

# The mare as a model for luteinized unruptured follicle syndrome: intrafollicular endocrine milieu

S T Bashir, M O Gastal, S P Tazawa<sup>1</sup>, S G S Tarso, D B Hales<sup>2</sup>, J Cuervo-Arango<sup>3</sup>, A R Baerwald<sup>4</sup> and E L Gastal

Department of Animal Science, Food and Nutrition, Southern Illinois University, 1205 Lincoln Drive, MC 4417, Carbondale, Illinois 62901, USA, <sup>1</sup>EquiBreed Oz, Warnervale, New South Wales, Australia, <sup>2</sup>Department of Physiology, Southern Illinois University, Carbondale, Illinois, USA, <sup>3</sup>Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad CEU Cardenal Herrera, Valencia, Spain and <sup>4</sup>Department of Obstetrics, Gynecology and Reproductive Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Correspondence should be addressed to E Gastal; Email: egastal@siu.edu

## 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 Day 7 to Day 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 human chorionic gonadotropin (hCG) (1500 IU) were administered after a follicle  $\geq 32$  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,  $PGF_{2\alpha}$ , 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.

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## 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.* 2007a, 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.* 2007a). 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.* 2008a, 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.* 2007a, Cuervo-Arango & Newcombe 2009, 2010). Therefore, recurrent LUFs result in prolonged periods of anovulation and long interovulatory intervals (Ginther *et al.* 2007a).

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.* 2007a,b). 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 LH 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 has been associated with LUFs 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<sub>2</sub>α 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<sub>2</sub>α 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.* 2008b). 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.* 2008a, 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 & Greenwich 1972), rabbits (Salhab *et al.* 2003), mares (Watson & Sertich 1991, Cuervo-Arango & Domingo-Ortiz 2011, Ginther *et al.* 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 (Athanasίου *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<sub>2</sub>α 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: i) reLH, when administered during diestrus alone or along with PGF<sub>2</sub>α, would increase the incidence of LUFs; ii) higher doses of FM would increase the incidence of LUFs; iii) inhibition of intrafollicular prostaglandin synthesis would be associated with LUF formation; iv) intrafollicular

prostaglandins (PGF<sub>2</sub>α and PGE<sub>2</sub>) concentrations would be decreased during systemic FM treatment; and v) 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.

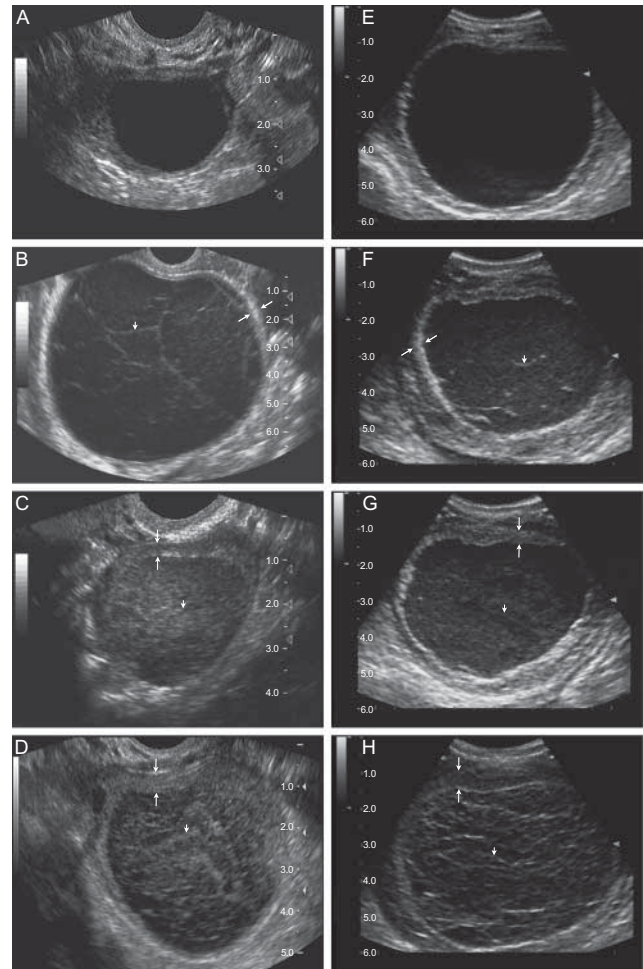
### Experiment 1. Effect of reLH and PG on LUF formation

#### 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<sub>2</sub>α (5 mg i.m.; Lutalyse, Pfizer Animal Health), reLH (0.5 or 1.0 mg i.v.; AspenBio Pharma, Inc., Castle Rock, CO, USA), or a combination of PGF<sub>2</sub>α 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<sub>2</sub>α), PG (5 mg PGF<sub>2</sub>α), LH 1.0 (1 mg reLH), and LH 1.0 + PG (1 mg reLH and 5 mg PGF<sub>2</sub>α). Mares were treated with PGF<sub>2</sub>α (5 mg/mare) only once on Day 7 and with reLH once every day from Day 7 to Day 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. An 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 convex-array transducer of 3.5–10 MHz was employed.



