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Evaluation of methods for suppressing estrus and ovulation in mares: sustained release injections of altrenogest versus deslorelin acetate

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**EVALUATION OF METHODS FOR SUPPRESSING ESTRUS AND OVULATION IN
MARES: SUSTAINED RELEASE INJECTIONS OF ALTRENOGEST VERSUS
DESLORELIN ACETATE**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in
the School of Animal Sciences

by
Thomas J. Stevens
B.S., Louisiana State University, 2007
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While this thesis was in preparation, a good friend of the LSU Agricultural Center Horse Farm and long time supporter of its programs, Dr. Patrick J. Burns, of Lexington, Kentucky, passed away unexpectedly at his home. Dr. Burns was Vice President of BioRelease Technologies of Lexington, Kentucky, and Birmingham, Alabama, and was an active scientist in the horse world for over 25 years. Dr. Burns' contributions to the projects described herein and to others over the years at the Horse Farm have always been greatly appreciated. He will be sorely missed.

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ABSTRACT

Two experiments assessed the potential of altrenogest, an artificial progestogen, and deslorelin acetate, a gonadotropin-releasing hormone (GnRH) agonist, for suppressing estrus and ovulation in cyclic mares. In the first experiment, mares were administered a luteolytic dose of prostaglandin-F_{2α} on d 6 of diestrus, and were then treated with 1 of 3 formulations of altrenogest in slow-release vehicle (6 mares/group): 1) Biorelease altrenogest LA150 (225 mg total as a 1.5 mL injection); 2) Biorelease altrenogest LA225 (225 mg total as a 1.0 mL injection); or 3) Biorelease altrenogest LA225 (450 mg total as a 2.0 mL injection). Six control mares received vehicle. Compared to control mares (10.0 days), time to ovulation was greater ($P < 0.01$) for all mares receiving altrenogest (17.5 days overall); there was no difference ($P > 0.5$) among groups receiving altrenogest. Control mares (6 out of 6) first displayed estrus an average of 4.5 days after prostaglandin-F₂ injection; of the 18 mares receiving altrenogest, 11 displayed first estrus at an average of 13.5 days ($P < 0.02$ relative to controls) and there was no difference ($P > 0.19$) among groups. It was concluded that the 3 formulations of altrenogest were equally effective in delaying estrus and ovulation in cyclic mares. It is suggested that a 10-day injection interval could be used to keep mares out of heat for extended periods of time. The second experiment was similar to the first, except that there were 2 groups: 1) control mares that received a vehicle injection and 2) mares that received an i.m. injection of 1.5 g of deslorelin acetate in biodegradable microparticles. Administration of deslorelin did not affect ($P > 0.1$) the day of first onset of estrus or the day of ovulation. There was a tendency ($P = 0.08$) for deslorelin treated mares (3 out of 7 vs. 0 out of 6 controls) to not show estrus at the expected time, even though they experienced luteolysis and ovulation. It was concluded that injection of 1.5 g of deslorelin acetate was not an effective method for suppressing estrus and ovulation in cyclic mares.

INTRODUCTION

Considerable research has been performed over the years in an effort to induce seasonally anovulatory mares to display estrus and ovulate in winter. In contrast, less effort has been expended in devising a reliable method for inhibiting the normal displays of estrus and occurrence of ovulation in mares. Two practical situations in the horse industry exist when owners desire this inhibition: 1) in performance mares, behavioral estrus can interfere with a mare's training and response, and is thus unwanted, and 2) in recipient mares in embryo transfer programs, it is more efficient to have mares in a neutral state (noncyclic) so that they can be manipulated more easily by hormone therapy. The first situation (keeping mares out of estrus) might be achieved by endocrine manipulation, such as the long-term use of a progestogen, or perhaps by a total shut down of the reproductive system, analogous to the seasonal anovulatory state. The second situation, however, would likely require a total shut down, such that the mare would present a "blank slate" to be manipulated hormonally.

The research presented herein was designed to test the possibility of using one of two commonly available hormonal analogs, altrenogest and deslorelin acetate, as a means of inhibiting behavioral estrus displays and suppressing ovulation in normally cyclic mares. Altrenogest is an orally active progestogen originally developed for synchronizing estrus in mares, and deslorelin acetate is a GnRH agonist that is commonly used to hasten ovulation in cyclic mares.

CHAPTER I

REVIEW OF LITERATURE

Equine Hypothalamic-Pituitary-Gonadal Axis

Previous research described the relationship between the hypothalamic-pituitary-gonadal axis and reproductive function (Ginther, 1992). The reproductive axis is controlled by the brain via chemical neurotransmitters that impinge on the hypothalamus, which contains neurons that produce and secrete gonadotropin releasing hormone (GnRH; Guyton and Hall, 1996). In the mare, this neuropeptide is released in a pulsatile manner in response to the change in season and position in the estrous cycle. Upon stimulation, GnRH is released from nerve endings at the median eminence of the hypothalamus, where it is stored in secretory granules; it then travels through portal vessels to reach the adenohypophysis where it binds to receptors on gonadotropes in the pars distalis of the hypothalamus (Ginther, 1992; Guyton and Hall, 1996). Gonadotropin releasing hormone stimulates gonadotropes to synthesize and secrete lutenizing hormone (LH) and follicle stimulating hormone (FSH; Irvine and Alexander, 1993; Guyton and Hall, 1996). Concentration of these hormones depends on the pulsatile fashion in which GnRH is released (Alexander and Irvine, 1993). If the pulse frequency is between 2 and 4 pulses per day, FSH is the predominant hormone, causing follicular recruitment. If the pulse frequency high (intervals of every 2 h or less), LH predominates and ovulation is induced (Alexander and Irvine, 1993). The ovary is dependent on the secretion of FSH and LH for development and ovulation. Receptors for FSH are found on the granulosa cells in the ovary, whereas LH receptors are found on the thecal cells (Guyton and Hall, 1996). Maturing follicles produce estradiol, which is released into the peripheral circulation and causes the surge of LH that will result in ovulation (Morel, 2008).

