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Repeatability of prolactin responses to sulpiride in mares and geldings and the effect of pergolide and cabergoline

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REPEATABILITY OF PROLACTIN RESPONSES TO SULPIRIDE IN MARES AND GELDINGS AND THE EFFECT OF PERGOLIDE AND CABERGOLINE

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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 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

Four experiments were conducted in an effort to develop a method, based on prolactin secretion, for assessing the efficacy and duration of activity of dopaminergic agonists for the treatment of pituitary pars intermedia dysfunction (PPID) in horses. In the first experiment, prolactin response to a low dose of the dopamine antagonist, sulpiride, was generally repeatable in estrogen-primed geldings in winter over 8 every-other-day challenges. It was concluded that estrogen-primed, sulpiride-challenged geldings in winter could serve as a model for the study of potential dopaminergic drugs for the treatment of PPID in horses. The second experiment was performed in the summer with mares, and again tested the repeatability of the prolactin responses over a 30-day period. The responses in mares were generally repeatable, and there was no effect due to stage of the estrous cycle. It was concluded that mares could serve as a model for the study of potential dopaminergic drugs as well as geldings, and stage of the estrous cycle did not have to be taken into account. In the third experiment, two formulations of the dopamine agonist, pergolide, were tested (oral administration versus injection) against a single formulation of cabergoline (injected) and control injections (vehicle) for their efficacy to reduce unstimulated plasma prolactin concentrations in geldings. Oral pergolide reduced prolactin concentrations for a few hours, whereas injected pergolide suppressed prolactin concentrations for 24 hours. Cabergoline suppressed prolactin concentrations for up to 5.5 days. It was concluded that the injectable formulations had potential for further study as possible treatments for PPID in horses. The last experiment tested the efficacy of daily pergolide injection versus a single injection of cabergoline, for suppressing the prolactin secretion induced by low dose sulpiride injections in mares. Daily injection of pergolide suppressed prolactin responses as long as the injections were given, plus another 2 days. The single cabergoline injection suppressed prolactin responses for a

minimum of 10 days. Based on these results, cabergoline in slow-release vehicle seems to provide an excellent possibility for administering dopaminergic activity to horses with PPID. Whether these results are directly applicable to PPID horses needs to be determined.

INTRODUCTION

Pituitary pars intermedia dysfunction (PPID), also referred to as equine Cushing's disease, is a common disease in older horses caused by an abnormal stimulation of the adrenal glands by products from the cells of the intermediate lobe of the pituitary gland (McFarlane, 2011). The hypersecretion of cortisol from the adrenal cortex results in several undesirable symptoms, including constantly long, curly hair coat (hirsuitism), insulin resistance, excessive drinking and urination (polyuria and polydipsia), laminitis, lethargy, excessive sweating, loss of muscle mass, repeated infections such as sole abscesses, tooth root infections, and sinusitis, as well as infertility (Messer, 1999). The presence and severity of symptoms generally depends upon the length of time the disease goes untreated (McFarlane, 2011).

The cells of the intermediate lobe that are purported to be responsible for the disease are the melanotropes, cells that normally produce and secrete α-melanocyte stimulating hormone (α-MSH, hereafter referred to as MSH), which is involved with skin and hair coloring in most species (Hadley and Levine, 2007). The activity of the melanotropes is known in other species to be regulated by the hypothalamic secretion of dopamine, an inhibitory neurotransmitter that keeps MSH secretion within normal limits (Hadley and Levine, 2007). Prolactin, a hormone produced by the lactotropes of the adenohypophysis, is regulated by the hypothalamus in the same manner, i.e., by dopamine, and its secretion is constantly under negative feedback (Moore, 1987; Saiardi and Borrelli, 1998). The nature of this negative tone is revealed when horses are administered a dopamine receptor antagonist, such as domperidone or sulpiride; plasma concentrations of both hormones (MSH and prolactin) increase within minutes (Johnson and Becker, 1987; Brendemuehl and Cross, 2000; Beech et al., 2011).

The current understanding of the cause of PPID involves a reduced dopamine secretion from the hypothalamus in older horses (McFarlane et al., 2005), thereby increasing the activity

of the melanotropes and perhaps shifting their production from MSH to adrenocorticotropic hormone (ACTH; Saiardi and Borrelli, 1998). The currently accepted treatment for PPID therefore is based on drugs that mimic the activity of dopamine, thereby suppressing the activity of the melanotropes. Pergolide, a drug previously used in human medicine for treatment of Parkinson's disease, is now approved for use in horses to treat PPID (Schott, 2002). Cabergoline, another dopamine agonist, has been suggested as a longer acting, and perhaps less expensive, alternative to pergolide in humans (Godbout et al., 2010), and may be applicable to PPID horses.

The experiments described herein were conducted in an effort to devise a model, using prolactin secretion, to study the efficacy and duration of action of dopaminergic drugs for the treatment of PPID. Even though pergolide is currently sold for the treatment of PPID, it is expensive, needs to be administered daily, and is likely of lower activity as a dopamine agonist relative to cabergoline. The rationale for using prolactin, rather than MSH or ACTH, as the monitor of the effects of the drugs being tested is primarily cost. Commercial kits for ACTH and MSH are expensive, and their measurement requires the use of aprotinin as an inhibitor of protease activity in the blood samples collected. Prolactin measurement in the LSU Agricultural Center School of Animal Sciences is more cost effective compared to the costs of the commercial kits.

CHAPTER 1

REVIEW OF LITERATURE

Prolactin

In 1928, Stricker and Grueter reported that milk secretion could be stimulated in rabbits by giving them extract of the anterior pituitary gland (cited by Hadley and Levine, 2007). However, upon injecting the extract directly into the mammary ducts, only the alveoli attached to those treated ducts produced milk. This result indicated that more hormones acted jointly with prolactin in controlling mammary gland development (Hadley and Levine, 2007). Prolactin was the name Riddle, Bates, and Dykshorn had given to a fraction of bovine pituitary extracts that had stimulated crop sac growth in pigeons (cited by Hadley and Levine, 2007). The majority of prolactin production and secretion occurs in the adenohypophysis by the cells referred to as lactotropes, but it can also be produced from the mammary glands, uterus, placenta, and T-lymphocytes (Bachelot and Binart, 2007). Prolactin's structure, in most mammals, consists of between 197 and 199 amino acids in one single chain (Sinha, 1995). Most often regarded as the lactogenesis and mammary growth and development hormone, prolactin actually plays a very diverse role in reproduction in both males and females, giving it the title of the "hormone of maternity" (Hadley and Levine, 2007).

Control of Prolactin Secretion

Prolactin secretion, unlike that of most other adenohypophyseal hormones, increases after removal of hypothalamic input to the pituitary gland (e.g., in dispersed cell suspensions, or after transection of the stalk-median eminence; Hadley and Levine, 2007). This led to the discovery that the neurotransmitter found throughout the brain and peripheral structures in the body, dopamine, was the main, if not sole, source of the tonic inhibition of prolactin secretion in most mammals (Ben-Jonathan, 1985). Dopamine is released into the hypophysial portal system

through the tuberoinfundibular neurons located in the mediobasal hypothalamus (Moore, 1987). These dopaminergic neurons are unique from the others of their kind; they are not directly regulated by dopaminergic receptor-mediated mechanisms, but by concentrations of prolactin in blood and cerebrospinal fluid (Moore, 1987). After dopamine has been released into the portal system, it attaches to type-2 dopamine (D2) receptors that are linked to membrane channels, as well as G proteins, and suppresses the secretory activities of the lactotropes (Ben-Jonathan and Hnasko, 2001). Not only does dopamine inhibit prolactin secretion through control of calcium fluxes in this manner, it also activates intracellular signaling pathways and represses prolactin gene expression and the proliferation of lactotropes (Ben-Jonathan and Hnasko, 2001).