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

The color-flow mode was used to display follicle, corpus luteum (CL), and LUF blood flow as previously described (Ginther *et al.* 2007a,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  $\geq 28$  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).

## Experiment 2. Optimum dose of flunixin meglumine (FM) to induce LUF

### Animals and treatments

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

### Ultrasound evaluations

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

Follicle diameter, follicle blood flow, LUFs, and endometrial echotexture were evaluated using the same ultrasound methodology as in Experiment 1. The thickness of the follicle wall (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: i) presence of echoic foci floating in the antrum, ii) detection of follicle wall serration (i.e., irregular or notched surfaces of the granulosa layer; Gastal *et al.* 2007), and iii) loss of spherical shape.

## Experiment 3. Systemic and intrafollicular hormones and growth factors

### Animals and treatments

Cycling mares ( $n=23$ ) with a growing follicle  $\geq 28$ -mm were scanned daily after Day 15 using B-mode and color-Doppler ultrasonography (Gastal *et al.* 1998, 2006) until a follicle  $\geq 32$  mm was detected. When the largest follicle reached  $\geq 32$  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, 1999b). The aspirated follicular fluid was immediately processed in a refrigerated centrifuge ( $1500 \times g$  for 10 min), and 10 ml of the supernatant was stored at  $-20^\circ\text{C}$  until hormone assays were performed. Follicle diameter, follicle blood flow, and endometrial echotexture were measured, as described in Experiment 1.

### Blood samples and hormone assays

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

Plasma P4 concentrations were determined using a solid-phase radioimmunoassay kit containing antibody-coated tubes and  $^{125}\text{I}$ -labeled P4 (Coat-A Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) as described and validated for mare plasma (Ginther *et al.* 2005). Plasma LH was assayed using an equine ELISA kit (Endocrine Technologies, Inc., Fremont, CA, USA). Plasma and follicular fluid PGFM,  $PGF_2\alpha$ ,  $PGE_2$ , and E2 concentration was determined using ELISA kits (Neogen Co., Lexington, KY, USA) after 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., Fremont, 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<sub>2</sub>, 2.6% and 0.1 ng/ml; E<sub>2</sub>, 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**

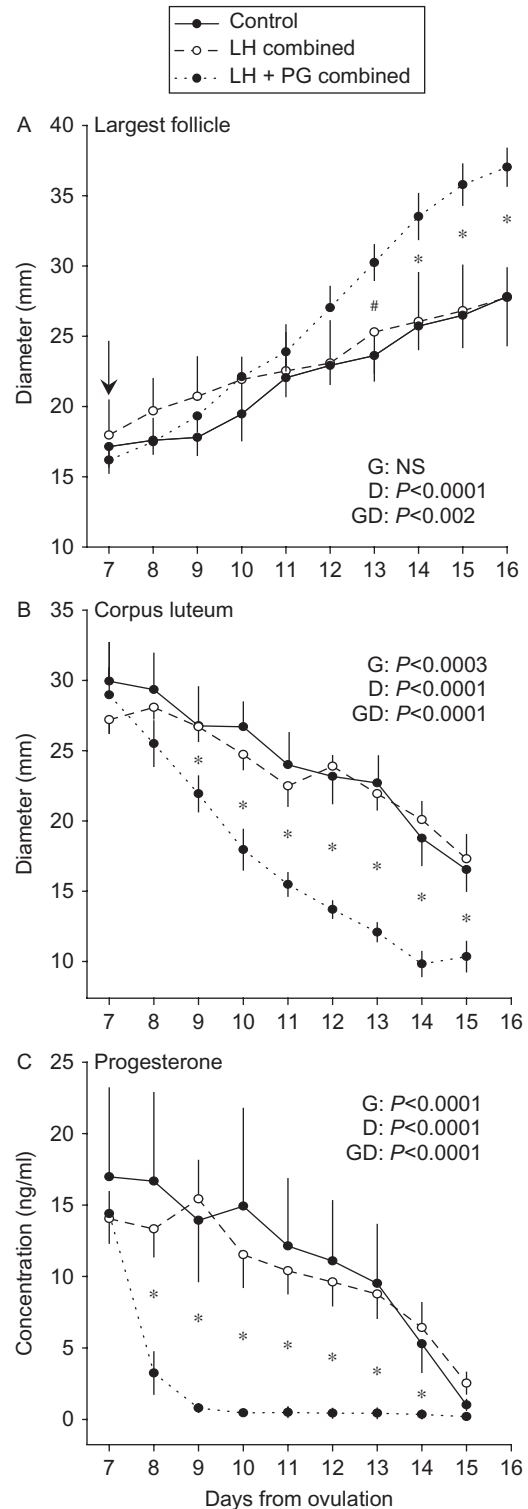
The Shapiro-Wilk test was used for testing normal distribution of the data. Data not normally distributed were transformed to log or rank before any statistical analyses. Dixon's test was used to identify outlier observations, which were excluded from any statistical analyses. Sequential data were analyzed 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., Cary, NC, USA) 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 among groups and between time points within a group to identify significant differences. A probability of  $P < 0.05$  indicated that a difference was significant and  $P > 0.05$  or  $< 0.1$  indicated that results tended to be different. Data are presented as mean ± S.E.M.

