**Factors Affecting the Ovarian Response to a Combined Estradiol-Sulpiride Treatment in
Seasonally Anovulatory Mares**

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Approved for publication by the Director of the Louisiana Agricultural Experiment Station as
manuscript number 2016-230-30593.

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Abstract

Twenty-three seasonally anovulatory mares, housed at two separate farms, were treated with 50 mg of estradiol cypionate (ECP) and 3 g of sulpiride in January to study factors that contributed to success of the treatment (response = ovulation within 28 days). Every-other-day blood samples and pretreatment secretagogue challenges were used to characterize prolactin, LH, IGF-I, leptin, and insulin concentrations. Ovaries of each mare were scanned via ultrasound regularly until detection of a 32-35 mm follicle, at which time the mare was artificially inseminated. Prolactin was stimulated in all treated mares and was similar ($P > 0.05$) in responding and non-responding mares. Nine mares, all at the same farm (BH; farm effect, $P = 0.006$), responded with pre-ovulatory sized follicles within 20 days of treatment. Five of the 9 were inseminated and 3 conceived. Retrospective analysis revealed that of the mares responding, body condition score ($P = 0.03$), body weight ($P = 0.02$), plasma concentrations of insulin ($P = 0.01$) and leptin ($P = 0.09$), and pretreatment response of LH to GnRH ($P = 0.106$) were higher in responding than in non-responding mares. In general, factors that differed and may contribute to whether a given mare responds to this ECP-sulpiride protocol were mainly characteristics pointing towards well-nourished mares. Minor nutritional differences between farms likely played a role in the lack of success on the one farm. Also, the LH response to GnRH prior to treatment may be indicative of the subsequent LH response to ECP-sulpiride and hence the ovarian response.

Key words: estradiol cypionate, mares, ovulation induction, seasonal anovulation, sulpiride

1. Introduction

Treatment with exogenous prolactin or an induced increase in endogenous prolactin concentrations can advance the first ovulation in seasonally anovulatory mares [1-5]. Nequin et al. [1] hastened follicular growth in deep anestrous mares with one treatment of ovine prolactin. Thompson et al. [2] advanced the date to first ovulation in pony mares in winter by administration of recombinant porcine prolactin, although antibodies to the injected prolactin were eventually detected in those mares. In lieu of exogenous prolactin, various dopaminergic antagonists such as sulpiride [3, 5-7], domperidone [4], and fluphenazine [1] have been used to stimulate secretion of endogenous prolactin. Sulpiride and domperidone have been used most frequently to stimulate prolactin secretion, a response greatly enhanced by pretreatment with estradiol [6-8], which was also shown to stimulate luteinizing hormone (LH) in seasonally anovulatory mares [6].

In a series of experiments, Mitcham et al. [8-10] compared prolactin and ovarian responses in mares receiving various doses of estradiol cypionate (ECP) combined with either domperidone or sulpiride, generally at 1.5 or 3.0 g injected 1 to 11 days after ECP injection. Throughout 10 years of experimentation, success rates (percentage of mares ovulating within a specified period of time) have varied from a high of 89% [6] down to as low as 50% for the more simplified protocols (treatment with ECP and one injection of sulpiride). In general, mares that respond do so within 28 days. Non-responding mares typically ovulate much later, interspersed with control mares. The question that arises after each experiment is why some mares respond to the selected treatment, whereas others do not. That is, what factor(s), either internal or external, affect(s) whether a mare responds or not?

The aim of the current experiment was to describe and measure as many factors as possible that could potentially affect the response of a given mare. Towards this end, all available anovulatory mares were treated in January with a standard protocol of ECP followed by a single injection of sulpiride, and pretreatment assessments as well as real-time hormonal data were retrospectively used to determine any differences between mares that responded and those that did not respond in the first 28 days. Because previous experiments had not assessed fertility of early ovulations induced by ECP and sulpiride, responding mares were also artificially inseminated with semen from a single stallion once a 32-mm or larger follicle was detected.

2. Materials and Methods

Procedures used in these experiments were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Agricultural Center.

