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Abstract

Luteinized unruptured follicle (LUF) syndrome is a recurrent anovulatory dysfunction that affects up to 23% of women with normal menstrual cycles and up to 73% with endometriosis. Mechanisms underlying the development of LUF syndrome in mares were studied to provide a potential model for human anovulation. The effect of extended increase in circulating LH achieved by administration of recombinant equine LH (reLH) or a short surge of LH and decrease in progesterone induced by prostaglandin $F_2\alpha$ ($PGF_2\alpha$) on LUF formation (Experiment 1), identification of an optimal dose of COX-2 inhibitor (flunixin-meglumine, FM; to block the effect of prostaglandins) for inducing LUFs (Experiment 2), and evaluation of intrafollicular endocrine milieu in LUFs (Experiment 3) were investigated. In Experiment 1, mares were treated with reLH from Days 7−15 (Day 0 = ovulation), $PGF_2\alpha$ on Day 7, or in combination. In Experiment 2, FM at doses of 2.0 or 3.0 mg/kg every 12 h and hCG (1500 IU) were administered after a follicle ≥32 mm was detected. In Experiment 3, FM at a dose of 2.0 mg/kg every 12 h plus hCG was used to induce LUFs and investigate the intrafollicular endocrine milieu. No LUFs were induced by reLH or $PGF_2\alpha$ treatment; however, LUFs were induced in 100% of mares using FM. Intrafollicular $PGF_2\alpha$ metabolite (PGFM), $PGF_2\alpha$, and PGE_2 were lower and the ratio of PGE_2:$PGF_2\alpha$ was higher in the Induced LUF group. Higher levels of intrafollicular E2 and total primary sex steroids were observed in the Induced LUF group along with a tendency for higher levels of GH, cortisol, and T; however, LH, PRL, VEGF-A, and NO did not differ between groups. In conclusion, this study reveals part of the intrafollicular endocrine milieu and the association of prostaglandins in LUF formation, and indicates that the mare might be an appropriate model for studying the poorly understood LUF syndrome.

Introduction

Anovulation is one of the main causes of infertility in women and females of many domestic species. One of the types of anovulatory dysfunction is luteinized unruptured follicle (LUF) syndrome, which has been reported in women (Marik & Hulka 1978, Koninckx *et al.* 1981, Hamilton *et al.* 1985, Hulka 1985, Katz 1988, Check 2007), mares (Kaiser *et al.* 1999, Gastal *et al.* 2006, Ginther *et al.* 2007*a*, Cuervo-Arango & Newcombe 2009), cattle (Peter 2004), llamas (Adams *et al.* 1991), rhinoceroses (Stoops *et al.* 2004), and elephants (Lueders *et al.* 2011). LUFs, also known as hemorrhagic anovulatory follicles (HAFs) in veterinary medicine, occur when the preovulatory follicle fails to rupture or ovulate and the antrum gets increasingly filled with blood. LUF/HAF is the most common form of anovulation in mares. LUF syndrome during the breeding season is considered a serious economic concern for the equine industry. Similarly, anovulation can have significant financial implications for women undergoing assisted reproductive techniques (Eijkemans *et al.* 2005). Therefore, it is important to investigate the mechanisms of development of LUF syndrome. Greater knowledge about the pathophysiology of LUFs can be applied to prevent their occurrence and thus to develop safe and effective treatments to optimize reproductive health in both animals and humans.

The ultrasonographic morphological characteristics of naturally occurring or induced LUFs are similar in women (Priddy *et al.* 1990, Zaidi *et al.* 1995) and mares (Coetsier & Dhont 1996, Cuervo-Arango & Newcombe 2012). LUF formation involves the development of well-vascularized luteal tissue, as indicated by echotexture and color Doppler signals in both women and mares (Zaidi *et al.* 1995, Ginther *et al.* 2007*a*). In the absence of ovulation, the antrum of the follicle becomes permeated with blood, which appears as echogenic foci and fibrin-like strands on a B-mode ultrasonogram. Due to similarities in antral follicular dynamics (Ginther *et al.*

2004, Baerwald 2009, Gastal 2011) and LUF morphology between species, we have proposed the mare as an appropriate model for understanding LUF syndrome in women.

The reported incidence of LUFs in women is highly variable. LUFs have been documented to occur in 11-23% of women with normal menstrual cycles (Vanrell *et al.* 1982, Kerin *et al.* 1983, Dal *et al.* 2005), 13-73% of women with endometriosis (Kaya & Oral 1999), and 4-58% of women with unexplained infertility (Koninckx & Brosens 1983, Kugu *et al.* 1991). In addition to this, LUFs have been reported in women with pelvic inflammatory disease (Hamilton *et al.* 1986) and inflammatory arthritis (Smith *et al.* 1996). LUFs are highly repeatable across cycles (79-90%), resulting in recurrent anovulation (Hamilton *et al.* 1986, Qublan *et al.* 2006) and infertility.

In cycling mares, the incidence of LUFs is also highly variable (5-25%; Lefranc & Allen 2003, Ginther *et al.* 2008*a*, Cuervo-Arango & Newcombe 2009, 2010). A 5% incidence of LUFs has been reported during the early ovulatory season, followed by 20% during the late reproductive season (Gastal *et al.* 1998). Similarly, LUF syndrome has been shown to occur more often in older mares and be recurrent in some individuals (>50% of the estrous cycles), encompassing much or all of the breeding season (Ginther *et al.* 2007*a*, Cuervo-Arango & Newcombe 2009, 2010). Therefore, recurrent LUFs result in prolonged periods of anovulation and long interovulatory intervals (Ginther *et al.* 2007*a*).

The systemic and intrafollicular endocrine milieu associated with LUF syndrome is poorly understood. Knowledge about the endocrine changes associated with LUF formation is lacking (Hamilton *et al.* 1985, Ginther *et al.* 2007). The use of luteinizing hormone (LH) during early proestrus in rodents has successfully induced LUFs (Plas-Roser *et al.* 1985, Mattheij & Swarts 1995). Similarly, the use of luteinizing hormone releasing hormone, human chorionic

gonadotropin (hCG), and human menopausal gonadotropin has been associated with LUFs in women (Bergquist & Lindgren 1983, Ghanem *et al.* 2009), and hCG in guinea pigs (Westfahl 1988). Continued investigations are required to determine the effects of chronic administration of LH on the incidence of LUFs in mares and women. It has been well documented that a $PGF_2\alpha$ injection causes immediate release of LH, resulting in induction of ovulation in several species such as cows (Hafs *et al.* 1975), sows (Srikandakumar & Downey 1989), and mares (Gastal *et al.* 2005). In a recent study, administration of $PGF_2\alpha$ with or without complete ablation of antral follicles increased LH concentrations early in the ovulatory wave and also during the preovulatory period and were associated with a high incidence of LUFs in mares (Ginther *et al.* 2008*b*). Although it is not known exactly what physiological mechanism (interference with luteinization or maturation of granulosa cells) might be disturbed when LH levels are affected at the beginning of the follicular wave or during the ovulatory period, there is enough evidence to allow the test of a hypothesis of LH participation in LUF formation.

