Research Report

Reproductive hormones, antral follicle count, and transvaginal oocyte aspiration outcomes in mares receiving intravenous clomiphene citrate

Hayley Moore,[a] Jessica Klabnik,[a] Hannah Maxwell,[a] Jayachandra Babu,[b] Manjusha Annaji,[b] Nur Mita,[b] Bernardo Chaves-Cordoba,[c] Pouya Dini,[d] Alejandro De La Fuente,[d] Katelyn Waters,[a] Richard Hopper,[a] Lexie Russell,[e] Candace Lyman[a]

[a]Department of Clinical Sciences, College of Veterinary Medicine, [b]Drug Discovery and Development Unit, Harrison College of Pharmacy, [c]College of Agriculture, Auburn University, Auburn, AL, USA

[d]Department of Population Health and Reproduction, School of Veterinary Medicine, University of California Davis, Davis, CA

[e]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK, USA

Abstract

The likelihood of creating an in vitro produced foal is much lower in horses than in other species because of inefficient in vitro embryo production. Inability to superstimulate ovaries is a major factor that could be addressed by choosing a right compound; clomiphene citrate (CC), a selective estrogen receptor modulator, is a potential agent. Therefore, 2 experiments were performed; mares received 2,000 mg of intravenous CC, every 24 hours for 5 days to assess the effects on reproductive hormone concentrations, uterine and ovarian responses, and oocyte parameters. In Experiment 1, 6 mares received CC and were monitored for plasma CC concentrations, serum reproductive hormone concentrations, and ovarian responses. Transvaginal oocyte aspirations were performed and in vitro embryo production parameters were collected. In Experiment 2, 6 mares received CC; reproductive hormone concentrations and ovarian responses were compared to control group (n = 6). Concentrations of CC had positive correlations with antral follicle count and uterine edema (r = 0.60, p = 0.0004 and r = 0.47, p = 0.009, respectively), and a moderate negative correlation with follicle-stimulating hormone (r = – 0.48, p = 0.001). Compared to controls, there were no differences in reproductive hormone concentrations, ovarian responses, or oocytes recovered in mares receiving CC; average blastocyst rate of matured oocytes was 47.5% in treated mares.

Keywords: Clomiphene citrate, mares, antral follicle count, oocyte aspiration, embryo, intracytoplasmic sperm injection

Introduction

The demand for transvaginal aspiration (TVA) of immature oocytes and intracytoplasmic sperm injection (ICSI) produced foals is increasing.[1,2] Unfortunately, the likelihood of creating an in vitro produced (IVP) embryo and a subsequent live offspring is comparatively much lower in horses than in other species where in vitro fertilization (IVF) is also in high demand (e.g. cattle). On average, only 61.2% of mares yielded > 1 embryos, with 1.4-2.1 blastocysts per TVA session per mare.[3,4] Overall, increasing oocyte aspiration efficiency and embryo production process would greatly benefit the equine industry. There are 2 potential ways IVP can be improved: increasing antral follicle counts (AFC) and improving oocyte quality or their developmental competence.

Attempts to superstimulate or superovulate equine ovaries using equine chorionic gonadotropin, gonadotropin-releasing hormone (GnRH), GnRH agonists, dopamine antagonists, human chorionic gonadotropin, inhibin immunization, various follicle-stimulating hormone (FSH) preparations, and aromatase inhibitors have had very limited, poor, or variable success.[5,6] Selective estrogen receptor modulators (SERMs) have

CONTACT Jessica Klabnik, Candace Lyman **jlk0066@auburn.edu, clyman@eqmedical.com**

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the potential to increase antral follicle counts and improve IVP parameters, thereby increasing reproductive efficiency in the mare. Clomiphene citrate (CC) is a SERM that has been used to treat infertility, anovulatory follicles, and enhance follicular recruitment by altering the pulsatility of GnRH in women and ruminants.[7,8] In women, CC treatments increased GnRH secretion, thereby increased FSH and luteinizing hormone (LH) release, due to its ability to block the negative feedback of estrogen by competitively binding to estradiol (E_2) binding sites in the hypothalamus.[9] This cascade leads to ovarian follicular recruitment and ovulation. Moreover, CC treatment significantly increased AFC in ewes.[9] To authors' knowledge, CC effects on oocytes and IVP production has not been studied in mares.

The pharmacokinetics of intravenous CC in the mare were described by our group.[10] It was identified that 2,000 mg intravenous CC in the mare achieved plasma CC concentrations that have been reported to be therapeutic in women. The purpose of this experiment was to explore CC use as a novel pharmaceutical support of fertility and to determine the effects on reproductive hormone concentrations, uterine and ovarian responses, and IVP parameters.

Objectives of this experiment were to: 1. Characterize hormone profiles (LH, FSH, E_2, progesterone (P_4), and antimüllerian hormone [AMH]), reproductive parameters (AFC, uterine edema, largest follicle size), and IVP parameters (oocyte recovery rate, maturation rate, blastocyst rate) in mares receiving CC; and 2. Compare hormonal profiles and reproductive parameters between mares receiving CC and controls. We hypothesized that plasma concentrations of CC are positively correlated with AFC, uterine edema, and increases in FSH.

Materials and methods

Clomiphene citrate formulation preparation and storage

An intravenous sterile CC aqueous solution was formulated (20 mg/ml) and stored at 4 °C[11]; 1 unexpired batch was used for each experiment. Strength, quality, purity, and stability of the compound were assured through established quality control measures.[11]

Experiment 1

Animal use was approved by the Institutional Animal Care and Use Committee of Oklahoma State University. Healthy; university-owned Quarter horse-type mares (n = 6), 5-15 years, formed treatment group and were housed and fed on pasture.

