicle reached 30 mm, ultrasound examinations were performed for that mare at 24-hour intervals until the follicle reached 35 mm in diameter. At that time, 3,500 units hCG was administered (i.v.) to induce ovulation. All mares that responded with visible ovulation/luteinization (via

ultrasound) were additionally monitored for 10 days following ovulation to assess luteal formation and to verify circulating progesterone levels.

Experiment 4.2

Experimental Design

Experiment 4.2 was conducted to determine if the ovulations induced by the protocol implimented in Experiment I were fertile and capable of producing a viable pregnancy. A total of 14 seasonally anovulatory mares were randomly allocated into one of two treatment groups. Treatment A consisted of mares (n=9) administered 25 μg/kg eST once daily beginning on day 1 and continuing until day 28 or until ovulation was achieved. Mares in Treatment B (n=5) were similarly administered the vehicle. All mares were administered the same GnRHa as those in Experiment 4.1, at a concentration of 50 ng/kg once daily beginning on day 10 and continuing until day 28 or until ovulation was achieved. Follicular development and circulating plasma hormone concentrations were assessed at 72-hour intervals as previously described with the following exception: (1) follicles were not assigned to developmental categories since follicles of large size (>30 mm), ovulation and pregnancy status were the only endpoints of importance in this experiment. Once a follicle reached 30 mm in diameter, follicular growth was assessed at 24-hour intervals until the follicle reached 33 mm in diameter. At that time hCG (5,000 units) was administered (i.v.) and the mare was inseminated with at least 500 million motile spermatozoa. All mares were inseminated with semen collected from a single stallion of proven fertility, which was the same for all mares. Oral altrenogest (Regu-Mate®, Hoechst Roussel Vet, Warren NJ) was administered once daily at a concentration of 0.044 mg/kg to all mares that ovulated to

ensure that luteal insufficiency would not inhibit the pregnancy status. All mares that were confirmed pregnant were maintained on oral altrenogest until day 50 of pregnancy. Pregnancy diagnosis was performed via transrectal ultrasonography at 14 and 20 days post-ovulation in all mares that ovulated.

Experiments 4.1 and 4.2

Experimental Conditions

These experiments were conducted during the calendar months of December, 1997 – January, 1998 (Experiment 4.1) and January, 1999 (Experiment 4.2) in south Louisiana. The average daylength (sunrise to sunset) for both experiments ranged from 9.5 to 10.6 hours. Also, the daily low temperature ranged from -3°C to 17°C for Experiment 4.1 and from -7°C to 19°C for Experiment 4.2. In addition the daily high temperature ranged from 10°C to 23°C for Experiment 4.1 and from 4°C to 27°C for Experiment 4.2. All mares came from the same experimental herd and were penned within the same pasture throughout the experimental period for both experiments. All mares were maintained on ryegrass pastures and Bahia grass hay, free-choice, and supplemented with 2.2 kg of a commercial 8% protein feed per mare per day throughout the experimental periods. The body condition scores for the mares used in these experiments ranged from 4 to 7 on a scale of 1 to 9, and were not different between treatment groups. All mares used in Experiments 4.1 and 4.2 were determined to be seasonally anovulatory by monitoring plasma progesterone concentrations as well as ovarian follicular activity for at least 4 weeks prior to the initiation of treatment. All mares had plasma progesterone levels of <0.5 ng/ml in addition to having no follicles >15 mm in diameter for the 4-week period prior to the initiation of treatment.

Statistical Analyses.

In Experiment 4.1, the number of follicles per mare, as well as the number of follicles within size category per mare were analyzed using an analysis of variance procedure (ANOVA). Numbers of mares ovulating per treatment group were analyzed using a similar ANOVA procedure for both experiments. Circulating concentrations of LH, FSH and IGF-I were analyzed across treatment groups using a split-plot ANOVA procedure (Cochran *et al.*, 1999).

RESULTS

Experiment 4.1

Follicular Development and Plasma Hormone Profiles

Results from Experiment 4.1 are presented in Figures 4.1 to 4.3 and Table 4.1. There was a significant increase ($P < 0.05$) in the total number of ovarian follicles per mare for mares treated with eST plus GnRHa when compared with GnRHa treatment alone (Figure 4.1). This increase was due mostly to a significant increase ($P < 0.05$) in the number of Category I (<10 mm) follicles per mare within treatment group (Figure 4.2). More importantly, the number of mares that responded to once daily eST plus GnRHa by growing Category IV (>30 mm) follicles was greater when compared with GnRHa treatment alone (7/10 *vs.* 1/10, respectively, $P < 0.05$) (Table 4.1). In addition, 50% of eST treated mares ovulated, which was significantly greater than the 10% ovulating in the control group ($P < 0.05$). Also, two additional mares luteinized without apparent ovulation in the eST treated group, which resulted in an overall 70% of mares in this group giving rise to functional luteal tissue formation. This was a significant increase over the 10% of mares responding in the control group ($P < 0.05$).

Plasma IGF-I concentrations were significantly elevated (P <0.05) by day 3 of treatment in the eST treated group when compared with control mares (Figure 4.3). However, there was no increase in either circulating LH or FSH concentrations between the two treatment groups throughout the experimental period. Plasma progesterone concentrations were monitored in all mares that ovulated/luteinized across treatment groups for 10 days following ovulation/luteinization to determine functional status of the forming luteal tissue. All luteal tissue formed in all mares across treatment groups were determined to be functional at least through the first 10 days post-ovulation. However, reproductive cyclic activity was not continued in any mare once treatment was stopped. In addition, there was no apparent effect of treatment on cyclic recrudescence later in the year (spring) for mares in either group compared with untreated mares from the same experimental herd.

Experiment 4.2

Follicle Development, Pregnancy Status and Plasma Hormone Profiles

In Experiment 4.2, the total number of follicles and the number of follicles within follicle size categories were not recorded, since the only endpoints of practical importance were (1) development of large (>30 mm) follicles, (2) the number of mares ovulating within treatment group and (3) the number of mares with a positive pregnancy diagnosis. There was a significant treatment effect of eST in conjunction with GnRHa on the number of mares with large follicle development (7/9 *vs.* 1/5, respectively, P <0.05) (Table 4.2). Also, there was a tendency for more eST treated mares to ovulate (P=0.1) when compared with control mares (6/9 *vs.* 1/5, respectively). In addition, while there was no statistical difference (P=0.2) in the number of mares confirmed

pregnant between treated and control mares (5/9 *vs.* 1/5, respectively), the addition of eST in conjunction with GnRHa did not adversely effect fertility, as demonstrated by the fact that 5 of the 6 mares that ovulated in the eST treated group were confirmed pregnant by 14 days post-ovulation.

As in Experiment 4.1, plasma IGF-I concentrations were elevated in eST treated mares when compared with control mares ($P < 0.05$, Figure 4.4). Again, no difference was detected in plasma FSH or LH levels between treatment groups throughout the experimental period. As before, all mares which ovulated had elevated plasma progesterone concentrations for at least 10 days post-ovulation, verifying the functionality of the forming luteal tissue in these mares. Also, there was no noticeable effect of treatment with eST plus GnRHa or GnRHa alone on resumption of seasonal reproductive cyclicity when compared with untreated mares from the same experimental herd.

DISCUSSION

It is well known that, during the winter, most mares exhibit an anovulatory period. The direct cause for this can be attributed to the fact that hypothalamic GnRH content is severely reduced causing LH output by the pituitary to be almost nonexistent (Hart *et al.*, 1984). It is suspected that melatonin output from the pineal gland in response to increasing periods of darkness plays some function in reducing GnRH secretion, however, the exact mechanism of this action has yet to be determined, as there are no melatonin receptors found in the hypothalamus of the domestic mare (McKinnon and Voss, 1993). It has long been known that increasing the period of

Table 4.1. Number of mares per treatment group that exhibited growth of large follicles (>30 mm), ovulation and luteal tissue formation (Experiment 4.1)

Treatment	>30 mm follicle	Ovulation	Luteal function
eST plus GnRHa	7/10[a]	5/10[a]	7/10[a]
Control plus GnRHa	1/10[b]	1/10[b]	1/10[b]

[a,b]Means within columns with different superscripts are different ($P < 0.05$).

Table 4.2. Number of mares per treatment group that exhibited growth of large follicles (>30 mm), ovulation and pregnancy status (Experiment 4.2)

Treatment	>30 mm follicle	Ovulation	Pregnant
eST plus GnRHa	7/9[a]	6/9	5/9
Control plus GnRHa	1/5[b]	1/5	1/5

[a,b]Means within columns with different superscripts are different ($P < 0.05$).

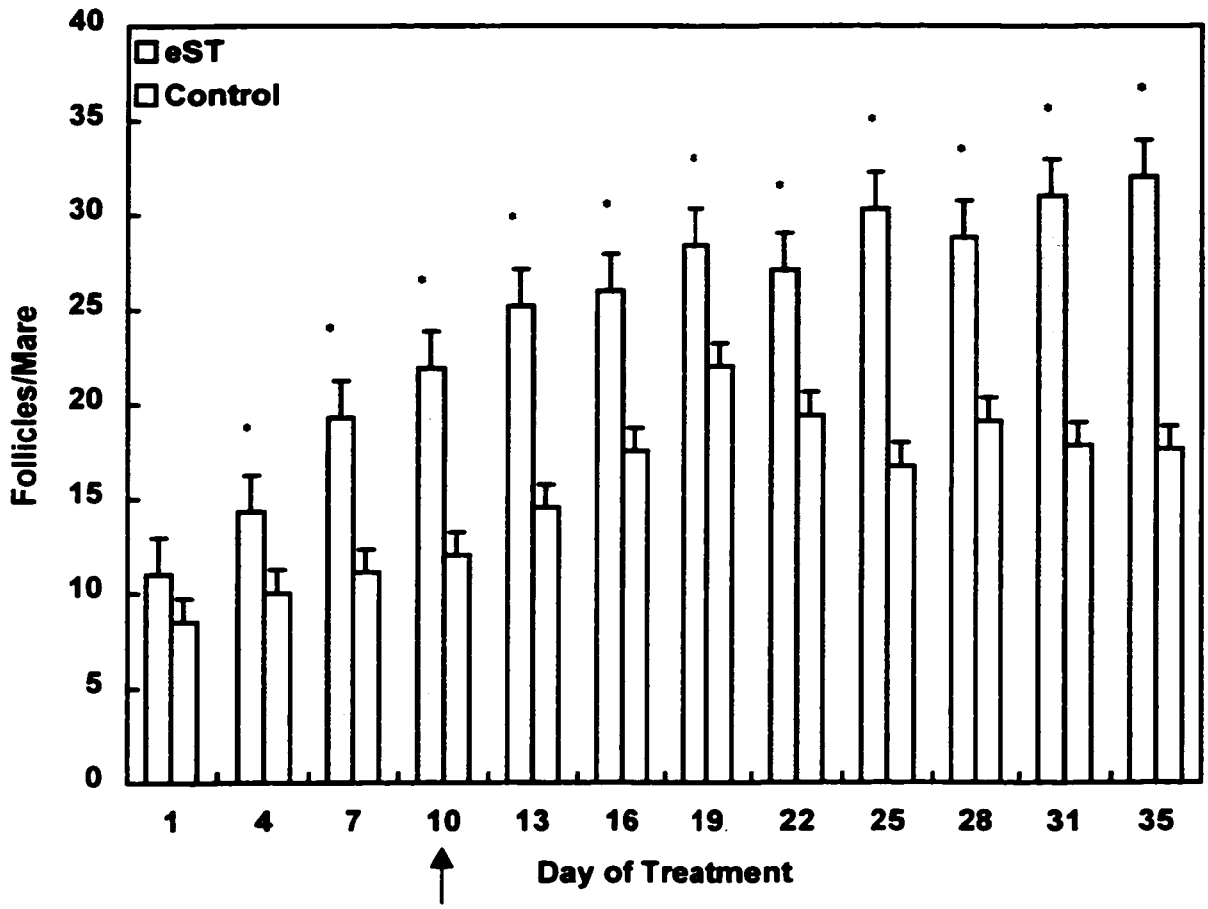

Figure 4.1. Total number of follicles (±SEM) per mare for anovulatory mares treated once daily with eST plus GnRHa. Day 10=start of GnRHa treatment (Experiment 4.1). (*) Denotes significant differences within days.

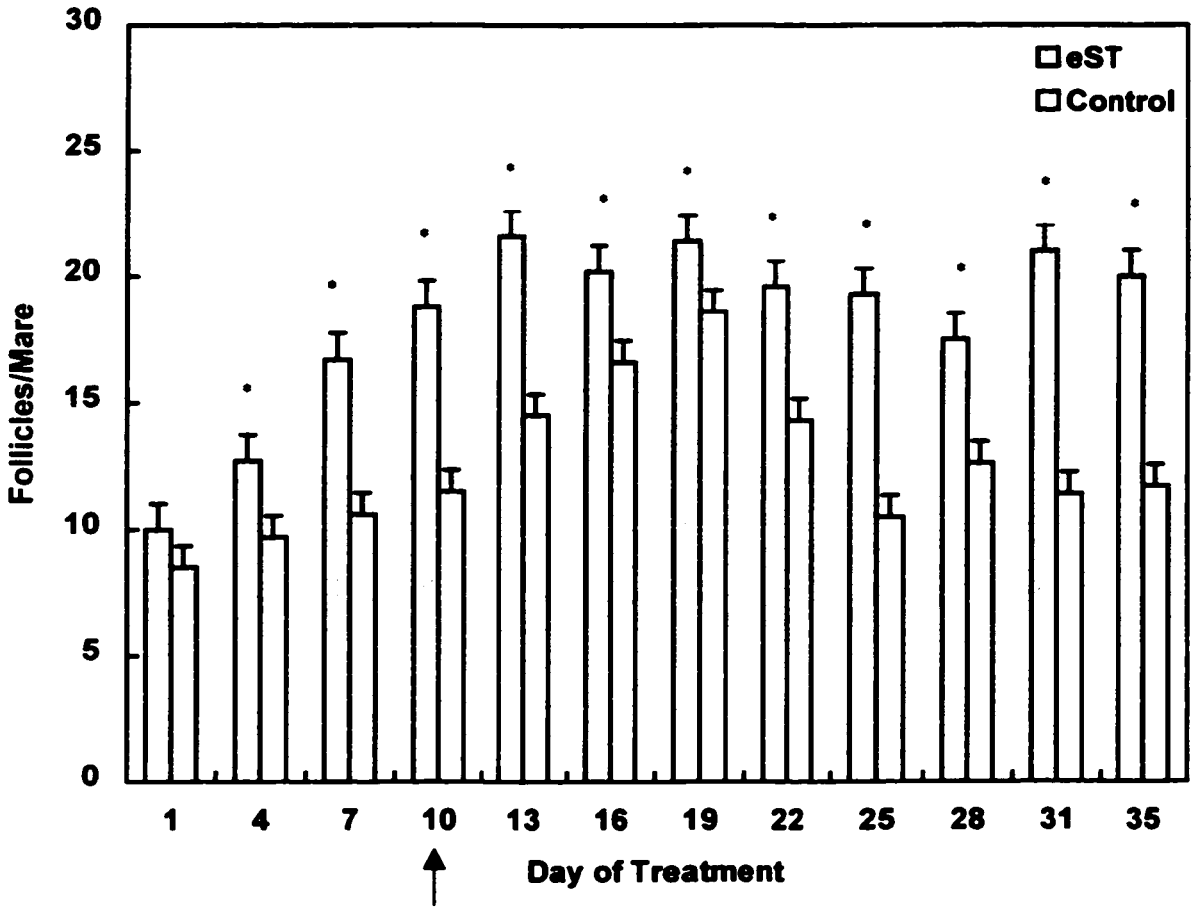

Figure 4.2. Number of Category I (<10 mm) follicles (±SEM) per mare for anovulatory mares treated once daily with eST plus GnRHa. Day 10=start of GnRHa treatment (Experiment 4.1). (*) Denotes significant differences within days.