The advances in knowledge regarding LUF syndrome in women and animals have been slow, in part because of the difficulty of predicting the occurrence of such ovarian structures. Therefore, the use of pharmacologically induced LUFs is a promising way to study this syndrome. Use of pharmacological agents in fertility or superovulatory treatments has increased the occurrence of LUFs and/or anovulatory follicles in women (Martinez *et al.* 1991, Ghanem *et al.* 2009) and mares (Lefranc & Allen 2003, Ginther *et al.* 2008*a*, Cuervo-Arango & Newcombe 2010, Meyers-Brown *et al.* 2011). Similarly, intrafollicular or systemic administration of prostaglandin inhibitors has been shown to cause luteinization of follicles in rats (Armstrong & Grinwich 1972), rabbits (Salhab *et al.* 2003), mares (Watson & Sertich 1991, Ginther *et al.* 2008*a*, Cuervo-Arango & Domingo-Ortiz 2011), and women (Killick & Elstein 1987, Priddy *et*

al. 1990, Jesam *et al.* 2010, 2014). In mares, flunixin meglumine (FM) at a dose rate of 1.7 or 2.0 mg/kg body weight has been shown to induce LUFs in 73-83% of mares, respectively (Cuervo-Arango *et al.* 2011, Cuervo-Arango & Domingo-Ortiz 2011). Furthermore, intrafollicular administration of prostaglandins has successfully prevented FM-induced LUF formation, allowing subsequent ovulation and conception in mares (Martínez-Boví & Cuervo-Arango 2015). In women, NSAIDs have been used experimentally to inhibit ovulation, and a dose-dependent effect has been observed (Athanasiou *et al.* 1996, Bata *et al.* 2006, Jesam *et al.* 2010, 2014). Therefore, pharmacological approaches to inhibit ovulation and induce LUF formation, such as the use of COX-2 inhibitors, may serve as an effective model to elucidate the pathophysiology of LUF syndrome.

The objectives of the experiments conducted in this study were to investigate: the role of reLH and $PGF_2\alpha$ in the formation of LUFs (Experiment 1), the optimum dose of FM required to experimentally induce LUFs (Experiment 2), and the intrafollicular endocrine milieu (Experiment 3) in induced LUFs in the mare. The hypotheses tested were that: (1) reLH, when administered during diestrus alone or along with $PGF_2\alpha$, would increase the incidence of LUFs; (2) higher doses of FM would increase the incidence of LUFs; (3) inhibition of intrafollicular prostaglandin synthesis would be associated with LUF formation; (4) intrafollicular prostaglandins ($PGF_2\alpha$ and PGE_2) concentrations would be decreased during systemic FM treatment; (5) imbalance in the intrafollicular endocrine milieu is associated with LUF formation.

Materials and Methods

Animals

Mares (*n* = 36) were evaluated during two ovulatory seasons (April to October) in the northern hemisphere and handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. This study was approved by the Southern Illinois University Institutional Animal Care and Use Committee (IACUC, 10-041). The mares were Quarter-Horse type breed, 5−15 years of age, weighed 400−550 kg, had docile temperament, and did not present any apparent abnormality of the reproductive tract as determined by ultrasonographic examination. Mares were reused in subsequent experiments after ultrasonographically confirming at least two normal ovulatory cycles in between experiments. All mares had good body condition score (average score ~6-7; score 1 = emaciated and score 9 = obese; Henneke *et al.* 1983) throughout the study. Mares were kept under natural light in pasture with free access to water and trace-mineralized salt.

Experiment 1. Effect of reLH and PG on LUF formation

Animals and treatments

On Day 7 (Day 0 = ovulation), mares (*n* = 30) were randomly divided into six different treatment groups to receive either saline, $PGF_2\alpha$ (5 mg i.m.; Lutalyse, Pfizer Animal Health, Kalamazoo, MI, USA), reLH (0.5 mg or 1.0 mg i.v.; AspenBio Pharma Inc., Castle Rock, CO), or a combination of $PGF_2\alpha$ and reLH. The groups were: Control (10 ml saline, i.v.), LH 0.5 (0.5 mg of reLH), LH 0.5 + PG (0.5 mg reLH and 5 mg $PGF_2\alpha$), PG (5 mg $PGF_2\alpha$), LH 1.0 (1 mg reLH), and LH 1.0 + PG (1 mg reLH and 5 mg $PGF_2\alpha$). Mares were treated with $PGF_2\alpha$ (5 mg/mare) only once on Day 7 and with reLH once every day from Days 7−15.

Ultrasonographic examinations and end points

Transrectal ultrasonographic examinations were performed daily from Day 0 until 4 days after the next ovulation or the beginning of LUF formation. A LUF was detected ultrasonographically by the presence of a thick and echoic follicle wall, suggestive of luteinization, decreased turgidity, echoic foci and/or fibrin septae in the antrum, a gel-like substance in antrum, and/or pronounced serration of the granulosa layer around the whole follicle, as previously described (Ginther *et al.* 2006, Cuervo-Arango & Newcombe 2012). A series of comparative ultrasound images of LUFs in women and mares is given for illustration purposes regarding the similarities shown by both species (Fig. 1). For transrectal scanning, a duplex B-mode (gray-scale) and pulsed-wave color-Doppler ultrasound instrument (Aloka SSD-3500; Aloka America, Wallingford, CT, USA) equipped with a finger-mounted 3.5−10 MHz convex-array transducer was employed. The color-flow mode was used to display follicle, corpus luteum (CL), and LUF blood flow as previously described (Ginther *et al.* 2007*a*, 2007*b*). Constant color-gain, velocity, and filter settings were used for all Doppler examinations. The entire follicle, CL, and LUF were scanned in a gradual, steady motion several times.

Follicle diameter was calculated from the average of height and width of the antrum at the apparent maximal area from two frozen images. The largest follicle was measured on each day of examination. CL diameter was measured throughout the study. In addition, endometrial echotexture was scored from 1 to 4 (minimal to maximal) during each examination, based on the extent of anechoic areas of the endometrial folds (Ginther & Pierson 1984). Follicle wall blood flow was quantified in follicles ≥28 mm until ovulation or LUF formation, using color-flow Doppler as previously described in mares (Acosta *et al.* 2004) and women (Campbell *et al.* 1993).

185

186 ***Experiment 2. Optimum dose of flunixin-meglumine (FM) to induce LUF***

187

188 *Animals and treatments*

189 At the beginning of estrus, cycling mares (n = 18) with a growing follicle ≥32 mm (Hour 0), in
190 the presence of endometrial edema (echotexture score ≥3) were administered 1500 IU of hCG
191 (Chorulon®, i.v.; Intervet Inc, Millsboro, DE). Immediately, mares were randomly divided (n =
192 6 mares/group) into three treatment groups (FM-2, FM-3, and Control), and treatments were
193 started. All groups were treated every 12 h with FM or saline until Hour 36. FM-2 and FM-3
194 groups received 2.0 or 3.0 mg/kg of body weight of FM (Flunixiject™, i.v.; Henry Schein®
195 Animal Health, Dublin, OH), respectively, and the Control group received 10 ml of saline (i.v.)
196 solution. Mares were monitored for any adverse effects from the above mentioned doses of FM,
197 as the normal dose was exceeded (1.1 mg/kg of body weight per day).

198

199 *Ultrasound evaluations*

200 Mares were scanned every other day from Day 12 after ovulation until a follicle reached 25−27
201 mm in diameter; subsequently, scans were conducted daily. Mares with a ≥32-mm follicle were
202 randomly allocated to a treatment group and scans were performed: every 12 h from Hours 0−36,
203 every 2 h between Hours 36−48, and every 12 h from Hours 60-96. Mares with more than one
204 ≥32-mm follicle at the beginning of treatment were not included in the study.