Prolactin Secretion and Actions in Horses

Most research in horses has dealt with factors that affect prolactin secretion, including season (Johnson, 1986; Thompson et al., 1986a), meal consumption (Nadal, et al., 1997; McManus and Fitzgerald, 2000), exercise or stress (Colborn et al., 1991; Thompson et al., 1994), endophyte-infected tall fescue consumption (McCann et al., 1992), sex and gonadal presence (Thompson et al., 1986a), treatment with dopaminergic antagonists (Donadeau and Thompson, 2002; Thompson and DePew, 1997) or thyrotropin releasing hormone (TRH; Johnson, 1986; Thompson et al., 1986b), and estradiol pretreatment (Kelley et al., 2006). Plasma prolactin concentrations in horses, like in most mammals, are stimulated by the long days of summer, and are lowest during the short days of winter (Nequin et al., 1993). Most physical stimuli, such as exercise or other forms of stress, and administration of sulpiride, domperidone (dopamine receptor antagonists), or TRH all cause increases in prolactin secretion within a matter of minutes. Geldings have significantly lower plasma prolactin concentrations in spring than mares or stallions, which have similar concentrations, however the response to exercise is similar in all three types of horses (Thompson et al., 1994).

Apparently the increasing prolactin concentrations in the spring are involved with loss of the winter hair coat (Thompson et al., 1997) as well as the return of ovarian activity in mares (Nequin et al., 1993). Treatment of seasonally anovulatory mares with prolactin stimulated ovarian activity (Nequin et al., 1993) and induced ovulation (Thompson et al., 1997); subsequent production of anti-prolactin antibodies, which would neutralize the mares' endogenous prolactin, resulted in failure to shed the winter coat well into the spring (Thompson et al., 1997).

Strong evidence for the need for prolactin in mares for mammary development and subsequent milk production is derived from the research on fescue toxicity in mares (Redmond et al., 1994). Consumption of endophyte-infected tall fescue in the last 3 months of pregnancy is associated with greatly reduced plasma prolactin concentrations and a failure of udder development in mares (Cross et al., 1995), and treatment with a dopamine antagonist (domperidone) has been shown to reverse those effects (Cross et al., 1995).

Adrenocorticotropic Hormone

Adrenocorticotropic hormone, also referred to as corticotrophin, is produced in the basophils of the pars distalis of the adenohypophysis, and is the smallest adenohypophyseal hormone, consisting of only 39 amino acids in a single chain (Hadley and Levine, 2007). Due to the similarity of the first 13 amino acids in ACTH and MSH, ACTH has considerable melanotropic activity (Hadley and Levine, 2007). The major role of ACTH is stimulation of steroid biosynthesis (corticosteroids) in the adrenal cortex. The two glucocorticoids that are produced from the adrenal gland in response to ACTH are cortisol and corticosterone, which are both important to carbohydrate metabolism (Hadley and Levine, 2007). Cortisol is the more commonly produced corticosteroid in most mammals.

Adrenocorticotropic hormone, which is dependent upon the trophic hypothalamic peptide corticotrophin releasing hormone (CRH; Hadley and Levine, 2007) for its production and

secretion, is further regulated by negative feedback control of the adrenal glucocorticoids. Elevated cortisol concentrations, or exogenous treatment with the glucocorticoid analog, dexamethasone, inhibit further ACTH secretion until the high concentrations abate. It is this feedback loop that is the basis of the dexamethasone suppression test for Cushing's syndrome (hyperadrenalism; Hadley and Levine, 2007).

Intracellular production of ACTH is via a large prohormone referred to as proopiomelanocortin, or POMC (Hadley and Levine, 2007). This 241 amino acid peptide is cleaved from a pre-prohormone (pre-proopiomelanocortin) that is 285 amino acids in length. Within the corticotrope, ACTH is cleaved from POMC by proteases specific to the corticotrope (Chrétien and Seidah, 1981). This latter specificity is important, because POMC contains within its sequence the sequences of ACTH, β-lipotropin, α-MSH, β-MSH, γ-MSH, and β-endorphin (Hadley and Levine, 2007).

Cushing's Syndrome

Cushing's syndrome is the complex series of changes that occur in animals due to hypersecretion of adrenal glucocorticoids (Guyton and Hall, 2006). In humans, Cushing's syndrome is often associated with hypothalamic or pituitary adenomas resulting in excessive CRH or ACTH secretion, or alternatively, with ectopic secretion by adenomas in other parts of the body, or by adenomas in the adrenal gland itself (Guyton and Hall, 2006). Hyperadrenalism due to excessive ACTH secretion by the adenohypophysis is specifically referred to as Cushing's disease (Guyton and Hall, 2006).

Diagnosis of the cause of Cushing's syndrome usually starts with measurement of both plasma cortisol and ACTH concentrations. If cortisol concentrations are high, but ACTH concentration is low or undetectable, it is likely that the normal feedback loop sites in the hypothalamus and pituitary are intact and functioning normally. If both ACTH and

glucocorticoid concentrations are high, it is likely that the problem arises from the hypothalamus or adenohypophysis, or perhaps from an ectopic source of ACTH. The dexamethasone suppression test, mentioned above, is used to determine whether the normal glucocorticoid feedback effect on the hypothalamic-adenohyphyseal axis is intact and functional. That is, dexamethasone treatment in the evening is expected to cause a significant drop in corticosteroid concentration in blood by the following morning in normal (non-Cushing's) horses.

Melanocyte Stimulating Hormone

Although three forms of MSH have been described (α, β, and γ), α-MSH is the predominant form and is believed to be the biologically relevant form in most animals (Hadley and Levine, 2007). Melanocyte stimulating hormone is produced and secreted by the pars intermedia of the adenohypophysis, which lies between the pars distalis and pars nervosa in most vertebrates (a distinct pars intermedia is notably absent in the humans and birds; Hadley and Levine, 2007). Melanocyte stimulating hormone is comprised of 13 amino acids and plays an important role in skin coloration for many vertebrates (Hadley and Levine, 2007). Like prolactin, disruption of hypothalamic input to the pituitary increases, rather than decreases, MSH secretion (Hadley and Levine, 2007), indicating a tonic inhibitory action by the hypothalamus. Dopamine is now known to be the hypothalamic factor inhibiting MSH by interacting with the dopaminergic receptors on the cell membranes of the pars intermedia cells. Release of dopamine holds the cells in a hyperpolarized state and causes the inhibitory effect (Hadley and Levine, 2007).

Aside from aiding in skin or coat pigmentation, MSH has been known to have other physiological roles. For example, a large dose of MSH given subcutaneously results in an erection in men, aids in satiety and energy homeostasis and thermoregulation in some species, induces positive behavioral effects (arousal, increased motivation, longer attention span, memory

retention, and increased learning ability), and neuroplasticity, causing alterations in neurotransmitter synthesis, electrophysiological parameters of neurotransmission, excitability in the spinal cord and peripheral nerves, and behavioral responses (Hadley and Levine, 2007).

Like ACTH in corticotropes, MSH in the melanotropes is derived from POMC. Proteases specific to the melanotropes cleave POMC such that the predominant product is MSH, although in mice, β-endorphin has also been shown to be produced (Saiardi and Borrelli, 1998). Treatment with glucocorticoids or dexamethasone does not perturb the production of POMC or MSH by the melanotropes (Hadley and Levine, 2007), whereas the absence of dopaminergic control (via D2 receptor gene knockout) in mice resulted in pars intermedia hypertrophy, excessive POMC production, and ACTH secretion (Saiardi and Borrelli, 1998). This unusual ACTH production likely indicates a change in the proteases produced in the cell as a result of the lack of dopaminergic input.

Pituitary Pars Intermedia Dysfunction in Horses

Pituitary pars intermedia dysfunction is a slow progressing disorder with characteristic clinical signs in horses 15 years and older (Messer, 1999; McFarlane, 2011). It has also been referred to as equine Cushing's disease, due to the similarities is has with human Cushing's syndrome. However, unlike the human disease, the cause of equine PPID is adenomas within, or adenomatous hypertrophy of, the pars intermedia of the pituitary gland (Messer, 1999). Tumor formation within the intermediate lobe causes an increase in production of POMC peptides, due to an abnormal stimulation of the melanotropes. Often, there is a loss of function in the adjacent tissues due to compression on the tissues by the swelling lobe (McFarlane et al., 2006). The increase in POMC production also results in large amounts of MSH and β-endorphin-related peptides, as well as a small amount of ACTH. Melanocyte stimulating hormone and the β-endorphin-related peptides can result in a six-fold potentiation of ACTH activity; that, combined

with the small increase of ACTH synthesis from POMC, is enough to stimulate adrenocortical steroidogensis, resulting in an increase in plasma cortisol concentrations and the loss of the diurnal cortisol secretion pattern (Messer, 1999). McFarlane et al. (2003), based on a review of the available literature, suggested that PPID may result from dopaminergic neurodegeneration due to oxidative stress. Oxidative stress leads to a modification in cellular components, such as proteins, DNA, and cell membrane lipids, caused by excessive exposure to exogenous sources of oxidants. The damage to the cells leads to cell death, or neurodegeneration for neurons; with the loss of function of the dopaminergic neurons, dopamine inhibition on the pars intermedia is reduced or lost (McFarlane et al., 2003).