Experiment was conducted from August to September of 2022 in the Northern Hemisphere. As per the experimental design (Figure 1), mares underwent estrous cycle synchronization via transvaginal follicle ablation (day – 10) followed by prostaglandin $F_{2\alpha}$ (5 mg intramuscular dinoprost tromethamine, Lutalyse®, Zoetis, Parsippany, NJ, USA) treatment on day – 4. Intravenous CC (2,000 mg) was given on days 0-4, where day 0 was the first day of CC treatment; AFC, uterine edema (uterine edema was assigned a score utilizing a scale from 0-5, a score of 0 = no uterine edema, 1 = slight, 2 = mild, 3 = moderate, 4 = heavy, and 5 = excessive uterine edema) and the largest ovarian follicle were determined via transrectal ultrasonography. On day 7, mares underwent TVA for immature oocyte collection. Oocytes were shipped counter-to-counter to a collaborator for processing and fertilization via ICSI; a highly trained embryologist documented oocyte maturation, cleavage, and blastocyst development rates.

On days 0-4 and days 7, 10, and 14, blood samples were collected for serum P_4, LH, FSH, E_2, and AMH assays. Additional blood was collected for serum P_4 on days – 10, – 6, – 4, and – 3 during the synchronization period. Blood samples were also collected in an EDTA tube for plasma CC concentrations on days 1-4 and on days 7, 10, and 14. Each dose of CC was given immediately after blood collection. After each blood sample was collected, they were centrifuged (1,500 g for 10 minutes) either immediately or after clotting and stored at – 80 °C until assayed. On days 0, 4, and 8, blood was collected for complete blood count and serum chemistry analysis (Oklahoma Animal Disease Diagnostic Laboratory of Oklahoma State University College of Veterinary Medicine).

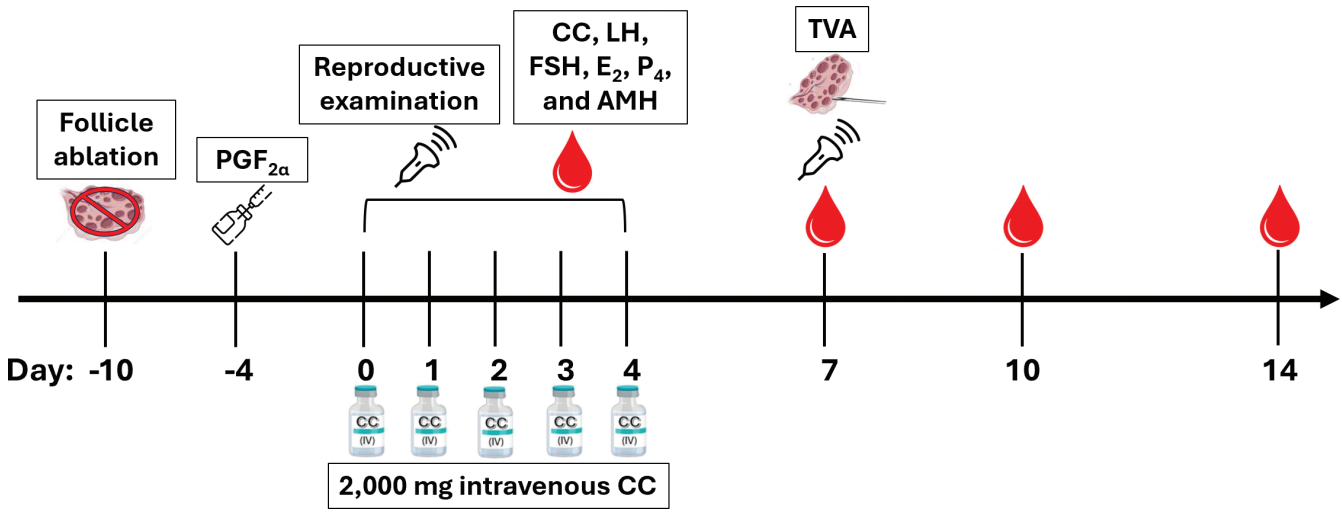

Figure 1. Experiment 1 design; reproductive examination included transrectal ultrasonography assessment of AFC, uterine edema, and largest follicle size and is denoted by the ultrasound probe icon; dates of blood drawn for plasma CC concentrations and serum hormones are denoted by the blood drop icon

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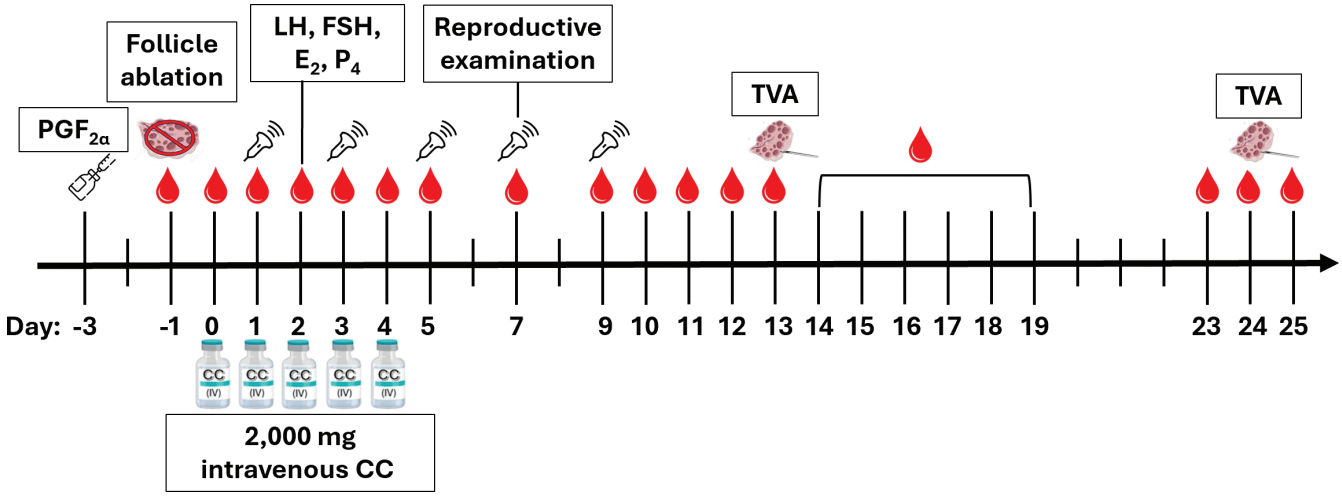