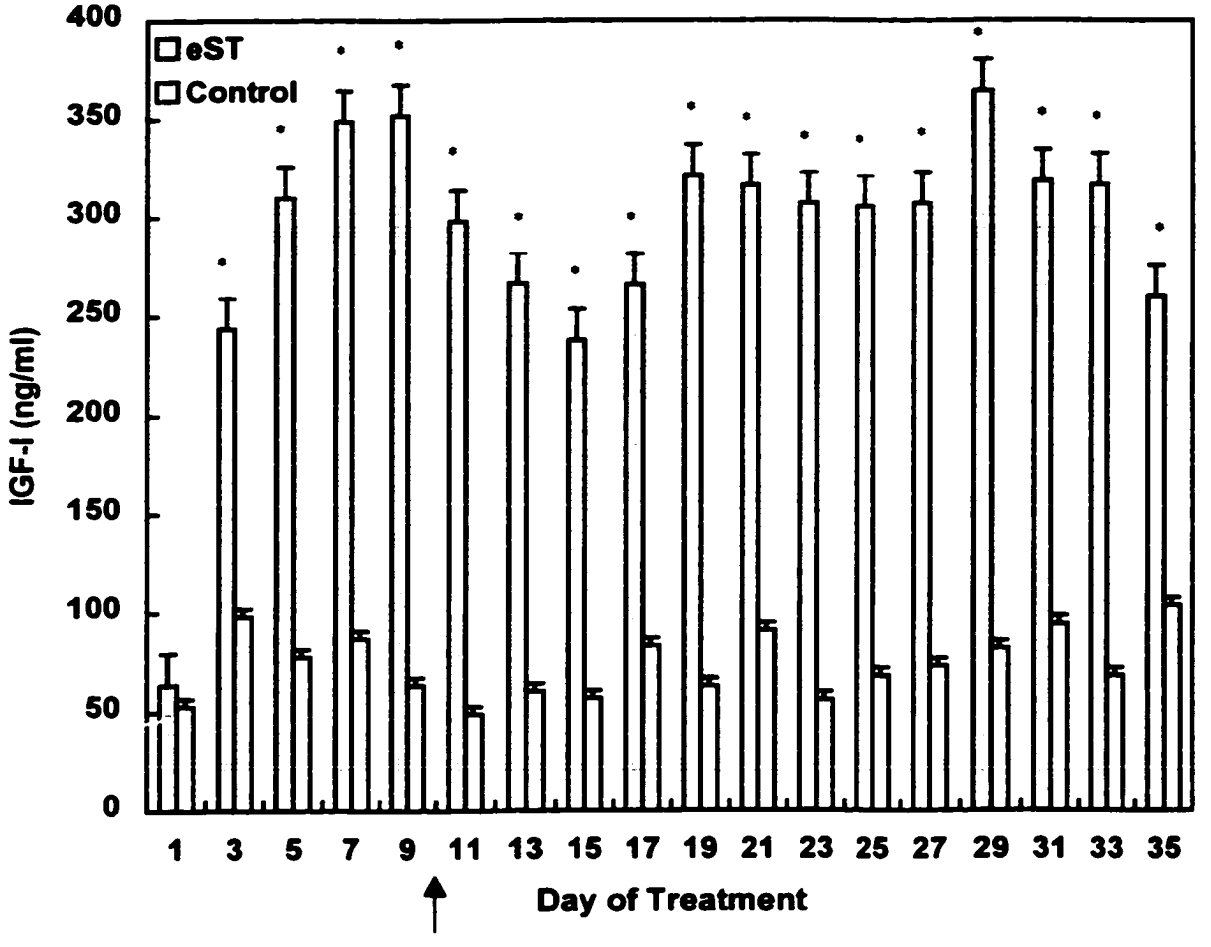

Figure 4.3. Plasma IGF-I concentrations (±SEM) for anovulatory mares treated once daily with eST plus GnRHa. Day 10=start of GnRHa treatment (Experiment 4.1). (*) Denotes significant differences within days.

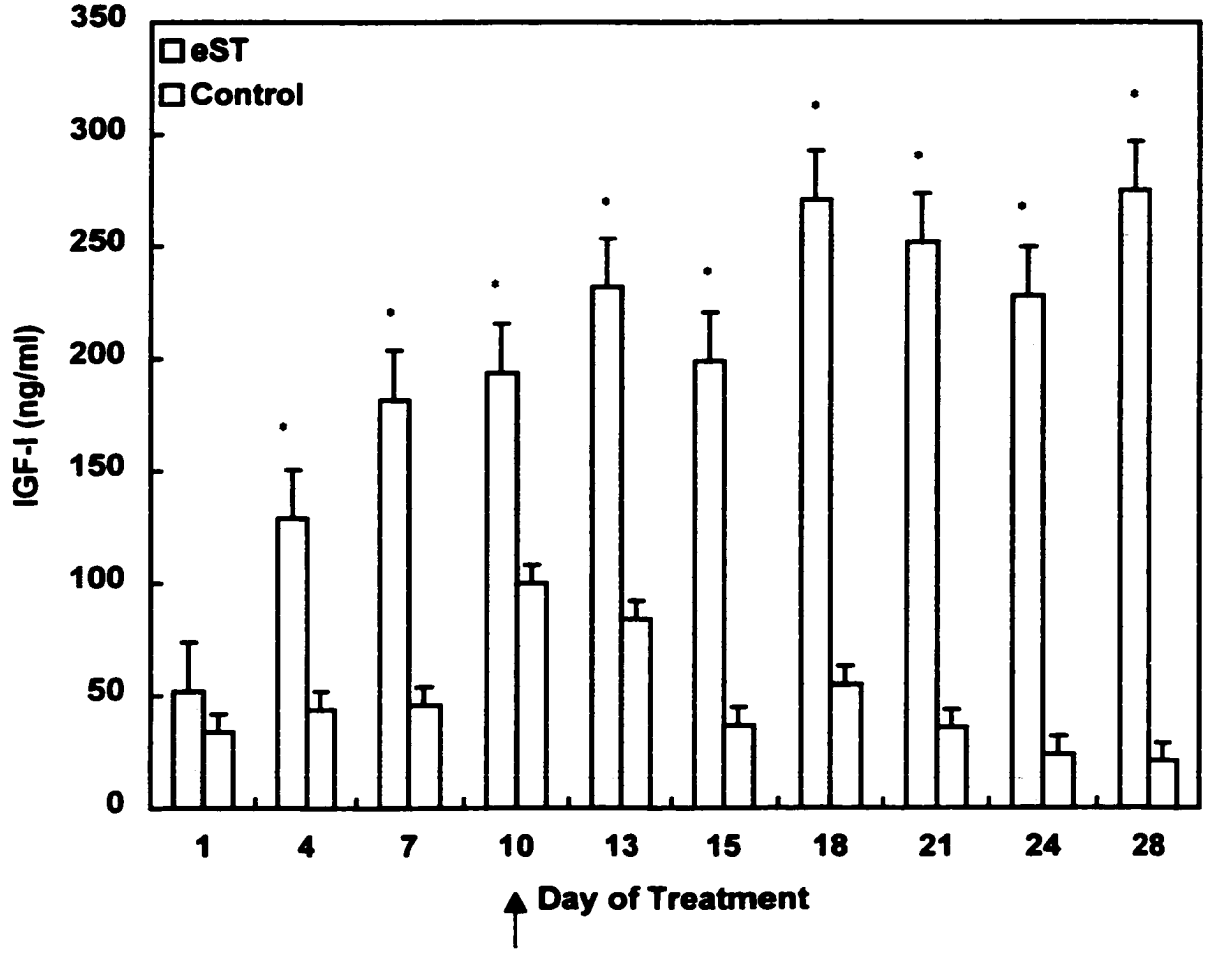

Figure 4.4. Plasma IGF-I concentrations (±SEM) for anovulatory mares treated once daily with eST plus GnRHa. Day 10=start of GnRHa treatment (Experiment 4.2). (*) Denotes significant differences within days.

daylight can enable mares to resume normal reproductive cyclicity (Kooistra and Ginther, 1975). At present, the most repeatable method of hastening the onset of the breeding season is to increase the daylength that the mares are subjected to by exposing them to artificial daylight (Kooistra and Ginther, 1975, Sharp *et al.*, 1975, Cleaver *et al.*, 1991). Typically, mares are exposed to 16 hours per day of "daylight" by placing them in a lighted stall for a period of ~ 60 days (Sharp *et al.*, 1975).

There are, however, two major setbacks associated with this type of protocol, which include: (1) the initial cost of setting up and (2) the length of time required for the desired results to be realized. Essentially, when mares are placed under artificial daylight, a 60 day period is required because the mares must go through the process of vernal transition, which is a period when the reproductive system of the mare is preparing itself for the onset of the breeding season (Sharp *et al.*, 1975). Exposing mares to increased periods of light per day results in increased output of GnRH from the hypothalamus, thus increasing LH production and secretion by the pituitary (Cleaver *et al.*, 1991). It is interesting to note that although circulating levels of LH are elevated, no change was detected in FSH secretion (Cleaver *et al.*, 1991). This increase in LH results in increased ovarian activity and resumption of reproductive cyclicity (Sharp *et al.*, 1975; Oxender *et al.*, 1977; Freedman *et al.*, 1979).

It has been shown repeatedly that administration of GnRH and/or one of its analogs can induce ovulation in seasonally anovulatory mares (Johnson, 1986; Hyland *et al.*, 1987; Johnson, 1987; Becker and Johnson, 1992; Swinker *et al.*, 1993). For example, Johnson (1987) demonstrated that hourly pulses of GnRH cause elevated LH levels by 11 days and ovulation within 15 days. In addition, continuous infusion of

GnRH can also induce ovulation in seasonally anovulatory mares as much as 50% of the time, within 15 to 30 days (Hyland *et al.*, 1987). It has been determined that a pulsatile mode of GnRH administration is preferrable to continuous infusion, as ovulation is induced in a faster, more repeatable manner (Becker and Johnson, 1992). However, both of these methods of treatment are cumbersome and labor intensive, and are not a feasible method of inducing ovulation in a commercial sense. Therefore, other studies have been undertaken in an effort to find a more suitable method of GnRH administration (Ginther and Bergfelt, 1990; Harrison *et al.*, 1990; Fitzgerald *et al.*, 1993).

Harrison *et al.* (1990) have shown that induction of ovulation in seasonally anovulatory mares can be achieved via multiple daily injections of GnRHa (2x daily). This method of treatment was sufficient to increase pituitary LH output and induce ovulation in 57% of treated mares (Ginther and Bergfelt, 1990). Also, constant administration of GnRHa via a subcutaneous depot has been shown to be effective in inducing ovulation in these mares as well (Harrison *et al.*, 1990; Fitzgerald *et al.*, 1993). However, the substantial variation in response makes these methods currently unreliable for commercial purposes (Mumford *et al.*, 1994). It has proposed that the primary reason for the variation observed in the ovulatory response to these various methods of GnRH administration is the depth of anestrous exhibited by the experimental mares under treatment, with less results realized from mares in "deep" anestrus (Hyland *et al.*, 1987; Mumford *et al.*, 1994). "Deep" anestrus can be classified as the mare(s) in question having (1) no follicles >15 mm in diameter and (2) plasma progesterone concentration of <1 ng/ml for at least four consecutive weeks. As most mares do have

some follicular activity during the anovulatory period, it is crucial to quantify the depth of anestrous in order to determine the potential for success in these mares. Other factors, including nutrition and body condition, have also been implicated in the frequency and depth of seasonal anestrus exhibited in the domestic mare (Swinker *et al.*, 1993; Fitzgerald, 1996; Fitzgerald and Davison, 1997).

It has been demonstrated that the addition of ST can enhance the ovarian response to gonadotropin administration in cattle (Rieger *et al.*, 1991) and clinically infertile women (Owen *et al.*, 1991b). In addition, administration of ST increases the number of follicles on the ovaries of mares (Cochran *et al.*, 1999), pigs (Spicer *et al.*, 1992), cows (Gong *et al.*, 1991) and women (Owen *et al.*, 1991a,b). It has been proposed that these effects are caused, at least in part, by the ability of ST to stimulate both local (intrafollicular) as well as systemic IGF-I production (De La Sota *et al.*, 1993; Bergh *et al.*, 1994). Owen *et al.* (1991a) reported that IGF-I caused an increase in the follicular response to FSH stimulation of the granulosa cells. Also, addition of IGF-I to the culture medium of bovine granulosa cells causes an increase in the steroidogenic index of these cells (Schams *et al.*, 1987). Unpublished studies from our laboratory have demonstrated a similar effect in cultured equine granulosa cells, where addition of IGF-I caused a significant increase in progesterone production. It is therefore likely that the administration of ST, either directly or indirectly through increased IGF-I, enhances the ability of the follicle to respond to available gonadotropins via stimulation of the granulosa cells, possibly through increasing the number of FSH and LH receptors (Homburg *et al.*, 1988; European and Australian Multicenter Study, 1995).

With this hypothesis in mind, Experiment 4.1 was conducted to determine if the beneficial effect reported for ST administration could be demonstrated in the anovulatory mare. Since one of the major problems associated with GnRH induction of ovulation in these mares is the extreme variability in response, it was hypothesized that eST administration may augment the stimulatory effects of the gonadotropins secreted in response to administration of GnRHa on follicular growth and development and thereby decrease the variability seen in the response. There was a definite beneficial effect of treatment of eST in conjunction with once daily GnRHa administration, as witnessed by the increase in large follicle growth (7/10 *vs.* 1/10, $P < 0.05$), ovulation (5/10 *vs.* 1/10, $P < 0.05$) and luteal formation (7/10 *vs.* 1/10, $P < 0.05$) in eST-treated mares when compared with control mares, respectively (Table 4.1). Also, as previously reported in the horse (Cochran *et al.*, 1999), the cow (Gong *et al.*, 1991) and the pig (Samaras *et al.*, 1994), plasma IGF-I levels were elevated in mares treated with eST plus GnRHa (Figures 4.3 and 4.4). We hypothesize that this increase in systemic IGF-I concentrations is responsible, at least in part, for the follicular stimulatory effects caused by eST treatment to seasonally anovulatory mares.

The effects of ST administration on circulating gonadotropin concentrations have been previously reported (Gong *et al.*, 1991; Gong *et al.*, 1996). It has been demonstrated that Hereford x Friesian heifers treated with recombinant bovine somatotropin (rbST) had no increase in circulating LH or FSH concentrations (Gong *et al.*, 1991; Gong *et al.*, 1996). This is in agreement with the present study for the anovulatory mare, as no differences were detected in either plasma LH or FSH levels between treated and control mares. This further reinforces our hypothesis that ST/IGF-I

causes its effects by acting synergistically with available gonadotropins to induce follicular growth and development.

Based on the results observed in Experiment 4.1, Experiment 4.2 was conducted to determine if the ovulations caused by eST plus GnRHa treatment were indeed fertile and capable of producing a viable pregnancy. Although there was no statistical difference in the number of mares conceiving per treatment group (5/9 *vs.* 1/5) (Table 4.2), it is clear that eST administration had no detrimental effect on oocyte quality, as 5 of the 6 mares which ovulated in the treament group were confirmed pregnant by 14 days post-ovulation. This is in agreement with previous reports in the human, where women administered ST along with human menopausal gonadotropin (hMG) had no detrimental effect on the fertilizability of oocytes collected for *in vitro* fertilization procedures, but actually had an increase in the number of fertilized oocytes per female (Owen *et al.*, 1991a; Bergh *et al.*, 1994).

In the present study, we have demonstrated that the addition of eST to a protocol including once daily injections of a potent GnRHa can enhance follicular development and the ovulatory response in seasonally anovulatory mares. The increase in the ovulatory response seen in eST treated mares has potential commercial applications, since this protocol (1) decreases the variation typically seen when administering GnRH to anovulatory mares as well as (2) allow for a more manageable protocol of once daily injections to be employed. It is important to note that all mares utilized in the present study were determined to be in "deep" anestrus based on the mares having plasma progesterone concentrations of <0.5 ng/ml as well as only follicles <15 mm in diameter present on their ovaries for a minimum of four consecutive weeks prior to the initiation

of treatment. Given the initial expense and labor involved in artificial lighting regimens, as well as the variability and labor involved with traditional GnRH therapy, a protocol including once daily injections of eST plus GnRH could be beneficial to commercial horse breeders.

CHAPTER V

PRODUCTION OF LIVE FOALS FROM SPERM-INJECTED OOCYTES HARVESTED FROM PREGNANT MARES

INTRODUCTION

In vitro fertilization (IVF) has become a frequently used treatment for infertility in human couples (Cha *et al.*, 1991; Trounson *et al.*, 1994a; Palermo *et al.*, 1996). Also, IVF is now a commercially feasible method to recapture the reproductive potential of valuable clinically infertile cows (Stroud and Myers, 1992; Looney *et al.*, 1994). The consistent production of IVF embryos in the horse has not yet become reality, even though adequate success rates have been reported for *in vitro* oocyte maturation (Del Campo *et al.*, 1990; Zhang *et al.*, 1990; Hinrichs *et al.*, 1993a,b) and sperm cell capacitation (Varner *et al.*, 1987; Samper *et al.*, 1989). To date, only one foal has been reported as a result of conventional IVF (Palmer *et al.*, 1991) and there has been one foal produced after intracytoplasmic sperm injection (ICSI) of abattoir oocytes (E.L. Squires, J.M. Wilson, H. Kato, A. Blaszczyk, unpublished data).

The reason(s) for poor IVF and subsequent development rates of equine oocytes remains unclear. Equine oocytes appear to have a thick zona pellucida compared with those of other species, and the time necessary for *in vitro* maturation (IVM) is longer than for domestic ruminant species (Hinrichs *et al.*, 1993a,b). Therefore, it was suspected that the zona pellucida of the IVM oocyte is, in part, a barrier to *in vitro*-prepared sperm cells (Li *et al.*, 1995). The potentially altered zona pellucida of IVM oocytes (Chan, 1987; Cohen *et al.*, 1990; Trounson *et al.*, 1994b), in addition to less than adequate sperm cell preparation for IVF, likely contributes to poorer than expected IVF rates in the mare.

Live offspring have been produced in laboratory animals (Gordon and Talansky, 1986) and humans (Cohen *et al.*, 1991, 1992) by using a simple, prefertilization zona-drilling procedure to aid sperm cells in crossing the zona pellucida. Recently, acceptable fertilization and later stage embryo development rates were obtained consistently from oocytes of pregnant mares by applying a similar prefertilization zona drilling procedure (Li *et al.*, 1995; Meintjes *et al.*, 1995). Pregnant mares were utilized as oocyte donors in these studies because multiple, good quality germinal vesicle-stage oocytes could be harvested repeatedly from a single mare, which is often not the case when using cyclic mares as oocyte donors. However, the size of the opening drilled into the zona pellucida, the concentration of sperm cells used for *in vitro* insemination and the time period of zona-drilled oocyte exposure to sperm cells may influence normospermic fertilization rates (Choi *et al.*, 1994).