Clinical signs of PPID can include hirsutism (excessive hair coat that fails to shed in the spring and summer months), muscle atrophy, lethargy, bulging supraorbital fat pads, recurrent or chronic laminitis, susceptibility to infections (e.g. recurring sole abscesses), polyuria and polydipsia (excessive urination and thirst), and infertility (McFarlane et al., 2003; Donaldson et al., 2004). Hirsutism and abnormal fat deposits are the most common symptoms of this disease.

The current treatment for equine PPID is pergolide, a dopaminergic agonist (Donaldson et al., 2004). Melanotropes are kept under tonic suppression through the hypothalamic axis, via D2 receptors on their cell surface, and are inhibited by dopamine. Due to dopaminergic neurodegeneration in older animals, dopamine can no longer be produced or secreted to inhibit production (McFarlane et al., 2003). Based on this model, Sojka et al. (2006) suggested that pergolide would be the most effective treatment for PPID.

Rational for Present Experiments

The goal of the experiments presented herein was to determine whether the measurement of prolactin, or perhaps its response to the dopamine antagonist, supliride, could be used in horses as a model for potential efficacy tests on dopamine agonists, pergolide, and an alternative,

cabergoline for the treatment of PPID in horses. Because of the mutual regulation (tonic inhibition) of MSH and prolactin by hypothalamic dopamine, secretion and changes in secretion of the two hormones would be expected to occur in parallel. That is, doses and vehicle formulations of pergolide and cabergoline that inhibit prolactin may at the same time indicate the effect they would have on MSH. Because prolactin can be measured easily and cheaply, relative to MSH, in the LSU School Animal Sciences laboratory, it is presently the desired route for such assessments. Thus, the experiments described herein were specifically designed to 1) determine the repeatability of prolactin responses to a low dose of sulpiride given over an extended period of time in geldings, 2) to determine similarly the repeatability of responses of mares and any effect of the estrous cycle, 3) to determine the relative inhibitory effect (degree and time span) of pergolide and cabergoline formulations on resting prolactin concentrations in geldings, and 4) to determine the relative inhibitory effect (degree and time span) of specific pergolide and cabergoline formulations on the sulpiride-induced prolactin response in mares.

CHAPTER 2

REPEATABILITY OF PROLACTIN RESPONSES TO A LOW DOSE OF SULPIRIDE IN GELDINGS

Introduction

Prolactin secretion in horses is under tonic suppressive control by hypothalamic dopamine secretion, and thus can be temporarily stimulated by an intravenous injection of a dopamine receptor antagonist such as domperidone or sulpiride (Johnson and Becker, 1987; Brendemuehl and Cross, 2000; Donadeu and Thompson, 2002). Given that melanotropes, the cells that produce and secrete MSH in the intermediate lobe of the pituitary gland, are also under tonic, suppressive control by hypothalamic dopamine secretion (Hadley and Levine, 2007), it is possible that prolactin secretion in response to sulpiride may also reflect simultaneous MSH secretion. If so, then monitoring prolactin secretion could be used in lieu of measuring MSH concentrations in horses for the study of the efficacies and durations of activity of dopaminergic drugs (e.g., pergolide).

For long-term studies, prolactin response to injections of sulpiride for such use would require a relatively repeatable response from shot-to-shot, and even day-to-day, in the absence of any competing drug. Because repeated injections of sulpiride at or above the known saturating dose (about 50 mg of the +/- racemic mixture of sulpiride in a 500 kg horse) are known to result in a rapid decreases in prolactin response in geldings in late winter (Thompson and DePew, 1997), it is likely that a lower dose, perhaps 20 to 40% of maximal, would be needed to avoid the depletion of pituitary prolactin reserves. Moreover, to ensure easily measured plasma prolactin concentrations in winter, when prolactin secretion is naturally low, pretreatment with estradiol, which has been shown to enhance prolactin responses to sulpiride (Kelley et al., 2006; Thompson et al., 2008), might be of benefit.

The present experiment was designed to determine whether prolactin responses to a low dose of sulpiride in estrogen-primed geldings would be consistent enough to provide a basis for further development of a model for the future assessment of dopaminergic drugs in horses.

Materials and Methods

Six light horse, long-term geldings housed at the LSU Agricultural Center horse farm were used. They were between 6 and 20 years old, weighed between 410 and 526 kg, and had body condition scores between 5 and 8 (Henneke et al., 1983). They were routinely kept on native grass pasture most of the year, and on winter ryegrass pasture when native grasses were dormant. They remained on pasture except when experimental procedures were being performed.

On March 31, 2011, all geldings received a single intramuscular injection of 100 mg of estradiol cypionate (ECP; Biorelease estradiol cypionate LA, 50 mg/mL; BetPharm Pharmacy, www.betpharm.com) to stimulate prolactin. Sulpiride challenges were started on April 5, 2011, and were continued every-other-day through April 19, 2011. For each day of treatment and blood sampling, the geldings were brought in from pasture the evening before and were kept in a small lot with native grass hay and water available for ad libitum consumption. At approximately 08:00 the morning of blood sampling, the geldings were tethered loosely either in an outdoor chute or under an open–sided shed. A single sample of jugular blood was obtained via venipuncture from each of the geldings, and then sulpiride was injected intravenously. The dose of the +/- racemic mixture of sulpiride (Sigma Chem. Co., St. Louis, MO) was 5 μg/kg BW. The sulpiride was dissolved in sterile saline with sufficient NaOH added to result in complete solubilization; the final concentration of the solution was 0.5 mg/mL.

Samples of jugular blood were collected subsequently at 10, 20, 40, and 60 min after sulpiride injection. All blood samples were collected through 22-guage needles into tubes

containing 100 units of sodium heparin. The samples were placed at 5°C until centrifugation at 1200 x g for 15 min; plasma was harvested and was stored at -15°C.

The procedures described above were repeated on alternate days for a total of 8 sulpiride challenges. When all plasma samples had been collected, prolactin concentration was measured by radioimmunoassay (Colborn et al., 1991). From the raw prolactin data, two additional data sets were derived: 1) net changes in prolactin concentration from time 0, and 2) net areas under the response curve. The net change in concentration, which should be proportional to the amount of prolactin released in the first few minutes after sulpiride injection, was the difference between the highest prolactin concentration achieved after sulpiride injection (usually the 10 min sample) minus the time 0 (pre-injection) concentration. The net areas under the response curve were calculated by first subtracting the pre-injection concentration from all subsequent values, and then summing the concentration x time increments (rectangle summation). The net differences and the net areas were analyzed by ANOVA with SAS (SAS Instit., Cary, NC) with horse and day as the main factors; the horse x day interaction served as the error term. Differences among days were assessed by the least significant difference test (Steel et al., 1997). Linear and quadratic trends in means for days were assessed by appropriate "contrast" statements in the SAS program.

Results

All geldings responded to the low dose of sulpiride with robust increases in plasma prolactin concentrations (Figure 2.1).