Therefore, without control of the gonads by the hypothalamic-hypophyseal axis, the mare would have minimal follicular growth and no cyclic estrous activity.

Estrus Cycle of the Mare

The estrous cycle is the repetitive sequence of events that prepare the mare for conception (Daels and Hughes, 1993). The estrus cycle of the mare begins at puberty and last an average of 21.7 days per cycle, being slightly longer early and late in the breeding season, and consistently around 21 days at the peak of the breeding season (April through September; Ginther, 1992). The cycle is a pattern of events, both physiological and behavioral, which are under hormonal control (Daels and Hughes, 1993). Control of the estrous cycle is governed by the hypothalamic-pituitary axis. The cycle is divided into two phases, estrus and diestrus. The estrous phase lasts on average 6.5 days and is the period in which a dominant follicle emerges on one ovary and the mare becomes sexually receptive (Ginther 1992). Ovulation occurs an average of 24 h prior to the end of sexual receptivity (i.e., on the penultimate day of the estrous period). The diestrus phase typically lasts 15 to 16 days and is the period of elevated plasma progesterone concentrations and nonreceptivity to the stallion. Diestrus ends when the corpus luteum (CL) regresses and estrus begins again 1 to 2 days later (Daels and Hughes 1993).

The hypothalamic-pituitary axis is controlled by day length, which is perceived by the pineal gland (Sharp and Cleaver, 1993). The pineal gland secretes melatonin, which is thought to affect dopaminergic neurons in the hypothalamus that control prolactin secretion by the pituitary gland in other species (Lincoln and Tortonese, 1995). Either directly on GnRH secreting neurons, or indirectly through other pathways, elevated melatonin concentrations during the long nights of winter also result in reduced gonadotropin production and secretion by the pituitary gland (Sharp and Davis, 1993) via reduced production and secretion of GnRH (Silvia et al.,

1987). As day length increases in late winter (in the northern hemisphere), the melatonin influence diminishes and GnRH is produced and secreted in greater amounts, resulting in a stimulation of pituitary LH and FSH and a continuation of estrous cycling.

Plasma LH concentrations are low from days 5 to 16 of diestrus and begin to rise with luteolysis. After luteolysis, the negative feedback by progesterone on pituitary LH secretion is removed and the increasing estrogen secretion from the follicles, particularly the emerging dominant follicle, greatly stimulates LH secretion throughout the estrous period. This LH stimulates development and maturation of the primary follicle in a positive feedback loop (Daels and Hughes, 1993); the high LH concentrations eventually lead to ovulation and subsequent CL formation.

Plasma FSH concentrations are suppressed by two ovarian factors from the dominant follicle: estrogen (primarily estradiol) and inhibin, a protein hormone (Ginther, 1992). The high plasma FSH during diestrus stimulates the growth and maturation of follicles, as well as estrogen secretion. Secretion of FSH during diestrus is sometimes biphasic, with peaks occurring soon after ovulation and again towards the end of diestrus (Alexander and Irvine, 1993). Such biphasic secretion is not observed consistently in all mares (Ginther, 1992). Once a follicle reaches the preovulatory stage, the high secretion rates of estradiol and inhibin greatly suppress FSH secretion, which is low albeit measurable throughout the estrous period.

The behavioral changes associated with estrus are thought to be primarily due to estrogens secreted by the developing follicles. Granulosa cells within the follicle convert androgens produced by the thecal cells (an LH dependent action) into estradiol 17-beta via the aromatase enzyme system, and this action is FSH dependent (Daels and Hughes 1993). Plasma estradiol concentrations peak just prior to ovulation, and return to very low levels shortly after

ovulation. The decline in estradiol concentrations prior to ovulation are due to the release of the granulosa cells into the follicular fluid, which is part of the ovulation process (Morel, 2008). Formation of the CL occurs shortly post ovulation, and progesterone concentrations are measurably elevated in jugular plasma within approximately 24 h (Ginther, 1992). The luteal tissue contained within the CL is in part derived from the follicular thecal cells (Morel, 2008), although granulosa cells are thought to also contribute to the luteal cells population (Squires, 1993). Plasma progesterone concentrations rise for 5 to 6 days post ovulation and reach their maximum near 10 ng/mL. These concentrations are maintained until day 15 to 16 of the cycle, and drop 4 to 5 days prior to the next ovulation (Daels and Hughes, 1993). The rise in progesterone concentrations, which are typically inhibitory to LH secretion (Thompson et al., 1991), causes a gradual reduction in LH concentrations in early diestrus. The elevated progesterone concentrations in diestrus are, if anything, slightly stimulatory on FSH secretions (McNeil-Wiest et al., 1988; Thompson et al., 1991).

The relatively rapid formation of the CL with its associated progesterone secretion is responsible for the shift from estrous displays to those of diestrus (i.e., nonreceptivity to stallion; Ginther, 1992). Progesterone blocks displays of estrus in mares, even in the presence of estradiol. Similarly, even though estrogen is stimulatory to LH concentrations in mares in the absence of progesterone, the combination of estrogen and progesterone acts synergistically to inhibit LH secretion more so than a similar dose of progesterone alone (Garcia and Ginther, 1978).