Single intracytoplasmic or multiple subzonal microinsemination of sperm cells may circumvent the variables associated with prefertilization zona drilling and minimize the possibility of polyspermic fertilizations. In humans, ICSI has become the method of choice for treating male factor infertility (Ng *et al.*, 1993; Palermo *et al.*, 1996) and, because of its high efficiency and circumvention of polyspermic fertilization, is preferred over the subzonal sperm injection (SUZI) procedure (Palermo *et al.*, 1993; Van Steirteghem *et al.*, 1993; Abdalla *et al.*, 1995). However, in domestic cats, SUZI first seemed to be more effective than ICSI, with one kitten being born as a result of SUZI (Pope *et al.*, 1995). It was later determined by the same researchers that ICSI was more efficient than SUZI in domestic cats (C.E. Pope, personal communication) with one report of live kittens delivered from ICSI (Pope *et al.*, 1997).

Little is known about the optimum developmental requirements for early-stage equine embryos. It has been shown in several species that exposure to relatively high levels of glucose in the culture medium can be inhibitory to embryonic development during the first 48 hours of culture, and that culture in a medium containing little or no glucose can enhance early embryonic development and viability (Chatot *et al.*, 1989; Ellington *et al.*, 1990; FitzGerald and DiMattina, 1992).

The objectives of this study were (1) to circumvent the possibility of inadequate *in vitro* sperm cell capacitation, *in vitro* zona pellucida hardening and polyspermic fertilizations by performing ICSI or SUZI procedures on IVM equine oocytes, (2) to evaluate the effects of a glucose-free, phosphate-free medium on the development and viability of early stage equine embryos and (3) to test the viability of these *in vitro*-derived embryos by nonsurgical embryo transfer at the morula stage or by surgical transfer procedures at earlier developmental stages.

MATERIALS AND METHODS

Experiment 5.1

Experimental Design

Mature oocytes, as evidenced by the presence of a first polar body in the perivitelline space under an inverted microscope, were randomly allocated to five microfertilization treatments. In Treatment A, a single immobilized sperm cell was injected directly into the ooplasm. In Treatment B, two to three motile sperm cells were injected under the zona pellucida in the perivitelline space. Treatments C and D consisted of sham injection procedures, where a similar volume of 10% (w/v) polyvinyl pyrrolidone (PVP) in Earle's balanced salt solution (Medi-Cult, Copenhagen, Denmark)

was injected (without containing sperm cells) either directly into the ooplasm (Treatment C) or into the perivitelline space (Treatment D). Treatment E was a polyspermy control group where immobilized sperm cells (n=3 to 5) were injected directly into the ooplasm.

Oocyte Collection

During the months of April to August in the southern United States, immature oocytes were collected from early pregnant crossbred mares in good body condition (days 21 to 40 of gestation), using a repeatable noninvasive transvaginal ultrasound-guided follicular aspiration procedure as previously reported by this laboratory (Meintjes *et al.*, 1994, 1995). All follicles with a diameter ≥5 mm were punctured with a 12-gauge single lumen needle and then rinsed up to 10 times with oocyte flushing medium (by alternate filling and emptying). The oocyte flushing medium consisted of phosphate-buffered saline (Gibco, Grand Island, NY) supplemented with 1% heat-treated calf serum (Gibco), 2 USP units of heparin (Steris Laboratories, Phoenix, AZ), 100 μg of streptomycin (Gibco) and 100 units of penicillin-G (Gibco) per ml of medium. Follicular activity on the ovaries of these mares was monitored by transrectal ultrasound twice per week, and the follicles aspirated when three or more follicles >15 mm in diameter were detected. Several mares were aspirated on more than one occasion.

In Vitro Maturation and Oocyte Preparation

After oocyte isolation and evaluation under light microscopy (200x), all oocytes with an intact oolemma were washed in IVM medium. IVM medium consisted of tissue culture medium 199 (TCM-199, Gibco) with 10% estrual mare serum collected at this

laboratory. *In vitro* maturation was conducted in 100 μl microdroplets of IVM medium covered with mineral oil in a 35-mm petri dish for 36 hours at 38°C in an atmosphere of 5% CO_2 in air. After IVM, the oocytes were stripped from the majority of their cumulus cells by vigorous pipetting for ~5 minutes in a 0.025% (v/v) trypsin (Sigma, St. Louis, MO) solution to facilitate the sperm injection procedure. Mature oocytes with an extruded first polar body were identified under light microscopy (200X) and then placed separately in 5 μl sperm-injection microdroplets consisting of HEPES-buffered modified Earle's balanced salt solution (Medi-Cult, Copenhagen, Denmark) under oil in a 35-mm petri dish.

Semen Preparation

One ml (40 to 60 x 10^6 sperm cells) of cooled, extended semen from a single mature Arab stallion of proven fertility was washed twice in Ham's F-10 medium (Gibco) at 500 x g to remove the seminal plasma and semen extender. Resuspended sperm cells (2 ml) were treated with a 1 μM concentration of calcium ionophore A23187 (Sigma) for 5 minutes and then quenched with an equal volume of Ham's F-10 medium with 3% bovine serum albumin (Sigma). A 2 μl volume of this final sperm-cell suspension was placed in the same 35-mm petri dish as the prepared oocytes, but in a centrally situated 4 μl microdroplet of 10% PVP.

Microinsemination

A total of 94 mature oocytes were alloted to the five microinsemination treatments of this experiment. Microinsemination was performed on a heated stage under 400X magnification, using an inverted Nikon microscope (Tokyo, Japan) and two Leitz micromanipulator units. Each of the 5 μl microdroplets (n=8), that were arranged

in a circle around the sperm-PVP droplet in a prewarmed petri dish, contained a single mature oocyte. The holding pipette (100 μm outside diameter) was situated on the left and the sperm injection pipette (3-5 μm diameter) on the right. The tip of both microinstruments had a 30° angle to facilitate oocyte manipulation and sperm visibility during the injection procedure. The sperm-injection needle was prefilled with 10% PVP.

Sperm cells with good progressive motility, displaying simultaneous lateral head movement, were selected from the sperm-PVP droplet and immobilized prior to the two ICSI treatments (Treatments A and E). Sperm immobilization was conducted with the tip of the injection needle by performing a simultaneous down and lateral cut movement initially over the proximal third of the sperm tail. This crushing of the sperm tail was repeated several times further distal from the head. Interestingly, in this experiment using equine sperm, the sperm tail did not exhibit the characteristic kinked pattern as previously described for human spermatozoa (Dozortsev *et al.*. 1995; Van den Bergh *et al.*, 1995).

The sperm was loaded tail first into the injection pipette by applying gentle suction through the injection pipette. The injection pipette with the sperm cell was now moved to an oocyte droplet without raising it from the mineral oil. Using the holding and injection pipette, the oocyte was secured with the polar body or area of polar granularity at the 6 or 12 o'clock position (Palermo *et al.*, 1996). The sperm head was now positioned very close to the tip of the injection needle just before penetrating the zona pellucida. After piercing the zona, but before pushing into the ooplasm, any excess PVP in front of the sperm head was expelled into the perivitelline space. Once the

injection needle was pushed into the ooplasm, gentle suction was applied until the ooplasmic membrane ruptured, enabling a small amount of ooplasm to be aspirated freely into the needle (Vanderzwalmen *et al.*, 1996). The sperm cell and the micro-volume of aspirated ooplasm was then expelled into the ooplasm and the injection pipette was swiftly withdrawn.

The SUZI procedure (Treatment B) was similarly performed, but two or three motile sperm cells were inserted subzonally into the perivitelline space, as has been described previously in humans (Fishel *et al.*, 1990). Also, sham-injection procedures were performed similar to the ICSI and SUZI procedures, but only an equivalent amount of PVP (<1 μl) was injected into the ooplasm (Treatment C) or under the zona (Treatment D) without any sperm cells.

Embryo Co-Culture and Transfer

After sperm injection, the injected oocytes were washed and cultured in groups on a monolayer of bovine oviduct epithelial cells in 50 μl droplets of TCM-199 with 10% fetal bovine serum (Gibco) under mineral oil at 38°C in an atmosphere of 5% CO_2 in air. The culture medium was changed every other day and the injected oocytes were co-cultured for up to 7 days. Embryo development was evaluated under inverted light microscopy (200 to 400X) at 48-hour intervals after microinsemination. All oocytes that did not exhibit evidence of cleavage by 72 hours after the injection procedure were selectively removed from culture, fixed and stained with a 2% (w/v) aceto-orcein solution (Sigma) to assess fertilization or maturation status (Meintjes *et al.*, 1995).

All morula stage embryos (n=4) were nonsurgically transferred to synchronized recipients (one embryo per recipient) on day 5.5 after sperm injection, using a standard

transcervical embryo transfer procedure (Imel *et al.*, 1981). In addition, a set of two embryos (one 2-cell and one 4-cell embryo) was surgically transferred to the oviduct of each of two recipient mares at 48 hours after sperm injection. Surgical transfers were performed by exteriorizing the oviduct through a flank incision, and subsequently threading a small catheter (Wallace Embryo Transfer Catheter, Simcare Ltd., Peter Road, Lancing, West Sussex, England) containing the embryos ~4 to 6 cm into the lumen of the oviduct. The embryos were then expelled into the oviduct along with 10 to 20 µl of embryo transfer medium (HEPES-buffered TCM-199 supplemented with 10% fetal bovine serum).

Statistical Analyses

Fisher's Exact Two-tailed Test (Metha and Patel, 1983) was used to compare the ratios of oocytes that cleaved and proceeded to further developmental stages for the five microinsemination treatments. In selecting this test, it was assumed that the distribution of oocytes collected from the mares was equal and that each individual oocyte acted independently of the other oocytes collected.

Experiment 5.2

Experimental Design

Experiment 5.2 was conducted in a similar manner to that of Experiment 5.1, with the following exceptions, only ICSI was used to fertilize mature oocytes in the second experiment. Mature, injected oocytes (n=86) derived from pregnant crossbred mares in good body condition were randomly allocated within mare to one of two culture treatment groups (Treatments A and B). Treatment A consisted of injected oocytes (n=43) cultured for 48 hours in TCM-199 (with glucose) supplemented with

15% fetal bovine serum at 38°C in an atmosphere of 5% CO_2 in air. Injected oocytes in Treatment B (n=43) were similarly cultured in a glucose-free, phosphate-free medium (P-1™, Irvine Scientific, Santa Ana, CA) supplemented with 15% fetal bovine serum for 48 hours at 38°C in an atmosphere of 5% CO_2 in air. Co-culture was not incorporated into in this experiment, since P-1™ medium does not maintain the development of bovine oviduct epithelial cells.

Oocyte Collection.

Oocytes were collected during the 1997 breeding season from pregnant mares on days 14 to 70 of pregnancy via transvaginal ultrasonography, as in Experiment 5.1, except that 12-gauge double-lumen needles (Cook® Veterinary Products Inc., Bloomington, Indiana) were used to recover oocytes in this experiment. With this method, a slight modification of the collection procedure was necessary. Negative pressure (90 mm Hg) was constantly applied via a vacuum pump while oocyte collection medium (PBS plus 1% calf serum with 2 USP units of heparin, 100 μg of streptomycin and 100 units of penicillin-G per ml) was "pulsed" through the follicular cavity 10 to 15 times. Adequate force was required in pulsing the incoming medium to provide sufficient pressure to expand the follicle and to allow for the detachment of the oocyte from the follicular wall.

In Vitro Maturation and Oocyte Preparation

IVM of oocytes was performed as in Experiment 5.1. However, oocytes in this experiment were stripped of the majority of their cumulus cells by gentle pipetting in a solution of 80 units/ml hyaluronidase (Sigma) in TCM-199, rather than in a solution of 0.025% trypsin as in Experiment 5.1.

Semen Preparation

Cooled, extended semen (40 to 60 x 10^6 sperm cells/ml) from the same Arab stallion was used for the ICSI procedure in this experiment. A microvolume of this semen (<1 μl) was placed, unwashed, into the central PVP droplet (3 μl) in the microfertilization dish, without any prior exposure to calcium ionophore as in Experiment 5.1.

Microinsemination

Only ICSI was performed in Experiment 5.2, and the ICSI procedure utilized was similar to that of Experiment 5.1. Only one sperm cell was injected into the ooplasm of an *in vitro* matured oocyte. Care was taken in assuring that the tail of the sperm cell was adequately crushed and that the sperm cell was completely immobilized prior to its insertion into the ooplasm of the recipient oocyte.

Embryo Transfer

A total of 31 embryos were surgically transferred into the oviducts of synchronous recipient females at 48 hours post-ICSI. The embryos were loaded into a Wallace catheter (Simcare, Ltd.) (1 to 4 embryos/catheter) and the soft, inner portion of the catheter was threaded 4 to 6 cm into the surgically exposed oviduct of the recipient female. The embryos were then gently expelled along with 10 to 20 μl of transfer medium (HEPES-buffered TCM-199 supplemented with 10% fetal bovine serum). All embryos were transferred into the oviduct ipsilateral to the previously ovulated follicle. Recipient females were subsequently monitored by ultrasonography at day 14 post-fertilization to determine pregnancy status. All recipients were treated with altrenogest (Regu-Mate®, Hoechst Roussel Vet, Warren NJ) daily, beginning on the day of embryo

transfer and continuing until pregnancy was confirmed 12 to 14 days later. Pregnant recipients were maintained on altrenogest supplementation until 120 days of gestation.

Statistical Analyses

The statistical analyses performed in this experiment were similar to Experiment 5.1, with the ratio of embryos cleaved and the embryonic development stages being the parameters compared across the two culture treatments.

RESULTS

Experiment 5.1

A cleavage rate of 39% (2-cell embryos) was obtained for ICSI-derived oocytes and this was greater ($P<0.05$) than the 6% for SUZI oocytes (Table 5.1). Similarly, more ($P<0.05$) ICSI oocytes developed to the 4- to 8-cell stage than did oocytes subjected to the SUZI procedure. When more than one sperm were injected directly into the ooplasm, 33% of injected oocytes cleaved. This was not different from the oocytes subjected to the ICSI or SUZI procedures, but these polyspermic fertilized oocytes failed to develop further than the 2-cell stage. Furthermore, none of the sham-injected oocytes cleaved. Aceto-orcein staining of the oocytes that did not undergo cleavage revealed that at least 25% of SUZI-derived oocytes did not fertilize and that an additional 13% contained a metaphase plate and a single pronucleus.

Two embryos at the 2-cell stage and two embryos at the 4- to 8-cell stage (n=4) were surgically transferred to the oviducts of recipient mares and, thus, did not have the opportunity to develop to later stages *in vitro*. None of the morulae transferred nonsurgically resulted in an ultrasonic detectable pregnancy by day 16 after sperm injection. However, one of the surgical embryo transfers (2- to 4-cell embryo) resulted

in a 16 mm embryonic vesicle in the body of the uterus on day 14 after sperm injection, but was lost (23 mm diameter) between days 16 and 18 following sperm injection.

Experiment 5.2

A total of 263 follicles were punctured from 20 aspiration procedures, yielding an average of 13 follicles punctured per procedure. Of these, 174 oocytes were collected giving a recovery rate of 66% (8.7 oocytes/procedure). Overall, 86 oocytes were deemed mature by the presence of a first polar body after maturation in TCM-199 supplemented with 10% estrous mare serum (49%). However, when degenerate oocytes were excluded from the analysis, the maturation rate was 73%.

Of the 86 matured oocytes exposed to ICSI, 47 cleaved giving an overall cleavage rate of 55%. Cleavage rates differed between the two media treatment groups (47% *vs.* 63% for Treatments A and B, respectively) ($P<0.10$) (Table 5.2). However, there was no difference in the number of 2-cell, 3-cell, 4-cell, 6-cell or 8-cell embryos developing *in vitro* between Treatments A and B.

Since most of the embryos produced were subsequently transferred into synchronous recipients, it was important to establish a system of grading these embryos prior to transfer to determine which embryos had the greatest chance of developing into a viable pregnancy. The grading system was based on the rate of embryonic development as evaluated at 48 hours post-ICSI, and was as follows: Grade-1 = 4- to 8-cell stage, with blastomeres of even size and shape and <15% perivitelline fragments, Grade-2 = 4- to 8-cell stage, with blastomeres of even size and shape and 15 to 30% perivitelline fragments, Grade-3 = 4- to 8-cell stage, with blastomeres of uneven size and shape and >30% perivitelline fragments or 2- to 3-cell stage with <15%

perivitelline fragments and blastomeres of uneven size and shape, Grade-4 = 2- to 3-cell stage, with ≥15% perivitelline fragments and blastomeres of uneven size and shape.

A total of 13 embryos were transferred from Treatment A (one Grade-1, three Grade-2 and nine Grade-3 embryos). In Treatment B, 18 embryos were transferred (three Grade-1, five Grade-2, seven Grade-3 and three Grade-4 embryos). The one mare receiving the Grade-1 embryo from Treatment A was diagnosed pregnant by the presence of an embryonic vesicle at day 14 post-ICSI, which resulted in the birth of a healthy female foal, weighing 29.1 kg, at 319 days of gestation. Similarly, two of the three mares receiving Grade-1 embryos from Treatment B were confirmed pregnant by 16 days post-ICSI. One of these mares lost her pregnancy between day 20 and day 35 post-ICSI, and the other pregnancy resulted in the birth of a healthy, 27.7 kg female foal at 328 days of gestation.