Analysis of the net changes in prolactin concentrations and the areas under the response curves provided essentially the same information. Both dependent variables were affected ($P < 0.001$) by day of sulpiride challenge. Moreover, both variables showed a linear ($P < 0.002$)

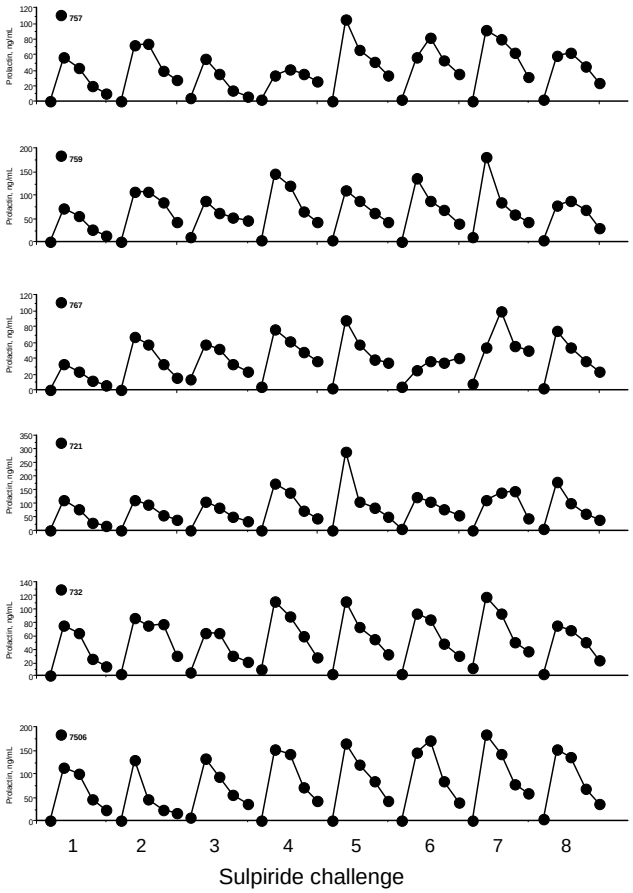

Figure 2.1. Plasma concentrations of prolactin in 6 geldings administered sulpiride at 5 µg/kg BW on 8 alternate days beginning April 5, 2011. All geldings received a single injection of estradiol cypionate on March 31, 2011.

and a quadratic ($P < 0.003$) component in the means of the 8 days of challenge (Figure 2.2), with an upward trend in the first few days followed by a plateau.

The within-gelding coefficients of variation ranged from 16 to 41% for the net increase in prolactin concentrations and from 20 to 35% for the net areas under the response curves.

Discussion

The prolactin responses to sulpiride in these geldings were relatively robust over the 8 challenges compared to geldings administered a saturating dose of sulpiride (100 mg of the +/-

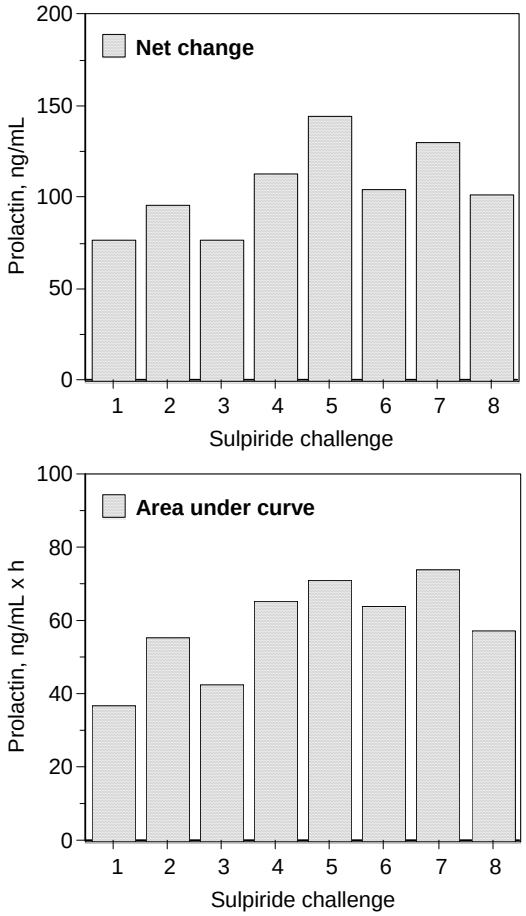

Figure 2.2. Mean net changes in prolactin concentrations and net areas under the response curves for geldings administered sulpiride at 5 µg/kg BW on 8 alternate days beginning April 5, 2011. All geldings received a single injection of estradiol cypionate on March 31, 2011. There were significant linear ($P < 0.001$) and quadratic ($P < 0.002$) components for both dependent variables. The pooled SEM were 15.5 ng/mL and 5.9 ng•mL^{-1}•h for net changes and areas, respectively.

racemic mixture, or approximately 160 to 200 µg/kg BW) daily starting on February 8 (Thompson and DePew, 1997). In that experiment, the geldings were not pretreated with estradiol, and the mean prolactin response (area under curve) decreased from 25 ng•mL^{-1}•h in the first week to 10 ng•mL^{-1}•h in the second week of treatment, indicating a 60% depletion of pituitary reserves of prolactin. Treatment of the geldings in the present experiment with estradiol

stimulated prolactin to the extent that the average response to sulpiride at 5 μg/kg BW was greater than the average response to the saturating dose in the geldings of Thompson et al. (1997). Moreover, rather than decreasing from the first day to the last, the prolactin responses in these estradiol-primed geldings increased gradually to a plateau. This stimulation of prolactin secretion by the estradiol-sulpiride combination is in fact dependent upon both components (estradiol and a dopamine antagonist) being present. The results of Kelley et al. (2006) demonstrated how prolactin secretion in seasonally anovulatory mares is greatly stimulated by the simultaneous treatment with estradiol and sulpiride, but how prolactin secretion begins to decrease soon after estradiol treatment stops. Thus, the prolactin responses to the low dose injections of sulpiride in the present experiment would likely have started diminishing in magnitude over time if no further ECP injection was given, based on the plasma estradiol patterns reported by Thompson et al. (2008) after a 100-mg injection of ECP to geldings in November. In that experiment, mean plasma estradiol concentrations after ECP injection increased to approximately 20 pg/mL in 3 days and returned to baseline at approximately 20 days.

Although a perfectly repeatable situation would be one in which the prolactin response was unchanged over time and the variation among responses was close to zero, the results obtained here do seem to have potential as a means to study the efficacy and duration of activity of dopamine agonists. That is, successful dopaminergic agonist treatment would be one in which the prolactin response to low-dose sulpiride injection was completely blocked (basically no response). Even with the 20 to 35% intra-gelding coefficient of variations for net areas under the response curves, the prolactin responses to sulpiride would still be easily differentiated from no response, and would thus provide information as to the efficacy of a given dose of agonist and its duration of action.

Compared to daily, oral administration of pergolide, which is the current standard treatment for PPID horses (Schott, 2002), formulations of pergolide or cabergoline in vehicles that provide several days to perhaps weeks of drug release are possible. Biodegradable polymers are commonly used to deliver drugs by intramuscular injection for slow release over time (Jeonga et al., 1999). Polymer-based in-situ formings (solutions that form hydrophobic depots in aqueous environment after injection) are another class of vehicle used for slow-release drug delivery, of which sucrose acetate isobutyrate (SAIB) is a prime example. Both SAIB and biodegradable microparticle vehicles have been successfully tested at the LSU Agricultural Center horse farm (Storer et al., 2009) for the extended delivery of altrenogest, a progestogen used in the horse industry for the synchronization of estrus and the long-term suppression of estrus and ovulation in mares (Webel and Squires, 1982). The application of these technologies to delivery systems for dopamine agonists for the treatment of PPID certainly deserves further study.

CHAPTER 3

REPEATABILITY OF PROLACTIN RESPONSES TO A LOW DOSE OF SULPIRIDE IN MARES: EFFECT OF THE ESTROUS CYCLE

Introduction

It was shown in Chapter 2 that treating estrogen-primed geldings with low dose sulpiride injections every other day resulted in relatively consistent prolactin responses. Like geldings, mares can be kept in groups on pasture and may also serve as convenient test animals in experiments testing the efficacy and duration of action of dopaminergic agonists such as pergolide and cabergoline. Unlike geldings, mares have cyclic changes in their internal steroid milieu during the breeding season, with elevated progesterone concentrations during diestrus and elevated estrogen concentrations during estrus (Ginther, 1992). Although Johnson (1986) reported that plasma prolactin concentrations did not vary across the estrous cycle in mares, there is no information on the possible interaction of estrogen secretion during estrus and administration of dopamine antagonists such as sulpiride or domperidone. Thus, the present experiment was designed to determine whether low dose sulpiride injections administered every other day to cyclic mares would result in similar plasma prolactin responses, similar to geldings, or whether the changing steroidal conditions would alter the responses.