Progestogens and Equine Reproduction

Suppression of the estrous cycle is often desired by owners of performance horses to decrease signs of estrus that are often detrimental to the ability of their horse to compete at the

highest level. Previous research revealed that oil based injections of progesterone were able to block estrus and ovulation (Loy and Swan, 1966). There have been many attempts at using synthetic progestogens to circumvent the estrous cycle with minor successes. The failure of most synthetic progestogen preparations is the inefficiency in binding to the progesterone receptor. Previous studies tested the effects of medoxyprogesterone acetate on quarter horses mares and found that it had no significant effects on the estrus cycle of the mare (McCue, 2003). These results were later validated by Storer et al. in 2009.

Currently, the only FDA approved method to suppress estrous is altrenogest, which is an orally active progestogen marketed as Regumate (Intervet, Inc., Millsboro, DE). Altrenogest is sold as a solution in oil and is fed at 0.044 mg/kg of body weight per day (McCue, 2003). Altrenogest is the 17 α -allyl derivative of trenbolone, an anabolic agent similar to testosterone. Regumate is approved by the Federal Drug Administration for the following uses: 1) to facilitate attainment of regular cycles during the transition period from winter anestrus to the physiological breeding season, 2) to facilitate management of the mare exhibiting prolonged estrus during the transition period, and 3) to permit scheduled breeding of mares during the physiological breeding season (<http://www.regu-mate.com/label.asp>). Behavioral suppression of estrus occurs within 2 to 3 days of administration (Hodgson et al., 2005), and will continue as long as the drug is administered at the recommended dose. One drawback of Regumate as sold is that it requires daily application. A means of delivering altrenogest, which is now off patent, without daily feeding is highly desirable.

Use of GnRH and GnRH Agonists in Equine Reproduction

With the revelation of the amino acid structure of GnRH in the 1970's (Nair and Schally, 1972), peptide chemists soon began testing various modifications to the original structure in an

effort to produce longer acting, more active analogs (Coy et al., 1973). Naturally occurring GnRH is a decapeptide synthesized and stored in terminal granules in the median eminence. Substitutions of the sixth amino acid, glycine, and deletion of the carboxyl terminal amino acid, also glycine, with the addition of an ethyl amide group resulted in analogs with much greater half-lives in peripheral circulation and much greater biologic activity (Coy et al., 1974). Several of these analogs were extensively tested for their efficacy to induce ovulation in estrous mares (Irvine, 1993), and a biodegradable capsule formulation of deslorelin acetate was eventually marketed as Ovuplant in the late 1990's.

A major problem with the use of GnRH analogs in other species has been the development of refractoriness, or down regulation of pituitary responsiveness to GnRH (D'Occhio et al., 2000). Fitzgerald et al. (1993) first reported the inhibitory effect of goserelin acetate on ovulation in seasonally cyclic mares, but with variable results. Although mares seem to be less amenable to down regulation (Porter et al., 1997), the widespread use of Ovuplant after its appearance on the market revealed that it had undesirable side-effects in mares not conceiving to an Ovuplant-induced ovulation (Johnson et al., 2000). Those mares displayed a delayed return to estrus and delayed follicular growth (Johnson et al., 2000; Farquhar et al., 2001; Blanchard et al. 2002). Johnson et al. (2002) subsequently reported that mares treated with Ovuplant had greatly reduced plasma LH and FSH concentrations and a reduced sensitivity to GnRH challenges for up to 10 days after ovulation. About the same time, McCue et al. (2002) and Wendt et al., (2002) showed that if the implant was removed within 48 h after insertion, the beneficial effect on ovulation was retained, but the lengthening of the interovulatory interval was avoided. Similar to anecdotal evidence among horse breeders at the time, Johnson et al. (2000)

also found that a small percentage of mares became totally anovulatory for the entire season after Ovuplant administration, a fact that eventually led to Ovuplant being pulled from the U.S. market.

It is due to this down-regulation by deslorelin acetate that it is now being considered as a means of inhibiting, rather than stimulating, reproductive processes in mares. Deslorelin acetate is readily available in large quantities now due to its poor performance as a commercial product in Ovuplant. Ovuplant capsules contained 2.1 mg of deslorelin acetate. The question is whether much larger doses of deslorelin, particularly in slow-release formulations, could cause complete down regulation of the reproductive processes of mares.

Slow-release Drug Delivery Systems

Various approaches for slowing down the release of drugs, when injected into the body or taken orally, have been developed over many years of research. Many texts and reviews are available on the subject, and one recent online article covers the history and present state of the science very well (Rhee et al., 2010). Basically, slow-release formulations can be divided into several types: oil-based injectable solutions, injectable-drug suspensions, polymer-based microspheres, and polymer-based in-situ formings (form hydrophobic depots in aqueous environment after injection). The oil-based injectable solutions and injectable drug suspensions are capable of releasing drug for weeks after a single injection, while polymer-based microspheres and in-situ gels may last for months (Rhee et al., 2010).