2.1. Animals

Mares used in this experiment were housed at one of two Louisiana Agricultural Experiment Station farms: the Central Research Station Horse Unit on the Ben Hur Plantation (BH) and the Reproductive Biology Center (RBC). The two farms were located approximately 9 miles apart south of the Louisiana State University main campus in Baton Rouge. All mares were maintained outdoors throughout the year. They grazed native grass pasture during the warmer months and were supplemented with hay prepared from the same native grasses for *ad libitum* consumption as needed in the fall and winter. In addition to this routine maintenance, mares at

the BH farm were also supplemented in winter with 18% protein tubs (Positive Feed, Ltd.; Sealy, TX) and were limit-grazed on winter ryegrass pasture for 1 to 2 hours daily starting in January.

Starting in early December, 2015, all non-pregnant mares housed at the two farms were assessed by ultrasonic scanning of the ovaries once a week, and samples of jugular blood were collected approximately weekly. Anovulation was defined as the absence of any follicle >20 mm, the absence of any corpora lutea, and plasma progesterone concentrations consistently less than 1 ng/mL.

Twenty-three mares were identified that matched the criteria for anovulation: 14 from BH and 9 from RBC. They were all light horse mares, mainly Quarter Horses, Thoroughbreds, and Arabians. Prior to the start of any treatments (first week of January), body weight (BW) and body condition score (BCS; as described by Henneke et al. [11]), were recorded for each mare.

2.2. Pretreatment Assessments, ECP-Sulpiride Treatment, and Blood Sampling

On January 9, 2016, all mares were challenged intravenously with a combination of gonadotropin releasing hormone (GnRH; 0.5 µg/kg of BW) and sulpiride (racemic mixture; 4 µg/kg of BW) obtained from Sigma-Aldrich, St. Louis, MO. Samples of jugular blood were drawn and 0, 10, 20 and 30 minutes relative to injection to characterize the LH, follicle stimulating hormone (FSH), and prolactin responses. The blood samples were collected into 6-mL evacuated tubes containing sodium heparin as an anticoagulant (Vacurette, Greiner Bio-One, Monroe, NC). The following day (January 10, 2016), all mares received an intramuscular injection in the neck of 50 mg of ECP (BET Pharm, LLC, Lexington, KY). This was considered as day 0 of the experiment.

A comparison of timing of the sulpiride injection component of the standard protocol was overlaid on the experiment, such that approximately half the mares at each farm received sulpiride on day 1 (10 mares, 6 at BH and 4 at RBC) versus the rest that received sulpiride on day 6 (13 mares, 8 at BH and 5 at RBC). Sulpiride injections consisted of 3 g of the racemic mixture of sulpiride suspended in 5 mL of vegetable shortening (Crisco; J.M. Smucker Company, Orrville, OH) administered subcutaneously in the girth area as previously described by Thompson et al. [12]. Jugular blood samples were drawn at 0, 1, 3, 6 and 24 hours relative to injection of sulpiride, and then again every other day until February 28 (day 49). Plasma from these samples was used for the determination of plasma prolactin, leptin, insulin, and insulin-like growth factor-I (IGF-I) concentrations.

An additional jugular blood sample was collected from each mare on the day of ovulation and again for 5 successive days thereafter for determination of plasma LH and progesterone concentrations. For all blood samples collected throughout the experiment, plasma was harvested by centrifugation at 1200 x g for 15 minutes and was stored at -20°C.

2.3. Ultrasonography and Artificial Insemination

Ovarian follicular activity was monitored via ultrasonography (Aloka 550V with 5-Mhz linear-array transducer; Hitachi-Aloka, Wallingford, CT) every 3 to 4 days until a follicle 25 mm or greater was detected, after which the mare was scanned daily until the follicle either reached 32 mm or greater, or regressed to < 25 mm. Upon detection of a 32-mm follicle or greater, the mare was artificially inseminated with at least 500×10^6 progressively motile sperm from a stallion of proven fertility. After insemination, the mare was administered 0.75 mg deslorelin (BET Pharm, Lexington, KY) intramuscularly to induce ovulation. Inseminated mares were scanned for pregnancy at 12 days after ovulation. If an embryonic vesicle was detected, the mare

was scanned again at 16 and 22 days (+/- 1 day). Upon detection of a heartbeat at 22 days or later, the pregnancy was terminated with a luteolytic dose of dinoprost (Lutalyse; Zoetis Inc., Kalamazoo, MI). Some mares ovulated before obtaining a 32-mm follicle, and were not inseminated. Those mares, plus inseminated mares that did not conceive, were scanned every 3 to 4 days to determine their subsequent ovulation.