205 Follicle diameter, follicle blood flow, LUFs, and endometrial echotexture were evaluated
206 using the same ultrasound methodology as in Experiment 1. The thickness of the follicle wall
207 (granulosa layer) was determined by averaging measurements made on three different locations

(Gastal *et al.* 1998, 2006). In addition, the following qualitative B-mode characteristics of the preovulatory follicle were recorded from Hour 0 until ovulation or LUF formation: 1) presence of echoic foci floating in the antrum, 2) detection of follicle wall serration (i.e., irregular or notched surfaces of the granulosa layer; Gastal *et al.* 2007), and 3) loss of spherical shape.

Experiment 3. Systemic and intrafollicular hormones and growth factors

Animals and treatments

Cycling mares (n = 23) with a growing follicle ≥28-mm were scanned daily after Day 15 using B-mode and color-Doppler ultrasonography (Gastal *et al.* 1998, 2006) until a follicle ≥32 mm was detected. When the largest follicle reached ≥32 mm (Hour 0) and the score for endometrial echotexture was between 3 and 4 (estrus-like), mares were randomly assigned into two treatment groups: Control (n = 11) and Induced LUF (n = 12). Mares received an injection of 1500 IU of hCG and were treated twice daily with saline (10 ml, i.v.; Control group) or FM (2.0 mg FM/kg of body weight; Induced LUF group) until 36 h after hCG injection.

Collection of follicular fluid and end points

Follicular fluid was collected from the preovulatory follicle in the Induced LUF and the Control groups by ultrasound-guided transvaginal follicle aspiration at Hour 36 after hCG injection as described (Gastal *et al.* 1995, 1999*b*). The aspirated follicular-fluid was immediately processed in a refrigerated centrifuge (1500 xg for 10 min), and 10 ml of the supernatant was stored at −20ºC until hormone assays were performed. Follicle diameter, follicle blood flow, and endometrial echotexture were measured, as described in Experiment 1.

231

232 ***Blood samples and hormone assays***

233 Jugular blood samples were collected in heparinized tubes, immediately placed in ice water bath,
234 processed in a refrigerated centrifuge (1500 xg for 10 min), decanted, and stored (−20ºC) until
235 analyzed. For Experiment 1, samples were collected daily between Days 7−15. For Experiment
236 2, samples were collected every 12 h between Hours 0−36 and then every 2 h until ovulation or
237 LUF formation. For Experiment 3, samples were collected every 12 h between Hours 0−36. For
238 Experiment 1, systemic LH was assayed from Days 7−9 to investigate the effect of treatment on
239 the increase in circulating LH, and progesterone (P4) was assayed from Days 7−15. For
240 Experiment 2, systemic PGFM (prostaglandin $F_2\alpha$ metabolite) and P4 were assayed from Hours
241 0−38. For Experiment 3, PGFM was assayed in both plasma and follicular fluid; additionally,
242 follicular fluid was assayed for $PGF_2\alpha$, prostaglandin E_2 (PGE_2), estradiol 17-β (E2), P4,
243 testosterone (T), LH, nitric oxide (NO), vascular endothelial growth factor-A (VEGF-A),
244 cortisol, prolactin (PRL), and growth hormone (GH). Furthermore, the ratios of PGE_2:$PGF_2\alpha$,
245 E2:P4, E2:T, and P4:T were calculated. Total primary sex steroids were calculated by combining
246 the E2, P4, and T.

247 Plasma P4 concentrations were determined using a solid-phase radioimmunoassay kit
248 containing antibody-coated tubes and I^{125}-labeled P4 (Coat-A Count Progesterone, Diagnostic
249 Products Corporation, Los Angeles, CA, USA) as described and validated for mare plasma
250 (Ginther *et al.* 2005). Plasma LH was assayed using an equine ELISA kit (Endocrine
251 Technologies, INC., Freemont, CA, USA). Plasma and follicular fluid PGFM, $PGF_2\alpha$, PGE_2, and
252 E2 concentration was determined using ELISA kits (Neogen Co., Lexington, KY, USA) after
253 extraction with diethyl-ether, as previously described (Ginther *et al.* 2010). Intrafollicular LH,

GH, cortisol, PRL, and testosterone concentrations were determined by ELISA kits (Endocrine Technologies, INC., Freemont, CA, USA). Intrafollicular NO was estimated using a colorimetric kit (Cayman Chemical Company, Ann Arbor, MI, USA). Intrafollicular VEGF-A was assayed by ELISA kit (Kingfisher Biotech, Inc., Saint Paul, MN, USA). All the assays were performed following the manufacturers' protocol and were validated for equine follicular fluid by using multiple dilutions and pilot assays to determine the optimal dilutions required for the hormone concentration to be within the detection range of the assay. The intra-assay CVs and sensitivities for the different hormone assays were as follows: P4, 8.7% (experiment 1), 6.3% (experiment 2), 6.6% (experiment 3), and 0.02 ng/ml; PGFM, 6.3% (experiment 2) and 3.2% (experiment 3), and 20 pg/ml; $PGF_2\alpha$, 6.1% and 2 pg/ml; PGE_2, 2.6% and 0.1 ng/ml; E2, 4.9% and 0.1 ng/ml; NO, 5.1% and 20 µM/ml; LH, 4.5% (plasma) and 8.9% (follicular fluid), and 0.25 ng/ml; GH, 5.2% and 0.25 ng/ml; cortisol, 9.5% and 1 ng/ml; testosterone, 6.1% and 0.1 ng/ml; and VEGF-A, 3.6% and 28.5 pg/ml.

Statistical analyses

The Shapiro-Wilk test was used for testing normal distribution of the data. Data not normally distributed were transformed to log or rank before any statistical analyses. Dixon's test was used to identify outlier observations, which were excluded from any statistical analyses. Sequential data were analyzed by one-way ANOVA for main effects of group, time, and group by time interaction. The analyses were done using SAS PROC MIXED (Version 9.2; SAS Institute, Inc.) with a REPEATED statement to minimize autocorrelation between sequential measurements. When a group effect or interaction was obtained, differences among groups within time points were further analyzed. Tukey's test was used between time points within a group to identify

277 significant differences. A probability of $P < 0.05$ indicated that a difference was significant and
278 $P > 0.05$ or < 0.1 indicated that results tended to be different. Data are presented as mean ±
279 S.E.M.
280
281 **Results**
282
283 ***Experiment 1. Effect of reLH and PG on LUF formation***
284 No LUFs were observed in this experiment, regardless of treatment. More specifically, reLH
285 (0.5−1.0mg) given for 9 days during the diestrous phase and/or PG administered on Day 7 did
286 not induce LUF formation. Furthermore, reLH had no effect on any other endpoint. The diameter
287 of the largest follicle increased ($P < 0.0001$) for all groups, but no overall group effect was
288 observed during the treatment period (data not shown). A group-by-day interaction ($P < 0.02$)
289 was potentially caused by the greater diameters in the PG groups when compared with the
290 Control and LH groups. After combining the reLH (LH 0.5 + LH 1.0) groups and PG (LH
291 0.5+PG, LH 1.0+PG, and PG) groups, a larger ($P < 0.05$) follicle diameter was seen on Days
292 13−16 for the PG group when compared with the Control and LH groups (Fig. 2A). The growth
293 rate of the largest follicle from Days 7−16 was greater ($P < 0.001$) in the PG group than in the
294 Control and reLH groups (Table 1). In addition, maximum follicle diameter during the treatment
295 period and on Day 16 was greater ($P < 0.01$) in the PG group compared to the Control and reLH
296 groups. The percentage of blood flow of the dominant follicle 3 days before ovulation did not
297 differ ($P > 0.05$) among groups.
298 $PGF_2\alpha$ had a marked effect on CL diameter and half-life, which affected the plasma P4
299 concentrations and shortened the interovulatory interval (IOI; Table 1). As expected, CL