Figure 2. Experiment 2 design. Reproductive examination includes transrectal ultrasonography assessment of antral follicle count, uterine edema, and largest follicle size and is denoted by the ultrasound probe icon. Blood drawn for CC concentrations and hormone analysis is denoted by the blood drop icon

Experiment 2

Animal use was approved by the Institutional Animal Care and Use Committee of Oklahoma State University. Healthy client-owned Quarter horse-type mares (n = 12), 6-10 years, were used.

Experiment was conducted from July to August of 2023 in the Northern Hemisphere. Control (n = 6) and treatment (n = 6) groups were chosen based on the order in which mares were caught and brought into the treatment area for follicle ablation on day – 1, with the first 6 mares assigned to the treatment group and the remaining 6 mares to the control group. Mares underwent estrous cycle synchronization via prostaglandin $F_{2\alpha}$ (5 mg intramuscular dinoprost tromethamine [day – 3]) treatment and transvaginal follicle ablation (day –1). Treatment group mares received 2,000 mg intravenous CC on days 0-4 (Figure 2) and control group received placebo.

On days 1, 3, 5, 7, and 9, AFC, uterine edema (uterine edema was assigned a score utilizing a scale from 0-3 (different from Experiment 1 based on evaluators' experience); with a score of 0 denoting no uterine edema; 1 = mild uterine edema; 2 = moderate uterine edema; and 3 = heavy uterine edema), and the largest ovarian follicle were determined via transrectal ultrasonography. On days 13 and 24, mares had TVA for immature oocyte collection and total oocytes collected were recorded.

On days outlined (Figure 2), blood samples were collected in blood tubes without additive for LH, FSH, P_4, and E_2 assays. After each sample was collected, they were centrifuged (1,500 g for 10 minutes) after clotting and serum was frozen, and then shipped to Auburn University for storage at – 80 °C until assayed.

Hormone analysis

Serum FSH, LH, P_4, and E_2 were determined (Dr. Erin Oberhaus laboratory of Louisiana State University) in duplicate by radioimmunoassay (RIA). Assays utilized 125-I labeled LH and FSH. Antibodies for LH and FSH were generated in rabbits; LH antibodies were generated against pregnant mare serum gonadotropin and FSH antibodies were generated against equine FSH.[12,13] Antibody-antigen complexes were precipitated with antirabbit second antibody. Serum FSH intraassay coefficients of variation were 2.6% for Experiment 1 and 2.4% for Experiment 2. Serum FSH interassay coefficients of variation were 6% for Experiment 1 and 5% for Experiment 2 with a concentration of detection of 1.4 ng/ml. Serum LH intraassay coefficients of variation were 4.2% for Experiment 1 and 4% for Experiment 2. Serum LH interassay coefficients of variation were 8.5% for Experiment 1 and 8% for Experiment 2, with a concentration of detection of 0.2 ng/ml.

Serum P_4 was determined using commercially available kit reagents (ImmuChem Double Antibody, 125-I RIA Kit, MP Biomedicals, Costa Mesa, CA, USA). Intraassay coefficients of variation were 2.3% for Experiment 1 and 2.8% for Experiment 2, with a concentration of detection of 0.05 ng/ml. Serum E_2 was determined using commercially available kit reagents (Estradiol Double Antibody, 125-I RIA Kit, MP Biomedicals). Intraassay coefficients of variation 3.1% for both experiments with a concentration of detection of 0.03 pg/ml. Parallelism of the standard curve and recovery of added mass in the commercial kits for P_4 and E_2 was demonstrated to validate the assays for equine samples.[14,15] Estradiol was measured in 2 ml ether extracted samples.

For Experiment 1 mares, serum was shipped overnight (Clinical Endocrinology Laboratory, University of California, Davis School of Veterinary Medicine) on dry ice; AMH was determined using a commercial (AL-115, Ansh Labs, Webster, TX, USA) equine AMH enzyme linked immunosorbent assay. Intra- and interassay coefficients of variation and concentrations of detection were 8.9%, 4.8%, and 0.009 ng/ml, respectively. Results were analyzed on Softmax Pro 7.1 software (Molecular Devices) using a 4 Parameter Logistic curve fit.