Overall, the pregnancy rate was low for these mares (25% pregnancy/transfer, 8% pregnancy/embryo). However, when only Grade-1 embryos are considered, 75% (three of four) of the mares were diagnosed pregnant by 16 days post-ICSI, and 50% (two of four) resulted in live births.

DISCUSSION

The consistent production of IVF embryos in the horse will likely have valuable commercial, research and conservational applications. Recent studies, applying prefertilization zona-drilling (Li *et al.*, 1995; Meintjes *et al.*, 1995) and partial zona removal (Choi *et al.*, 1994), for the first time indicated that equine IVF rates may approach those of other domestic species and possibly humans. These studies also implicated that inadequate sperm capacitation and/or the presence of an *in vitro* zona

Table 5.1. *In vitro* development of subzonal and intracytoplasmic sperm-injected horse oocytes

Treatment	Oocytes	2-cell	4- to 8-cell	8- to 16-cell	Morula	ET
ICSI	36	$14^{a\dagger}$	$9^{a\dagger}$	3	3	3
SUZI	32	2^{b}	1^{b}	1	1	1
ICSI (s)	10	-	-	-	-	-
SUZI (s)	10	-	-	-	-	-
ICSI (psc)	6	$2^{a,b}$	-	-	-	-

ET = embryo transfer; (s) = sham injected, PVP without sperm; (psc) = polyspermy control, three to five sperm cells injected per oocyte.

[†]Two embryos from each of these groups (n=4) were surgically transferred to the oviducts of recipient mares and, thus, did not have the oppurtunity to develop to later stages *in vitro*.

[a,b]Different superscripts within the same column are different ($P<0.05$).

Table 5.2. *In vitro* development of intracytoplasmic sperm-injected equine oocytes following 48 hours of culture in either TCM-199 or P-1™ culture medium

Characteristic	(n)	Treatment group	
		TCM-199	P-1™
Cleavage rates	47	20 (47%)[a]	27 (63%)[b]
Cell stage:			
2-cell		12 (60%)	14 (52%)
3-cell		4 (20%)	5 (19%)
4-cell		4 (20%)	6 (22%)
6-cell		0 (0%)	1 (4%)
8-cell		0 (0%)	1 (4%)
Embryo grade:			
Grade 1		1 (5%)	3 (11%)
Grade 2		3 (15%)	5 (19%)
Grade 3		10 (50%)	8 (30%)
Grade 4		6 (30%)	11 (41%)

[a,b]Different superscripts within the same column are different (P = 0.09).

pellucida barrier may contribute to the less than anticipated conventional IVF rates in the horse. Although promising, these techniques will always have the possibility of producing polyspermic embryos. Many factors such as the size of the opening drilled into the zona pellucida, the concentration of sperm cells used for *in vitro* insemination, the time period of zona-drilled oocyte exposure to sperm cells and the individual stallion semen characteristics may be difficult to standardize and hamper consistent normospermic IVF rates.

ICSI is now a standard procedure used for the treatment of male factor infertility in humans (Ng *et al.*, 1993; Palermo *et al.*, 1996) even when sperm cells have to be obtained from the epididymides (Mansour *et al.*, 1996) or testicle (Kahraman *et al.*, 1996). The ICSI technique also circumvents the difficulties encountered with fertilizing human oocytes with a hardened zona pellucida, such as IVM oocytes or oocytes obtained from patients with severe endometriosis (Ng *et al.*, 1993).

ICSI was considered in the present study in an effort to bypass the necessity of effective *in vitro* sperm cell capacitation, as well as to circumvent the zona pellucida and oolemma barrier, while still ensuring normospermic fertilization. There was no guarantee that SUZI may not perform better in the horse than ICSI, therefore, SUZI was also included in the microinsemination treatment groups in Experiment 5.1. It was clear from the data in this experiment that ICSI is the microinsemination technique of choice in the horse, as also reported for humans (Palermo *et al.*, 1993; Van Steirteghem *et al.*, 1993). It seemed that SUZI oocytes were either not penetrated by the perivitelline inserted sperm (25% without a single pronucleus and a normal appearing metaphase II plate) or were fertilized but not activated (13% with one pronucleus, presumably from

sperm origin and an intact metaphase II plate). It was encouraging to note that no polyspermic fertilized oocytes developed further than the 2-cell stage and that none of the sham-injected oocytes were parthenogenically activated by the injection procedure.

The cleavage rate of ICSI oocytes in this study (39% and 55% for Experiments 5.1 and 5.2, respectively) seemed to be lower than the cleavage rate of the best sperm-treatment group (79%) in a previous prefertilization zona-drilling study by our laboratory (Li *et al.*, 1995). Reasons for this lower cleavage rate may include the learning curve necessary to perform ICSI efficiently with adequate crushing of the sperm tail (Dozortsev *et al.*, 1995; Van den Bergh *et al.*, 1995), damage to the injected oocytes (Palermo *et al.*, 1996) and the possibility that some cleaved polyspermic embryos were included in the prefertilization zona-drilling group. However, the cleavage and embryo developmental rates obtained by ICSI in this study appear to be an improvement on rates from previous conventional equine IVF studies (Del Campo *et al.*, 1990; Palmer *et al.*, 1991; Grøndahl *et al.*, 1995) and compare favorably with other recent ICSI studies in the horse (Dell'Aquila *et al.*, 1997), the cow (Goto *et al.*, 1996; Mutsuro *et al.*, 1996) and the cat (Pope *et al.*, 1995, 1997). The data presented in Table 5.1 suggests that there was a marked decline in ICSI embryos between the 4- to 8-cell stage and the 8- to 16-cell stage. This is due to the removal of four embryos (two 2-cell and two 4-cell embryos) from culture for the surgical intraoviductal transfer to synchronized recipients. For the same reason, the morula/blastocyst development rate between this and the previous prefertilization zona-drilling studies (Li *et al.*, 1995; Meintjes *et al.*, 1995) cannot be compared.

In Experiment 5.2, cleavage rates for ICSI oocytes were higher when the oocytes were cultured for 48 hours in P-1™ medium when compared with TCM-199 (63% and 47%, respectively) (P=0.09). This is in agreement with previous reports on the detrimental effects of high levels of glucose present in the culture medium for early stage mouse (Chatot *et al.*, 1989), cow (Ellington *et al.*, 1990), hamster (Seshagiri and Bavister, 1989) and human embryos (FitzGerald and DiMattina, 1992). For example, Chatot *et al.* (1989) found that medium containing a relatively high level of glucose inhibited the development of 1-cell mouse embryos during the first 48 hours of culture, but not after 48 hours. In fact, the addition of glucose to the culture medium on day 3 of culture significantly improved development of mouse embryos to the blastocyst stage, suggesting that the toxic effects of glucose is short-lived in murine embryos (Chatot *et al.*, 1989).

Similarly, Ellington *et al.* (1990) determined that a high glucose level in the culture medium is inhibitory to bovine embryos during the first 48 hours of culture and that culturing these embryos in a glucose-free medium facilitated the development of the embryos through the 8-cell stage. In addition, it has been shown in both cattle (Ellington *et al.*, 1990) and humans (FitzGerald and DiMattina, 1992) that culturing early stage embryos in medium without glucose significantly improves embryo quality. In contrast, Watson *et al.* (1994) found no difference in cleavage rates of ovine embryos cultured in medium without glucose. In addition, it has been demonstrated that glucose levels had no effect on the development of ovine embryos past the *in vitro* block developmental stage (McGinnis and Youngs, 1992).

Even though no statistical differences were detected between the culture treatments for early embryonic development and embryo quality, it is apparent that embryos cultured in a low glucose environment were not inhibited in their development, since this was the only group to produce both 6- and 8-cell embryos at 48 hours post-ICSI (Table 5.2). Also, two pregnancies were established from the transfer of two Grade-1 embryos cultured in the P-1™ medium (low glucose), with one pregnancy resulting in a live birth.

Results from this study indicate that intracytoplasmic sperm injection should not be overlooked as the method of choice for the production of *in vitro* equine embryos. Although no pregnancies resulted from the nonsurgical morula-stage embryo transfers, oocytes aspirated from pregnant mares were viable and capable of establishing four pregnancies after *in vitro* maturation, ICSI, *in vitro* culture and intraoviductal embryo transfer, with two live births resulting at days 319 and 328 of gestation, respectively.

Also, previous reports have suggested that multiple, good quality oocytes can be collected repeatedly from early pregnant mares (Li *et al.*, 1995; Meintjes *et al.*, 1995). The benefit of using the pregnant mare as an oocyte donor is that the oocytes can be collected uniformly at the germinal vesicle stage, which allows for known, fixed IVM intervals resulting in larger quantities of good quality oocytes available for IVF. In addition, oocyte retrieval in this study was improved using the double-lumen needles when compared with previous reports using a single-lumen needle system (Meintjes *et al.*, 1995).

The present study provides strong evidence that ICSI of oocytes derived from pregnant mares can be a viable alternative for the production of gametes for assisted

reproduction and the treatment of clinical infertility in valuable mares, and that ICSI may have application in the conservation of endangered equids. This study, to our knowledge, is the first report of the production of live foals derived from *in vitro*-matured equine oocytes collected from pregnant mares.

CHAPTER VI

DEVELOPMENT OF A PROTOCOL USING ALTRENOGEST AND eST FOR OVARIAN STIMULATION IN THE DOMESTIC MARE FOR USE IN TRANSVAGINAL OOCYTE ASPIRATION PROCEDURES FOR THE PRODUCTION OF *IN VITRO*-DERIVED EQUINE EMBRYOS

INTRODUCTION

The routine production of *in vitro*-derived equine embryos has not yet become a reality, even though an acceptable number of oocytes can be repeatedly collected from live mares, with successful *in vitro* maturation (IVM) of the oocytes followed by intracytoplasmic sperm injection (ICSI) (Cochran *et al.*, 1998, 2000). Since the first report of a foal being born from *in vitro* fertilization (Palmer *et al.*, 1991), only one other report has been published on the production of IVF-derived foals (Cochran *et al.*, 1998). In that study, Cochran *et al.* (1998) demonstrated that pregnant mares could be used as oocyte donors, and that ICSI could be performed on these oocytes in order to produce live foals. Since conventional IVF rates for horses remains low (Palmer *et al.*, 1991; Dell'Aquila *et al.*, 1996), ICSI appears to be the method of choice for the production of equine embryos *in vitro* (Dell'Aquila *et al.*, 1997; Cochran *et al.*, 1998; 2000).

However, one of the biggest obstacles to establishing an efficient IVF procedure for horses has been the inability to "superstimulate" domestic mares to produce high numbers of good quality oocytes, which is routinely performed in many domestic animals and in women. It has been demonstrated in cows (Manikkam *et al.*, 1997) and horses (Ginther and Bergfelt, 1992) that follicular waves continue throughout early pregnancy, and that these animals may serve as oocyte donors for IVF (Meintjes *et al.*,

1995). Meintjes *et al.* (1995) first demonstrated that pregnant mares could be effectively used as oocyte donors, and that they could provide high numbers of good quality oocytes when the oocytes were collected repeatedly using an ultrasound-guided approach. One possible explanation for the success realized when using oocytes derived from pregnant mares is that follicular waves that occur during pregnancy show a reduced occurrence of follicular dominance (Ginther and Bergfelt, 1992), caused by the continuously elevated levels of progesterone, which in turn reduces the negative effects that the dominant follicle exerts on its subordinates. Oral altrenogest, a synthetic progestin, has been shown to suppress the occurrence of dominant follicles in mares (Lofstedt *et al.*, 1989), with no effect on subsequent fertility of the treated mares once administration was discontinued (Sigler *et al.*, 1989). It is believed, therefore, that by treating mares daily with altrenogest, a "pseudopregnant" state could be induced during which follicular dominance would be minimized and oocyte collections could be performed with a high rate of success.

It has been well established that administration of somatotropin (ST) increases follicle number in horses (Cochran *et al.*, 1999), cattle (Carter *et al.*, 1998), pigs (Spicer *et al.*, 1992), rabbits (Yoshimura *et al.*, 1994) and women (Owen *et al.*, 1993). It is believed that the ability of ST to increase follicle number is caused, at least in part, through its ability to increase circulating IGF-I concentrations (Owen *et al.*, 1993; Yoshimura *et al.*, 1994), as it is well known that ST is a potent stimulator of IGF-I production (Owen *et al.*, 1993; Gong *et al.*, 1997; Cochran *et al.*, 1999).

Therefore, the purpose of the present study was twofold: (1) to develop a protocol for IVF in horses using oral altrenogest treatment to reduce follicular

dominance and allow for a higher number of good quality oocytes to be collected per mare, followed by IVM and ICSI of the collected oocytes and (2) to determine if daily administration of equine somatotropin (eST) could increase the number of follicles available from these altrenogest-treated mares.

MATERIALS AND METHODS

Experimental Design

A total of 18 mares in good body condition (body condition scores ranged from 5 to 8 on a scale of 1 to 9), from the same experimental herd, were randomly allocated to one of two treatment groups during the breeding season of 1998. Treatment A consisted of mares (n=9) treated with 0.044 mg altrenogest (Regu-Mate®, Hoechst Roussel Vet., Warren, NJ) per kg body weight for a total of 51 days (day 1 = first day of treatment). Mares in Treatment A also received a single daily injection of 25 μg eST (EquiGen™, BresaGen, Thebarton, South Australia) per kg body weight beginning on day 1 and ending on day 51. Mares in Treatment B (n=9) were similarly treated with altrenogest in addition to the eST vehicle beginning on day 1 and continuing for 51 days. All experimental animals were administered either altrenogest plus eST or vehicle for a preliminary period of 21 days. This was performed to allow for a possible "priming" effect of eST on follicular development, as well as to subject the mares to a period of time (which is approximately equal to one estrous cycle) of progesterone dominance. This time of progesterone dominance was used in order to allow for more synchronous development of follicles within a follicular wave.

On day 21, all mares were subjected to a follicle ablation procedure, during which all follicles ≥5 mm in diameter were punctured follicular fluid was collected via a

transvaginal ultrasonographic procedure. This was performed to (1) remove all atretic follicles that may be present on the ovaries of each mare and (2) induce a new wave of follicular growth for subsequent oocyte collection procedures. After the follicle ablation procedure was performed, mares from both treatment groups were maintained on their respective treatment protocols, and oocyte collection procedures (n=3/mare) were performed at 10-day intervals. Plasma samples were collected at 72-hour intervals on all mares via jugular venipuncture for the duration of the treatment period to assess plasma IGF-I concentrations in response to eST administration.

Oocyte Collection

Oocyte collection procedures were performed at 10-day intervals following the follicle ablation procedure on day 21 of treatment. This time interval was chosen based upon the approximate interval of follicular wave emergence and growth in the domestic mare. Oocyte collections were performed as previously described by Cochran *et al.* (1998). Briefly, a 12-gauge double-lumen stainless steel needle (Cook® Veterinary Products Inc., Bloomington, IN) was inserted through the vaginal wall via a transvaginal ultrasonic transducer attached to a needle guide. Negative pressure (90 mm Hg) was constantly applied using a vacuum pump while oocyte collection medium (PBS plus 2% calf serum with 2 USP units of heparin and 100 units of penicillin-G/ml) was pumped through the follicle cavity up to 10 times. Sufficient pressure was applied by the incoming medium to expand the follicle and to allow for the detachment of the oocyte from the follicular wall. The flushing medium was then collected into a 500 ml container and the contents were searched for oocytes contained therein.

Oocyte Maturation and Micromanipulation

After oocyte recovery, all oocytes with an intact oolemma were washed in *in vitro* maturation (IVM) medium. IVM medium consisted of tissue culture medium 199 (TCM-199, Gibco) with 10% estrual mare serum collected at this laboratory. *In vitro* maturation was conducted in 35 μl microdroplets of IVM medium covered with mineral oil in a 35-mm petri dish for 36 hours at 38°C in an atmosphere of 5% CO_2 in air. After IVM, the oocytes were stripped from the majority of their cumulus cells by vigorous pipetting for 2 to 3 minutes in TCM-199 containing 80 units of hyaluronidase per ml (Sigma, St. Louis, MO) to facilitate micromanipulation. Oocytes with an extruded first polar body (MII) were identified under light microscopy (200X) and then placed separately into 5 μl microdroplets consisting of HEPES-buffered TCM-199 under mineral oil (Sigma). All MII oocytes were then subjected to the ICSI procedure as previously described (Cochran *et al.*, 1998).

Embryo Culture and Transfer

After micromanipulation, all oocytes were placed in 35 μl microdrops of P-1™ medium (Irvine Scientific, Santa Ana, CA) and cultured for 48 hours at 38°C in an atmosphere of 5% CO_2 in air under oil. Embryonic development was then assessed and embryos were either surgically transferred into synchronous recipient mares (n=3) or allowed to develop additionally *in vitro* for up to 3.5 days (n=71). After 72 hours of culture, all remaining embryos were transferred into Blastocyst™ medium (Irvine Scientific) for the remainder of the culture period. At day 5.5, all morula-stage embryos (n=4) were transferred nonsurgically into synchronous recipient mares.

Statistical Analysis

The numbers of small- (5 to 10 mm), medium- (11 to 25 mm) and large- sized (>25 mm) follicles were recorded per mare during each oocyte collection procedure and were analyzed across treatment groups. In addition, the number of oocytes collected per mare was analyzed for all mares across treatment groups using ANOVA (Cochran *et al.*, 1999). Also, the percentage of oocytes reaching metaphase II after 36 hours of culture, as well as the cleavage rate of injected oocytes and embryonic development, were analyzed across treatment groups for mares in Treatments A and B via ANOVA (Cochran *et al.*, 1999).