**Results**

**Experiment 1. Effect of reLH and PG on LUF formation**

No LUFs were observed in this experiment, regardless of treatment. More specifically, reLH (0.5–1.0 mg) given for 9 days during the diestrous phase and/or PG administered on Day 7 did not induce LUF formation. Furthermore, reLH had no effect on any other end point. The diameter of the largest follicle increased ( $P < 0.0001$ ) for all groups, but no overall group effect was observed during the treatment period (data not shown). A group-by-day interaction ( $P < 0.02$ ) was potentially caused by the greater diameters in the PG groups when compared with the control and LH groups. After combining the reLH (LH 0.5 + LH 1.0) groups and PG (LH 0.5 + PG, LH 1.0 + PG, and PG) groups, a larger ( $P < 0.05$ ) follicle diameter was seen on Days 13–16 for the PG group when compared with the control and LH groups (Fig. 2A). The growth rate of the largest follicle on Days 7–16 was greater ( $P < 0.001$ ) in the PG group than in the control and reLH groups (Table 1). In addition, maximum follicle diameter during the treatment period and on Day 16 was greater ( $P < 0.01$ ) in the PG group compared to the control and reLH groups. The percentage of blood flow of the dominant follicle 3 days before ovulation did not differ ( $P > 0.05$ ) among groups.

PGF<sub>2α</sub> had a marked effect on CL diameter and half-life, which affected the plasma P4 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



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

**Table 1** Mean ( $\pm$ S.E.M.) follicular and luteal end points in various treatments (Experiment 1).

End points	Treatment Groups			P value
	Control	LH (combined)	LH+PG (combined)	
Number of mares	5	9	13	
IOI	21.8 $\pm$ 0.7 <sup>a</sup>	21.3 $\pm$ 0.9 <sup>a</sup>	16.6 $\pm$ 0.5 <sup>b</sup>	<0.0001
Follicle				
Growth rate (mm/day) from Day 7–16*	1.1 $\pm$ 0.2 <sup>a</sup>	1.1 $\pm$ 0.3 <sup>a</sup>	2.2 $\pm$ 0.3 <sup>b</sup>	<0.001
Maximum diameter (mm) during treatment	28.6 $\pm$ 1.9 <sup>a</sup>	29.8 $\pm$ 3.2 <sup>a</sup>	37.7 $\pm$ 1.3 <sup>b</sup>	<0.01
Diameter at Day 7	17.1 $\pm$ 1.4	18.0 $\pm$ 2.5	16.2 $\pm$ 0.9	NS
Diameter at Day 16	27.8 $\pm$ 2.0 <sup>a</sup>	27.8 $\pm$ 3.4 <sup>a</sup>	37.0 $\pm$ 1.4 <sup>b</sup>	<0.009
Blood flow (%) from Days –3 to –1	87.0 $\pm$ 3.5	78.6 $\pm$ 3.4	76.9 $\pm$ 3.6	NS
Corpus luteum				
CL diameter at Day 12	23.2 $\pm$ 1.9 <sup>a</sup>	23.9 $\pm$ 0.8 <sup>a</sup>	13.6 $\pm$ 0.6 <sup>b</sup>	<0.0001
Progesterone (ng/ml)				
At Day 12	11.1 $\pm$ 4.2 <sup>a</sup>	9.6 $\pm$ 1.7 <sup>a</sup>	0.4 $\pm$ 0.3 <sup>b</sup>	<0.0002
At beginning of luteolysis <sup>†</sup>	2.5 $\pm$ 0.9 <sup>a</sup>	4.0 $\pm$ 0.6 <sup>a</sup>	9.1 $\pm$ 1.4 <sup>b</sup>	<0.004
Beginning of luteolysis (day)	14.2 $\pm$ 0.4 <sup>a</sup>	14.2 $\pm$ 0.4 <sup>a</sup>	7.4 $\pm$ 0.2 <sup>b</sup>	<0.0001