Materials and Methods

Six light horse mares with previous histories of displaying normal estrous cycles were used. They ranged in age from 6 to 15 years, weighed between 498 and 556 kg, and had body condition scores between 6 and 8 (Henneke et al., 1983). They were routinely housed on native grass pastures at the LSU Agricultural Center horse farm. They remained on pasture except when experimental procedures were being performed.

Beginning on July 3, 2011, and continuing every other day thereafter through July 31, 2011, all mares received an intravenous injection of sulpiride in saline in the morning. For each day of injection, the mares were brought in from pasture the evening before and were kept in a small lot with native grass hay and water available for ad libitum consumption. At approximately 08:00 the morning of blood sampling, the mares were tethered loosely either in an outdoor chute or under an open-sided shed. A single sample of jugular blood was obtained via venipuncture from each of the mares, and then sulpiride was injected intravenously. The dose of the +/- racemic mixture of sulpiride (Sigma Chem. Co., St. Louis, MO) was 5 µg/kg BW. The sulpiride was dissolved in sterile saline with sufficient NaOH added to result in complete solubilization; the final concentration of the solution was 0.5 mg/mL.

Blood was obtained via jugular venipuncture into heparinized, evacuated tubes immediately before injection (time 0) and at 10, 20, 40, and 60 min after injection. Plasma was harvested by centrifugation (1200 x *g* for 15 min) and was stored at -15°C. Prolactin was measured in all plasma samples as described by Colborn et al. (1991). Although the mares were not subjected to heat detection, the initial plasma sample on each day of sulpiride challenge was used to measure LH concentration (Thompson et al., 1983) to help identify the stage of the estrous cycle of each mare throughout the experimental period.

The raw prolactin data were used as described in Chapter 2 to calculate the net changes in plasma prolactin concentrations and the net areas under the response curves for each sulpiride challenge for each mare. These data were subjected to ANOVA in SAS (SAS Instit., Cary, NC) with mare and day of challenge as main effects, and the interaction served as the error term. Based on LH concentrations, the net changes and areas were further characterized as occurring in 1) the follicular phase, 2) early diestrus, or 3) late diestrus. The period of rising and highest LH concentrations were considered to denote the follicular phase (Ginther, 1992); the period of

decreasing LH up to the next LH rise was considered the diestrous phase, which was further divided approximately in half and denoted early and late diestrus. These data were analyzed by ANOVA with mare, stage of the estrous cycle, and their interaction as factors; the replicated challenges and their interaction with mare and stage of the cycle made up the error term.

Results

Like in geldings in Chapter 2, the prolactin responses to the low dose of sulpiride were generally robust throughout the 30-day period in these mares (Figure 3.1). Analysis of the net

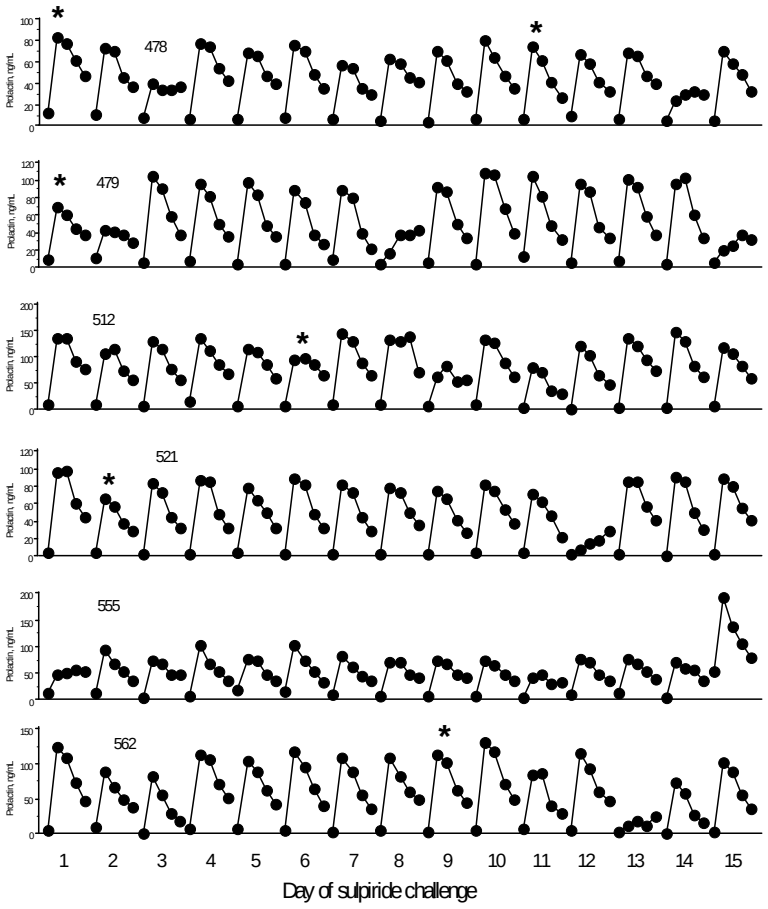

Figure 3.1. Plasma concentrations of prolactin in 6 mares administered sulpiride at 5 μg/kg BW on 15 alternate days beginning July 5, 2011. The days of peak LH concentrations are indicated with an asterisk; mare 555 had low LH concentrations throughout the 30-day period.

change in prolactin concentrations and the net areas under the response curves indicated no effect ($P > 0.1$) of day of sulpiride challenge (Figure 3.2).

There were occasional low responses in some mares, however they were not associated with any of the 3 estrous cycle categories, as evidenced by the lack of effect of estrous cycle status ($P > 0.1$) for both net change in prolactin concentrations and net area under the response curves (Figure 3.3).

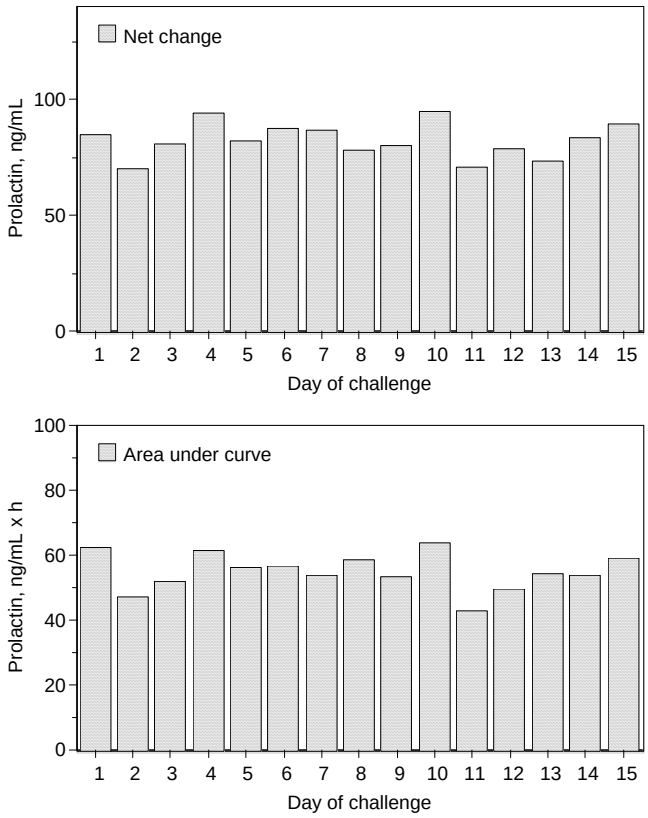

Figure 3.2. Mean net changes in prolactin concentrations and net areas under the response curves for mares administered sulpiride at 5 µg/kg BW on 15 alternate days beginning July 5, 2011. There was no effect ($P > 0.1$) of day of sulpiride challenge for either variable. The pooled SEM were 12.6 ng/mL and 7.2 ng•mL^{-1}•h for net changes and areas, respectively.

Intra-mare coefficients of variation ranged from 18 to 32% for net change in prolactin concentrations, and from 18 to 30% for net areas under the prolactin response curves.