2.4. Hormonal Assays

Frozen plasma samples were thawed and analyzed for prolactin, FSH, LH, leptin, IGF-I, insulin, and progesterone as appropriate. Prolactin, FSH, LH, leptin and IGF-I were measured with radioimmunoassays previously validated by our laboratory [13-17]. Intra- and interassay coefficients of variation and levels of detection were 7%, 12% and 0.2 ng/mL for prolactin; 6%, 9%, and 0.2 ng/mL for LH; 7%, 11%, and 1.4 ng/mL for FSH; 5%, 12%, and 8 ng/mL for IGF-I; and 4%, 8%, and 0.8 ng/mL for leptin. Insulin and progesterone concentrations were measured with commercially available kit reagents (ImmuChem Coated Tube ¹²⁵I RIA Kit and ImmuChem Double Antibody, ¹²⁵I RIA Kit, respectively; MP Biomedicals, Costa Mesa, CA). The intra- and interassay coefficients of variation and assay sensitivities were 5%, 8%, and 0.5 mIU/L for insulin and 5%, 9%, and 0.05 ng/mL for progesterone. Estradiol was measured in acetone-extracted, 0.2 mL samples of plasma with commercially available kit reagents (MP Diagnostics Estradiol 17-β Double Antibody, ¹²⁵I RIA Kit); intra- and interassay coefficients of variation and assay sensitivities were 7%, 12% and 1.2 pg/mL.

2.5. Statistical Analyses

Data for single-point dependent variables were analyzed by one-way analysis of variance (ANOVA) using the general linear model of SAS (SAS Instit., Cary, NC). Ovarian response was

156 coded as 0 (no response) or 1 (response) for analysis (see below for treatment groups). Data for
157 dependent variables collected over multiple time points were analyzed by one-way ANOVA with
158 repeated sampling (split-plot design [18]) with the same software. Retrospective analysis showed
159 that all mares responding with early ovulation were located at one farm (BH). Because of the
160 lack of success at RBC, analysis as a 2 x 2 factorial (with farm and success as the two factors)
161 was not possible, thus the treatments in the ANOVA were BH responding mares (BHR), BH
162 non-responding mares (BHN), and RBC non-responding mares (RBCN) run individually.
163 Subsequently, although there were no mares responding at RBC to the original ECP-sulpiride
164 treatment, there were 3 mares that displayed more ovarian activity than the rest of the RBC
165 mares, and came close to, but did not achieve, the 32-mm follicle size for breeding. Thus, these
166 mares were lumped together into a fourth treatment group as RBC transitional (RBCT) for
167 analyses of endpoints that might affect success.

168 All dependent variables were subsequently re-analyzed with the 4 groups in the main
169 analyses (treatments), as well as the timing of sulpiride (1 versus 6 days after ECP) and its
170 interaction with treatment. Time (minutes for the challenges, days for other hormonal
171 characteristics) was included as the repeated factor as appropriate. One-degree of freedom
172 contrasts [18] were used to assess the effect of farm (BHN+BHR versus RBCN+RBCT) and the
173 difference between responding and non-responding mares at BH (BHN versus BHR) and
174 between the two groupings at RBC (RBCN versus RBCT). The mares within group interaction,
175 which tested treatment in the repeated measures ANOVA, was used as the error term for these
176 comparisons.

177 ***3. Results***

178 ***3.1. Ovarian Responses and Pregnancies***

179 An ovarian response was defined as the occurrence of a pre-ovulatory follicle at least 32
180 mm in diameter within 28 days after sulpiride injection, which either ovulated spontaneously or
181 was induced to ovulate with deslorelin. Of the 23 mares treated with ECP and sulpiride, 9
182 responded with early ovulation (Table 1). The average days to ovulation for those mares
183 responding was 11.7 +/- 1.0, and ranged from 7 to 18 days post-sulpiride injection. There was no
184 difference ($P > 0.1$) in days to ovulation between mares receiving sulpiride on day 1 versus 6
185 after ECP. Mares not responding to the ECP-sulpiride treatment were scanned weekly until mid-
186 March; no ovulation was detected in any of these mares up to that point.