diameter and P4 concentration decreased ($P < 0.007$) faster in PG-treated groups, compared to the other groups (data not shown). The Control and reLH groups did not differ throughout treatment, whereas PG-treated groups had a smaller CL diameter on Day 12 ($P < 0.0001$). Therefore, the reLH (0.5 and 1.0 mg) groups and the PG groups were combined for further analyses (Fig. 2B). The Control and reLH-treated groups had a larger ($P < 0.0001$) CL diameter and greater P4 concentration from Days 9−15 than the PG group (Fig. 2B, C). The reLH treatment had no effect on CL diameter or P4 concentration when compared to the Control group. P4 concentration was greater ($P < 0.0002$) on Day 12 and lower ($P < 0.004$) on the day of the beginning of luteolysis in the Control and reLH groups compared to the PG-treated group (Table 1). Also, the mean day of the beginning of luteolysis (day before P4 was <1.0 ng/ml) was earlier ($P < 0.0001$) in the PG-treated group compared to the Control and reLH-treated groups (Table 1). Endometrial echotexture was greater ($P < 0.002$) in the PG (3.3 ± 0.1) versus Control (2.9 ± 0.1) and reLH (2.8 ± 0.1) groups. Plasma LH concentrations from Days 7−9 increased ($P < 0.03$) for reLH (3.7 ± 0.7 ng/ml) and PG (3.0 ± 0.6 ng/ml) groups; however, an increase was not observed in the Control group (1.8 ± 0.2 ng/ml).

Experiment 2. Optimum dose of flunixin-meglumine to induce LUF

In the Control group, ovulation was detected at 38.7 ± 0.7 h (range, 36 to 40 h) in all mares. In FM groups, both doses (2 and 3 mg/kg of body weight) resulted in induction of LUFs in 100% of the animals; no complications were observed in any animal after FM treatment. LUFs were first observed in treated mares at 49.2 ± 1.9 h (range, 44 to 60 h). No difference was observed in the time of first detection of LUFs and plasma PGFM concentration between FM treated groups. Therefore, FM groups were combined for further analyses (i.e., Induced LUF group). The plasma

PGFM concentration was lower ($P < 0.05$) in the Induced LUF group when compared to the Control group at Hour 24 (Fig. 3). Plasma P4 concentration was not different ($P>0.05$) between groups from Hour 0 to 36 (data not shown).

Follicle diameters of Induced LUF and Control groups were not different ($P > 0.05$) between Hours 0−38. However, Induced LUFs grew to a larger ($P < 0.009$) diameter from Hours 0−60 (Fig. 4A). Follicle wall serration, follicle wall thickness, and follicular shape (round/irregular) were not different between Induced LUF and Control groups up to Hour 38. The first increase ($P < 0.05$) in follicle wall serration occurred between Hours 0−24 in both groups (Fig. 4B). Follicle wall thickness and follicle wall serration continued to increase between Hours 38−60 for the Induced LUF group (Fig. 4B, C).

Follicle blood flow (Fig. 5D) increased differentially between groups after the beginning of treatment (time effect, $P < 0.001$; interaction, $P < 0.02$). The follicle blood flow of the largest follicle was greater ($P < 0.02$) in the Induced LUF group at Hour 38 than in the Control group. Follicle blood flow increased earlier in the Control group (Hour 12) compared to the Induced LUF group (Hour 24).The number of echoic foci in the follicular fluid before ovulation or LUF formation was lower ($P < 0.0001$) in the Control group (0.8 ± 0.3) than in the Induced LUF group (3.5 ± 0.2). Endometrial echotexture (overall score, 3.8 ± 0.1) was not different between groups.

Experiment 3. Systemic and intrafollicular hormones and growth factors

Plasma PGFM concentration was lower ($P < 0.0002$) at Hour 24 in the Induced LUF group versus the Control group (Fig. 5A). Overall, the follicle diameter tended ($P < 0.07$) to be greater in the Induced LUF group when compared with the Control group at Hours 0−36 (Fig. 5B). The

follicle diameter was greater in the Induced LUF group at Hour 24 ($P < 0.02$) and Hour 36 ($P < 0.05$). Follicle diameters at Hours 12, 24, and 36 increased in both groups, when compared to Hour 0. Follicle blood flow did not differ between groups (Fig. 5C), but increased earlier in the Control group (Hour 12) compared to the Induced LUF group (Hour 24). In addition, endometrial echotexture did not differ between groups; however, a faster decrease ($P < 0.05$) in edema score was detected between Hours 24−36 in the Control group (Fig. 5D).

Differences in follicular fluid hormone concentrations were observed between the Control and Induced LUF groups (Fig. 6). PGFM concentration was lower ($P < 0.004$) in the Induced LUF group (49.6 ± 2.0 pg/ml) versus the Control group (102.3 ± 20.5 pg/ml; Fig. 6A). $PGF_2\alpha$ was lower ($P < 0.0006$) in the Induced LUF group (0.013 ± 0.005 ng/ml) compared to the Control group (22.4 ± 6.1 ng/ml; Fig. 6B). Also, PGE_2 concentration was lower ($P < 0.004$) in the Induced LUF group (0.35 ± 0.05 ng/ml) compared to the Control group (24.7 ± 8.5 ng/ml; Fig. 6C). The PGE_2:$PGF_2\alpha$ ratio was higher ($P < 0.03$) in Induced LUF (93.0 ± 43.0) compared to Control (3.6 ± 1.5) mares (Fig. 6D).

Differences in follicular fluid primary sex steroids and their ratios were detected between treatment groups (Fig. 7). Intrafollicular E2 concentration was greater ($P < 0.02$) in the Induced LUF group (1993.0 ± 325.6 ng/ml) compared to the Control group (1114.3 ± 173.0 ng/ml). P4 was not different between groups (Fig. 7B); however, T concentration tended ($P < 0.1$) to be greater in the Induced LUF group (Fig. 7C). Furthermore, the total primary sex steroid concentration was greater ($P < 0.009$) in the Induced LUF group (3340.6 ± 350.4 ng/ml) versus the Control group (2047.4 ± 320.4 ng/ml; Fig. 7D). The ratios of E2:P4 and P4:T tended ($P < 0.1$) to be lower in the Induced LUF group (Fig. 7E, F); however, the E2:T ratio was not different between groups (Fig. 7G).

The follicular fluid LH, PRL, VEGF-A, and NO concentrations did not differ between the Control and Induced LUF groups (Fig. 8A, B, E, F). However, the concentration of cortisol tended ($P < 0.09$) to be higher in the Induced LUF group (6.8 ± 1.9 ng/ml) compared to the Control group (3.2 ± 0.5 ng/ml; Fig. 8D). Also, the follicular GH levels tended ($P < 0.07$) to be higher in the Induced LUF (0.59 ± 0.05 ng/ml) versus the Control group (0.48 ± 0.05 ng/ml; Fig. 8C).