IOI, interovulatory interval; NS, not significant.

<sup>a,b</sup>Means with different superscripts within an end point are different ( $P<0.05$ ). \*Day 0=ovulation. <sup>†</sup>Beginning of luteolysis=day before plasma P4 was <1.0 ng/ml

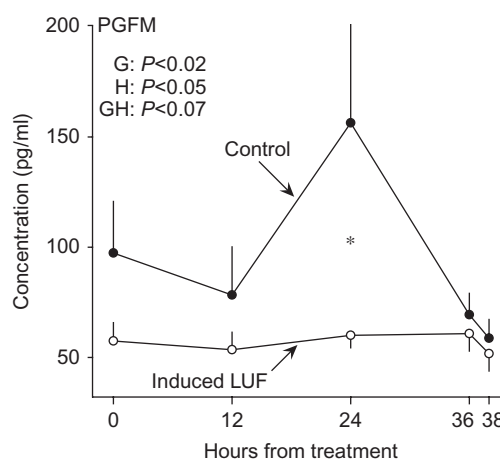
groups did not differ throughout treatment, whereas PG-treated groups had a smaller CL diameter on Day 12 ( $P<0.0001$ ). Therefore, the reLH (0.5 and 1.0 mg) groups and the PG groups were combined for further analyses (Fig. 2B). The control and reLH-treated groups had a larger ( $P<0.0001$ ) CL diameter and greater P4 concentration on Days 9–15 than the PG group (Fig. 2B and 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 $\pm$ 0.1) vs control (2.9 $\pm$ 0.1) and reLH (2.8 $\pm$ 0.1) groups. Plasma LH concentrations on Days 7–9 increased ( $P<0.03$ ) for reLH (3.7 $\pm$ 0.7 ng/ml) and PG (3.0 $\pm$ 0.6 ng/ml) groups; however, an increase was not observed in the control group (1.8 $\pm$ 0.2 ng/ml).

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

In the control group, ovulation was detected at 38.7 $\pm$ 0.7 h (range, 36–40 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 $\pm$ 1.9 h (range, 44–60 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 Hour 36 (data not shown).

Follicle diameters of induced LUF and control groups were not different ( $P>0.05$ ) between Hour 0 and Hour 38. However, induced LUFs grew to a larger ( $P<0.009$ ) diameter in 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 Hour 0 and Hour 24 in both groups (Fig. 4B). Follicle wall thickness and follicle wall serration continued to increase between Hour 38 and Hour 60 for the induced LUF group (Fig. 4B and C).



**Figure 3** Mean ( $\pm$ S.E.M.) PGF<sub>2</sub> $\alpha$  metabolite (PGFM) concentration for the induced LUF group (flunixin meglumine treatments combined) vs 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.