187 Of the 9 mares that responded to treatment, all 9 were residents of the BH farm. At RBC,
188 there were 3 mares that grew follicles up to 28 to 30 mm without ovulation. Given that these
189 were the only mares at RBC to show any ovarian response (albeit below threshold), they were re-
190 administered a second injection of sulpiride (same dose as previous) on day 42 (February 22) to
191 determine if perhaps a potentiation had been induced that might lead to a quick response. They
192 indeed responded with ovulatory sized follicles 12, 13, and 16 days after that second sulpiride
193 treatment. Given that these mares stood out from the rest of the mares at RBC, they were placed
194 in a separate group of their own (RBCT) for further analyses.

195 In the ANOVA for ovarian response, the RBCT mares were included as non-responding,
196 given that they did not ovulate within the original 28-day period. Accordingly, there were only
197 three treatment groups and no orthogonal contrast for RBCN versus RBCT. In that analysis,
198 there was an effect ($P = 0.0059$) of farm but no effect ($P > 0.1$) of day of sulpiride injection nor
199 any interaction with treatment.

200 Of the 9 BH mares responding to treatment, 5 were artificially inseminated and induced
201 to ovulate with deslorelin while the remaining 4 ovulated on their own without insemination or

deslorelin treatment. Of the 5 that were bred, 3 conceived and carried a pregnancy to 22 days, at which time the pregnancy was terminated. All pregnancies had morphologically normal embryonic vesicles and formation of embryo proper with cardiac activity. The 3 additional mares that received a second sulpiride treatment (RBCT group) were also treated with deslorelin and inseminated, and all 3 became pregnant, again with normal embryonic vesicles and cardiac activity. Thus, the overall pregnancy rate was 6 of 8 mares that were inseminated, or 75%.

3.2. Physical and Hormonal Characteristics of Responding and Non-responding Mares

Mean body condition scores, ages, and BW are presented in Table 2. The BHR mares had a higher BCS ($P = 0.06$) than the non-responding mares at both farms, but did not differ from RBCT mares. Age and weight were not different overall; however, contrast analyses (Table 3) indicated that BHR mares had higher BCS ($P = 0.03$) and weighed more ($P = 0.02$) than BHN mares.

Prior to ECP treatment, FSH and prolactin responses to the GnRH and sulpiride challenges were not different ($P > 0.1$; Fig. 1) across the four groups of mares. Averaged over the three post-GnRH sampling periods, mean concentrations of LH tended to be higher ($P = 0.106$; Fig. 1) in BHR mares than in BHN mares.

Mean prolactin concentrations in every-other-day samples before and after treatment with ECP plus sulpiride are presented in Fig. 2. Because there was no effect ($P > 0.1$) of day of sulpiride treatment on ovarian response, the data for all mares were normalized to day of sulpiride treatment (rather than ECP injection). Overall, there was no effect ($P > 0.1$) of farm, no difference between responding and non-responding mares at BH, and no difference between mares in the RBCN and RBCT groups.

Mean plasma LH concentrations before ECP treatment and over the next 18 days are presented in Fig. 3. A significant effect of treatment ($P = 0.004$) and an interaction ($P < 0.0001$) between response and day were present between responding, non-responding, and RBCT mares, with LH concentrations being greatest in BHR mares on days 10, 12, 14, 16, and 18 after ECP. Luteinizing hormone concentrations were also greater ($P < 0.05$) in BHN mares than in RBCN or RBCT mares on days 14, 16 and 18 post ECP treatment.

Plasma estradiol concentrations after treatment with ECP were measured in BH mares only, given that no RBC mare ovulated within 28 days. There was no difference ($P > 0.1$) in plasma estradiol concentrations between the responding and non-responding mares at BH (Fig. 4). Plasma estradiol peaked 1 to 2 days post ECP treatment and started a slow decline through day 16 after ECP.

Means for plasma IGF-I, leptin, and insulin concentrations are presented in Table 4. Concentrations of IGF-I did not differ ($P > 0.1$) among the four groups, nor was there any effect of farm. There was an effect of treatment ($P = 0.01$) on leptin concentrations as well as an interaction between treatment and day ($P < 0.0001$). Leptin concentrations were highest in RBCT mares; however, the difference was due primarily to two of the mares in particular with higher than normal leptin concentrations. Contrast analyses (Table 5) revealed that leptin concentrations were higher ($P = 0.09$) in the BHR mares than in the BHN mares, and were higher ($P = 0.003$) in the RBCT mares than in the RBCN mares. There was also an effect of treatment on insulin concentrations ($P = 0.05$), which were highest in RBCT and in BHR mares. Contrast analyses (Table 5) showed that concentrations of insulin were higher ($P = 0.01$) in BHR mares than in BHN mares.