Discussion

The pathophysiologic mechanisms underlying LUF syndrome in mares, women, or other species are not known. This series of experiments was conducted to gain insight about the effects of exogenous and endogenous LH and $PGF_2\alpha$ on LUF formation, as well as the effects on intrafollicular prostaglandins and other hormones in induced LUFs when a COX-2 inhibitor (FM) was used. This study is apparently the first to report and compare a wide range of intrafollicular biomarkers between ovulatory follicles and LUFs. Thus, the results herein presented are clinically relevant to veterinary and human medicine.

Low levels of follicular fluid prostaglandins and PGFM in mares with induced LUFs were the most consistent and novel finding in this study (Experiment 3). Our hypotheses that intrafollicular prostaglandins would be inhibited by systemic FM administration and that inhibition of intrafollicular prostaglandin synthesis would be associated with LUF formation were therefore substantiated. Intrafollicular $PGF_2\alpha$, PGFM, and PGE_2 were consistently decreased in mares of the Induced LUF group. Similar results have been shown in the follicular fluid of women treated with various NSAIDs (Priddy *et al.* 1990). Prostaglandins are vital for the ovulatory process in vertebrates (reviewed in Murdoch *et al.* 1993). It has been reported that

intrafollicular prostaglandins increased 36 h after hCG treatment in mares (Watson & Sertich 1991, Sirois & Dore 1997). A recent study (Martínez-Boví & Cuervo-Arango 2015) demonstrated the importance of prostaglandins in the ovulation process in mares by using intrafollicular injection of a supra-physiological cocktail of PGE_2 and $PGF_2\alpha$ (500 μg and 125 μg, respectively) to reverse the anovulatory effects of FM. Another novel finding from our study was the increased intrafollicular PGE_2:$PGF_2\alpha$ ratio in mares with induced LUFs. High intrafollicular PGE_2:$PGF_2\alpha$ ratio has been associated with lower pregnancy rates in humans (Smith *et al.* 1991). These findings corroborate the role of lowered intrafollicular prostaglandins in LUF formation and support the concept that an altered PGE_2:$PGF_2\alpha$ ratio may be caused by a compensatory mechanism when prostaglandin synthetase is blocked. These results highlight the importance of the mare as a possible animal model to study LUF syndrome in women.

Increased follicular fluid E2 in mares with induced LUFs was another novel finding in this study. We have previously reported that mares had higher E2 concentrations 3 days before the beginning of LUF formation (Ginther *et al.* 2007a). Likewise, higher plasma E2 concentrations have been described in women with spontaneous LUFs (Hamilton *et al.* 1985). However, no differences in intrafollicular E2 concentrations were previously documented in women following NSAID treatment (Priddy *et al.* 1990). Estradiol-mediated stimulation of PGE_2 synthesis in preovulatory follicles in mice (Toda *et al.* 2012), may explain the increased PGE_2:$PGF_2\alpha$ ratio in mares of the Induced LUF group. Furthermore, intrafollicular total primary sex steroids were higher in the Induced LUF group, although no differences were found in P4 concentrations. Greater overall steroid concentrations can be due to low levels of intrafollicular prostaglandins since prostaglandins decrease intracellular transport of cholesterol, reduce cellular cholesterol uptake, and lower the activity of steroidogenic enzymes (reviewed in Niswender *et al.* 2000).

Higher intrafollicular E2 levels in Induced LUF mares were consistent with a more differentiated endometrium with higher uterine edema.

LH, VEGF-A, NO, and PRL do not seem to be involved in LUF formation as the levels were not different between Induced LUF and Control groups. Cortisol and GH levels only tended to be higher in the Induced LUF group. Cortisol is thought to be involved in controlling the inflammatory process of ovulation (Espey & Lipner 1994, Andersen 2002). The role of cortisol in LUF formation is unclear at this point. It is possible that over-inhibition of the inflammatory process results in anovulation. Growth hormone has been shown to increase steroidogenesis in granulosa cells, and GH receptors (GHR) have been detected in granulosa cells in mice (Silva *et al.* 2009).

In this study, higher doses of FM were used in an attempt to increase the incidence of LUFs. Our hypothesis was not substantiated because both 2 mg and 3mg doses of FM induced LUFs in all mares (100% success rate). Nevertheless, the use of FM and hCG provides a reliable model for the study of LUF syndrome (Experiment 2). FM treatments were administered every 12 h and no adverse effects were noted in any animal. The percentage of LUFs in our study was higher than the 83% incidence of LUFs reported following FM treatment in mares (Cuervo-Arango & Domingo-Ortiz 2011) and the 36% incidence following treatment with higher doses of COX-2 inhibitor (meloxicam) in women (Jesam *et al.* 2014). The increased incidence of LUFs in the present study was, in part, attributed to a higher dose of FM in our study compared to previous study (Cuervo-Arango *et al.* 2011), and/or to a lower dose (1500 IU) of hCG in our study versus 2500 IU in a previous study (Cuervo-Arango *et al.* 2011, Cuervo-Arango & Domingo-Ortiz 2011). Therefore, it seems that for proper experimental induction of LUFs, an optimum balance

between ovulatory stimulus (hCG) and an adequate decrease of intrafollicular prostaglandin (by the use of COX-2 inhibitor) must be achieved.

The systemic concentration of PGFM was reduced in FM treated mares at Hour 24 in Experiment 2 and at Hours 12 and 24 in Experiment 3. These results were consistent with previous reports in mares (Ginther *et al.* 2011, Cuervo-Arango *et al.* 2011). Plasma PGFM concentrations are indicative of systemic $PGF_2\alpha$ concentrations, since $PGF_2\alpha$ is rapidly metabolized in the body (Shrestha *et al.* 2012). Lower systemic PGFM levels were also an indicative of the effectiveness of FM treatment in blocking prostaglandin synthesis in our study.

In Experiment 1, we aimed to induce LUFs by injecting reLH between Days 7−15 with or without treating with $PGF_2\alpha$ on Day 7. However, our hypothesis was not supported because reLH did not induce any LUFs. Furthermore, no differential effect on dominant follicle growth, CL development, or plasma P4 was seen by the use of reLH. $PGF_2\alpha$ decreased the CL lifespan and plasma P4, and shortened the IOIs. LH has been shown to be important for the establishment of follicle dominance in mares (Gastal *et al.* 1999*a*, 2000). Daily injections of eLH at the preovulatory phase, after a 32-mm follicle was detected, failed to induce anovulatory follicles in mares (Schauer *et al.* 2013). Similarly, in our study, the reLH treatment did not affect follicular growth before and after deviation phases, nor did it induce anovulation (LUF). Although the results of our experiment are not similar to what has been described in women (Bergquist & Lindgren 1983) and rats (Mattheij & Swarts 1995), it seems to be premature to assume that LH does not affect LUF formation in mares. The dose and frequency of LH administrations were not evaluated in our study. Continued titration studies are required to fully understand the role of LH in LUF formation.