Sucrose acetate isobutyrate (SAIB) is an organic polymer that is soluble in ethanol and falls into the latter category (polymer-based in situ formings) of slow-release formulations. It can be easily mixed with drugs dissolved in ethanol, and when some or all of the ethanol is evaporated under reduced pressure, it forms a solution that becomes very hydrophobic when

injected into the body (Tipton, 2002). In 2000, Southern BioSystems, Inc. and Thorn BioScience, LLC, announced the issuance of U.S. Patent 6,051,558, "Compositions Suitable for Controlled Release of the Hormone GnRH and its Analogs," based on the SAIB system. That patent was partially based on research from the LSU Agricultural Center in which three deslorelin formulations were assessed for efficacy of induction of ovulation and for resulting deslorelin concentrations produced in jugular blood over time. A copy of this patent can be viewed online at www.everypatent.com/comp/pat6051558.html. Application of this same technology for altrenogest injections more recently has led to the development of LA 150, an injectable altrenogest formulation that delivers 225 mg of altrenogest in a 1.5-mL i.m. dose. Storer et al. (2009) reported that application of this formulation to mares, when given simultaneously with a luteolytic dose of Lutalyse (prostaglandin- $F_{2\alpha}$) during diestrus, resulted in the occurrence of first estrus 12.7 days later, compared to 3.9 days for control mares. Moreover, Storer et al. (2009) pointed out that the C.V. for days to ovulation (mean = 16.5 d) was 11%, indicating a relatively tight grouping of the data around the mean, which meant that the LA 150 formulation should provide reliable timing of ovulation for prediction of breeding dates. The LA 150 formulation is, and has been, commercially available through BetPharm, a Lexington, KY, based pharmacy and can be purchased online (www.betpharm.com).

Rationale for Present Experiments

Today's horse owners often have the desire to circumvent the horse's naturally occurring physiological events (e.g., estrus) due to the need alter the horses temperament, behavior, or reproductive cycle. Mares in heat are often unruly and behave poorly during show events. Pharmaceutically affecting the natural heat cycles may provide the best solution to these problems. The experiments described herein were designed to test two possible methods to

suppress estrus and ovulation in normally cycling mares: 1) treatment with altrenogest in long-acting (slow-release) vehicle formulations, and 2) treatment with a high dose of the potent GnRH agonist, deslorelin acetate, also in a slow-releasing injection vehicle.

CHAPTER II

EVALUATION OF SUSTAINED RELEASE ALTRENOGEST FORMULATIONS FOR SUPPRESSING ESTRUS AND OVULATION IN MARES

Introduction

Progestogens are frequently used to prevent the expression of estrus in race, show, and broodmares for periods of a week or longer. Unfortunately, daily treatments can be impractical for the administration of altrenogest or progesterone to mares, being inconvenient and time consuming. Recent advances in biodegradable, controlled-release drug delivery systems that allow single administration products to replace prolonged daily progestin treatment protocols have shown promise in mares (Storer et al., 2009). Such formulations can reduce labor and the associated handling stress to the animals and offer veterinarians an important means of maintaining effective compliance rates on farms with wide varieties of management systems. The present experiment was designed to evaluate the effectiveness of a new formulation of altrenogest (BET Pharm BioRelease LA225; www.betpharm.com) at two doses (225 versus 450 mg in 1 versus 2 mL) to the currently available altrenogest formulation (BET Pharm BioRelease LA150, 225 mg in 1.5 mL; www.betpharm.com) for suppression of estrus following PGF_{2α} treatment of cyclic mares. It was hypothesized that the new formulation would be as effective as the currently available formulation. For comparison, a dose of the new formulation twice that of the first was included to determine whether it extended the period of suppression even further.

Materials and Methods

Twenty-four mares of light horse breeds, maintained on native grass pasture at the LSU Agricultural Center horse farm in Baton Rouge, were used in May and June of 2010 for this study. They were in good body condition (scores of at least 6; Henneke et al., 1993), and ranged in age from 5 to 16 years. Verification of normal reproductive cyclicity was determined

by transrectal ultrasound scanning of the ovaries and daily detection for estrus with a vigorous stallion.

For all mares, once ovulation was detected, Lutalyse (10 mg; Pfizer Animal Health; <https://animalhealth.pfizer.com/sites/pahweb/Pages/Global.aspx>) was administered (i.m.) on the 7th day following ovulation (designated as day 0). Mares were simultaneously administered (i.m.) a single injection of altrenogest in a delay-release vehicle (treated groups; n = 6 each) or vehicle only (controls; n = 6). The treated groups received: 1) a single injection of Biorelease altrenogest LA150, currently available commercially (www.betpharm.com; 225 mg total; 22.5 mg/day for 10 days as a 1.5-mL solution); 2) a single injection of a new formulation, altrenogest LA225 (225 mg total; 22.5 mg/day for 10 days as a 1-mL solution); or 3) a single injection of the new altrenogest LA225 at twice the dose (450 mg total; 45 mg/day for 10 days as a 2-mL solution). As mares became available for treatment, group assignments were selected at random, but with the limitation that each successive set of four mares would contain one mare in each treatment group.

After injection, mares were checked for estrus daily and their ovaries were scanned for assessment of follicular sizes every 3 days until a follicle of 30 mm or larger was detected. Mares achieving 30-mm follicles were then scanned daily until ovulation. The disappearance of a preovulatory follicle (>35 mm) and the presence of a bright echogenic structure at the location of the previously observed follicle were taken to indicate ovulation. The day of ovulation was considered the day that the preovulatory follicle was no longer present. In addition, jugular blood samples were drawn daily, once a mare achieved a 30-mm follicle, until day 5 post-ovulation to confirm a rise in progesterone concentrations.

Estrous behavior was scored based on behavioral signs at each assessment. Mares nonreceptive to the stallion (diestrus) received scores of -3, -2, or -1, based on the degree of negativity they displayed; behavioral signs including kicking, biting, holding ears back, switching of tail, or holding tail down tightly. Passive mares (mares failing to show either positive or negative signs) were scored as 0. Mares displaying signs typical of estrus (lordosis reflex, urinating, raising of the tail, or clitoral eversion) were scored as 1, 2, or 3, based on the intensities of the signs.