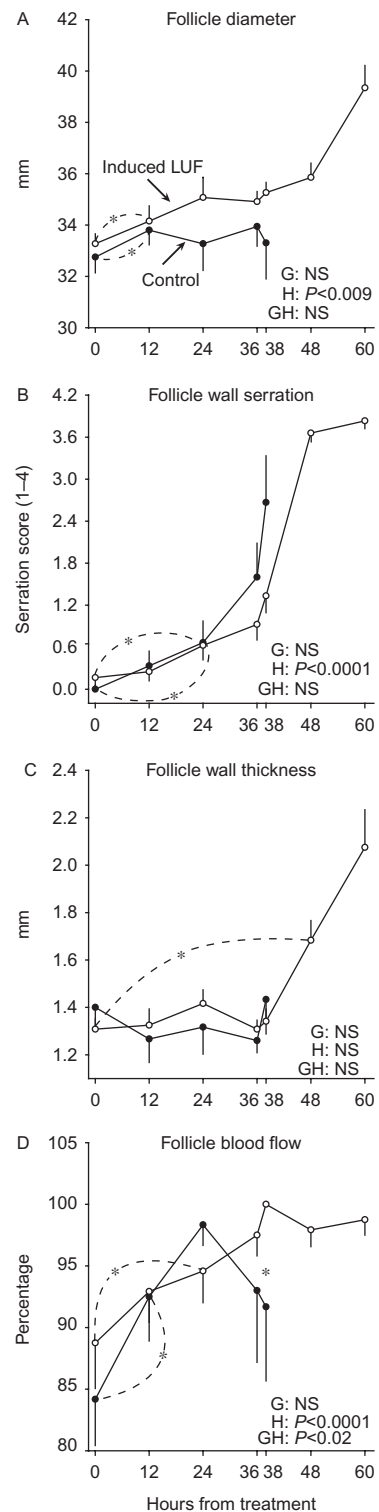
Follicle blood flow (Fig. 4D) increased differentially between groups after the beginning of treatment. Follicle blood flow increased earlier in the control group (Hour 12) compared to the induced LUF group (Hour 24). The follicle blood flow of the largest follicle was greater ( $P<0.02$ ) in the induced LUF group at Hour 38 than in the control group. The number of echogenic foci in the follicular fluid before ovulation or LUF formation was lower ( $P<0.0001$ ) in the control group ( $0.8\pm 0.3$ ) than in the induced LUF group ( $3.5\pm 0.2$ ). Endometrial echotexture (overall score,  $3.8\pm 0.1$ ) was not different between groups.

### Experiment 3. Systemic and intrafollicular hormones and growth factors

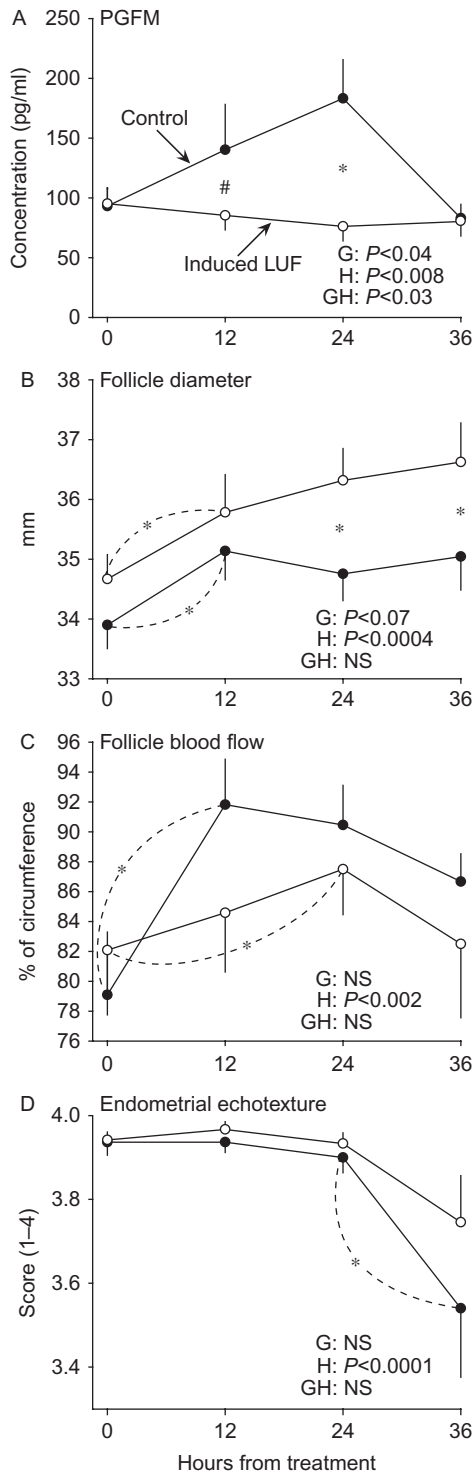
Plasma PGFM concentration was lower ( $P<0.0002$ ) at Hour 24 in the induced LUF group vs the control group (Fig. 5A). Overall, the follicle diameter tended ( $P<0.07$ ) to be greater in the induced LUF group when compared with the control group at Hours 0–36 (Fig. 5B). The follicle diameter was greater 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 Hour 24 and Hour 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 ( $49.6\pm 2.0$  pg/ml) vs the control group ( $102.3\pm 20.5$  pg/ml; Fig. 6A).  $\text{PGF}_2\alpha$  was lower ( $P<0.0006$ ) in the induced LUF group ( $0.013\pm 0.005$  ng/ml) compared to the control group ( $22.4\pm 6.1$  ng/ml; Fig. 6B). Also,  $\text{PGE}_2$  concentration was lower ( $P<0.004$ ) in the induced LUF group ( $0.35\pm 0.05$  ng/ml) compared to the control group ( $24.7\pm 8.5$  ng/ml; Fig. 6C). The  $\text{PGE}_2:\text{PGF}_2\alpha$  ratio was higher ( $P<0.03$ ) in induced LUF ( $93.0\pm 43.0$ ) compared to control ( $3.6\pm 1.5$ ) mares (Fig. 6D).