RESULTS

Follicle Populations, Oocyte Collections and Plasma IGF-I Levels

The effects of eST on altrenogest-treated mares are presented in Table 6.1. There was no effect of collection procedure (first, second or third collection) on any parameter measured, thus, data were pooled across the three collection procedures per mare per treatment. Daily administration of eST to altrenogest-treated mares had no effect on the number of small (1.40 ± 0.30 for both Treatments A and B), medium (3.3 ± 0.42 *vs.* 3.7 ± 0.64, Treatments A and B, respectively) or large follicles (1.37 ± 0.23 for both Treatments A and B) per mare per oocyte collection (10-day intervals). Furthermore, there was no difference in the total number of follicles per mare per oocyte collection for altrenogest-treated mares with or without eST (6.15 ± 0.65 *vs.* 6.52 ± 0.76 for Treatments A and B, respectively). However, when the total follicle number per mare was analyzed following 21 consecutive days of treatment (first follicle ablation), mares treated with altrenogest plus eST had significantly more follicles per

mare when compared with contemporary control mares (22.9 ± 2.5 *vs.* 13.9 ± 2.5 for Treatments A and B, respectively).

Unfortunately, this increase in follicle number was not maintained once oocyte collections began at 10-day intervals (Table 6.1). Also, there was no difference in the number of oocytes collected per mare per oocyte collection for mares treated with altrenogest with or without eST (4.4 ± 0.56 for both Treatments A and B). Finally, there was a definite increase in circulating plasma IGF-I levels for altrenogest-treated mares given eST when compared with contemporary control mares ($P<0.05$, Figure 6.1).

Oocyte Maturation, Embryo Cleavage and Embryonic Development

Results of oocyte maturation, embryo cleavage and embryonic development are summarized in Table 6.2. There was no effect on the percent of oocytes reaching metaphase II after 36 hours of IVM for mares with or without eST (73% *vs.* 58% for Treatments A and B, respectively). In addition, cleavage rates were similar (44% *vs.* 58%) for altrenogest-treated mares with or without eST, respectively. A total of 74 ICSI-derived equine embryos were produced from this experiment (35 and 39 from Treatments A and B, respectively).

The majority of these embryos (n=71) were cultured *in vitro* for up to 5.5 days, with three embryos being surgically transferred into the oviducts of two synchronous recipient mares after 48 hours of culture in order to attempt to establish that oocytes collected from mares treated in this fashion were fertile and capable of producing live foals. The pregnancy did not continue in the mare receiving the single embryo at 48 hours post-ICSI. However, the mare that received two embryos at 48 hours post-ICSI

was diagnosed pregnant with twins at day 14 post-ICSI, and one embryonic vesicle was immediately crushed to prevent complications related to twin conceptuses in the mare. Incidentally, both embryos were collected from a single mare in the control group. This pregnancy resulted in the birth of a healthy female foal, weighing 30.5 kg, after 339 days of gestation.

To our knowledge, this is the first report of a foal produced from oocytes collected from altrenogest-treated mares, followed by IVM and ICSI. Also, this represents the first incidence, to our knowledge, of a twin pregnancy resulting from the transfer of *in vitro*-produced equine embryos. There was no treatment effect on subsequent embryo development past the 2-cell stage (Table 6.2). Furthermore, none of the mares receiving morula-stage embryos at day 5.5 (n=4) resulted in a pregnancy.

DISCUSSION

One of the largest obstacles to developing a repeatable protocol for IVF in the horse is the inability to collect high numbers of good quality oocytes from donor mares. The major problem is that most attempts to superstimulate the equine ovary to produce a large number of follicles has met with very limited success (for review see Ginther, 1992). Therefore, if multiple oocytes are to be collected from a single collection procedure, researchers must utilize the available follicles present at the time of collection to achieve the desired results.

In mares, the ovaries undergo periods of follicular growth and development termed "follicular waves". This is true for other farm animal species as well (Lucy *et al.*, 1993; Pursley *et al.*, 1993), except for the pig (Ryan *et al.*, 1994). The majority of mares have 1 to 2 waves of follicular growth per interovulatory interval, each lasting 10

Table 6.1. Effect of eST administration to altrenogest-treated cyclic mares on follicular populations

Treatment	Small follicles	Medium follicles	Large follicles	Total follicles	Follicle ablation*
eST	1.40 (±0.30)	3.30 (±0.42)	1.37 (±0.23)	6.15 (±0.65)	22.9[a] (±2.5)
Control	1.40 (±0.30)	3.70 (±0.64)	1.37 (±0.23)	6.52 (±0.76)	13.9[b] (±2.5)

[a,b]Means within columns with different superscripts are different ($P < 0.05$).

*The follicle ablation procedure was performed at day 21 of treatment to remove any atretic follicles and to promote a new wave of follicular growth.

Table 6.2. Oocyte maturation, cleavage and early embryonic development for altrenogest-treated cyclic mares with and without eST

Treatment	Maturation	Cleavage
eST	73%	44%
Control	58%	58%

Treatment	2 to 4 cell	4 to 8 cell	8 to 16 cell	16 to 32 cell	Morula
eST	35	25	19	7	2
Control	39	28	19	6	2

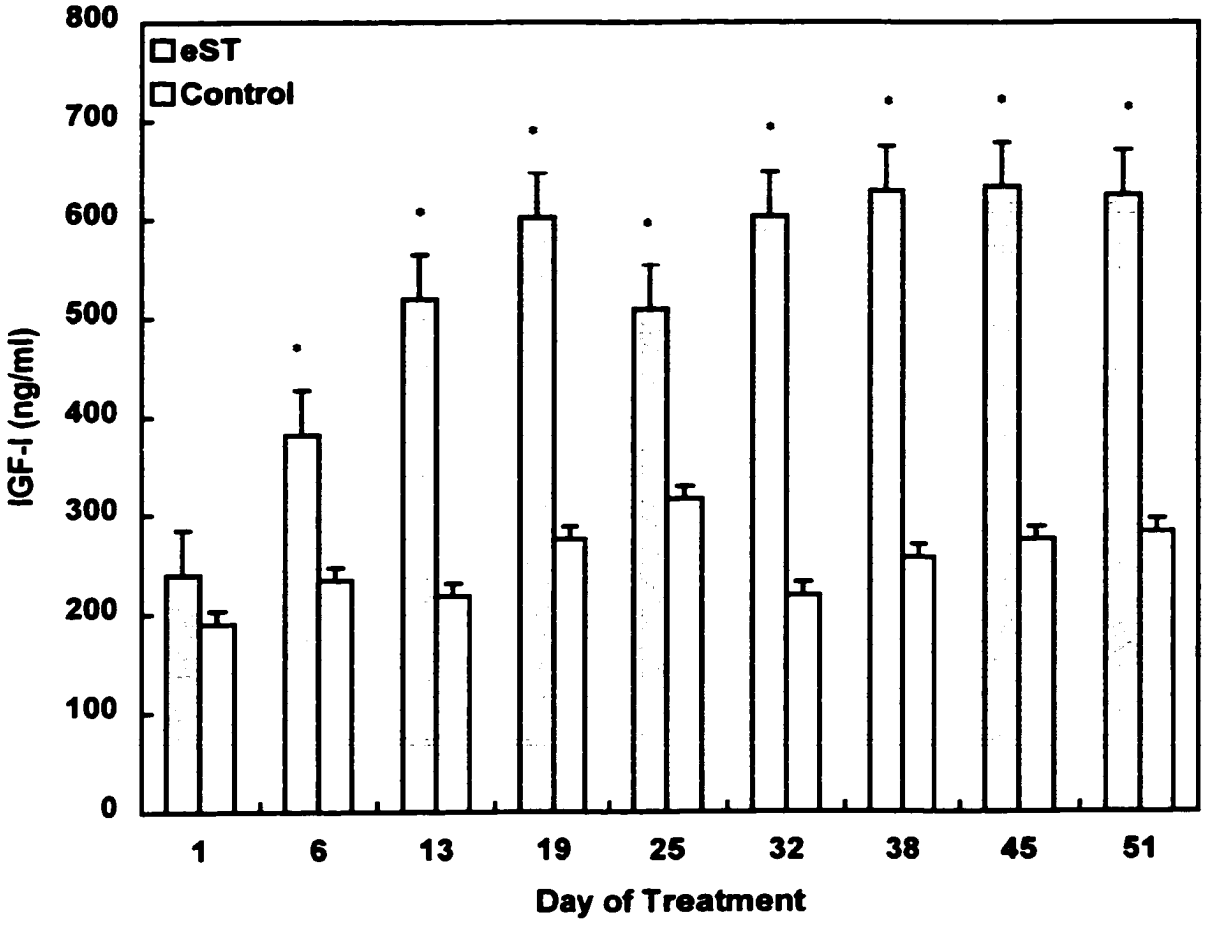

Figure 6.1. Plasma IGF-I concentrations (±SEM) for altrenogest-treated mares administered once daily injections of eST. (*) Denotes significant differences within days.

days (Ginther, 1992; Buratini *et al.*, 1997). One particular problem with attempting to collect oocytes from the follicles of cyclic mares is that, during most waves of follicular growth, one follicle is selected to become dominant over the other follicles within that wave, and that dominant follicle (DF) produces substances that inhibit the growth and development of the subordinate follicles (SF) within the same follicular wave (Ginther, 1992). Therefore, if one is to collect multiple oocytes from a cyclic mare, one must realize that the majority of the oocytes will come from these SF and may or may not be viable.

During periods of progesterone dominance, such as pregnancy, both cattle (Ginther *et al.*, 1989), and mares continue to show follicular wave emergence and growth (Ginther and Bergfelt, 1992). However, the incidence of follicular dominance is reduced, possibly due to decreased LH pulse frequency from the pituitary caused by elevated plasma progesterone concentrations (Adams *et al.*, 1992a). Bergfelt *et al.* (1991) demonstrated that, in cattle, anovulatory follicular waves continued at least to day 100 in both pregnant and progesterone-treated control cows. The fact that these follicular waves were anovulatory demonstrates the ability of progesterone treatment to inhibit DF development. Similar findings were reported for cows by Ginther *et al.* (1989), where the diameter of the largest follicle was lower in pregnant cows compared with nonpregnant controls. In addition, other studies have demonstrated the ability of pregnant cattle to continue follicular development through to parturition (Manikkam *et al.*, 1997).

In mares, follicular waves have been documented to occur during vernal transition (Ginther, 1990), during reproductive cyclicity (Buratini *et al.*, 1997) and

during pregnancy (Ginther and Bergfelt, 1992). Also, it has been shown that oocytes collected from pregnant donor mares are viable and capable of producing live foals (Cochran *et al.*, 1998). However, utilizing pregnant donors is of little use in subfertile mares, since it is very difficult to attain a viable pregnancy in this population of mares. Therefore, in the present study, we attempted to induce a "pseudopregnant" state in cyclic mares by administering altrenogest once daily. Although we did not attempt to identify individual waves of follicular activity in these mares, it was evident that follicular growth and development continued through to day 51 of treatment, as evidenced by the number of follicles available for aspiration per oocyte collection procedure in the present study. In addition, it has been demonstrated in cattle that follicle ablation can induce a new wave of follicular growth (Bergfelt *et al.*, 1997; Bo *et al.*, 1995), so it is not unreasonable to assume a similar phenomenon occurs in mares. Also evident from this work is that oocytes collected from altrenogest-treated mares are viable and capable of producing live offspring.

The administration of ST has been shown to increase the number of follicles in cattle (Lucy *et al.*, 1993), pigs (Spicer *et al.*, 1992), women (Owen *et al.*, 1993), rabbits (Yoshimura *et al.*, 1994) and mares (Cochran *et al.*, 1999). It is believed that ST causes these effects, at least in part, through its ability to increase circulating IGF-I concentrations (Owen *et al.*, 1993; Gong *et al.*, 1997; Cochran *et al.*, 1999). In the present study, eST administration to altrenogest-treated mares did cause a significant increase in plasma IGF-I levels (Figure 6.1). However, this treatment did not increase the number of aspiratable follicles per mare when mares were subjected to oocyte collections at 10-day intervals.

Interestingly, when data were analyzed for the number of aspiratable follicles following 21 consecutive days of eST treatment (first follicle ablation), there was a marked increase in the total number of follicles ≥5 mm in diameter on the ovaries of altrenogest-treated mares administered daily injections of eST (Table 6.1). The reasons for this are unclear, but possibly include a prolongation of the atretic phase of follicle development (Cochran *et al.*, unpublished data). Results supporting this hypothesis are reported by Chun *et al.*, 1996, where the authors show that IGF-I is a potent regulator of granulosa cell apoptosis, decreasing the apoptotic index of cultured granulosa cells by 45%. This then could lead to the follicle remaining on the ovary for a longer period of time, thereby, resulting in increased follicle numbers after a prolonged period of treatment. Unfortunately, the increase in follicle numbers was not maintained once oocyte collections were begun at 10-day intervals, which may limit the effectiveness of eST administration for purposes of oocyte collections at regular intervals in the domestic mare. Further studies are needed in this area of follicular growth and development.

Another explanation for the apparent lack of eST treatment to effect follicle numbers in the present study may revolve around the production of IGF-I binding proteins (IGFBP), which limit the bioavailability of circulating, as well as follicular, IGF-I. There are at least 5 IGFBP currently identified, each of which having a different molecular size and a different binding capacity. In general, the low molecular size IGFBP (IGFBP-2, -4, -5) seem to have a higher affinity to IGF-I than does the larger IGFBP-3 (Mondschein *et al.*, 1991). Interestingly, follicular fluid from small, as well as atretic, follicles contain higher amount of low molecular weight IGFBP, and larger,

dominant follicles contain more IGFBP-3 (Mondschein *et al.*, 1991; Echternkamp *et al.*, 1994b). Therefore, changes in follicle growth and atresia could be regulated more by IGFBP ratios than by IGF-I itself (Monget *et al.*, 1993; Echternkamp *et al.*, 1994b).

Increasing IGFBP in follicular fluid have been shown to inhibit IGF-I-stimulated progesterone production by the granulosa cells in pigs (Samaras *et al.*, 1995) and in cattle (Spicer *et al.*, 1999). Also, ST administration has been shown to not only increase circulating and follicular IGF-I, but also to increase the production of IGFBP (Armstrong *et al.*, 1996a). In the mare, at least 4 IGFBP have been identified, corresponding to IGFBP-2, -3, -4, and –5 (Gerard *et al.*, 1998). It is therefore possible that prolonged administration of eST to altrenogest-treated mares in the present study could have led to a build up of IGFBP, which would have inhibited the effects of IGF-I on follicle growth and development.

In the present study, oocyte collections performed at 10-day intervals on altrenogest-treated mares with or without eST yielded an average of 4.4 (± 0.56) oocytes per mare per collection procedure. This appears to be an improvement over previous reports using pregnant donors (Meintjes *et al.*, 1995). However, Cochran *et al.* (2000) reported a recovery of 8.7 oocytes per mare per collection using pregnant mares as oocyte donors. In the latter study, however, mares were not subjected to repeated collection procedures at regular intervals and therefore many of the collected oocytes came from atretic follicles, which is one of the points of concern in developing an efficient protocol for oocyte collection in the mare.

However, when compared with the traditional approach of using cyclic donor mares, with collection of only the pre-ovulatory follicle of an *in vivo*-matured oocyte,

this technique seems favorable. This is due to the fact that, with ~80% recovery rate for preovulatory follicle collections, and the fact that only ~80% of those oocyte collected would be at metaphase II, performing oocyte collections from 10 mares would yield only between 6 and 7 mature oocytes. With our approach, ~30 mature oocytes could be recovered from these same 10 mares, which would give a much higher chance of success. Also, there was no difference in the number of oocytes collected per mare from each collection procedure in the present study, so these numbers of oocytes could be attained for at least three consecutive collection procedures.

Another obstacle in improving equine IVF is the seemingly lowered success of IVM when compared with other domestic species. Successful IVM rates ranging from 15 to 60% have been reported, which is considerably lower than the 80 to 90% noted for cow IVM (Meintjes *et al.*, 1995; Goudet *et al.*, 1997; Goudet *et al.*, 1998a). It seems that there is a significant correlation in follicle size and success of IVM for equine oocytes, as oocytes collected from larger follicles have a better chance of reaching metaphase II in culture than those collected from small follicles (Goudet *et al.*, 1997). When using pregnant donor mares, 50 to 60% of the collected oocytes reached MII (Meintjes *et al.*, 1995), and the success rate was highest with oocytes collected from 10 to 15 mm follicles (Goudet *et al.*, 1998b).

The addition of serum to the IVM medium has been shown to increase the number of oocytes reaching metaphase II in culture in the mare (Willis *et al.*, 1991). In addition, adding 100 ng/ml of ST to the culture medium increases maturational rates of bovine oocytes (Izadyar *et al.*, 1997). In the present study, there was no effect of ST administration to altrenogest-treated mares on oocyte maturation following 36 hours of

culture *in vitro* (Table 6.2). However, the percent of oocytes reaching metaphase II tended to be higher (P=0.1) for altrenogest-treated mares receiving daily eST (73% *vs.* 58%, respectively). This suggests the possibility of an effect of either ST and/or IGF-I on oocyte competence in the mare. Further studies are needed to determine if there is any beneficial effect of increased ST/IGF-I on *in vitro* maturation in the domestic mare.