Injection sites were monitored for 5 days after injections for signs of swelling and sensitivity. Swelling was scored as 0 to 3 (0 = none, 1 = slight -diameter of swelling up to 12.5 mm, 2 = moderate- diameter of swelling 12.5 to 25 mm, 3 = significant diameter of swelling about 25 mm or larger). Sensitivity to touch (yes/no) and temperature elevation at site (yes/no) were also recorded.

Days to ovulation and days to first estrus were analyzed by one-way ANOVA (SAS, SAS Instit., Cary, NC). Injection site endpoints were analyzed by repeated measures ANOVA with treatment as the main effect and time as the repeated effect. Differences between means were assessed by the least significant difference test (Steel et al., 1997).

Results

The mean days to ovulation and mean days to first day of estrus are presented in Figure 2.1. All control mares ovulated after the injection of prostaglandin- $F_{2\alpha}$ at an average of 10.0 days (range 8 to 13). All mares treated with altrenogest ovulated after prostaglandin- $F_{2\alpha}$ injection. Means for the 3 treated groups ranged from 16.8 to 17.8 d, and all were greater ($P < 0.002$) relative to controls; there was no difference ($P > 0.59$) among the groups receiving altrenogest.

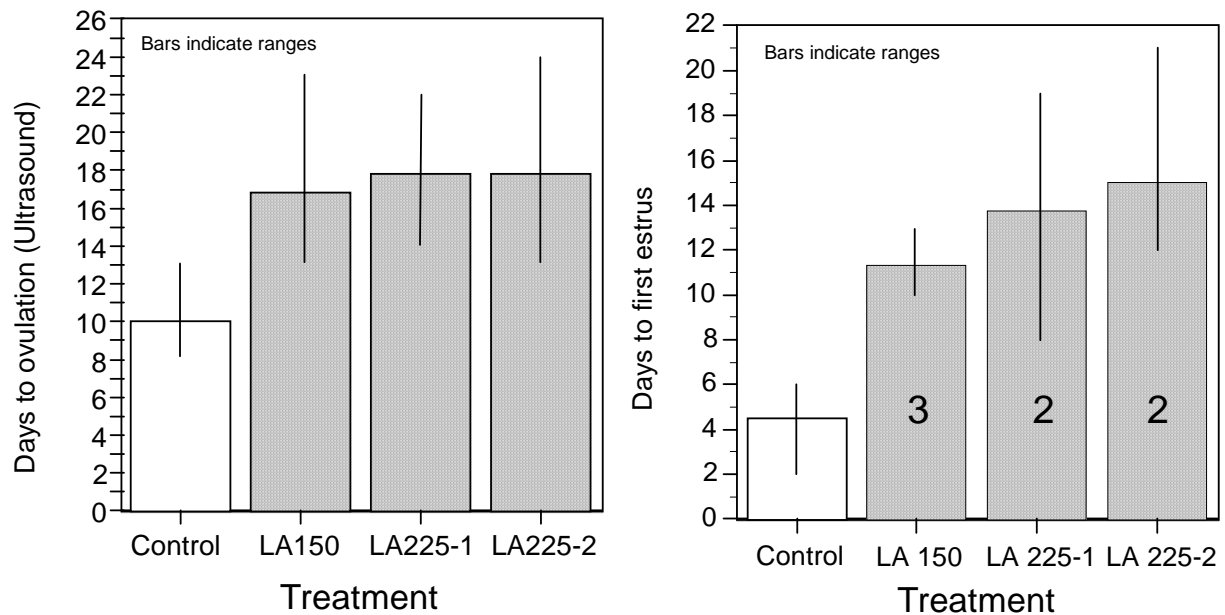


Figure 2.1. Days from treatment to ovulation (left panel) and to first estrus (right panel) for control mares and mares treated with a single injection of Biorelease altrenogest LA 150 (225 mg; 1.5 mL) versus Biorelease altrenogest LA 225 at 225 mg (1 mL) or 450 mg (2 mL). Vertical bars indicate ranges for each group. For both ovulation and estrus data, means for all treatment groups were greater ($P < 0.02$) than for controls; there was no difference among means for treated groups. Numbers inside bars for estrus means indicate the number of mares (of 6) that did not show estrus with their post-treatment ovulation; means are based on those mares that did display estrus. Pooled SEM were 2.0 days and 2.2 days for days to ovulation and days to first estrus, respectively.

All control mares displayed estrus after prostaglandin- $F_{2\alpha}$ injection, starting an average of 4.5 days after injection. Not all mares treated with altrenogest displayed estrus after prostaglandin- $F_{2\alpha}$ injection in association with the detected ovulation (2 or 3 mares in each group did not). Mean day of first estrus for the remaining mares in the treated groups ranged from 11.3 to 15.0 d, and were all greater ($P < 0.02$) relative to controls; there was no difference ($P > 0.19$) among the treated groups.