Plasma clomiphene citrate analysis for Experiment 1

Plasma CC were analyzed (Clinical Pharmacology Laboratory, Auburn University, College of Veterinary Medicine) with high-performance liquid chromatography.[10]

Transvaginal follicle ablation and oocyte aspiration

Transvaginal follicle ablation (pretreatment) and transvaginal ultrasound-guided aspiration of immature oocytes were performed as described.[16] Mares were restrained in stocks, the perineal region was cleaned with soap, and the tails were tied to the side of each mare. Mares were sedated with intravenous detomidine (5-7 mg; Dormosedan®, Zoetis), and intravenous butorphanol (5 mg; Torbugesic®, Zoetis); mares were treated with intravenous flunixin meglumine (500 mg; Banamine®, Merck Animal Health, Rahway, NJ, USA), and intravenous N-butylscopolammonium bromide (120 mg; Buscopan®, Boehringer Ingelheim, Duluth, GA, USA) was given immediately prior to the procedure. A transvaginal ultrasound probe (5-7 MHz) fitted with a needle guide was coated with contact gel and covered with a plastic sheath. Coated with sterile lubricant, the probe was inserted into the vagina and a 12-gauge double-lumen oocyte aspiration needle was placed into the needle guide. Transrectal palpation was implemented to bring each ovary to the ultrasound probe and visualize follicles available for puncture. For the follicle ablation on day – 10 for Experiment 1 and day – 1 for Experiment 2, all follicles ≥ 5 mm were punctured and follicular fluid was aspirated using a vacuum pump set at 145 mm Hg. For the TVAs in both experiments, each follicle ≥ 5 mm was flushed 6 times with medium (Vigro™ Complete Flush Solution, Vetoquinol®, Fort Worth, TX, USA) supplemented with 8 IU/ml heparin sodium salt. The fluid collected from the TVA was filtered through an embryo filter and placed in a Petri dish to search for oocytes under a stereoscope.

Collected cumulus-oocyte complexes (COCs) were placed in holding media, packaged in 1.5 ml glass vials at room temperature (22°C) and shipped (Veterinary Assisted Reproduction Laboratory, University of California, Davis School of Veterinary Medicine) overnight in an isothermic shipping device.

In vitro production of embryos for Experiment 1

On arrival at the laboratory, COCs were rinsed and transferred to droplets of maturation medium of 8-10 µl/oocyte under oil overlay and matured for 28-30 hours at 38.2°C in a humidified atmosphere (5.8% CO_2, 5% O_2, and 89.2% N_2). After maturation, COCs were stripped of cumulus cells by short culture in G-MOPS (Vitrolife, Gothenburg, Sweden), 2% hyaluronidase, and repeated pipetting. Oocytes were deemed mature if a polar body was observed in the perivitelline space under light stereoscopy. Frozen-thawed semen from 1 stallion with known fertility was used after the swim-up sperm selection procedure. Each sperm was immobilized and injected into a matured oocyte. After ICSI, injected oocytes were cultured at 38.2°C in a humidified atmosphere as above. Putative zygotes were examined for cleavage 24 hours after ICSI. Culture medium was changed on day 4 after ICSI and developing embryos were evaluated on days 7-10 after ICSI for blastocyst formation.

Data analysis

Descriptive data were analyzed and correlations were calculated using the CORR procedure with statistical analysis software package (SAS Learning Edition, 9.1; SAS Institute, Cary, NC, USA) to calculate Pearson correlation coefficients between variables. For Experiment 2, hormone data (P_4, E_2, FSH, LH) and ultrasonographic data (AFC, largest follicle, uterine edema) were analyzed by ANOVA using Glimmix procedure. Data were analyzed as a repeated measurements model, where the day and mare were random factors and treatment was a fixed factor. For both experiments, $p < 0.05$ was considered significant. Tukey-Kramer post-hoc was applied to test for pairwise comparisons. When an interaction was not significant, main effect means were reported.

Results

Experiment 1: hormone concentrations, ovarian response, and IVP outcomes

Hormone (LH, FSH, E_2, AMH, P_4) and CC concentrations, transrectal ultrasonographic assessment of uterine edema scores and largest follicle size profiles are presented (Figure 3); CC concentrations had a moderate positive correlation with AFC ($r = 0.60$, $p = 0.0004$), a moderate positive correlation with uterine edema ($r = 0.47$, $p = 0.009$), and a moderate negative correlation with FSH ($r = – 0.48$, $p = 0.001$). Correlations between the variables are summarized (Figures 4-6).

Median number of oocytes recovered per mare was 8.5 oocytes (interquartile range [IQR] = 3); median number of oocytes that matured and underwent ICSI per mare was 4.5 (IQR = 6); median number of oocytes cleaved per mare was 3.5 (IQR = 5); median number of blastocysts was 2.5 per mare (IQR = 3); average maturation rate of oocytes recovered was 51.3%; average blastocyst rate of matured oocytes was 47.5%; and average blastocyst rate of recovered oocytes was 25.3%.

Experiment 2: hormone concentrations, ovarian response, and oocyte recovery

For FSH, there were effects of day ($p < 0.0001$), treatment ($p = 0.1864$), and day by treatment interaction ($p = 0.0481$); however, Tukey-Kramer post-hoc of the data did not reveal any significant pairwise comparisons between treatments (Figure 7). The p values for others are presented (Figure 8): for LH for day ($p = 0.0074$), treatment ($p = 0.5995$), and day by treatment interaction ($p = 0.5379$); for AFC for day ($p < 0.0001$), treatment ($p = 0.7441$), and day by treatment interaction ($p = 0.1177$); and for largest follicle size day ($p < 0.0001$), treatment ($p = 0.6867$), and day by treatment interaction ($p = 0.8962$). Neither day nor treatment were different for E_2, P_4, uterine edema, or oocyte recovery.

Discussion

Experiments described explored the potential effects of CC on mare reproductive hormone concentrations, uterine and ovarian responses, and IVP parameters. Hormonal profiles and transrectal ultrasonographic assessment were characterized because of a 5-day treatment of CC; a compound readily used in human assisted reproductive techniques, but studies are lacking in mares. To authors' knowledge, this is the first report of CC treatment and its associated effects on reproductive outcomes in the mare.