Another problem with equine IVF is that conventional *in vitro* fertilization procedures have not shown much success to date. Only one foal has ever been reported from conventional IVF (Palmer *et al.*, 1991). However, in several studies in which relatively large numbers of oocytes were used, the fertilization rates for IVF in horses has remained low (less than 20%) (Palmer *et al.*, 1991; Li *et al.*, 1995; Dell'Aquila *et al.*, 1996). In humans, it has been stated that the best indicator of IVF outcome is related to ovarian response to gonadotropins (Roest *et al.*, 1996). Since the administration of exogenous gonadotropins has little effect on ovarian follicular development in the horse, this may be one indication of a species-specific problem associated with IVF.

Since the advent of ICSI in treating male-factor infertility in humans (Palermo *et al.*, 1992), ICSI has become a valuable tool in human infertility clinics. It has been shown in subfertile women that ICSI can lead to higher fertilization rates when compared with conventional IVF (94% *vs.* 72% respectively) (Pisarska *et al.*, 1999). Also, fertilization can be achieved independent of sperm characteristics (Palermo *et al.*, 1995), and there is no difference in detectable embryonic defects when comparing ICSI to conventional IVF in humans (Govaerts *et al.*, 1996). In the horse, ICSI has been shown to be the method of choice for IVF procedures compared with other forms of

micromanipulation (Cochran *et al.*, 2000), and it yields fertilization rates superior to that of conventional IVF (Dell'Aquila *et al.*, 1997).

Also, unlike other domestic species such as cattle, no additional oocyte activation procedures are necessary to induce acceptable fertilization rates. This is important since artificial activation of the oocyte can lead to the development of parthenogenic embryos, which have a severely limited chance of producing live offspring. Also, the injection procedure itself is inadequate in inducing parthenogenic embryonic division in the equine oocyte (Cochran *et al.*, 2000). In a previous study using pregnant oocyte donors, an overall cleavage rate of 55% was demonstrated when ICSI was the method of fertilization (Cochran *et al.*, 2000). Also, live foals have been produced from oocytes collected from pregnant mares and subjected to ICSI procedures (Cochran *et al.*, 1998). Thus, it seems that if a routine protocol for producing equine embryos *in vitro* is to become a reality, ICSI should be considered the method of choice for fertilization of the oocyte.

Finally, since there has been very limited success with equine IVF, developing a culture system for early equine embryos has not been feasible up to this point. Reports from other species have shown a detrimental effect of the presence of glucose in the culture medium during early embryonic development in mice (Chatot *et al.*, 1989), cattle (Ellington *et al.*, 1990), hamsters (Seshagiri *et al.*, 1989) and humans (Fitzgerald and DiMattina, 1992). Also, Cochran *et al.* (1998; 2000) have shown that utilization of a glucose-free medium (P-1™), which is currently used in human IVF procedures, yields superior cleavage rates when compared with TCM-199, which contains glucose. Although the difference noted may or may not be related to glucose content, since the

two media types exhibit other differences, P-1™ medium was chosen as the initial culture medium for the present experiment. This culture medium is designed only for the first 72 hours of culture following fertilization, at which time a second media type (Blastocyst™ medium) is used to allow for embryonic development up to the blastocyst stage.

There was no difference in either cleavage rates or embryonic development between the two treatment groups in the present experiment (Table 6.2). Unfortunately, no blastocysts were produced in the present study, and the nonsurgical transfer of four morula stage embryos did not result in any pregnancies. However, in an effort to verify that oocytes collected from altrenogest-treated mares were viable and capable of producing live offspring, three embryos were surgically transferred into the oviducts of two synchronous recipient mares at 48 hours of development. The mare receiving one embryo did not become pregnant. However, the mare that received two embryos was diagnosed pregnant with twins at 14 days post-fertilization via transrectal ultra-sonography. One embryonic vesicle was immediately crushed, and the other continued to develop and resulted in the birth of a healthy female foal at 339 days of gestation.

In summary, we have demonstrated that a protocol including treatment of mares with oral altrenogest, with a follicle ablation procedure performed at day 21 followed by oocyte collections at 10-day intervals, with IVM and ICSI performed on the collected oocytes, can be utilized to produce *in vitro*-derived equine embryos with a high degree of success. Furthermore, while culturing these *in vitro*-produced embryos to the morula stage did not result in a successful pregnancy being established, transfer of these embryos at earlier embryonic developmental stages can result in the production of

normal, healthy offspring. This is the first report, to our knowledge, of successful *in vitro* fertilization of equine embryos following a protocol of daily oral altrenogest to induce pseudopregnancy. In addition, this is the first report of twin pregnancies resulting from the transfer of IVF-derived equine embryos.

CHAPTER VII

EFFECTS OF eST ON EQUINE GRANULOSA CELL PROLIFERATION AND PROGESTERONE PRODUCTION *IN VITRO*

INTRODUCTION

In recent years, the effects of somatotropin (ST) administration on reproductive function in domestic species has become a major area of investigation. Currently, it is not know whether these effects are caused as a direct result of ST enhancement, or indirectly through elevated IGF-I. It has been shown, however, that both ST (Sirotkin *et al.*, 1998a) and IGF-I (Foster *et al.*, 1995) are stimulatory to granulosa cells cultured *in vitro*. For example, Behl and Pandey (1999) demonstrated that IGF-I increases progesterone production by caprine granulosa cells, and IGF-I has similar effects in porcine (Xia *et al.*, 1994) and bovine granulosa cells (Spicer and Echternkamp, 1995). Also, the addition of IGF-I to the culture medium causes a significant increase in granulosa cells proliferation in cattle (Armstrong *et al.*, 1996b) and sheep (Mariana *et al.*, 1998). In addition, it has been demonstrated that ST causes similar effects (Spicer and Enright, 1991), and it increases the production of IGF-I from the granulosa cells (Siroikin *et al.*, 1998a). It has been shown that granulosa cells do contain receptors for ST (Sharara and Nieman, 1994), but it has yet to be determined if the effects of ST administration to cultured granulosa cells are entirely IGF-I mediated.

In the horse, there is little information available as to the effects of ST and/or IGF-I on reproductive activity. It has been shown that daily administration of equine ST (eST) to increases the number of small follicles visible on the ovaries of cyclic mares, with a concomitant increase in circulating IGF-I concentrations (Cochran *et al.*, 1999). However, whether these effects are caused directly by eST, indirectly through

IGF-I, or through a combination of both are unclear. Therefore, the purpose of the present study was to determine the effects of eST and IGF-I on cell proliferation and steroid production of equine granulosa cells cultured for 24 hours *in vitro*.

MATERIALS AND METHODS

Granulosa cells were collected from all follicles ranging from 20 to 40 mm in diameter from six cyclic pony mares. Granulosa cells samples were then pooled, and the total number of viable cells available for culture was calculated from a subsample of the pooled collection. Treatments for the present study consisted of (A) TCM-199 (Gibco, Grand Island, NY) plus 3 mg/ml BSA (Sigma, St. Louis, MO), which served as the control medium, (B) control medium plus 100 ng/ml eST (EquiGen, BresaGen, Thebarton, South Australia), (C) control medium plus 100 ng/ml eST plus 30 μg/ml anti-IGF-I antibody (Sigma), (D) control medium plus 100 ng/ml human recombinant IGF-I (Sigma) and (E) control medium plus 100 ng/ml human recombinant IGF-I plus 30 μg/ml anti-IGF-I antibody. Treatments were replicated using Nunc four-well culture dishes (n=4 wells/Treatment). Granulosa cells were seeded at a concentration of 150,000 viable cells/well for each of the five Treatment groups and allowed to culture for 24 hours at 38°C in an atmosphere of 5% CO_2 in air. A total volume of 500 μl of each medium type was placed into the respective wells.

After 24 hours of *in vitro* culture the medium was removed from each culture well and centrifuged at 300 x g to remove all granulosa cells remaining in suspension. Each medium sample was then stored in individually labeled 0.05 ml centrifuge tubes and frozen until analysis of progesterone concentrations by RIA. After removal of the medium, each well was treated with 0.025% trypsin to facilitate the detachment of all

proliferating granulosa cells from the bottom of each culture well. The granulosa cells were then collected to determine the number of proliferating granulosa cells per well per treatment. The concentration of progesterone, as well as the number of proliferating granulosa cells per well per treatment, were analyzed using a split-plot ANOVA procedure (Cochran *et al.*, 1999).

RESULTS

The results of *in vitro* culture of equine granulosa cells with eST and IGF-I are summarized in Table 7.1. There was an increase in the number of proliferating granulosa cells after 24 hours of culture in all media supplemented with either eST or IGF-I relative to control media ($P<0.05$). In addition, the steroidogenic activity of equine granulosa cells as measured by *in vitro* progesterone production was enhanced in all media supplemented with either eST or IGF-I ($P<0.05$), irrespective of anti-IGF-I antibody content.

Interestingly, there was no detectable increase in IGF-I production in equine granulosa cells treated with eST alone when compared with control medium, as RIA analysis revealed no detectable IGF-I in either treatment group. The fact that the anti-IGF-I antibody had no effect on neither eST nor IGF-I stimulated cell proliferation or progesterone production suggests that there was insufficient antibody present to bind all available IGF-I, and that eST stimulated cell proliferation and progesterone production may be caused by a direct effect of eST alone.

DISCUSSION

Results from the present study clearly indicate that both eST and IGF-I enhance granulosa cell proliferation and steroid production in equine granulosa cells cultured *in*

Table 7.1. Effects of eST and IGF-I treatment to equine granulosa cells cultured for 24 hours *in vitro*

Treatment	Number of Cells	P_4 Production
A. Control	43,750[a] (±15581)	2.13[a] (±0.93)
B. eST	117,500[b] (±15581)	6.72[b] (±0.93)
C. eST plus anti-IGF-I	129,375[b] (±15581)	7.14[b] (±0.93)
D. IGF-I	113,437[b] (±15581)	6.05[b] (±0.93)
E. IGF-I plus anti-IGF-I	150,562[b] (±15581)	5.97[b] (±0.93)

[a,b]Means within columns with different superscripts are different ($P<0.05$).

vitro (Table 7.1). These data are in agreement with previous reports in rats (deMoura *et al.*, 1997), pigs (Xia *et al.*, 1994), cattle (Schams *et al.*, 1988; Armstrong *et al.*, 1996; Khamsi and Armstrong, 1997), sheep (Mariana *et al.*, 1998) and goats (Behl and Pandey, 1999). For example, Armstrong *et al.* (1996) demonstrated that in cultured bovine granulosa cells, IGF-I addition to the culture medium markedly increased both cell proliferation as well as progesterone production. In this study, it was also determined that IGF-I acted in synergism with FSH, as addition of FSH and IGF-I enhanced the response in the granulosa cells over that of either FSH or IGF-I alone (Armstrong *et al.*, 1996). This same synergistic effect of IGF-I with FSH has also been demonstrated in the rat, where addition of IGF-I caused a 3-fold increase in progesterone production by cultured granulosa cells over FSH alone (deMoura *et al.*, 1997). It was concluded from that experiment that the primary role for intrafollicular IGF-I is to enhance the responsiveness of the granulosa cells to available gonadotropins (deMoura *et al.*, 1997).

In humans, IGF-I has been shown to increase estradiol, but not progesterone, production by granulosa cells cultured *in vitro* (Mason *et al.*, 1993; Foster *et al.*, 1995). This same effect has been shown in cultured porcine follicles (Siroikin *et al.*, 1998a) and granulosa cells (Howard and Ford, 1994), where intrafollicular estradiol production was increased with the addition of IGF-I to the culture medium. The reason for the difference in which steroid is produced by *in vitro* cultured granulosa cells remains unclear, but is likely due to the specific culture conditions and/or the duration of culture of the granulosa cells. For instance, Berndtson *et al.* (1995) reported that the gonadotropin concentrations must be carefully regulated to maintain follicular-phase

steroid production (estradiol) by cultured bovine granulosa cells. Also, these authors state that *in vitro*-cultured granulosa cells usually produce more progesterone than estradiol due to a change in cellular type during culture, as the granulosa cells generally more closely resemble luteal cells (Berndtson *et al.*, 1995). In addition, it is well known that during follicular atresia and/or luteinization, estradiol production by the granulosa cells is diminished, while progesterone production increases (Gerard and Monget, 1998). It was for this reason that, in the present study, progesterone production was chosen as a representation of the steroidogenic activity of cultured equine granulosa cells. Regardless of which specific steroid is measured, the fact remains that IGF-I does enhance steroid production in *in vitro*-cultured granulosa cells of virtually every species studied to date.

It has been well documented that granulosa cells contain receptors for both ST (Sharara and Nieman, 1994), as well as for IGF-I (Spicer and Echternkamp, 1995; Stewart *et al.*, 1997). Therefore, it is likely that many of the effects on ovarian follicular growth and development are IGF-I mediated, since treatment of granulosa cells with ST causes increased IGF-I production by the granulosa cells (Xia *et al.*, 1994; Samaras *et al.*, 1996; Siroikin *et al.*, 1998a,b). However, it has yet to be clearly demonstrated whether these effects are entirely IGF-I mediated, or whether there is a direct effect of ST on granulosa cells proliferation and steroid production. In the present study, while cellular proliferation as well as steroid production were enhanced by ST and IGF-I relative to control medium (Table 7.1), the apparent lack of sufficient binding of the anti-IGF-I antibody to available IGF-I leaves this question unanswered. However, the inability of eST to increase the concentration of IGF-I in the medium in

this study suggests that some of these effects may be independent of IGF-I and could be caused by direct stimulation of the granulosa cells by ST. Alternatively, an increase in intracellular IGF-I to be utilized in an autocrine fashion by the granulosa cells cannot be ruled out.

Apoptosis, or regulated cell death, is thought to be the primary mechanism through which granulosa cells are destroyed within the follicle, which then results in follicular atresia (Kaipia and Hsueh, 1997). One possible explanation for the observed effects on follicle numbers in live animals treated with ST could include a model for attenuation of granulosa cells apoptosis through increased intrafollicular IGF-I, thereby, increasing the functional life of the follicles. This hypothesis is supported by the results of Guthrie *et al.* (1998), where it was demonstrated that IGF-I prevented spontaneous apoptosis in cultured porcine granulosa cells. Also, while FSH is the best suppressor of apoptosis in the granulosa cells (60% suppression), IGF-I has been shown to be very effective in suppressing apoptosis of cultured granulosa cells in the rat (45% suppression) (Chun *et al.*, 1996). This provides yet another possible model for the influence that ST and IGF-I have on reproductive function in mammalian species, and may provide a partial explanation for the results seen with *in vitro* culture of granulosa cells, since the prevention of apoptosis would lead to a greater number of viable granulosa cells available to then cause an increase in cellular proliferation and steroid production.

In conclusion, results from the present study in equine granulosa cells confirm those data from earlier reports in other domestic species that both ST and IGF-I enhance cell proliferation as well as steroid production following *in vitro* culture. However, the

question remains as to whether these effects are entirely IGF-I mediated or whether there is a direct effect of ST on the granulosa cells. Further studies are needed in order to clarify this. Also, an alternative method for studying the effects of ST and IGF-I on granulosa cells function, such as intrafollicular treatment as described by Gastal *et al.* (1995), may be more beneficial in determining the actual effects of these growth factors in the live animal when compared with those of *in vitro* studies centering around the culture of isolated granulosa cells in a foreign environment.

CHAPTER VIII

THE EFFECTS OF eST ON FOLLICULAR DEVELOPMENT AND PLASMA AND FOLLICULAR FLUID HORMONE PROFILES IN CYCLIC MARES TREATED FOR ONE ESTROUS CYCLE

INTRODUCTION

It is well known that somatotropin (ST) and insulin-like growth factor-I (IGF-I) are intricately involved in mammalian reproductive physiology. In recent years, this area of reproductive biology has come under intense investigation in most domestic species and in humans. It has been demonstrated that administration of exogenous ST increases follicle numbers on the ovaries of women (Owen *et al.*, 1991a), cattle (Lucy *et al.*, 1993), pigs (Echternkamp *et al.*, 1994a) and mares (Cochran *et al.*, 1999). The precise mechanism of action for ST on ovarian follicular development is not currently known, however, it is believed that ST causes these effects, at least in part, through stimulation of IGF-I, although a direct effect of ST on follicular growth is possible (Gong *et al.*, 1991). Furthermore, since many of the studies involving ST administration are representative of animals with supraphysiologic circulating concentrations of ST, a pharmacologic, rather than a physiologic, effect may have been seen.

However, in studies involving hypophysectomized ewes, where both gonadotropins and ST are absent, it was determined that gonadotropin treatment alone was insufficient in stimulating follicle growth, and that ST was required in addition to gonadotropins for normal ovarian folliculogenesis to take place (Eckery *et al.*, 1997). Also, in Ames dwarf mice, a condition in which no somatotrophs develop within the adenohypophysis, which further causes an absence of ST production, a significant reduction in gonadotropin secretion and follicular development was noted, and

administration of ST reversed these effects (Bartke *et al.*, 1996). It can therefore be said that ST production and secretion is required for normal reproductive processes to take place.