All mares that responded with ovulation experienced a normal rise in progesterone and decline in LH immediately following ovulation (Fig. 5). There was no difference ($P > 0.1$) between mares that were induced to ovulate with deslorelin and inseminated and mares that ovulated spontaneously and were not inseminated.

4. Discussion

The use of dopamine antagonist protocols to induce early ovulation in mares has met with varying results. Some mares respond similarly from season to season, while others either never respond or will respond one season, but not the next (E.L. Oberhaus, *personal observation*). The aim of this study was to retrospectively analyze factors that might contribute to or prevent a mare from responding to ECP and sulpiride with early follicular activity and ovulation. Also, while dopamine antagonist protocols to induce ovulation have repeatedly been studied in our lab and by others, it has not been determined whether ovulations induced by ECP-sulpiride are indeed fertile and whether the uterus would support early pregnancy.

In this study, all mares were treated with ECP and a single subcutaneous injection of sulpiride in vegetable shortening. Ten mares received sulpiride 1 day after ECP while 13 received sulpiride 6 days after ECP. Mitcham et al. [8] observed a tendency for prolactin response to be higher in mares receiving domperidone 1 day after ECP versus 6 days after ECP; however, in the present study, day of sulpiride treatment did not affect the degree to which prolactin concentrations were increased.

Ovarian success, as defined by ovulation within 28 days post sulpiride treatment, was relatively low in this experiment (9 of 23 mares, or 39%), whereas all 9 responding mares were housed at one farm. Considering only mares at the BH farm, success was 9 of 14 mares, or 64%.

Success rates of 50 to 89% have been achieved in previous experiments [6-10], depending upon the specific protocol used. The 89% success rate of Kelley et al. [6] was based on every-other-day injections of estradiol benzoate followed by daily sulpiride injections started 11 days later. Attempts to simplify that protocol into a single or double injection regimen is what has led to the lower percentages of success. In the last experiment reported by Mitcham [10], a single injection of ECP followed by three injections of sulpiride 5 days apart resulted in a success rate of 77%. Reducing the number of sulpiride injections to two, 5 days apart, produced a success rate of 63% (E.L. Oberhaus, *personal observation*). For the present experiment, the number of sulpiride injections was reduced to one in an effort to simplify the protocol even further.

Pre-trial assessments of the LH, FSH, and prolactin responses to a standardized GnRH-sulpiride challenge revealed no predictive information regarding FSH or prolactin responses prior to ECP-sulpiride treatment. There was a tendency for a difference in LH response. The tendency for a greater LH response to GnRH in BHR compared to BHN and RBCN may indicate that the adenohypophyses of mares that would go on to respond to treatment were already more competent in terms of LH synthesis and release, albeit low at that time of year. Given that no mares had ovarian follicle(s) greater than 15 mm before the experiment started, all mares were considered to be in a relatively “deep” anestrus-anovulatory state. This tendency of higher LH response was evident in the responses to ECP injection, with the BHR mares having a much greater rise in LH concentrations 10 to 18 days after ECP relative to BHN mares. The almost total lack of LH response to ECP in the mares at RBC may account for the lack (or relative lack) of ovarian stimulation.

Prolactin was stimulated in all mares for approximately 7 days by the injection of 3 g of sulpiride in vegetable shortening. The response to sulpiride did not differ between mares that

291 ovulated early and mares that did not, nor did it differ between the two farms. An important point
292 is that the responses were robust, with little variation, in contrast to what was often observed
293 with domperidone injections in previous experiments [7-9]. Thus, if there is indeed a threshold
294 level of prolactin concentrations that must be achieved for ovarian success, then it is likely that
295 all mares at both farms reached that threshold, whereas only mares at BH had sufficient LH
296 response to ECP to reach an ovulatory state. Previous injections of ECP alone, with no
297 dopaminergic antagonist to stimulate prolactin secretion, did not produce successful ovarian
298 responses [10]. In all our previous experiments with the estrogen-dopaminergic antagonist
299 combination, it has been consistent that both the prolactin response and the LH response to
300 estrogen must occur to some extent for the ovaries to respond. Failure of either results in zero
301 success.