These studies were designed to replicate common protocols used in women receiving CC for ovarian stimulation. A common protocol for women is to receive 50-150 mg total of CC orally per day, beginning on the second or third day of menses (i.e. early in the follicular phase) and continue for 5-6 days.[17] Based on the published pharmacokinetics study by our group,

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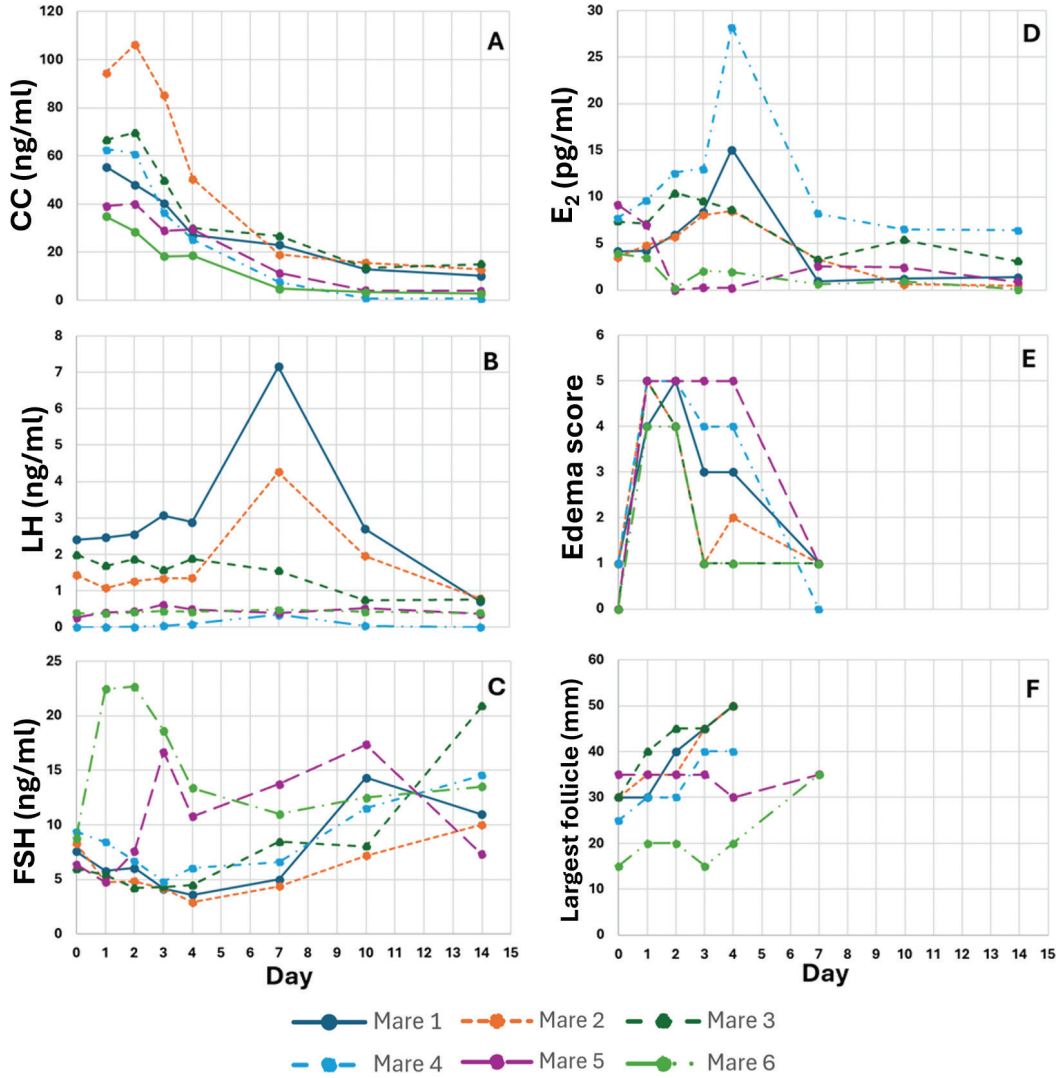

Figure 3. Experiment 1 mares' data: plasma CC concentrations from days 0-14; A. serum LH concentrations (ng/ml) for from days 0-14; B. serum FSH concentrations (ng/ml) from days 0-14; C. serum E_2 concentrations (pg/ml) from days 0-14; uterine edema score from days 0-7; and D. largest follicle (mm) from days 0-7

it was identified that CC has poor bioavailability when given orally in the mare, but a dose of 2,000 mg given intravenously achieves similar plasma concentrations in the mare that are known to be therapeutic in women.[10] The mares in these studies received transvaginal follicle ablations to initiate a new follicular wave and prostaglandin $F_{2\alpha}$ was given to lyse existing luteal tissue, in an attempt to replicate the physiologic state of women on the third day of the menstrual cycle with a new follicular wave developing and with no active luteal tissue. In Experiment 1, 4/6 mares ovulated prior to the TVA procedure and had a corpus luteum. The timing of the follicle ablation was altered in Experiment 2 to prevent ovulation from occurring prior to TVA.

The highest concentrations of plasma CC were observed on days 1 or 2, despite 5 days of treatment. Thereafter, all mares had a steady decline in CC over the remainder of the treatment period. This may be due, in part, to the fact that the elimination half-life of CC in the mare is relatively short, 4.95 +/- 1.10 hours, with a mean residence time of 7.14 +/- 1.59 hours and a mean accumulation ratio of 2.0 +/- 0.4 in mares receiving 2,000 mg of CC intravenously every 24 hours for 5 days.[10] Plasma CC in this experiment reflected these pharmacokinetic parameters given that the samples were trough samples, collected prior to CC treatment each day.[10] Because these were trough concentrations and not peak concentrations, care must be taken when interpreting the correlations between CC and other parameters.