Discussion

The prolactin responses to this low dose of sulpiride in mares were similar to, but perhaps slightly lower than, those observed in estrogen primed geldings in Chapter 2. The prolactin responses would be expected to be greater in July than in April, due to the naturally occurring

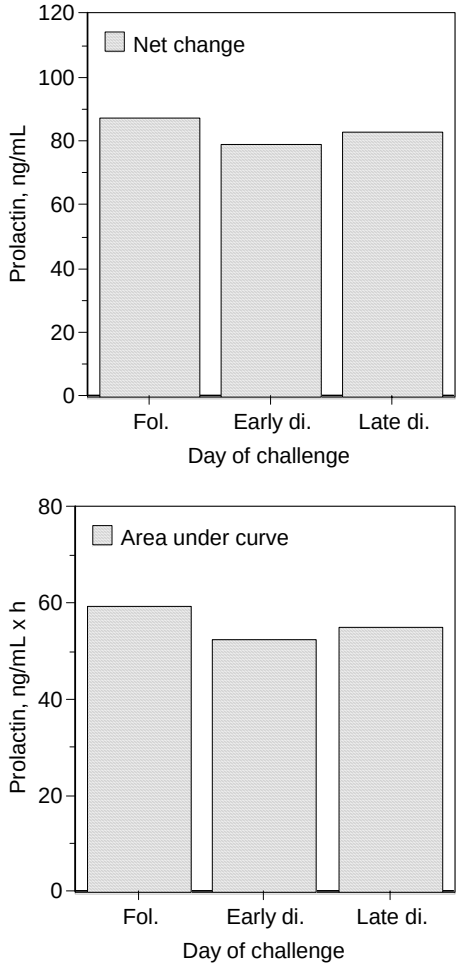

Figure 3.3. Mean net changes in prolactin concentrations and net areas under the prolactin response curves in mares during the follicular phase (Fol.), early diestrus (Early di.), and late diestrus (Late di.). There was no effect ($P > 0.1$) of estrous cycle status on either variable. Pooled SEM were 11.8 ng/mL for net changes and 16.0 ng•mL^{-1}•h for areas.

seasonal variations in plasma and pituitary prolactin concentrations (Johnson, 1986; Thompson et al. 1986a). Also, these mares received no estrogen priming before the start of sulpiride challenges. Apparently their own natural changes in plasma estradiol concentrations over the estrous cycle was insufficient to stimulate prolactin secretion, given the lack of effect of estrous cycle status on the net changes and net areas under the curves. This lack of estrous cycle stage effect on the prolactin responses to sulpiride is similar to the lack of estrous cycle stage effect on plasma prolactin concentrations reported originally by Johnson (1986).

Aurich et al. (1995) reported that both LH and prolactin were immediately released in response to the opioid antagonist, naloxone, in ovariectomized pony mares pre-treated for 8 days with estradiol or the combination of estradiol and progesterone. In contrast, there was no response to naloxone in mares not receiving steroid treatment or in mares treated with only progesterone. Whether the normally occurring changes in estrogen concentrations in mares over the estrous cycle might be sufficient to result in a naloxone-induced prolactin response needs to be determined.

Given that the prolactin response to low dose sulpiride challenge is generally repeatable across the estrous cycle, mares then provide another possible model for the testing of dopaminergic drugs for the possible treatment of PPID in horses. The occasional poor response observed in these mares (e.g., the 12th response in mare 521 and the 13th response in mare 562) stood out as exceptional but totally unexplained. One technical possibility would be failure to inject the entire sulpiride dose into the jugular vein, however this should have been obvious to the person doing the injecting. The poor responses occurred somewhat randomly and were not associated with stage of the estrous cycle.

CHAPTER 4

INHIBITORY EFFECTS OF PERGOLIDE AND CABERGOLINE ON PLASMA PROLACTIN CONCENTRATIONS IN GELDINGS: DURATION OF EFFECT

Introduction

Pergolide is a dopamine receptor agonist that is used in some countries as a treatment for Parkinson's disease. It was removed from the U.S. market in 2007 for human use due to an association of its use with heart valve dysfunction (Rasmussen et al., 2011), however it remains available for compounding by veterinary pharmacies to be sold as a treatment for PPID in horses (starting at 0.5 to 1.0 mg/day orally and gradually increasing the daily dose to up to 6 mg/day).

Cabergoline is another dopamine receptor agonist that is highly active on D2 receptors (Seeman, 2007). It was also commercially available for human use, and went off patent in 2005, but has the same potential side-effects as pergolide (Rasmussen et al., 2011). It may be a potential replacement for pergolide for use in horses due to its long acting nature (Godbout et al., 2010).

The objective of the present experiment was to determine and compare the effects of the current drug of choice, pergolide, in two possible formulations (oral administration and intramuscular injection) to cabergoline (injected), on the unstimulated daily plasma prolactin concentrations in geldings.

Materials and Methods

Sixteen light horse, long term geldings were used. They ranged in age between 6 and 20 years old, weighed between 410 and 616 kg, and had body condition scores between 5 and 8 (Henneke et al., 1983). All horses were located at the LSU Agricultural Center horse farm and were maintained on native grass pasture.

The 16 geldings were randomly assigned to one of the four treatment groups (n = 4/group): control, pergolide injection, cabergoline injection, and oral pergolide administration. On August 20, 2011, all geldings were treated at 08:00 in the morning. All injections (treatments and vehicle) were given intramuscularly and the pills (pergolide) were administered with the aid of a pill gun; geldings not receiving oral treatment had the pill gun placed into their mouth to equalize stress levels across treatments. The geldings were kept in a small pasture lot close to the site of treatment and blood collection. On the morning of treatment, and for every blood collection after treatment, horses were loosely tethered in an outdoor chute.

Blood samples were obtained via jugular venipuncture into heparinized, evacuated tubes 12 and 24 hours before treatment; immediately before injection (time 0 on day 0); at 1, 3, 6, 9, and 12 hours after injection; and every 12 hours thereafter until the morning of day 6. Plasma was harvested from all samples by centrifugation (1200 x *g* for 15 min) and was stored at -15°C. Prolactin was measured in all plasma samples as described by Colborn et al. (1991).

Prolactin concentrations were analyzed in a one-way ANOVA with repeated measures (sampling times) with treatment and time as main effects (SAS, SAS Instit., Cary, NC). The treatment effect was tested with the animal-within-treatment term, and time and the interaction were tested with residual error. The significance of differences between groups for each time period was tested by the least significant difference test (Steel et al., 1997).

Results

Mean plasma prolactin concentrations in the four groups of geldings are shown in Figure 4.1. The means for each treated group are plotted against the means for the vehicle-treated (control) geldings for clarity. There was an effect of treatment ($P = 0.061$) as well as a treatment x time interaction ($P = 0.0062$) in the ANOVA. Relative to controls, all treatments reduced ($P < 0.05$) prolactin concentrations, but the treatments varied as to the degree of reduction and to the

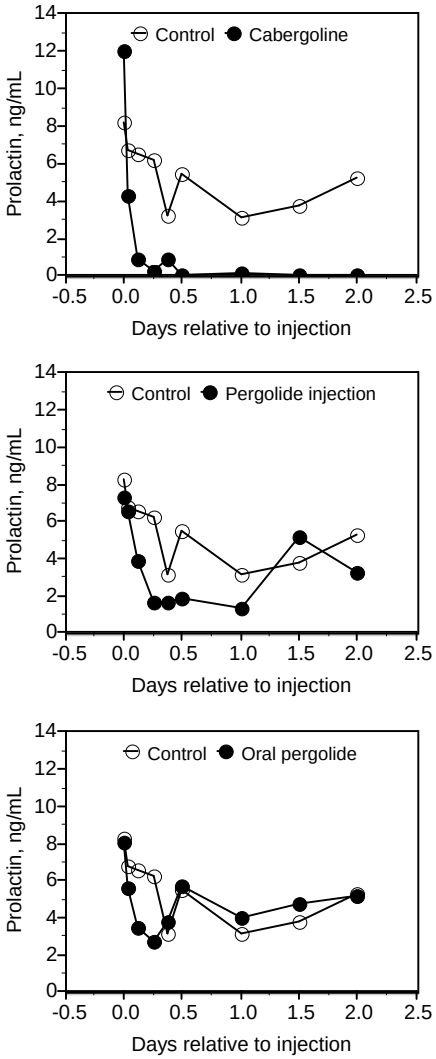

Figure 4.1. Mean plasma prolactin concentrations in the first 48 hours in vehicle-treated geldings (control) and those receiving an injection of 5 mg of cabergoline (top panel), an injection of 2 mg of pergolide (middle panel), or oral pergolide (2 mg; bottom panel). Pooled SEM was 1.6 ng/mL for prolactin concentrations.

duration of reduction. Oral pergolide reduced prolactin concentrations only at 3 and 6 hours, down to 2.7 ng/mL, after administration, whereas the injection of pergolide reduced prolactin concentrations from 6 to 24 hours after treatment, down to 1.6 ng/mL. Cabergoline injection reduced prolactin concentrations to <1 ng/mL for the first 2 days and to <1.6 ng/mL thereafter through day 6 (Figure 4.2).