The mean injection site swelling and sensitivity scores are presented in Figure 2.2. Injection site temperature scores were all zero, and are not presented. Swelling scores were

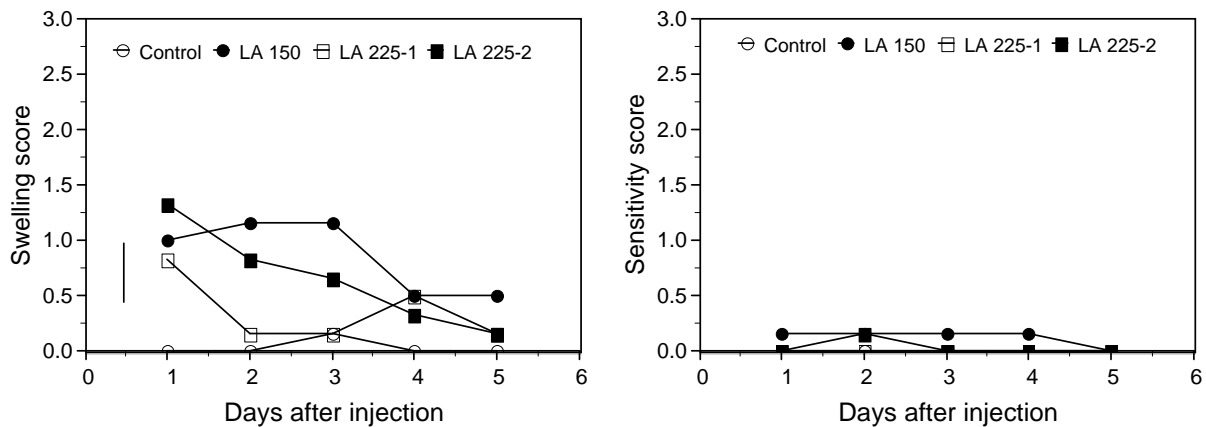


Figure 2.2. Swelling (left panel) and sensitivity (right panel) scores for the site of injection for control mares and mares treated with a single injection of Biorelease altrenogest LA 150 (225 mg; 1.5 mL) versus Biorelease altrenogest LA 225 at 225 mg (1 ml) or 450 mg (2 mL). The vertical line in the left panel represents the least-significant difference value for assessment of differences between means within any given day ($P < 0.05$). Swelling scores are based on a 0 to 3 scale: 0 = none; 1 = slight; 2 = moderate; and 3 = significant. Sensitivity scores are based on 0 = no and 1 = yes. Site of injection scores for temperature elevation taken on the same days were all 0 (none). Pooled SEM were 0.23 and 0.08 for swelling and sensitivity scores, respectively.

affected by treatment differentially over the first 5 days after injection (interaction, $P = 0.055$).

Swelling scores for mares receiving altrenogest averaged between 0.83 and 1.33 on day 1, and in general, decreased thereafter. Scores for the LA-150 and LA-225-2 treatments remained higher than controls on day 2, and the LA-150 again on day 3; no other comparisons were significant ($P > 0.077$). Sensitivity scores were not affected ($P = 0.3$) by treatment or day, and there was no interaction in the ANOVA.

Discussion

Treatment with all three altrenogest formulations in the current experiment resulted in similar delays in time to ovulation, averaging about 17 days overall. The mean for the group receiving LA 150 was 16.8 d, which is equivalent to the mean reported for mares administered

LA 150 in the experiment of Storer et al. (2009). Both injection volumes of the newer formulation (LA 225) resulted in ranges of response among mares similar to that in mares receiving LA 150. Unlike the results of Storer et al. (2009), the CV of days to ovulation for the LA 150-treated mares was 21% in the present experiment, twice that of the previous experiment (11%). Moreover, Storer et al. (2009) reported that doubling the volume (hence total dose) of LA 150 increased the days to ovulation by approximately 4 d, whereas doubling the amount of LA 225 in the present experiment did not alter the average days to ovulation, nor did it appear to have any effect on the range of responses among mares. Thus, for practical application, injection of 1 mL of LA 225 will provide results equivalent to injection of 1.5 mL of LA 150, and doubling the dose will not provide any better response.

Long-term altrenogest feeding (via Regumate) has been reported to have no detrimental effect on mares' health or breeding potential (Squires, 1993). Up to 88 days of feeding had no effect on clinical signs of health nor on various measurements of blood chemistry and biochemical endpoints in mares (Shideler et al., 1983). Further examination of mares fed altrenogest for 0, 15, 30, or 60 days in the breeding season revealed that subsequent pregnancy rates were unaffected (Squires et al., 1983). Thus, similar application of altrenogest via LA 225 as described herein should provide a method for long-term suppression of estrus and ovulation in mares that would not be expected to have any detrimental effect on the mares. Weekly injections of 1 mL of LA 225 would provide constant suppression, based on the results of this experiment, and this could likely be extended to every 10 days with the use of 2 mL of LA 225. From an economic standpoint, the 1 mL dose weekly would probably be chosen.

As depicted in Figure 2.1, all mares treated with altrenogest eventually ovulated, but 7 of the 18 did not exhibit estrus in association with the ovulation. Mares were not followed beyond 5

days after ovulation, but it is assumed that they would have returned to estrus after the end of the subsequent diestrus. Although lack of behavioral estrus has been noted to occur in 26% of mares treated with prostaglandin- $F_{2\alpha}$ (Munro et al., 1979), the time between prostaglandin- $F_{2\alpha}$ treatment prior to altrenogest administration and the eventual subsequent ovulation makes it unlikely that prostaglandin- $F_{2\alpha}$ was having any effect in this case. More likely is the possibility that the threshold concentration of altrenogest in the blood for suppression of ovulation is slightly greater than that for suppression of behavioral estrus. This means that use of LA 225 for suppression of estrus may require fewer injections (or longer spacing in time) than for suppression of ovulation, especially if a normal diestrus follows the delayed ovulation, which would provide another 14 days of diestrus without any further altrenogest injection.