In humans, CC acts on the hypothalamic-pituitary-gonadal axis by stimulating the production of FSH[18] and preventing a premature spike in LH through antiestrogenic effects on the pituitary.[18,19] Under CC treatment in Experiment 1, serum LH was not correlated with CC. In Experiment 2, LH was not impacted by treatment, but the main effect of day was significant on days 24 and 25. It is reasonable to assume that given mare's typical estrous cycle length this is likely the natural rise of LH prior to ovulation.

Although in humans CC resulted in a more sustained endogenous FSH release and increasing AFC,[18] data in our studies did

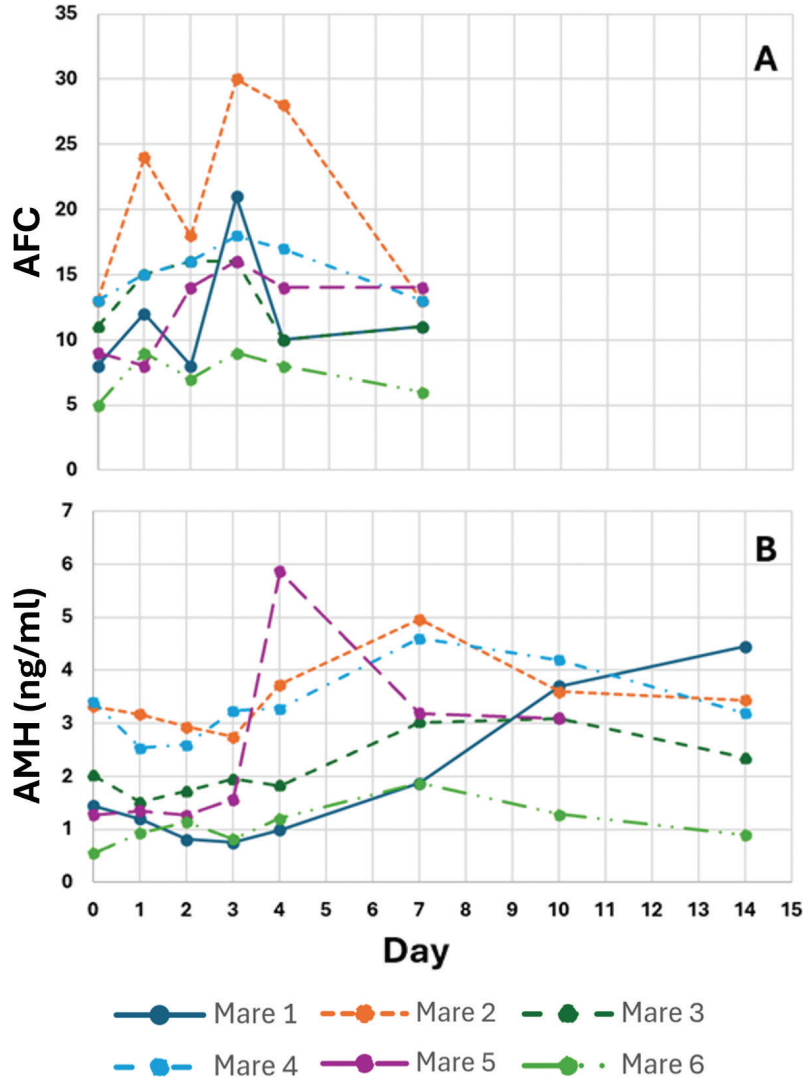

Figure 4. Experiment 1 mares' additional data: A. AFC from days 0-7; B. serum AMH (ng/ml) from days 0-14

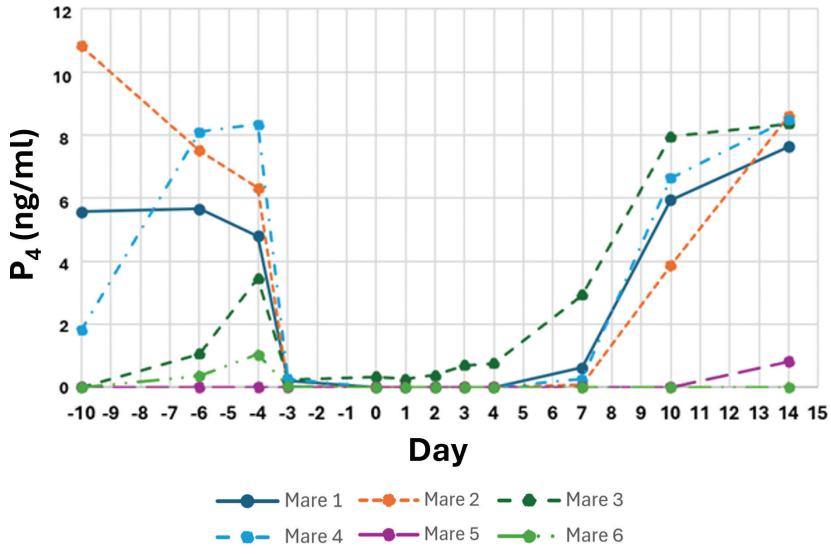

Figure 5. Serum P_4 concentrations (ng/ml) in Experiment 1 mares from days – 0-14