302 It is well documented that nutrition and body condition play an important role in
303 reproduction [19, 20]. Gentry et al. [20] demonstrated that low BCS of 3.0 to 3.5, produced by
304 nutrient restriction, resulted in low plasma leptin and IGF-I concentrations, low unstimulated
305 prolactin concentrations, a low LH response to GnRH, and an extended seasonal period of
306 anovulation with minimal follicular activity. Interestingly, the prolactin response to sulpiride
307 reported by Gentry et al. [20] did not differ between high and low BCS mares, much like that
308 observed in the present experiment. Albeit speculation, parallels can be seen between the
309 differences observed herein between responding and non-responding mares, and the results of
310 Gentry et al. [20]: low BW and BCS, low plasma leptin, low LH response to GnRH, and in this
311 experiment, low insulin concentrations.

312 In this experiment, plasma IGF-I concentrations did not differ between groups of mares,
313 but leptin and insulin were higher in the BHR than in the BHN mares even prior to treatment

with ECP and sulpiride. These hormones are generally thought of as indicators of nutritional status, with higher concentrations associated with better nutrition. For mares housed at the BH farm, the differences would be consistent with that concept. For the RBC mares, the trends are in the same direction, but did not meet statistical muster. Due to different winter management schemes at the two farms, differences in nutritional availability were present, even though all mares were housed on pasture without any grain supplementation. Retrospective investigation revealed that the mares at the BH farm had access to round bales of Alicia bermuda grass hay, winter ryegrass pasture, and *ad libitum* protein tubs (primarily cottonseed and soybean meals, molasses, and added vitamin A). Mares at the RBC farm had access to round bales of coastal bermuda and rye grass hay, but did not have access to winter ryegrass or protein supplementation. These differences in potential nutrient intake may have contributed to the failure of RBC mares to respond to the initial treatment.

The three RBC mares that were considered transitional due to significant follicular activity (relative to the other nonresponding mares) with no ovulation had the highest leptin concentrations of all mares in the four groups. Of those three mares, two had leptin concentrations ranging from 2 to 5.5 ng/mL compared to < 2 ng/mL for most other mares at both farms. It is possible that these mares were hyperleptinemic as a result of insulin insensitivity [16, 21], although no direct assessment of insulin sensitivity was performed. Hyperleptinemia is typically associated with hyperinsulinemia, due to compensated insulin resistance [16, 21]. However, the mean insulin concentrations in these three mares were not outstanding relative to the other mares at both farms. Insulin insensitivity per se is known to be associated with perturbations in estrous cyclicity [23, 24], thus studies involving the use of sulpiride in these mares deserve further research. The fact that these three mares responded so consistently and

quickly to the second sulpiride injection on February 22 may indicate that the first ECP-sulpiride treatment established conditions within the ovaries that potentiated the response to subsequent elevated prolactin concentrations.

The effect of ECP and sulpiride on LH concentrations seems to be somewhat complex. Clearly, estradiol has a stimulatory effect on LH secretion, and an increase in LH has not been observed in mares receiving a dopamine antagonist alone [6-10]. Garcia and Ginther [25] administered 1 mg of estradiol daily to ovariectomized pony mares in February and observed an increase in LH, but not until 6 days after initiation of treatment. Similarly, Kelley et al. [6] and Mitcham [10] administered 11 mg estradiol benzoate and 150 mg ECP, respectively, to seasonally anovulatory mares and did not observe an increase in LH until after treatment with sulpiride 6 to 11 days after estrogen treatment. Moreover, Mitcham [10] administered sulpiride or domperidone in biodegradable microparticles to mares simultaneously (same morning) as the ECP injection and observed no increase in plasma LH concentrations over the next 21 days. The question arises as to whether treatment with sulpiride after ECP enhances the LH response. In the present study, an increase in LH was not observed until 10 days after ECP treatment. At that point, concentrations of LH rose to levels similar to those seen around a typical, breeding season ovulation, and appeared to coincide with late stage follicular growth and ovulation in those (BHR) mares. Plasma estradiol in responding and non-responding mares at BH peaked one and two days, respectively, after ECP treatment and then began to decline. Four of the 9 responding mares exhibited a decline in circulating estradiol followed by a subsequent rise around the time of each mare's respective ovulation; therefore, it is possible that LH is being stimulated by endogenous follicular estradiol as well as the exogenous ECP.