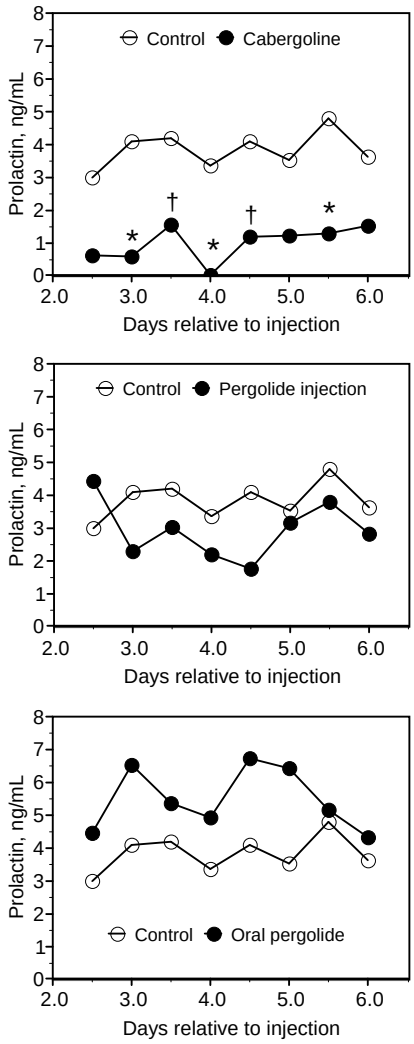

Figure 4.2. Mean plasma prolactin concentrations 2.5 to 6 days after treatment in vehicle-treated geldings (control) and those receiving an injection of 5 mg of cabergoline (top panel), an injection of 2 mg of pergolide (middle panel), or oral pergolide (2 mg; bottom panel). Only cabergoline reduced prolactin concentrations during this period (*P < 0.05; †P < 0.1). Pooled SEM was 1.6 ng/mL for prolactin concentrations.

Discussion

Although a starting dose of pergolide of 0.5 to 1.0 mg/day orally is recommended for horses suspected of having PPID (Donaldson et al., 2004; Schott, 2002), a single dose of 2 mg was used in the present experiment as a typical dose that would be given after a gradual ramp up to effect. It is not uncommon for the dose to be ramped up to as high as 6 mg/day (Donaldson et

al., 2004; McFarlane, 2011). This 2 mg dose, when administered orally, had a very short-lived effect on plasma prolactin concentrations. If the inhibitory effect on melanotropes in the intermediate lobe of the pituitary gland were similar in degree and duration as the effect on prolactin secretion, it seems that this mode of administration of pergolide is an unnecessary waste of drug. Intramuscular injection of an equal amount of pergolide produced a full 24 hours of suppression, and thus would be a more efficient and efficacious approach for treating PPID.

Cabergoline was administered as an injection, and at the 5 mg/horse dose, because it, in combination with the slow-release vehicle used, was expected to last considerably longer than the pergolide injections. The cabergoline injections definitely produced the greatest suppression of prolactin concentrations in these geldings, and the duration of action was much longer than the pergolide injections. Prolactin concentrations were generally suppressed by cabergoline even at 5.5 days after injection. If this efficacy and duration of activity can be shown to be consistently obtained with larger groups of horses, a 5 to 6 day injection regimen would be vastly superior to daily feeding or injection of pergolide.

Throughout the experiment, the geldings were observed while blood samples were collected for any adverse signs due to the dopaminergic agonist treatments. No adverse behaviors were noted in the 6 day period of sample collection, nor were there any signs of irritation or swelling at the site of injections. It was concluded that the pergolide and cabergoline formulations for injection deserved further study as a potential treatment for PPID in horses.

CHAPTER 5

INHIBITORY EFFECTS OF DAILY PERGOLIDE INJECTIONS VERSUS A SINGLE INJECTION OF CABERGOLINE ON THE DAILY PROLACTIN RESPONSES TO A LOW DOSE OF SULPIRIDE IN MARES

Introduction

The responses of unstimulated plasma prolactin concentrations in Chapter 4 indicated that the injectable pergolide and cabergoline formulations in slow release vehicle had potential as superior treatments for PPID in horses compared to daily oral dosing of pergolide. The ability of these formulations to suppress the prolactin response to low dose sulpiride stimulation as described in Chapters 2 and 3 was tested in mares.

Materials and Methods

Fifteen light horse mares were used. They ranged in age between 5 and 16 years old, weighed between 480 and 616 kg, and had body condition scores between 5 and 8 (Henneke et al., 1983). All horses were located at the LSU Agricultural Center horse farm and were maintained on native grass pasture.

Mares were initially assigned to one of three groups of five based on their ages, body weights, and body condition scores, such that the means for those characteristics in the three groups were similar. The groups were then randomly assigned treatment: controls (saline injected), daily pergolide injections, and a single injection of cabergoline. Control mares received single intramuscular injections of saline daily from day 0 through day 6. The cabergoline-injected mares received a single intramuscular injection of 5 mg of cabergoline in slow-release vehicle on day 0 and then injections of saline from day 1 through day 6. The pergolide-injected mares received single daily intramuscular injections of pergolide in slow-release vehicle on days 0 through 6. All injections were given in the morning between 07:00 and 08:00.

The low-dose sulpiride challenges (2 μg/kg BW of the +/- mixture in saline) were started on day -2 (October 19, 2011), and were repeated on days -1, 0, 1, 2, 3, 4, 6, 8, and 10. The sulpiride dose was reduced from 5 (Chapters 2 and 3) to 2 μg/kg BW because the prolactin responses in those earlier experiments were more robust than needed for monitoring treatment effects, and because the challenges in this experiment were to occur daily. The original experimental protocol called for daily sulpiride challenges through day 10, however several mares became averse to the injection and blood sampling regimen, and it was decided to go to an every-other-day challenge after day 4. That change alleviated the behavioral problems.

For each sulpiride challenge, mares were prepared as described in Chapter 3. On day 0, the day of first treatment injection, the sulpiride challenge was started 30 min after the treatment injections. Blood was obtained via jugular venipuncture into heparinized, evacuated tubes at time 0 (treatment), 10, 20, 40, and 60 minutes relative to sulpiride injection. Plasma was harvested by centrifugation (1200 x *g* for 15 min) and was stored at -15°C. Prolactin was measured in all plasma samples as described by Colborn et al. (1991).

Plasma prolactin concentrations in response to sulpiride were analyzed in a one-way ANOVA with two repeated measures (days and minutes within days). Also, areas under the response curves were calculated for each mare on each day as described in Chapter 2; these areas were analyzed in a one-way ANOVA with repeated measures (days). The significance of differences between groups for each time period was tested by the least significant difference test (Steel et al., 1997).