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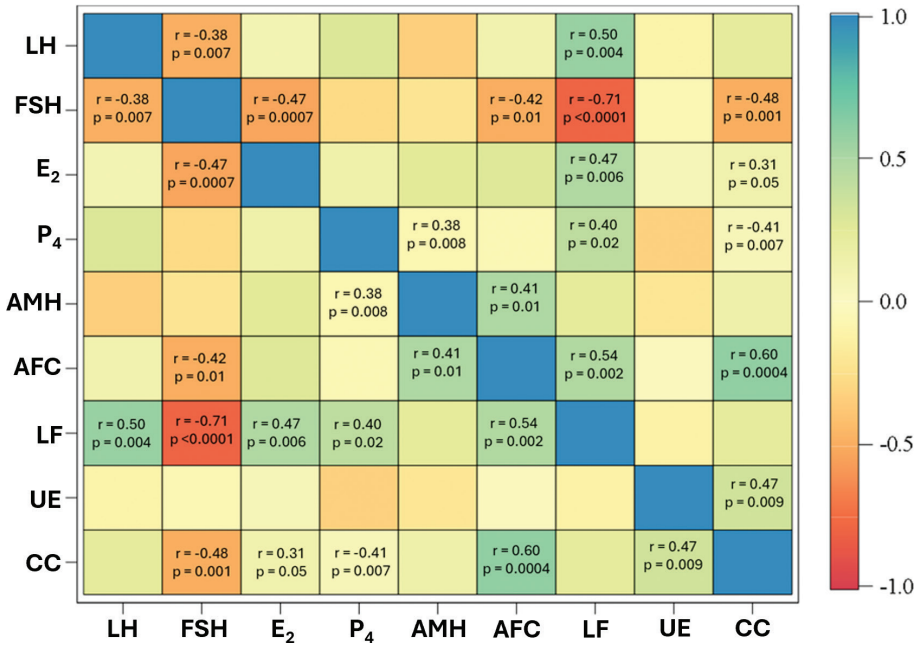

Figure 6. Correlation (heatmap) between data in Experiment 1; significant data points with their corresponding p values

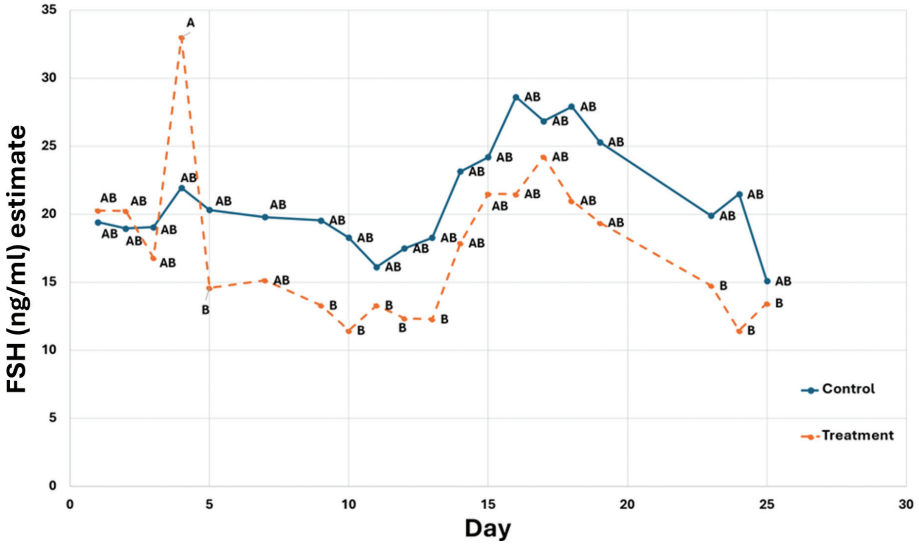

Figure 7. Estimates for FSH (ng/ml) in Experiment 2 mares; by day and by treatment; A-B differed ($p < 0.05$)

not support our hypotheses regarding AFC or FSH, as CC treatment did not increase AFC or FSH. In contrast to what was observed in women, FSH had a moderate negative correlation with plasma CC in Experiment 1 mares. In Experiment 2, pairwise comparisons after Tukey-Kramer post-hoc adjustment did not reveal any direct differences between treatment and controls. However, the pairwise differences within the treatment group had numerically, but nonsignificantly, decreased FSH after the discontinuation of CC. This prompts the need for a study to determine the effect of CC on FSH concentrations. This pattern is different to that was observed in women with polycystic ovarian syndrome receiving CC, where a transient rise in FSH was observed, followed by patterns similar to that of normal menstrual cycles.[20] This was also in contrast to what was observed in rats receiving CC, where FSH increased during and immediately after treatment.[21]

In Experiment 1, E_2 had a moderate positive correlation with plasma CC, but no differences were observed between treatment and control mares in Experiment 2. Further studies investigating the endogenous estrogen concentrations prior to treatment may be warranted as CC's predominant mode of action (i.e. estrogen agonist versus antagonist) in women varies based on the concentration of endogenous estrogen.[22] For example, systemic estrogen decreases as a result of CC treatment in women with polycystic ovary syndrome[23] but not in women that have history of poor response to ovarian stimulation.[24]

Although there was a moderate positive correlation with AFC and plasma CC in Experiment 1, CC treatment did not impact AFC in Experiment 2. For most mares, the number of follicles increased over time with minor fluctuations and the largest follicle generally increased over time. It was expected to have