Injection of all formulations/volumes of altrenogest resulted in temporary but slight swelling at the site of injection. Depending on the use of the mare being treated (e.g., show mare vs. a racing mare), this slight swelling may be a factor in choosing a treatment option. The injections in the present experiment were given in the base of the neck anterior to the shoulder. Although there was slight swelling, actual sensitivity in the area was rare, as indicated by the average scores all being < 0.25 . Again, depending on the use of the mare, alternate sites could easily be rotated such that any swelling would have minimal effect on performance.

CHAPTER III

EVALUATION OF A SUSTAINED RELEASE DESLORELIN ACETATE FORMULATION FOR INHIBITING ESTRUS AND OVULATION IN MARES

Introduction

High doses of GnRH or one of its analogs have been used to down-regulate the reproductive activity of women (Swerdlhoff and Heber, 1983) as well as cattle and other ruminants (D'Occhio et al., 2000). Apparently, the high concentrations of GnRH activity in the blood in such cases obliterates the normal hypothalamic GnRH signal to the adenohypophysis, down-regulates GnRH receptors on the gonadotropes, and results in little to no production of LH from these cells (Ortmann and Diedrich, 1999). Injection of highly active GnRH analog in biodegradable microspheres is a commonly used procedure for endometriosis in women (Schweppe and Hummelshoj, 2005).

Unlike human and ruminant females, mares have been less responsive to high-dose GnRH down-regulation. (Ginther, 1992; Porter et al., 1997). Given the temporary inhibition of LH and FSH secretion by deslorelin acetate in previous experiments (Johnson et al., 2000, 2002; Farquhar et al., 2001), it is possible that a larger dose of deslorelin acetate may cause long-term shut down. Thus, the aim of the present experiment was to test the efficacy of a newly formulated deslorelin preparation in a biodegradable, slow-release vehicle formulation for suppression of ovarian activity and estrous activity in mares.

Materials and Methods

Fourteen light horse mares of moderate to high BCS (5 to 8; Henneke et al., 1993) were used. The mares were long-term residents of the LSU AgCenter Horse Unit in Baton Rouge, Louisiana, were maintained on local native grass pastures, and received appropriate routine dewormings and vaccinations. The experiment was conducted in July and August, 2010.

Mares were checked daily for estrus with a vigorous stallion. On July 17, all mares received a single i.m. injection (10 mg) of prostaglandin- $F_{2\alpha}$ to lyse any luteal tissue they might have. Any mare not returning to estrus within 7 days was given a second prostaglandin- $F_{2\alpha}$ injection on July 24. When a mare came into heat, her ovaries were examined via transrectal ultrasound scanning to assess follicular sizes and possible ovulation. When a dominant follicle of at least 30 mm was achieved, a mare was randomly assigned to either 1) control group, and received an i.m. injection (4 mL) of vehicle ($n = 6$), or 2) deslorelin-treated group, and received an i.m. injection of 1.5 g deslorelin acetate in a biodegradable, slow-release vehicle formulation (4 mL; $n = 7$). One mare never showed estrus after the two prostaglandin- $F_{2\alpha}$ injections and was removed from the experiment.

After injections, mares were checked for estrus daily and their ovaries were scanned for assessment of follicular sizes every 3 days until a follicle of 30 mm or larger was detected. Mares achieving 30-mm follicles were then scanned daily until ovulation. The disappearance of a preovulatory follicle (>35 mm) and the presence of a bright echogenic structure at the location of the previously observed follicle were taken to indicate ovulation. The day of ovulation was considered the day that the preovulatory follicle was no longer present. In addition, jugular blood samples were drawn daily, once a mare achieved a 30-mm follicle, until day 5 post-ovulation to confirm the patterns in progesterone and LH concentrations. These data were used to estimate date of ovulation based on 1) the first progesterone increase (0.5 ng/mL or higher) occurs approximately 24 h after ovulation, and 2) the peak in LH concentrations occurs approximately 24 h after ovulation (Ginther, 1992). This approach was found to be a reliable backup to ultrasound scanning in a previous experiment (Storer et al., 2009).

Estrous behavior was scored based on behavioral signs at each assessment. Mares nonreceptive to the stallion (diestrus) received scores of -3, -2, or -1, based on the degree of negativity they displayed; behavioral signs including kicking, biting, holding ears back, switching of tail, or holding tail down tightly. Passive mares (mares failing to show either positive or negative signs) were scored as 0. Mares displaying signs typical of estrus (lordosis reflex, urinating, raising of the tail, or clitoral eversion) were scored as 1, 2, or 3, based on the intensities of the signs.

Days to ovulation were analyzed in a one-way ANOVA (SAS) with treatment as the single factor. Percentage of mares expressing heat at the expected time for return to estrus was analyzed by coding (0 = no expression, 1 = expression of estrus); these data were also analyzed by one-way ANOVA.

Results

Mean numbers of days until estrus, for those mares exhibiting a post-treatment estrus, are presented in Figure 3.1. Neither the days to first estrus nor the days to ovulation differed between groups ($P > 0.1$). The proportion of mares exhibiting estrus at the expected time did differ ($P = 0.77$) between groups; all 6 of the control mares (100%) exhibited estrus but only 4 of the 7 deslorelin-treated mares did (57%).