Mari et al. [5] assessed fertility following sulpiride treatment in anestrus mares. First ovulation of the season was advanced in sulpiride-treated mares and pregnancy rates on the first cycle were 40% (4/10). In addition, Mari et al. [5] reported normal foalings in all pregnant mares. Throughout the series of experiments with estrogen pretreatment coupled with antidopaminergic agents that we have completed [6-10], this is the first time to test the actual fertility of the ovulations induced by treatment. Although minimal in number, the percentage of mares becoming pregnant after insemination is certainly within the expected range for mares bred on one estrous cycle in spring and summer. More extensive studies are needed to confirm whether the fertility of these induced ovulations are indeed equivalent to later breedings. However, these data are encouraging for breeders who want to get their open mares pregnant early in the year.

In conclusion, treatment with 50 mg of ECP followed by 3 g of +/- sulpiride in vegetable shortening 1 or 6 days later elevated plasma prolactin concentrations in all mares for 6 to 8 days. Given the robust responses across all mares, prolactin response could not be a factor in determining whether a mare responded within 28 days or not (success). Assessments of plasma FSH and prolactin responses to low-dose sulpiride and GnRH challenges provided no predictive information as to future success. The tendency for higher LH response to GnRH in responding mares prior to treatment may be indicative of the subsequent LH response and eventually the ovarian response to ECP-sulpiride. Success was influenced by body condition and likely nutritional status as indicated by higher plasma leptin and insulin concentrations in the responding mares. We suspect that the likelihood of a seasonally anovulatory mare responding to the treatment protocol used herein is inversely correlated to the “depth” of the anestrus state. That depth of anestrus is known to be influenced by nutritional status as well as proximity to the

seasonal transition period, as we [20] and others [26] have suggested in previous reports for mares treated with only dopaminergic antagonists. Finally, from the limited inseminations performed with these mares, we suspect that these induced ovulations are indeed fertile and can produce at least 22-day embryos with heartbeats. Future research would be needed to better clarify the actual fertility rate and to confirm that the pregnancies can be carried to term.

Acknowledgements

This work was supported by the USDA National Institute of Food and Agriculture, Hatch project Accession Number 0224441.

The authors thank A. F. Parlow and the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program, Harbor-University of California Los Angeles Medical Center, Torrance, CA, for reagents.

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463

Table 1. Number of horses treated at each farm that responded with ovulation or a transition-like state.

Farm	Day of Sulpiride Treatment	n	No. Responding	No. Transition-like
RBC	1	4	0 (0%)	1 (25%)
	6	5	0 (0%)	2 (40%)
BH	1	6	5 (83%)	0 (0%)
	6	8	4 (50%)	0 (0%)

Abbrev: RBC, Reproductive Biology Center; BH, Ben Hur Farm.

Table 2. Mean body condition score (BCS), age, and body weight for horses of the four response groups at the two farms.

	Means				Treatment effect
	BHN	BHR	RBCN	RBCT	P-value
BCS (1-9)	5.2 ^a	6.5 ^b	5.2 ^a	5.3 ^{a,b}	0.06
Age (yrs)	11	15	11	13	0.23
Weight (kg)	420.6	488.9	478.5	478.0	0.11

Abbrev: BHN, Ben Hur non-responders; BHR, Ben Hur responders; RBCN, RBC non-responders; RBCT, RBC transition-like.

^{a,b} Means within rows with no like superscript differ ($P < 0.05$).

Table 3. One-degree of freedom contrast analyses for body condition score (BCS), age, and body weight between farms and within farms.

	Between Farms P - value	BHN vs. BHR P-value	RBCN vs. RBCT P-value
BCS	0.25	0.03	0.84
Age	0.56	0.11	0.44
Weight	0.30	0.02	0.99

Abbrev: BHN, Ben Hur non-responders; BHR, Ben Hur responders; RBCN, RBC non-responders; RBCT, RBC transition-like.

Table 4. Mean plasma concentrations of IGF-I, leptin and insulin.