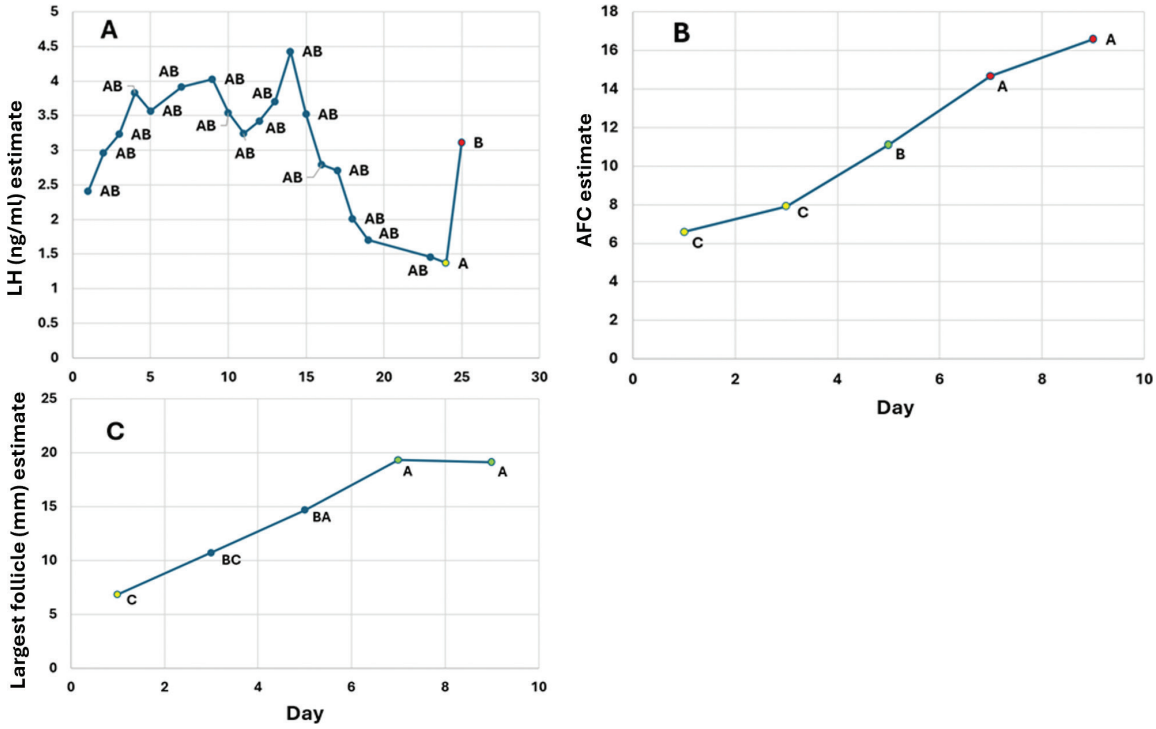

Figure 8. Estimates for Experiment 2 mares by day; A. LH (ng/ml); B. AFC; and C. largest follicle size (mm). A-C differed ($p < 0.05$) within each panel

an increase in AFC during the recruitment phase of the follicular wave and a decrease once a growing dominant follicle had been identified. In Experiment 1, AFC had a moderate positive correlation with the largest follicle size. This was an unexpected result and may be due to small sample size.

Given that CC is a selective estrogen receptor modulator, it is reasonable to hypothesize that the estrogen receptors in the mare endometrium respond to CC similarly in how the endometrium responds to estrogen. Uterine edema in Experiment 1 mares was moderately positively correlated with plasma CC, supporting the experiment hypothesis. This edema was not correlated with any other hormone or ovarian parameters, suggesting that it could be related to CC treatment. However, in Experiment 2, uterine edema was not significant in mares receiving CC compared to controls, in contradiction to the experiment hypothesis. Further studies are needed to help clarify discrepancies between these findings.

Treatment with CC did not impact oocyte recovery rate in Experiment 2. Unfortunately, these oocytes were utilized for other research purposes and were not developed into embryos. Average maturation rate of oocyte and average blastocyst rate of mature oocytes in Experiment 1, numerically exceeded ICSI outcomes from a study of 59 mares, in which the average blastocyst rate of matured oocytes was 24.1%, and the average blastocyst rate of recovered oocytes was 15.8%.[25] Although data have not revealed a difference in AFC or oocyte recovery in mares receiving CC, the blastocyst rate observed was compelling and warrants further study.

Major limitations to this study were that Experiment 1 did not have a control group and a larger sample size would be helpful when confirming the results of the IVP embryo data, and Experiment 2 lacked IVP embryo data. These studies assumed that the therapeutic plasma concentration range in women was appropriate for mares; however, this may not the case and changes in dosage, route, or frequency may be necessary. Further studies are needed to determine the true therapeutic range in the mare. A major difference in CC protocols in women was that the timing of oocyte aspiration was determined on a case-by-case basis by monitoring woman's ovaries closely to determine the optimal day for aspiration; this may also be applicable in mares. Despite these limitations, to our knowledge, this is the first study in mares that examined CC effect on reproductive hormone profiles and reproductive parameters.

Conclusion

In mares, intravenous CC given at suspected therapeutic concentrations had minimal impact on hormone profiles. Uterine edema and AFC were both positively correlated with plasma CC; however, they were not impacted by CC treatment. Although in these studies, CC did not improve AFC or oocyte recovery, CC may have effects at oocyte level, as the average blastocyst rate of matured oocytes was 47.5%. Embryo development data supported that CC has an impact on assisted reproductive technology. Further studies are warranted to assess oocyte quality, maturation, and embryo production.

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Conflict of interest

None to declare.

Authors' contribution and declaration

HM analyzed and interpreted data, drafted manuscript, reviewed and edited; JK analyzed and interpreted data, supervised, reviewed and edited; HM reviewed and edited; JB conceptualized, designed methodology, prepared drug, edited; MA designed methodology, prepared drug, edited; NM involved in methodology, prepared drug, edited; BCC analyzed data, reviewed, edited; PD & AF collected data, reviewed and edited; KW & RH reviewed and edited; LR collected data, edited; and CL conceptualized, acquired funding, designed methodology, collected data, supervised the project. Authors have read and approved final submission.

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