In summary, COX-2 inhibitors used in conjunction with hCG can be used to pharmacologically induce LUFs with 100% success in mares. We postulated that LUFs result from decreased intrafollicular prostaglandin concentrations and/or altered prostaglandin synthesis, as indicated by disparity in PGE_2:$PGF_2\alpha$ ratio. Increased intrafollicular E2 was associated with LUF formation; however, further studies are necessary to ascertain the cause-effect relationship and also to understand the role of testosterone, cortisol, and GH. The effect of LH on LUF formation remains unclear. This study further encourages the use of intrafollicular versus systemic biomarkers for evaluating ovulatory disorders. Finally, results from this study suggest the use of the mare as a potential model for investigating anovulatory infertility in women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Acosta TJ, Gastal EL, Gastal MO, Beg MA & Ginther OJ 2004 Differential blood flow changes between the future dominant and subordinate follicles precede diameter changes during follicle selection in mares. *Biology of Reproduction* **71** 502-507.

Adams GP, Sumar J & Ginther OJ 1991 Hemorrhagic ovarian follicles in llamas. *Theriogenology* **35** 557-568.

Andersen C 2002 Possible new mechanism of cortisol action in female reproductive organs: physiological implications of the free hormone hypothesis. *Journal of Endocrinology* **173** 211-217.

Armstrong DT & Grinwich DL 1972 Blockade of spontaneous and LH-induced ovulation in rats by indomethacin, an inhibitor of prostaglandin biosynthesis. I. *Prostaglandins* **1** 21-28.

Athanasiou S, Bourne TH, Khalid A, Okokon EV, Crayford TJ, Hagstrom HG, Campbell S & Collins WP 1996 Effects of indomethacin on follicular structure, vascularity, and function over the periovulatory period in women. *Fertility and Sterility* **65** 556-560.

Baerwald AR 2009 Human antral folliculogenesis: what we have learned from the bovine and equine models. *Animal Reproduction* **6** 20-29.

Bata MS, Al-Ramahi M, Salhab AS, Gharaibeh MN & Schwartz J 2006 Delay of ovulation by meloxicam in healthy cycling volunteers: A placebo-controlled, double-blind, crossover study. *Journal of Clinical Pharmacology* **46** 925-932.

Bergquist C & Lindgren PG 1983 Ultrasonic measurement of ovarian follicles during chronic LRH agonist treatment for contraception. *Contraception* **28** 125-133.

Campbell S, Bourne TH, Waterstone J, Reynolds KM, Crayford TJ, Jurkovic D, Okokon EV & Collins WP 1993 Transvaginal color blood flow imaging of the periovulatory follicle. *Fertility and Sterility* **60** 433-438.

Check JH 2007 Ovulation disorders: Part II. Anovulation associated with normal estrogen. *Clinical and Experimental Obstetrics and Gynecology* **34** 69-72.

Coetsier T & Dhont M 1996 Complete and partial luteinized unruptured follicle syndrome after ovarian stimulation with clomiphene citrate/human menopausal gonadotrophin/human chorionic gonadotrophin. *Human Reproduction* **11** 583-587.

Cuervo-Arango J, Beg MA & Ginther OJ 2011 Follicle and systemic hormone interrelationships during induction of luteinized unruptured follicles with a prostaglandin inhibitor in mares. *Theriogenology* **76** 361-373.

Cuervo-Arango J & Domingo-Ortiz R 2011 Systemic treatment with high dose of flunixin-meglumine is able to block ovulation in mares by inducing hemorrhage and luteinisation of follicles. *Theriogenology* **75** 707-714.

Cuervo-Arango J & Newcombe JR 2009 The effect of cloprostenol on the incidence of multiple ovulation and anovulatory hemorrhagic follicles in two mares: A case report. *Journal of Equine Veterinary Science* **29** 533-539.

Cuervo-Arango J & Newcombe JR 2010 Risk factors for the development of haemorrhagic anovulatory follicles in the mare. *Reproduction in Domestic Animals* **45** 473-480.

Cuervo-Arango J & Newcombe JR 2012 Ultrasound characteristics of experimentally induced luteinized unruptured follicles (LUF) and naturally occurring hemorrhagic anovulatory follicles (HAF) in the mare. *Theriogenology* **77** 514-524.

549 **Dal J, Vural B, Caliskan E, Ozkan S & Yucesoy I** 2005 Power Doppler ultrasound studies of
550 ovarian, uterine, and endometrial blood flow in regularly menstruating women with
551 respect to luteal phase defects. *Fertility and Sterility* **84** 224-227.
552 **Eijkemans MJ, Polinder S, Mulders AG, Laven JS, Habbema JD & Fauser BC** 2005
553 Individualized cost-effective conventional ovulation induction treatment in
554 normogonadotrophic anovulatory infertility (WHO group 2). *Human Reproduction* **20**
555 2830-2837.
556 **Espey LL & Lipner H** 1994 Ovulation. In *The Physiology of Reproduction*, pp. 725-813.
557 **Gastal EL** 2011 Ovulation: Part 2. Ultrasonographic morphology of the preovulatory follicle. In
558 *Equine Reproduction*, pp. 2032-2054.
559 **Gastal EL, Bergfelt DR, Nogueira GP, Gastal MO & Ginther OJ** 1999*a* Role of luteinizing
560 hormone in follicle deviation based on manipulating progesterone concentrations in
561 mares. *Biology of Reproduction* **61** 1492-1498.
562 **Gastal EL, Gastal MO & Ginther OJ** 1998 The suitability of echotexture characteristics of the
563 follicular wall for identifying the optimal breeding day in mares. *Theriogenology* **50**
564 1025-1038.
565 **Gastal EL, Gastal MO & Ginther OJ** 2006 Relationships of changes in B-mode echotexture
566 and colour-Doppler signals in the wall of the preovulatory follicle to changes in systemic
567 oestradiol concentrations and the effects of human chorionic gonadotrophin in mares.
568 *Reproduction* **131** 699-709.
569 **Gastal EL, Gastal MO & Ginther OJ** 2007 Serrated granulosa and other discrete ultrasound
570 indicators of impending ovulation in mares. *Journal of Equine Veterinary Science* **26** 67-
571 73.

572 **Gastal EL, Gastal MO, Nogueira GP, Bergfelt DR & Ginther OJ** 2000 Temporal
573 interrelationships among luteolysis, FSH and LH concentrations and follicle deviation in
574 mares. *Theriogenology* **53** 925-940.
575 **Gastal EL, Gastal MO, Wiltbank MC & Ginther OJ** 1999*b* Follicle deviation and
576 intrafollicular and systemic estradiol concentrations in mares. *Biology of Reproduction* **61**
577 31-39.
578 **Gastal EL, Kot K & Ginther OJ** 1995 Ultrasound-guided intrafollicular treatment in mares.
579 *Theriogenology* **44** 1027-1037.
580 **Gastal EL, Rodrigues BL, Gastal MO, Beg MA & Ginther OJ** 2005 Responsiveness of the
581 early corpus luteum to PGF2α and resulting progesterone, LH, and FSH interrelationships
582 in mares. *Animal Reproduction* **2** 240-249.
583 **Ghanem MES, Sadek EM, Helal AS, Al-Boghdady LA, Emam ME & Bakr NI** 2009 Effect
584 of stimulation protocol on the risk of luteinized unruptured follicle (LUF) in polycystic
585 ovarian syndrome (PCOS): does LUF affect luteal phase profile? *Fertility and Sterility* **92**
586 99.
587 **Ginther OJ, Cuervo-Arango J & Beg MA** 2011 Disruption of periovulatory FSH and LH
588 surges during induced anovulation by an inhibitor of prostaglandin synthesis in mares.
589 *Animal Reproduction Science* **126** 91-95.
590 **Ginther OJ, Gastal EL, Gastal MO & Beg MA** 2005 Regulation of circulating gonadotropins
591 by the negative effects of ovarian hormones in mares. *Biology of Reproduction* **73** 315-
592 323.
593 **Ginther OJ, Gastal EL, Gastal MO & Beg MA** 2006 Conversion of a viable preovulatory
594 follicle into a hemorrhagic anovulatory follicle in mares. *Animal Reproduction* **3** 29-40.