Mean plasma concentration (ng/mL)					Treatment effect	Interaction with day
	BHN	BHR	RBCN	RBCT	P-value	
IGF-I	138.40	172.19	84.97	127.25	0.16	0.91
Leptin	0.24 ^a	0.87 ^b	0.40 ^{a,b}	1.85 ^c	0.01	< 0.0001
Insulin	21.73 ^a	36.62 ^b	30.18 ^{a,b}	37.08 ^b	0.05	0.33

Abbrev: BHN, Ben Hur non-responders; BHR, Ben Hur responders; RBCN, RBC non-responders; RBCT, RBC transition-like.

^{a-c} Means within rows with no like superscript differ ($P < 0.05$).

Table 5. One-degree of freedom contrast analyses for plasma IGF-I, leptin, and insulin concentrations between farms and within farms.

	Between Farms P-value	BHN vs. BHR P-value	RBCN vs. RBCT P-value
IGF-I	0.14	0.42	0.40
Leptin	0.06	0.09	0.003
Insulin	0.29	0.01	0.29

Abbrev: BHN, Ben Hur non-responders; BHR, Ben Hur responders; RBCN, RBC non-responders; RBCT, RBC transition-like.

Fig. 1. Plasma luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin areas under the curve after intravenous injection of gonadotropin releasing hormone and sulpiride 24 hours before ECP injections. The farm-response groups were: mares at the Ben Hur farm that did not respond (BHN), those that did respond (BHR), and mares at the Reproductive Biology Center farm that did not respond (RBCN) and those that showed transitional-like ovarian activity (RBCT). Number of mares per group were 5, 9, 6, and 3, respectively, for the BHN, BHR, RBCN, and RBCT groups. ^{a,b}Means for LH areas differ ($P = 0.11$). Error variances from the analyses of variance were 0.096, 1218, and 287 for LH, FSH, and prolactin, respectively. The individual standard error of the means are shown by vertical lines with bars.

Fig. 2. Mean plasma concentrations of prolactin in response to subcutaneous sulpiride injections in January (day 0). The farm-response groups were: mares at the Ben Hur farm that did not respond (BHN), those that did respond (BHR), and mares at the Reproductive Biology Center farm that did not respond (RBCN) and those that showed transitional-like ovarian activity (RBCT). Number of mares per group were 5, 9, 6, and 3, respectively, for the BHN, BHR, RBCN, and RBCT groups. No differences ($P > 0.05$) were detected between any of the four groups of mares. Pooled standard errors of the means from the analysis of variance were 7.38, 5.5, 6.73, and 9.52 ng/mL, respectively.

Fig. 3. Mean plasma concentrations of LH before ECP treatment (day 0) and over the next 18 days in Ben Hur responding mares (BHR), Ben Hur non-responding mares (BHN), RBC non-responding mares (RBCN), and RBC transitional mares (RBCT). A significant effect of group (P

= 0.004) and an interaction ($P < 0.0001$) between group and day were present between responding, non-responding, and transitional mares, with LH concentrations being greater in BHR mares on days 10, 12, 14, 16, and 18 post ECP. Luteinizing hormone concentrations were also greater ($P < 0.05$) in BHN mares than in RBCN or RBCT mares on days 14, 16 and 18 post ECP treatment. Pooled standard errors of the means from the analysis of variance were 0.54, 0.41, 0.5, and 0.7 ng/mL, respectively. Asterisks (*) indicate differences between BHR and all other groups; double daggers (‡) indicate differences between BHN and all other groups.

Fig. 4. Mean plasma estradiol concentrations in mares at the Ben Hur farm after treatment with 50 mg of ECP on day 0. Estradiol concentrations in mares at the Reproductive Biology Center farm were not assessed given that no mare responded on that farm with a 32-mm or greater follicle within 28 days. Pooled standard error of the mean from the analysis of variance was 2.13 pg/mL.

Fig. 5. Mean plasma LH and progesterone concentrations on day of ovulation (day 0) and for 5 successive days after. No differences existed ($P > 0.1$) between mares that were induced to ovulate with deslorelin and inseminated and mares that ovulated spontaneously and were not inseminated. Pooled standard errors of the means from the analysis of variance were 1.7 ng/mL for LH and 0.99 ng/mL for progesterone.