It is well known that ST stimulates hepatic IGF-I production, and that animals receiving ST administration have elevated intrafollicular IGF-I (Gong *et al.*, 1991; Monget *et al.*, 1993), but whether these increases in intrafollicular IGF-I are a result of increased production of IGF-I within the follicle, or if the excess IGF-I is a product of the plasma is not fully understood. However, it has been demonstrated that granulosa cells do possess the capacity to produce IGF-I (Xia *et al.*, 1994), and that IGF-I mRNA is highest in pre-ovulatory follicles (Yuan *et al.*, 1998), which suggests that the increased intrafollicular IGF-I is, at least in part, produced locally within the follicle by the granulosa cells. This is important, since several studies have demonstrated the synergistic actions of IGF-I on gonadotropin stimulation of ovarian follicles in both humans and rats (Homburg *et al.*, 1988; European and Australian Multicenter Study, 1995; deMoura *et al.*, 1997). For example, in *in vitro*-cultured rat granulosa cells, the response to FSH administration was trebled when IGF-I was given (deMoura *et al.*, 1997). Also, IGF-I increases LH receptor numbers in rat granulosa cells, which would enhance the follicular response to available LH (Liu *et al.*, 1998). It is therefore likely that ST administration causes some of its effects on follicular development through increasing systemic and intrafollicular IGF-I, thereby, enhancing the ability of the growing follicle(s) to respond to available gonadotropins.

It is currently not known whether the increases noticed in ovarian follicle numbers resulting from ST administration are a result of increased follicular activation

of gonadotropin-independent follicles, or whether a different mechanism, such as delaying follicular atresia is involved. It is well known that follicular growth and development occurs in "waves" in many domestic species, and that by increasing the number of follicles recruited into a follicular wave one could then possibly increase the ovulation rate (Lucy *et al.*, 1993; Ginther and Bergfelt, 1993; Buratini *et al.*, 1997).

The problem with this approach is that the recruitment of small antral follicles into a follicular wave occurs at a time when the follicles are not gonadotropin-sensitive, and at the present time, it is not fully known what factors are involved in follicle activation and recruitment into a follicular wave. It is possible that ST and/or IGF-I may enhance follicular activation and recruitment, but if this is the case, then ST administration should be carried out for a relatively long (1 to 3 months) duration (Bergh *et al.*, 1994). Conversely, ST/IGF-I may act through a different mechanism to increase ovarian follicle numbers, such as by either rescuing follicles from the process of atresia, or by prolonging the atretic process through preventing apoptosis of the follicular granulosa cells. These possibilities should not be overlooked, since they are the two most common methods for FSH stimulation in ovarian superstimulation treatments (Mihm *et al.*, 1997).

In the domestic mare, little has been published as to the importance of ST/IGF-I in ovarian follicular growth and development. In one study, Cochran *et al.* (1999) demonstrated that cyclic mares receiving eST had increased numbers of small follicles present on their ovaries at 3 and 5 days after treatment and on the first day of standing estrus. However, this is the only study published to date demonstrating any effects of eST administration on reproductive function in cyclic mares. It was not known from

this study whether the effects seen by the authors were caused by an increase in the number of follicles recruited into a follicular wave or whether ST administration delayed or prolonged the follicular atretic process in eST-treated mares.

The purpose of the present study was, therefore, to determine the effects of eST administration to cyclic mares for one estrous cycle on (1) follicular wave parameters, (2) growth and atresia of individual follicles and (3) plasma and follicular fluid concentrations of IGF-I, estradiol and progesterone.

MATERIALS AND METHODS

A total of 10 reproductively sound cyclic mares of mixed breeds, all in good body condition, were randomly allocated into one of two treatment groups. Treatment A consisted of mares (n=5) administered 25 μg eST (EquiGen, BresaGen Ltd., Thebarton, South Australia) per kg body weight once daily for one complete estrous cycle (day 1 = first day of treatment; corresponds to day 1 post-ovulation). Mares in Treatment B (n=5) were similarly administered a sodium borate solution, which served as the vehicle for the eST. Follicle development and plasma and follicular fluid hormone profiles were monitored over a total of three estrous cycles in order to establish normal parameters for each mare, as well as to determine if any carryover effects would be seen from eST administration (Pre-Treatment = Cycle A, Treatment = Cycle B, Post-Treatment = Cycle C).

Experimental Procedures

Follicle growth and developmental patterns were monitored via transrectal ultrasonography in all mares as previously described by Cochran *et al.* (1999). Briefly, a 5 MHz linear transducer was inserted into the rectum of each mare and consecutive

sagittal sections of each ovary were photographed using a thermal printer attached to the ultrasound machine. Each follicle present was identified, and measurements were taken of each individual follicle at its maximum diameter three times per week. The diameter of each follicle was plotted over time for each mare in order to determine a follicular growth and development profile for each mare. These measurements were taken beginning on day 1 post-ovulation of Cycle A (pre-treatment) and continuing until the day of ovulation of Cycle C (post-treatment) for all mares.

A follicular wave was defined as all follicle development beginning with the time the largest follicle was retrospectively identified until this follicle reached its maximum diameter and ovulated. The number of follicular waves per mare per cycle and the number of follicles per wave per mare per cycle was analyzed to determine if eST administration would effect any of these parameters. Also, the interval of growth for each follicle, defined as the time in days that a follicle was retrospectively identified until it reached its maximum diameter, was analyzed for each mare within treatment. In addition, the interval of follicular atresia, which was defined as the time in days a follicle reached its maximum diameter until it was no longer identifiable by ultrasound, was analyzed per mare within treatment for each estrous cycle studied. Finally, the number of ovulations per mare per treatment group was analyzed for all estrous cycles as well.

Plasma and follicular fluid samples were collected 12 to 24 hours prior to ovulation for each estrous cycle (Cycles A, B and C; n=3 collection procedures/mare) for mares in both treatments. From each mare, 0.5 ml of follicular fluid was collected via transvaginal ultrasound-guided follicular aspiration from (1) two follicles <10 mm

in diameter, (2) one follicle 10 to 20 mm in diameter and (3) the pre-ovulatory follicle present at the time of collection. Plasma samples were collected via jugular venipuncture from each mare on the day of follicular fluid collection. All plasma and follicular fluid samples were placed in individually labeled 15-ml conical tubes, frozen within 30 minutes of collection and stored until RIA was performed to determine the concentrations of IGF-I (Sticker *et al.*, 1995), estradiol and progesterone.

Statistical Analysis

The number of follicular waves per mare per treatment group as well as the number of follicles per follicular wave were analyzed using ANOVA (Cochran *et al.*, 1999). In addition, the number of ovulations per mare per treatment and the number of days of follicular growth and atresia per follicle were analyzed with a similar ANOVA procedure (Cochran *et al.*, 1999). Finally, plasma and follicular fluid IGF-I concentrations were analyzed using a split-plot ANOVA (Cochran *et al.*, 1999).

RESULTS

Follicular Development

The effects of daily eST administration to cyclic mares on follicle populations are presented in Table 8.1. All mares exhibited one or two waves of follicle growth per estrous cycle regardless of treatment, and there was no effect of treatment with eST on the number of follicular waves per mare per cycle, the total number of follicles per follicular wave or the length (in days) of follicle growth in the present study. However, administration with eST did effect the number of days required for follicular atresia to be completed, as follicles from mares in Treatment A remained on the ovaries for a longer duration than those from mares in Treatment B during the treated cycle (Cycle

B), but not for the pre- or post-treated cycles (Cycles A and C) (8.78 ± 0.64 *vs.* 6.39 ± 0.56 for Treatments A and B, respectively, P<0.05).

Also, mares treated with eST ovulated more follicles (P<0.05) than contemporary control mares during Cycle B, but not during Cycles A and C, demonstrating a positive effect of eST on large follicle development (1.6 ± 0.1 *vs.* 1.0 ± 0.1, respectively). There were no detectable effects of eST administration on follicular parameters in the post-treated cycle (Cycle C) in the present study, suggesting a relatively short-lived effect of eST administration on ovarian follicular dynamics in the domestic mare.

Hormone Profiles

Analysis of plasma and follicular fluid steroid concentrations (estradiol and progesterone) revealed no detectable differences between treated and control mares for any of the estrous cycles studied. However, plasma IGF-I was elevated in mares receiving eST during Cycle B when compared with control mares (610.7 ± 56.1 *vs.* 221.3 ± 56.1 for mares in Treatments A and B, respectively), but not for Cycles A and C (Figure 8.1). Also, follicular fluid collected from pre-ovulatory follicles 12 to 24 hours prior to ovulation had higher IGF-I levels (471.1 ± 44.3 *vs.* 269.7 ± 44.3 for mares in Treatments A and B, respectively) in eST-treated mares than in control mares during Cycle B (Figure 8.1).

Finally, mares in Treatment A had higher IGF-I concentrations in the follicular fluid from pre-ovulatory follicles when compared with follicular fluid from small (<10 mm) or medium (10 to 20 mm) follicles within the same mares (Figure 2) during Cycle B. This was not detected in untreated control mares, as no differences were detected in

Table 8.1. Effect of eST administration to cyclic mares on follicular atresia and ovulatory response

Treatment	Time of atresia (days)	Ovulations/mare
A. eST	$8.78^{a} \pm 0.64$	$1.6^{a} \pm 0.1$
B. Control	$6.39^{b} \pm 0.56$	$1.0^{b} \pm 0.1$

[a,b]Means within columns with different superscripts are different ($P<0.05$).

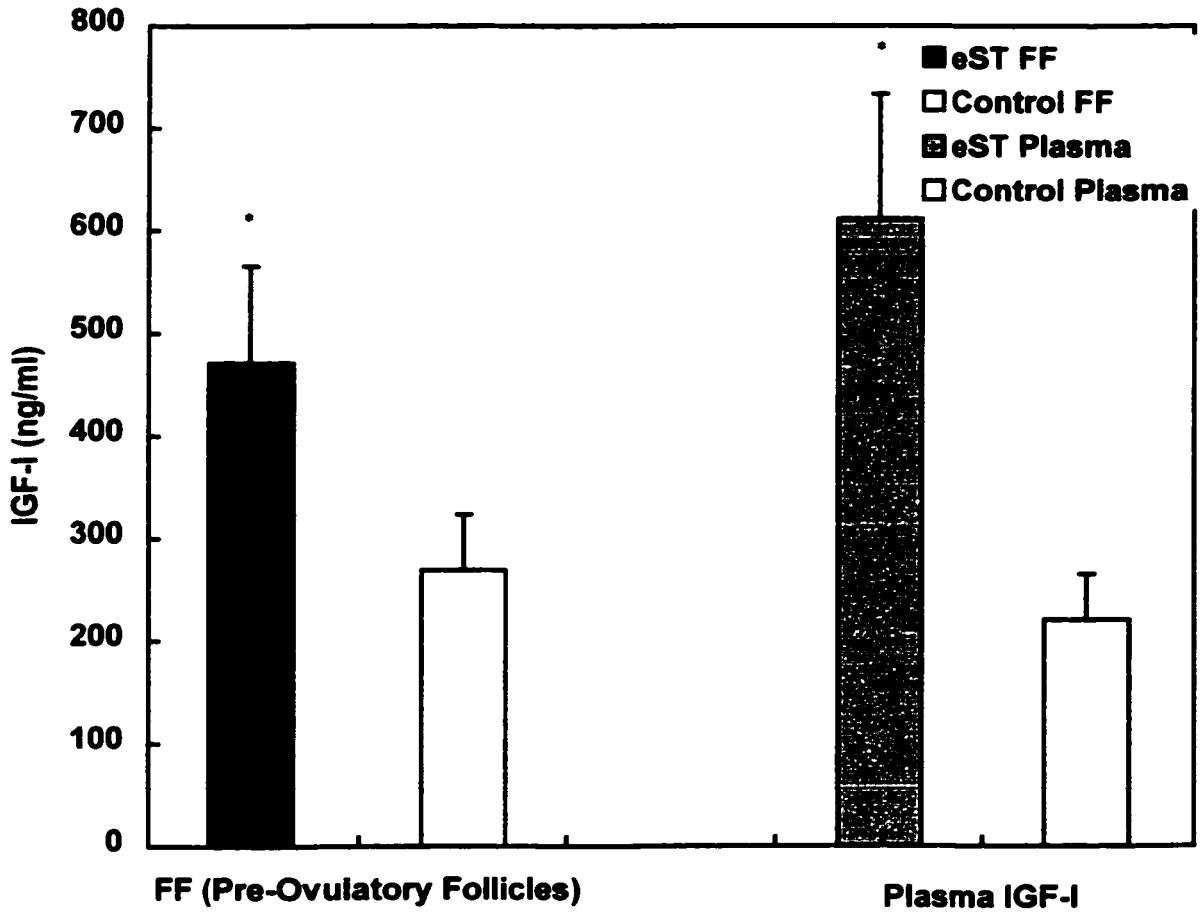

Figure 8.1. Plasma and follicular fluid IGF-I concentrations (±SEM) for cyclic mares administered once daily injections of eST. (*) Denotes significant differences between treatment groups.

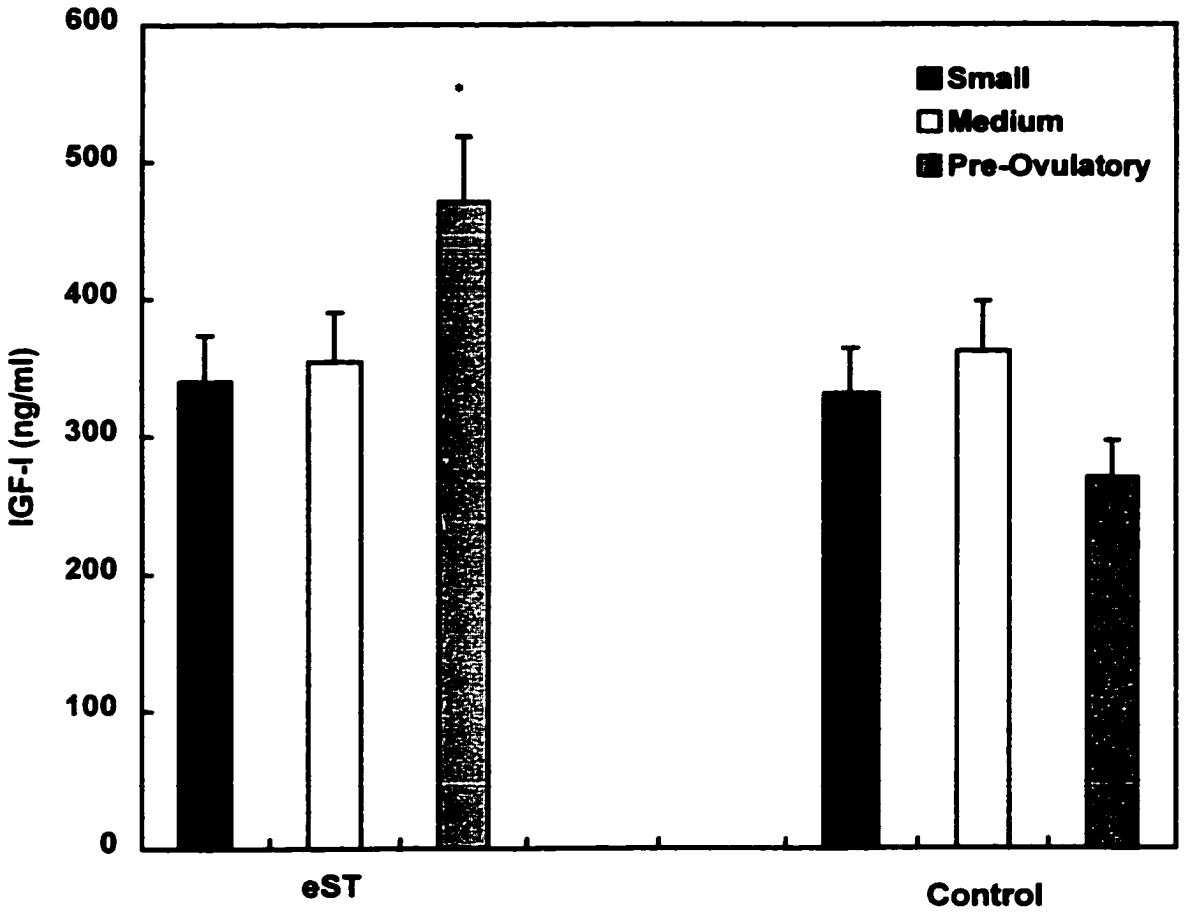

Figure 8.2. Follicular fluid IGF-I concentrations (±SEM) for small, medium and pre-ovulatory sized follicles from cyclic mares administered once daily injections of eST. (*) Denotes a significant difference.

follicular fluid IGF-I concentrations between small, medium or pre-ovulatory follicles (Figure 8.2).

DISCUSSION

The importance of ST and IGF-I on reproductive function in domestic species and humans has become a major research focus in reproductive biology over the past decade. It has been clearly demonstrated that ST administration increases the number of visible follicles on the ovaries of cows (Gong *et al.*, 1991; Lucy *et al.*, 1993; Gong *et al.*, 1996), women (Owen *et al.*, 199a1), pigs (Spicer *et al.*, 1992; Echternkamp *et al.*, 1994a) and mares (Cochran *et al.*, 1999). Also, perifused rabbit ovaries exposed to ST responded with increased follicle numbers, as well as with higher intrafollicular IGF-I concentrations (Yoshimura *et al.*, 1994). In addition, ST is a potent stimulator of both systemic and intrafollicular IGF-I in virtually all species studied to date (Hugues *et al.*, 1991; Owen *et al.*, 1991a,b; Echternkamp *et al.*, 1994a,b; Yoshimura *et al.*, 1994; Gong *et al.*, 1997).