595 **Ginther OJ, Gastal EL, Gastal MO & Beg MA** 2007*a* Incidence, endocrinology, vascularity,
596 and morphology of hemorrhagic anovulatory follicles in mares. *Journal of Equine*
597 *Veterinary Science* **27** 122-131.
598 **Ginther OJ, Gastal EL, Gastal MO, Bergfelt DR, Baerwald AR & Pierson RA** 2004
599 Comparative study of the dynamics of follicular waves in mares and women. *Biology of*
600 *Reproduction* **71** 1195-1201.
601 **Ginther OJ, Gastal EL, Gastal MO, Utt MD & Beg MA** 2007*b* Luteal blood flow and
602 progesterone production in mares. *Animal Reproduction Science* **99** 213-220.
603 **Ginther OJ, Gastal MO, Gastal EL, Jacob JC & Beg MA** 2008*a* Induction of haemorrhagic
604 anovulatory follicles in mares. *Reproduction Fertility and Development* **20** 947-954.
605 **Ginther OJ, Jacob JC, Gastal MO, Gastal EL & Beg MA** 2008*b* Follicle and systemic
606 hormone interrelationships during spontaneous and ablation-induced ovulatory waves in
607 mares. *Animal Reproduction Science* **106** 181-187.
608 **Ginther OJ & Pierson RA** 1984 Ultrasonic anatomy and pathology of the equine uterus.
609 *Theriogenology* **21** 505-516.
610 **Ginther OJ, Shrestha HK, Fuenzalida MJ, Shahiduzzaman AK & Beg MA** 2010
611 Characteristics of pulses of 13,14-dihydro-15-keto-prostaglandin f2alpha before, during,
612 and after spontaneous luteolysis and temporal intrapulse relationships with progesterone
613 concentrations in cattle. *Biology of Reproduction* **82** 1049-1056.
614 **Hafs HD, Louis TM, Stellflug JN, Convey EM & Britt JH** 1975 Blood LH after PGF2alpha in
615 diestrous and ovariectomized cattle. *Prostaglandins* **10** 1001-1009.

Hamilton CJ, Evers JL & Hoogland HJ 1986 Ovulatory disorders and inflammatory adnexal damage: a neglected cause of the failure of fertility microsurgery. *British Journal of Obstetrics and Gynaecology* **93** 282-284.

Hamilton CJ, Wetzels LC, Evers JL, Hoogland HJ, Muijtjens A & de Haan J 1985 Follicle growth curves and hormonal patterns in patients with the luteinized unruptured follicle syndrome. *Fertility and Sterility* **43** 541-548.

Henneke DR, Potter GD, Kreider JL & Yeates BF 1983 Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Veterinary Journal* **15** 371-372.

Hulka JF 1985 Luteinized unruptured follicle syndrome. *Fertility and Sterility* **44** 853-854.

Jesam C, Salvatierra AM, Schwartz JL & Croxatto HB 2010 Suppression of follicular rupture with meloxicam, a cyclooxygenase-2 inhibitor: potential for emergency contraception. *Human Reproduction* **25** 368-373.

Jesam C, Salvatierra AM, Schwartz JL, Fuentes A & Croxatto HB 2014 Effect of oral administration of a continuous 18 day regimen of meloxicam on ovulation: experience of a randomized controlled trial. *Contraception* **90** 168-173.

Kaiser B, Koene M, Swagemakers J, Bader H & Hoppen HO 1999 Diagnosis, therapy and endocrinologic parameters of persistent follicles in mares in comparison with preovulatory follicles. *Tierarztl Praxis Ausgabe G Grosstiere Nutztiere* **27** 180-186.

Katz E 1988 The luteinized unruptured follicle and other ovulatory dysfunctions. *Fertility and Sterility* **50** 839-850.

637 **Kerin JF, Kirby C, Morris D, McEvoy M, Ward B & Cox LW** 1983 Incidence of the
638 luteinized unruptured follicle phenomenon in cycling women. *Fertility and Sterility* **40**
639 620-626.
640 **Killick S & Elstein M** 1987 Pharmacologic production of luteinized unruptured follicles by
641 prostaglandin synthetase inhibitors. *Fertility and Sterility* **47** 773-777.
642 **Koninckx PR & Brosens IA** 1983 The luteinized unruptured follicle syndrome. *Fertility and*
643 *Sterility* **39** 249-250.
644 **Koninckx PR, Brosens IA, Verhoeven G & De Moor P** 1981 Increased postovulatory plasma
645 follicle stimulating hormone levels in the luteinized unruptured follicle syndrome: a role
646 for inhibin? *British Journal of Obstetrics and Gynaecology* **88** 525-529.
647 **Kugu K, Taketani Y, Kohda K & Mizuno M** 1991 Exaggerated prolactin response to
648 thyrotropin-releasing hormone in infertile women with the luteinized unruptured follicle
649 syndrome. *Archives Gynecology Obstetrics* **249** 27-31.
650 **Lefranc A-C & Allen WR** 2003 Incidence and morphology of anovulatory haemorrhagic
651 follicles in the mare. *Pferdeheilkunde* **19** 611-612.
652 **Lueders I, Taya K, Watanabe G, Yamamoto Y, Yamamoto T, Kaewmanee S, Niemuller C,**
653 **Gray C, Streich WJ & Hildebrandt TB** 2011 Role of the double luteinizing hormone
654 peak, luteinizing follicles, and the secretion of inhibin for dominant follicle selection in
655 Asian elephants (Elephas maximus). *Biology of Reproduction* **85** 714-720.
656 **Marik J & Hulka J** 1978 Luteinized unruptured follicle syndrome: a subtle cause of infertility.
657 *Fertility and Sterility* **29** 270-274.
658 **Martínez-Boví R & Cuervo-Arango J** 2015 Intrafollicular treatment with PGE2 and PGF2α
659 inhibits the formation of luteinised unruptured follicles (LUF) and restores normal

ovulation in mares treated with flunixin-meglumine. *Equine Veterinary Journal* (doi: 10.1111/evj.12396).

Martinez AR, Bernardus RE, Kucharska D & Schoemaker J 1991 Urinary luteinizing hormone testing and prediction of ovulation in spontaneous, clomiphene citrate and human menopausal gonadotropin-stimulated cycles. A clinical evaluation. *Acta Endocrinologica (Copenh)* **124** 357-363.

Mattheij JA & Swarts HJ 1995 Induction of luteinized unruptured follicles in the rat after injection of luteinizing hormone early in pro-oestrus. *European Journal of Endocrinology* **132** 91-96.

Meyers-Brown G, Bidstrup LA, Famula TR, Colgin M & Roser JF 2011 Treatment with recombinant equine follicle stimulating hormone (reFSH) followed by recombinant equine luteinizing hormone (reLH) increases embryo recovery in superovulated mares. *Animal Reproduction Science* **128** 52-59.

Murdoch WJ, Hansen TR & McPherson LA 1993 A review--role of eicosanoids in vertebrate ovulation. *Prostaglandins* **46** 85-115.