Results

Mean plasma prolactin concentrations in response to sulpiride challenges over the course of the experiment are presented in Figure 5.1. There was an effect of treatment, day, and minute of blood sampling ($P < 0.01$) as well as the three-way interaction ($P < 0.001$). Daily pergolide

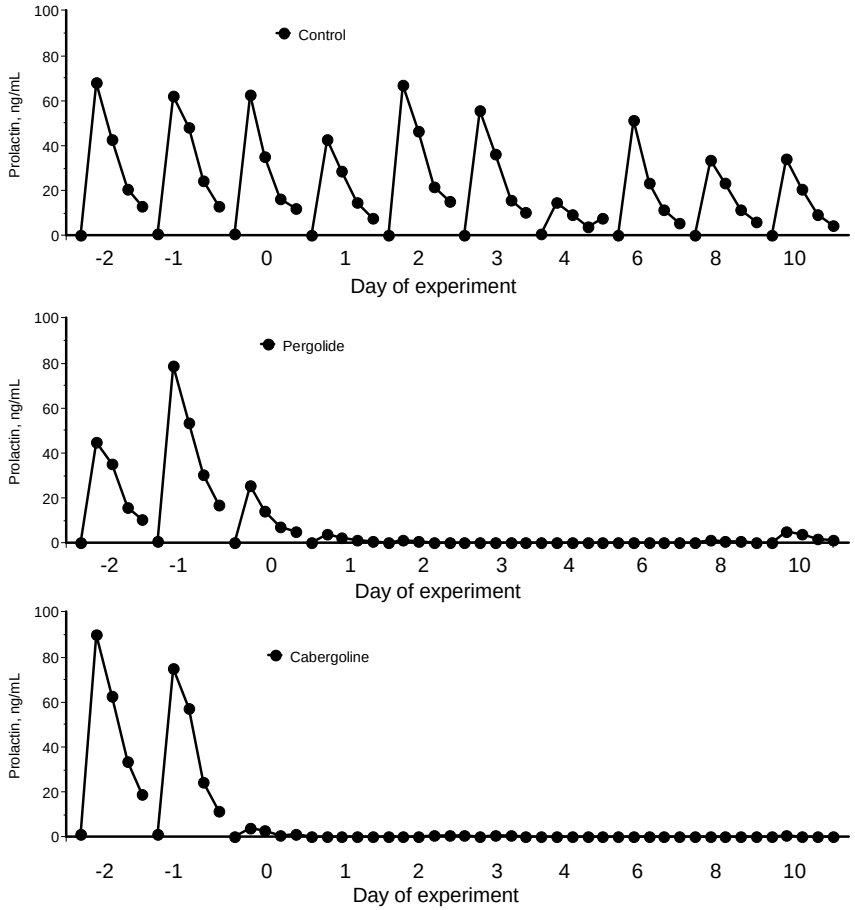

Figure 5.1. Mean plasma prolactin concentrations in response to low dose sulpiride challenges in control mares and mares receiving daily injections of 2 mg pergolide (days 0 through 6) or a single injection of 5 mg of cabergoline in slow-release vehicle on day 0. The sulpiride challenge on day 0 was started 30 minutes after the treatment injections. The pooled SEM was 6.6 ng/mL.

injections and the single cabergoline injection both suppressed ($P < 0.05$) the prolactin response to sulpiride through day 10. There was an effect of treatment even on day 0, just 30 minutes after the first (or only) treatment injection, with cabergoline almost totally suppressing the prolactin response to sulpiride challenge. When expressed as areas under the curves (Figure 5.2), the responses after cabergoline were essentially zero after day 0, whereas there was a small response in the pergolide-treated mares on day 0 and eventually on day 10 (which was 4 days after the last pergolide injection).

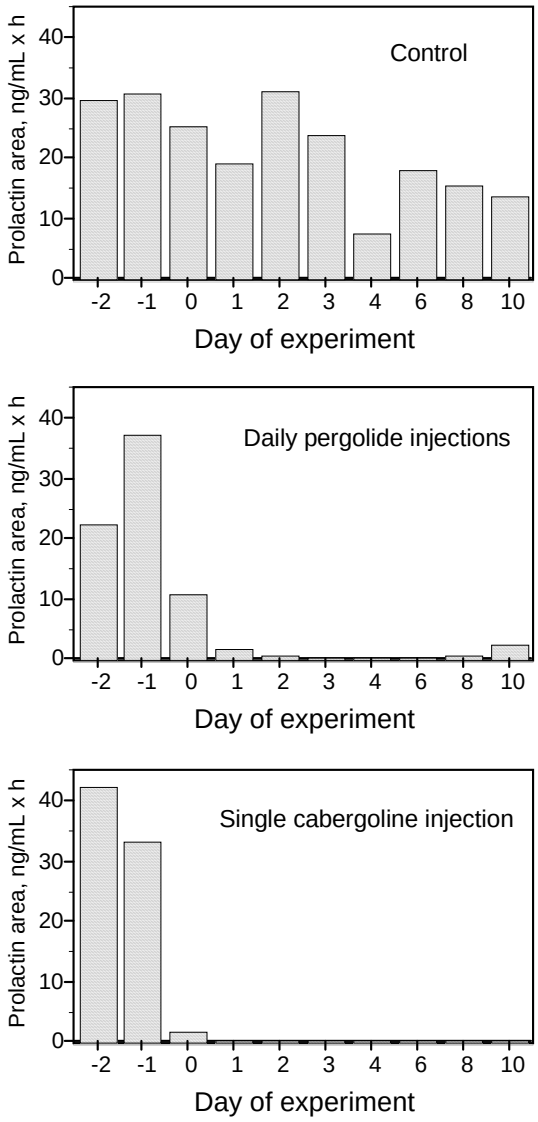

Figure 5.2. Mean areas under the prolactin response curves in control mares and mares receiving daily injections of 2 mg pergolide (days 0 through 6) or a single injection of 5 mg of cabergoline in slow-release vehicle on day 0. The sulpiride challenge on day 0 was started 30 minutes after the treatment injections. The pooled SEM was 5.9 ng•mL^{-1}•h.

Discussion

Daily oral pergolide administration is currently the treatment of choice for PPID in horses. However, from the results of this study and those reported in Chapter 4, both oral and injectable pergolide formulations have a relatively short duration of effectiveness of 24 hours or

less. Injecting pergolide daily was effective in suppressing prolactin response to low dose sulpiride challenge in the present experiment, whereas a single injection of 5 mg of cabergoline was just as effective, and lasted for at least 10 days. The effects of the cabergoline injection may last even longer, and the actual duration of effectiveness will have to be studied in future experiments. Regardless, the high effectiveness and longer duration of activity of the cabergoline formulation indicate that it is superior to pergolide in counteracting the antidopaminergic activity of the injected sulpiride. Whether the results obtained herein for prolactin in response to sulpiride will similarly apply to MSH and ACTH secretion in PPID mares needs to be determined in future research.

SUMMARY AND CONCLUSIONS

Four experiments were conducted in an effort to develop a method for assessing the efficacy and duration of activity of dopaminergic agonists for the treatment of PPID in horses. Prolactin secretion, which is under the same hypothalamic control (tonic dopaminergic suppression) as MSH secretion, was used as a proxy for MSH secretion.

In the first experiment, it was determined that the prolactin response to a low dose of the dopamine antagonist, sulpiride, was generally repeatable in estrogen-primed geldings in winter over 8 every-other-day challenges. It was concluded that estrogen-primed, sulpiride-challenged geldings in winter could serve as a model for the study of the efficacy and duration of activity of potential dopaminergic drugs for the treatment of PPID in horses.

The second experiment was performed in the summer with mares previously displaying estrous cycles, and again tested the repeatability of the prolactin responses to low dose sulpiride injection administered every-other-day for 30 days. The responses in mares were generally repeatable, as in geldings, and there was no effect due to stage of the estrous cycle (follicular phase, early diestrus, and late diestrus). It was concluded that mares could serve as a model for the study of potential dopaminergic drugs as well as geldings, and stage of the estrous cycle did not have to be taken into account.

In the third experiment, two formulations of the dopamine agonist, pergolide, were tested (2 mg administered orally and 2 mg injected in a slow-release vehicle) against a single formulation of cabergoline (5 mg injected in a slow-release vehicle) and control injections (vehicle) for their efficacy to reduce unstimulated plasma prolactin concentrations in geldings. Oral pergolide reduced prolactin concentrations for only a few hours, whereas injected pergolide suppressed prolactin concentrations for a full 24 hours. Injection of cabergoline suppressed

prolactin concentrations for up to 5.5 days. It was concluded that the injectable formulations had potential for further study as possible treatments for PPID in horses.

The last experiment tested the efficacy of daily pergolide injection (2 mg/day) versus a single 5 mg injection of cabergoline, both in slow-release vehicle, for suppressing the prolactin secretion induced by low dose sulpiride injections in mares. Daily injection of pergolide suppressed the prolactin responses to sulpiride as long as the injections were given, plus another 2 to 3 days. The single cabergoline injection suppressed the prolactin responses to sulpiride for a minimum of 10 days. Based on these results, cabergoline in slow-release vehicle seems to provide an excellent possibility for administering dopaminergic activity to horses with PPID. Whether these results are directly applicable to MSH and ACTH secretion in PPID horses needs to be determined.

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