In vitro studies using cultured granulosa cells have demonstrated that most of the effects seen with ST administration, such as steroid production and cellular proliferation, are IGF-I mediated, since the addition of anti-IGF-I antibodies can negate the positive effect of ST administration (Gong *et al.*, 1991). However, Izadyar *et al.* (1997) demonstrated that some of the positive effects demonstrated by ST administration on cumulus expansion and oocyte maturation were the result of a direct effect on ST and not mediated by IGF-I. It is therefore likely that the effects noted from administration of exogenous ST arise from both a direct effect of ST on follicular granulosa cells, as well as an indirect effect through increased IGF-I production.

Unfortunately, the exact mechanism through which ST/IGF-I exerts its effects on follicle development is not fully understood. Although it is known that both ST and IGF-I are mediators of gonadotropic actions on the follicular cells, enhancing the response of the granulosa cells to available gonadotropins (Yoshimura *et al.*, 1994), this is an insufficient explanation as to how ST/IGF-I is able to increase the number of small, presumably gonadotropin-independent follicles of treated animals.

Therefore, one of the goals of the present study was to determine if eST administration to cyclic mares would result in an increase in the number of follicles recruited into a follicular wave. The exact mechanism of early follicle activation and recruitment into a follicular wave is thought to be gonadotropin-independent, but is poorly understood at present (Britt, 1988). Although, growing evidence suggests that various growth factors and/or changes in local ovarian blood flow could begin the process (Staigmiller, 1982; Driancourt *et al.*, 1991; Gutierriz *et al.*, 1997; Bao and Garverick, 1998).

In this study, there was no effect of eST on the number of follicles per follicular wave between treated and control mares. Furthermore, there was no difference in the number of follicular waves per estrous cycle between treated and control mares, as all mares exhibited either one or two waves of follicle growth per cycle studied, as has been previously reported for cyclic mares (Buratini *et al.*, 1997). Therefore, it is unlikely that the increase in follicle numbers demonstrated previously for cyclic mares administered eST (Cochran *et al.*, 1999) is a result of an increase in the number of small, gonadotropin-independent follicles available for recruitment into a follicular wave.

Previous reports in rats (Chun *et al.*, 1996) and pigs (Guthrie *et al.*, 1998) have suggested that one mechanism of action for ST/IGF-I may be to decrease the apoptotic index of the granulosa cells, thereby, prolonging the time required for a follicle to complete the atretic process. Chun *et al.* (1996) demonstrated that, while FSH was the best suppressor of granulosa cell apoptosis (60% suppression), IGF-I was a close second in suppression (45%), with very minimal suppression seen from other growth factors and gonadotropins. It is well known that the major mechanism of action of FSH in superstimulation protocols is to "rescue" follicles already in the growth stage from the process of atresia, which is precipitated by spontaneous apoptosis of the granulosa cells. It is therefore likely that this could be one mechanism for the ability of ST/IGF-I to increase the number of follicles in treated subjects. In the present study, it was determined that eST administration caused a significant increase in the number of days required for the completion of atresia of the follicles in treated mares (Table 8.1). Therefore, we conclude that a prolongation of follicular atresia, rather than an increase in small follicle growth, is likely the mechanism through which ST/IGF-I causes greater numbers of small- and medium-sized follicles to be present on the ovaries of treated mares.

ST administration has also been shown to improve the ovulatory response in cattle (Rieger *et al.*, 1991), as well as women (Owen *et al.*, 1991a), undergoing ovarian superstimulation protocols. It has been demonstrated that these effects are partly the result of an ability of ST and IGF-I to work in synergy with available gonadotropins, enhancing the follicular response to available gonadotropins (Homburg *et al.*, 1988; Owen *et al.*, 1991b; Hugues *et al.*, 1991; Yoshimura *et al.*, 1994; European and

Australian Multicenter Study, 1995; deMoura *et al.*, 1997). This can partially be explained by the role of intrafollicular IGF-I on dominant follicle selection. It is currently believed that changes in bioavailable intrafollicular IGF-I are, in part, responsible for the process of dominant follicle selection in mammalian species (Owen *et al.*, 1991b). As follicles grow and mature, the ratio of IGF-I:IGF-I binding proteins (IGFBP) changes within the follicle, allowing more IGF-I to be available to bind to the granulosa cells and, thereby, enhance the response of these cells to available gonadotropins (Monget *et al.*, 1993; De La Sota *et al.*, 1996; Funston *et al.*, 1996; Yuan *et al.*, 1998).

One possible explanation for the synergy exhibited between IGF-I and gonadotropins could be due to an increase in the number of gonadotropin receptors on the granulosa cells of the growing follicles, allowing for more gonadotropin binding to the receptors and, thus, an increased growth response (Zhang *et al.*, 1987). Beam and Butler, (1997) demonstrated that in postpartum dairy cows that had ovulated during the first 2-week interval postpartum had at least 40% greater plasma concentrations of IGF-I compared with those cows not ovulating early postpartum, further suggesting the importance of IGF-I in reproductive function. In the present study, administration of eST to cyclic mares caused a significant increase in the number of ovulations per mare during Cycle B (treatment cycle) when compared with contemporary control mares or with the same mares during both the pre- and post-treatment cycles (Cycles A and C, respectively, [Table 8.1]). In addition, previous studies from our laboratory have demonstrated that seasonally anovulatory mares respond to gonadotropin therapy at a much greater frequency when eST is added to the treatment regimen. These results are

in agreement with the current hypothesis that increased intrafollicular IGF-I concentrations are able to enhance gonadotropic actions on follicular cells.

In the present study, only fluid from pre-ovulatory follicles showed increased IGF-I concentrations in response to eST treatment (Figure 8.1). The intrafollicular IGF-I concentrations of pre-ovulatory follicular fluid was parallel to, but less than, plasma IGF-I levels (Figure 8.1), as has been previously reported for humans (Monget *et al.*, 1993). Also, there were no differences in intrafollicular IGF-I concentrations from small, medium or pre-ovulatory follicles in untreated control mares (Figure 8.2). This is in agreement with previous reports in women (Tapanainen *et al.*, 1992; Thierry Van Dessel, *et al.*, 1996) and cattle (Spicer and Enright, 1991), where no difference was detected in intrafollicular IGF-I across follicle sizes. However, other reports in cattle (Mihm *et al.*, 1997) and women (Rabinovici *et al.*, 1990) have shown the opposite, that there is indeed higher amounts of IGF-I present in the follicular fluid of large follicles when compared with small- and medium-sized follicles. At present, it is still not known which case will prove to be correct, but the fact remains that, even if there are no differences in absolute values of intrafollicular IGF-I, there are differences in the amounts of bioavailable IGF-I between small, medium and pre-ovulatory follicles due to changes in intrafollicular IGFBP production (Owen *et al.*, 1991b).

It is currently believed that the increase in intrafollicular IGF-I seen during ST administration comes from an increase in both local as well as systemic production of IGF-I. This is due to the fact that, although ST administration causes increased hepatic production of IGF-I (Gong *et al.*, 1997), which could filter into the follicular cavity via circulation through the plasma, ST treatment also causes a significant increase in IGF-I

production by the granulosa cells within the follicle (Yoshimura *et al.*, 1996a). Using perifused rabbit ovaries, Yoshimura *et al.* (1996a) demonstrated that approximately three times more IGF-I mRNA was stimulated in follicular granulosa cells exposed to ST when compared with those follicles that were not exposed to ST. This verifies that follicular cells do have the capacity to produce and secrete IGF-I in response to ST stimulation, and that this production was at least partially responsible for the increase in follicular fluid levels of IGF-I in these perifused rabbit ovaries (Yoshimura *et al.*, 1996a). Interestingly, it has been demonstrated in cattle that granulosa cells from pre-ovulatory follicles contain much greater amounts of IGF-I mRNA than cells from smaller follicles, further suggesting an important role of intrafollicular IGF-I on dominant follicle selection (Yuan *et al.*, 1998). Also, it has been shown that immunization of cattle against somatotropin releasing factor lowers intrafollicular IGF-I, as a result of the diminished capacity to produce and secrete ST (Kirby *et al.*, 1993; Stanko *et al.*, 1994).

It has been demonstrated that ST administration can increase intrafollicular steroid concentrations in cattle (Spicer and Enright, 1991), women (Tapanainen *et al.*, 1992) and pigs (Echternkamp *et al.*, 1994a). Increases in intrafollicular estradiol and progesterone can be brought about through the ability of IGF-I to increase 3 β-hydroxysteroid dehydrogenase and aromatase enzyme mRNA in the granulosa cells of treated subjects (Tapanainen *et al.*, 1992). However, while some studies show an increase in intrafollicular estradiol (Echternkamp *et al.*, 1994b; Howard and Ford, 1994) or progesterone (Xia *et al.*, 1994), others have reported that treatment with ST produces no detectable increases in intrafollicular estradiol and progesterone secretion (Spicer *et*

al., 1992; Tapanainen *et al.*, 1992). In the present study, there was no effect of eST administration to cyclic mares on either plasma or follicular fluid concentrations of estradiol or progesterone. In addition, there was no effect of time (Cycle A *vs.* Cycle B *vs.* Cycle C) on either plasma or intrafollicular steroid levels in this study. Further studies are needed using cyclic mares to confirm these findings.

In summary, we have demonstrated that the administration of eST does effect reproductive function in cyclic mares. Although no effect was seen in the number of follicles recruited into a follicular wave or the number of follicular waves per estrous cycle, it was determined that eST treatment to cyclic mares did cause a prolongation of the follicular atretic process, allowing the regressing follicles to remain on the ovaries for a longer period of time.

As expected, plasma IGF-I concentrations were elevated in eST-treated mares, and intrafollicular IGF-I was higher in pre-ovulatory follicles as well. This could be the reason for the increase in ovulation rate noticed in mares administered eST during Cycle B (treatment cycle). There were, however, no differences in plasma or intrafollicular concentrations of estradiol or progesterone between treated and control mares in this study. The present study provides strong evidence as to the importance of ST and IGF-I on reproductive processes in the domestic mare, and partially explains some of the underlying mechanisms involved in dominant follicle selection and growth. Further studies are needed in this area of equine reproductive physiology to evaluate these effects further.

CHAPTER IX

SUMMARY AND CONCLUSIONS

Treatment of domestic species and women with somatotropin (ST) has been shown to have dramatic effects on follicular growth and development at the level of the ovary. To date, little has been reported as to the effects of ST administration to the domestic mare. In the present series of experiments, it was demonstrated that ST administration increases ovarian follicles in both cyclic as well as seasonally anovulatory mares. This is similar to the findings reported for other species, where ST administration caused a higher number of ovarian follicles to be detectable via ultrasound during the ST treatment period. The mechanism of action of ST on ovarian follicular dynamics has yet to be conclusively established. In the mare, as in other species, a significant rise in peripheral as well as intrafollicular IGF-I was detected as a result of ST treatment, and it is currently believed that this excess IGF-I is, at least in part, responsible for the ovarian effects that occur with ST administration. Since a higher number of ovarian follicles was found with ST administration to both cyclic and anovulatory mares, in the present study, we hoped to identify the mechanism of action of these effects. Also, we wished to attempt to incorporate these findings into a practical scheme to improve the fertility of the domestic mare.

In cyclic mares, it was noticed that administration of equine ST (eST) not only significantly increased the number of ovarian follicles present during the treatment interval, but it also increased the number of ovulations per mare when compared with untreated control animals. Since no effects were noticed in circulating plasma gonadotropin concentrations, it was hypothesized that ST administration, primarily through

increasing circulating IGF-I levels, enhanced the ability of the follicles to respond to available gonadotropins, thus enabling more follicles to respond to these gonadotropins by growing to larger sizes. In addition, it was determined that the ST treatment to cyclic mares did not effect the number of follicles recruited into the cohort of follicles that make up a follicular wave. Rather, ST administration caused a delay in the atretic response of the ovarian follicles, prolonging the period of time required for the follicles to completely regress.

This would explain why increased numbers of ovarian follicles are typically noted in animals treated with ST, since, after ST administration, the affected follicles would be present on the ovaries for a longer period of time and would therefore appear to be more numerous when evaluating the ovaries via ultrasound. This mechanism of action should come as no surprise, since this is very similar to the mechanism of action of FSH, which is thought to "rescue" follicles from the atretic process, so that increased numbers of follicles can be made available for the harvesting of multiple oocytes/embryos from treated individuals.

There was no clear relationship detected as to the ability of ST to stimulate above normal concentrations of steroid hormones circulating in the blood. In general, ST administration had no effect on circulating steroid hormone levels in treated mares, but did increase intrafollicular steroid concentrations in large-sized follicles. Also, as demonstrated in other species, both ST and IGF-I exposure significantly increased steroidal output by *in vitro* cultured equine granulosa cells, demonstrating the ability of both hormones to stimulate granulosa cell steroidogenesis. As to the effects of ST/IGF-

I on gonadotropin production and secretion, there was no evidence of any effect of elevated ST or IGF-I on circulating FSH or LH concentrations in any animal studied.

In seasonally anovulatory mares, it was determined that ST administration caused a significant increase in the number of ovarian follicles. However, only follicles less than 15 to 20 mm in diameter were detected in these mares, which would severely limit the use of this hormone in a practical setting. Therefore, since treatment of various species with ST has been shown to cause an enhancement of the growing follicles to available gonadotropins, it was hypothesized that treatment of seasonally anovulatory mares with GnRHa in addition to ST may cause an increase in the ovulatory response of these mares to the GnRHa.

This proved to be the case, since ST administration in addition to GnRHa treatment significantly increased the number of mares that grew large, pre-ovulatory-sized follicles, as well as increased the number of mares ovulating when compared to mares treated with GnRHa alone. In addition, the ovulations detected in mares treated with GnRHa plus ST were determined to be fertile, since pregnancies and live births were accomplished using this treatment regimen. It is also of importance that once daily ST treatment was utilized in conjunction with once daily GnRHa treatment to induce ovulation in seasonally anovulatory mares. This is the first report, to our knowledge, of the induction of ovulation in seasonally anovulatory mares using only once daily injections of GnRHa. We have demonstrated that ST administration can enhance the follicular response to available gonadotropins in seasonally anovulatory mares, and that treatment of these mares with ST allows for a more predictable, less variable ovulatory response.

One of the primary goals of this research was to develop an ovarian stimulation protocol for the collection of oocytes from mares for use in *in vitro* fertilization techniques. Once a repeatable method of producing equine embryos and foals with *in vitro* methods was developed (e.g., ICSI), a method of increasing the number of viable oocytes collected from individual mares was evaluated. It was determined that ST administration did not increase the number of available ovarian follicles in altrenogest-treated mares when oocytes were collected from these mares at regular 10-day intervals. However, when the mares were treated for 21 consecutive days prior to oocyte collection, there was a significant increase in the number of ovarian follicles available for collection when compared with control mares treated only with altrenogest.

This further implies that the ovarian effects noted with ST treatment are enhanced by a prolongation of follicular atresia, rather than an increase in the number of new, developing follicles within a follicular wave. If ST administration caused its effects by increasing the number of follicles within a follicular wave, then it would be expected that mares treated in this fashion would have a higher number of ovarian follicles available for aspiration at any time during the treatment period. Since oocyte collections were performed at 10-day intervals in the present study, which coincide with the approximate interval of follicular wave growth in the domestic mare, it can be concluded that no increase in the number of follicles within a follicular wave occurred in ST-treated animals, as no differences in ovarian follicle numbers were detected between treated and control mares. It should be noted, however, that a method of repeatably producing equine embryos *in vitro*, which were capable of producing live

foals, was developed in conjunction with a protocol that allows for multiple oocytes to be collected from individual mares at a given time.

In addition, it was demonstrated that most of the effects of ST administration at the level of the ovary is due primarily to increased IGF-I exposure of the follicular granulosa cells. This was shown *in vitro* by culturing equine granulosa cells in the presence of either ST or IGF-I. In both cases, the cellular response was similar, with both hormones stimulating cellular proliferation as well as cellular steroid production to a similar degree.

In summary, we conclude that ST administration to the domestic mare enhances the ovarian response both directly and indirectly through IGF-I by stimulating granulosa cell function and by enhancing the responsiveness of the granulosa cells to available gonadotropins, possibly by increasing gonadotropin receptors within these cells. Finally, it was determined that the increases in ovarian follicular numbers detected with ST administration were primarily a result of prolonging the atretic process by the ovarian follicles as a direct result of ST treatment. Further studies are needed in this area of equine follicular dynamics to elucidate more thoroughly the mechanism of actions of ST and IGF-I in the equine ovary.

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VITA

Richard Anthony Cochran was born December 10, 1973, in Richton, Mississippi, to parents Mark and Suzanne Cochran. Richard is the eldest of five siblings, including two brothers and two sisters. He was reared in Petal, Mississippi, where he attended elementary school and graduated from Petal High School in May of 1992. Richard then attended Jones County Junior College in Ellisville, Mississippi, majoring in agriculture. Richard was wed to Jill Denise Everett on December 25, 1992, while continuing his college education. In August of 1994, Richard and Jill moved to Baton Rouge, Louisiana, where Richard began his senior college study in the Department of Animal Science. He completed his bachelor of science degree in August of 1996, and immediately enrolled into the doctoral program within the Department of Animal Science under the direction of Professor Robert A. Godke. He will earn his doctorate in reproductive physiology in August of 2000.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Animal Science

Title of Dissertation: The Effects of Equine Somatotropin (eST) on Reproductive Function in the Domestic Mare

Approved:

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Date of Examination:

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