Niswender GD, Juengel JL, Silva PJ, Rollyson MK & McIntush EW 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews* **80** 1-29.

Peter AT 2004 An update on cystic ovarian degeneration in cattle. *Reproduction in Domestic Animals* **39** 1-7.

Plas-Roser S, Kauffmann MT & Aron C 1985 Prostaglandins involvement in the formation of luteinized unruptured follicles in the cyclic female rat. *Prostaglandins* **29** 243-253.

Priddy AR, Killick SR, Elstein M, Morris J, Sullivan M, Patel L & Elder M 1990 The effect of prostaglandin synthetase inhibitors on human preovulatory follicular fluid prostaglandin, thromboxane, and leukotriene concentrations. *Journal of Clinical Endocrinology and Metabolism* **71** 235-242.

Qublan H, Amarin Z, Nawasreh M, Diab F, Malkawi S, Al-Ahmad N & Balawneh M 2006 Luteinized unruptured follicle syndrome: incidence and recurrence rate in infertile women with unexplained infertility undergoing intrauterine insemination. *Human Reproduction* **21** 2110-2113.

Salhab AS, Amro BI & Shomaf MS 2003 Further investigation on meloxicam contraceptivity in female rabbits: luteinizing unruptured follicles, a microscopic evidence. *Contraception* **67** 485-489.

Schauer SN, Guillaume D, Decourt C, Watson ED, Briant C & Donadeu FX 2013 Effect of luteinizing hormone overstimulation on equine follicle maturation. *Theriogenology* **79** 409-416.

Shrestha HK, Beg MA, Burnette RR & Ginther OJ 2012 Plasma clearance and half-life of prostaglandin F2alpha: a comparison between mares and heifers. *Biology of Reproduction* **87** 18, 11-16.

Silva JR, Figueiredo JR & van den Hurk R 2009 Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology* **71** 1193-1208.

Sirois J & Dore M 1997 The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process. *Endocrinology* **138** 4427-4434.

Smith EM, Anthony F & Masson GM 1991 Follicular fluid prostaglandin E2:prostaglandin F2 alpha ratio in relation to outcome of matched oocyte. *Human Reproduction* **6** 519-523.

Smith G, Roberts R, Hall C & Nuki G 1996 Reversible ovulatory failure associated with the development of luteinized unruptured follicles in women with inflammatory arthritis taking non-steroidal anti-inflammatory drugs. *British Journal of Rheumatology* **35** 458-462.

Srikandakumar A & Downey BR 1989 Induction of ovulation in gilts with cloprostenol. *Theriogenology* **32** 445-449.

Stoops MA, Pairan RD & Roth TL 2004 Follicular, endocrine and behavioural dynamics of the Indian rhinoceros (Rhinoceros unicornis) oestrous cycle. *Reproduction* **128** 843-856.

Toda K, Ono M, Yuhki K, Ushikubi F & Saibara T 2012 17beta-Estradiol is critical for the preovulatory induction of prostaglandin E(2) synthesis in mice. *Molecular and Cellular Endocrinology* **362** 176-182.

Vanrell JA, Balasch J, Fuster JS & Fuster R 1982 Ovulation stigma in fertile women. *Fertility and Sterility* **37** 712-713.

Watson ED & Sertich PL 1991 Concentrations of arachidonate metabolites, steroids and histamine in preovulatory horse follicles after administration of human chorionic gonadotrophin and the effect of intrafollicular injection of indomethacin. *Journal of Endocrinology* **129** 131-139.

Westfahl PK 1988 Circulating sex steroids after induction of luteinized unruptured follicles in adult guinea pigs. *Steroids* **51** 101-114.

Zaidi J, Jurkovic D, Campbell S, Collins W, McGregor A & Tan SL 1995 Luteinized unruptured follicle: morphology, endocrine function and blood flow changes during the menstrual cycle. *Human Reproduction* **10** 44-49.

749 **Figure Legends**

750

751 **Figure 1.** A series of comparative ultrasound images of preovulatory follicles and LUFs in four
752 women (A–D) and one mare (E–H). Images were obtained before ovulation in women (A) and a
753 mare (E), and various stages of LUF formation. Large diameter, thicker and echogenic follicle
754 wall (luteinized; long arrows), and echoic foci and fibrin-like strands (short arrows) in the
755 follicle antrum can be observed in different degrees in LUFs in women (B–D) and mare (F–H).

756

757 **Figure 2.** Mean (± S.E.M.) diameters of the largest follicle and CL, and corresponding
758 concentrations of P4 for Days 7−15 after ovulation (Day 0) for the Control, LH combined, and
759 LH + PG combined groups. Arrow indicates beginning of treatments on Day 7. The probabilities
760 for a group effect (G), day effect (D), and group-by-day interaction (GD) are shown. An asterisk
761 (*) indicates days of a significant difference ($P < 0.05$) between groups and a pound mark (#)
762 indicates a difference that approached significance ($P < 0.1$).

763

764 **Figure 3.** Mean (± S.E.M.) $PGF_2\alpha$ metabolite (PGFM) concentration for the Induced LUF group
765 (flunixin meglumine treatments combined) versus the Control group (saline). The probabilities
766 for a group effect (G), hour effect (H), and group-by-hour interaction (GH) are shown. An
767 asterisk (*) indicates hours of a significant difference ($P < 0.05$) between groups.

768

769 **Figure 4.** Mean (± S.E.M.) follicle diameter, follicle wall serration, follicle wall thickness, and
770 follicle blood flow for the Induced LUF group (flunixin meglumine treatments combined) versus
771 the Control group (saline). The probabilities for a group effect (G), hour effect (H), and group-

by-hour interaction (GH) are shown for Hours 0−38. An asterisk (*) indicates the first increase ($P < 0.05$) within a group.

Figure 5. Mean (± S.E.M.) plasma $PGF_2\alpha$ metabolite (PGFM) concentration, follicle diameter, follicle blood flow, and endometrial echotexture score for the Induced LUF group (flunixin meglumine treatment) versus the Control group (saline). The probabilities for a group effect (G), hour effect (H), and group-by-hour interaction (GH) are shown. An asterisk (*) indicates hours of a significant difference ($P < 0.05$) between groups, and a pound mark (#) indicates a difference that approached significance ($P < 0.1$) between groups.

Figure 6. Mean (± S.E.M.) follicular fluid concentrations of $PGF_2\alpha$ metabolite (PGFM), $PGF_2\alpha$, PGE_2, and PGE_2:$PGF_2\alpha$ ratio for the Induced LUF group (flunixin meglumine treatment) versus the Control group (saline). Bars with different superscripts within an endpoint are different ($P < 0.05$).

Figure 7. Mean (± S.E.M.) follicular fluid concentrations of E2, P4, T, and total primary sex steroids for the Induced LUF group (flunixin meglumine treatment) versus the Control group (saline). Bars with different superscripts within an endpoint are different ($P < 0.05$), and a pound mark (#) indicates a difference that approached significance ($P < 0.1$) between groups. NS, non-significant.

Figure 8. Mean (± S.E.M.) follicular fluid concentrations of LH, cortisol, GH, VEGF-A, and NO for the Induced LUF group (flunixin meglumine treatment) versus the Control group (saline).

795 Bars with a pound mark (#) indicate a difference that approached significance ($P < 0.1$) between
796 groups.
797