Discussion

The dose of deslorelin acetate used in the present experiment (1.5 g) was approximately 700 times greater than that used in Ovuplant (2.1 mg deslorelin as deslorelin acetate). Although the inhibition of LH and FSH secretion caused by Ovuplant use in mares was generally temporary (Johnson et al., 2000, 2002; Farquhar et al., 2001), lasting approximately 10 days or less (Johnson et al., 2002), a small percentage of mares seemed to be hypersensitive to

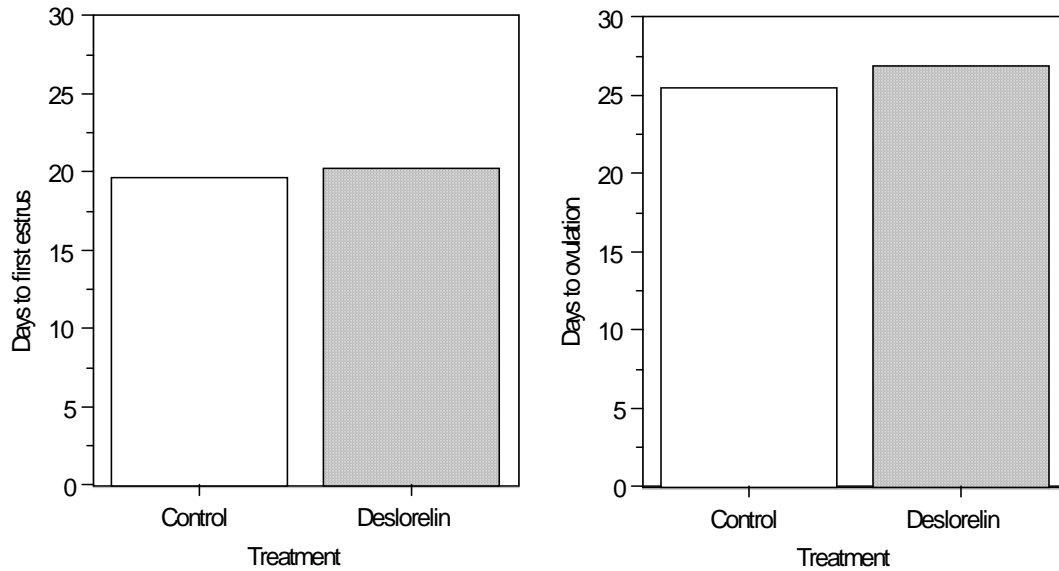


Figure 3.1. Mean days to first estrus (left panel) and ovulation (right panel) in control mares (n = 6) and mares treated with 1.5 g of deslorelin acetate in a biodegradable, slow-release formulation (n = 7). All 6 of the control mares, but only 4 of the treated mares, displayed estrus at the expected time of return to estrus. All mares ovulated around the expected time. There was no difference ($P > 0.1$) between groups for either endpoint. Pooled SEM were 1.0 and 0.9 days for days to first estrus and days to ovulation, respectively.

deslorelin, in the form of Ovuplant, and would go into an anestrous state and be lost for the breeding season (Johnson et al., 2000). McCue et al. (2002) subsequently reported that removal of the implant (Ovuplant) within 48 h of insertion resulted in normal interovulatory intervals in mares. Thus, the detrimental effect of Ovuplant seemed to be in the timing of the exposure to the peptide. More recent data (Stich et al., 2004) has confirmed that liquid formulations of deslorelin do not result in follicular suppression and extended interovulatory periods. The slow-release formulation used in the present experiment should have resulted in elevated plasma concentrations of deslorelin for a much longer time than those produced by Ovuplant. However, there was no effect of treatment in the present experiment on time to ovulation and little effect on

time to first estrus. Thus, it is apparent that this approach (i.e., dose of deslorelin and vehicle formulation) to inhibiting estrus and ovulation in mares has basically no promise as a practical application.

Why this approach had no effect on time to ovulation, when a much smaller dose of deslorelin in the form of Ovuplant consistently did in several experiments (Johnson et al., 2000, 2002; Farquhar et al., 2001), raises the question as to what actually was the basis of the Ovuplant inhibition. Given that the Ovuplant effect (lengthening) on interovulatory interval was avoided if the capsule was removed within 48 h after implantation (McCue et al, 2002), it must be assumed that the inhibitory effect on LH and FSH secretion reported by Johnson et al.(2000, 2002) was occurring in the first 48 h after implantation. This assumption really needs to be tested in an empirical experiment: treat a group mares with Ovuplant, and remove the implant from half the mares after 48 h. Measurement of daily LH and FSH concentrations, in a manner similar to that of Johnson et al. (2000), in those mares plus a control group would provide direct evidence of whether all the inhibitory effect was in the first 48 h.

SUMMARY AND CONCLUSIONS

Two experiments were performed to test the efficacies of altrenogest, an artificial progestogen, and deslorelin acetate, a potent analog of GnRH, in inhibiting estrus and ovulation in mares displaying normal estrous cycles. Treatment with two doses of a newly formulated solution of altrenogest in slow-release vehicle was compared to the currently commercially available formulation (LA 150, 1.5 mL injection) and vehicle injection in mares after induction of luteolysis. All groups receiving altrenogest had extended periods of diestrus relative to control mares as well as a delay to next ovulation; there was no difference among the groups receiving altrenogest for either endpoint. A similar experiment comparing treatment with deslorelin acetate (1.5 g) in a slow-release formulation to vehicle injection (controls) resulted in no change in days to first estrus or days to ovulation. Deslorelin treatment did reduce the percentage of mares displaying estrus at the expected time by about 40%. It is concluded that altrenogest, both in the established and in the newer formulations, can be used to delay the onset of estrus and ovulation in normally cycling mares. An injection schedule of approximately once every 10 days could be used to keep mares out of heat for extended periods of time.

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VITA

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