Differences in follicular fluid primary sex steroids and their ratios were detected between treatment groups (Fig. 7). Intrafollicular  $\text{E}_2$  concentration was greater ( $P<0.02$ ; Fig. 7A) in the induced LUF group ( $1993.0\pm 325.6$  ng/ml) compared to the control group ( $1114.3\pm 173.0$  ng/ml).  $\text{P}_4$  was not different between groups (Fig. 7B); however,  $\text{T}$  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 ( $3340.6\pm 350.4$  ng/ml) vs the control group ( $2047.4\pm 320.4$  ng/ml; Fig. 7D). The ratios of  $\text{E}_2:\text{P}_4$



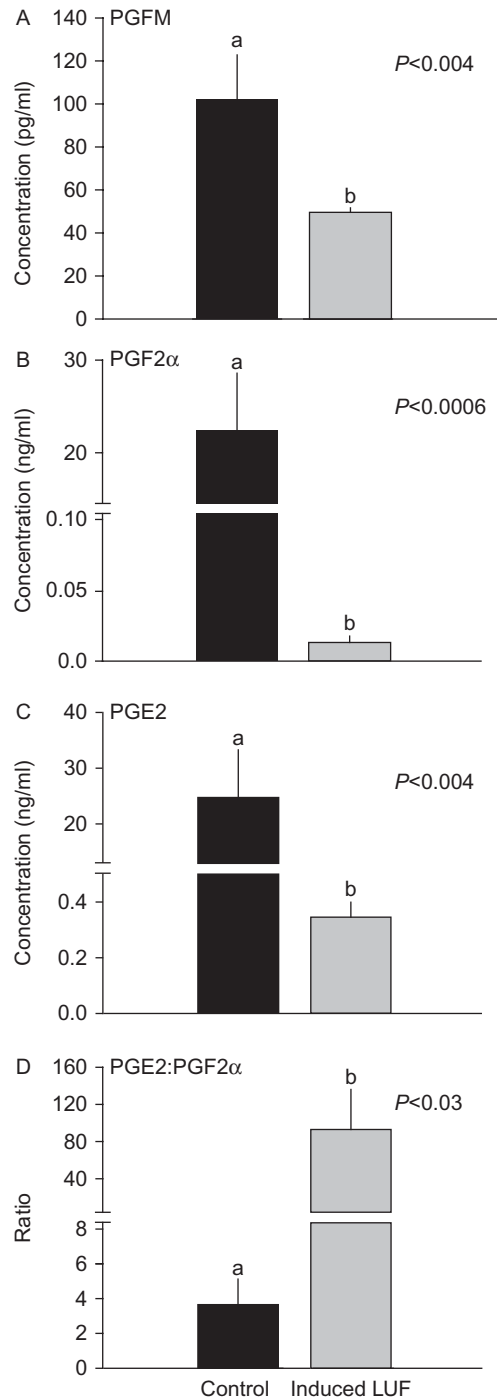
**Figure 4** Mean ( $\pm$  S.E.M.) follicle diameter, follicle wall serration, follicle wall thickness, and follicle blood flow for the induced LUF group (flunixin meglumine treatments combined) vs the control group (saline). The probabilities for a group effect (G), hour effect (H), and group-by-hour interaction (GH) are shown for Hours 0–38. An asterisk (\*) indicates the first increase ( $P<0.05$ ) within a group and hours of a significant difference ( $P<0.05$ ) between groups.



**Figure 5** Mean ( $\pm$ S.E.M.) plasma PGF<sub>2</sub> $\alpha$  metabolite (PGFM) concentration, follicle diameter, follicle blood flow, and endometrial echotexture score for the induced LUF group (flunixin meglumine treatment) vs 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 the first increase or decrease ( $P < 0.05$ ) within a group and 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.

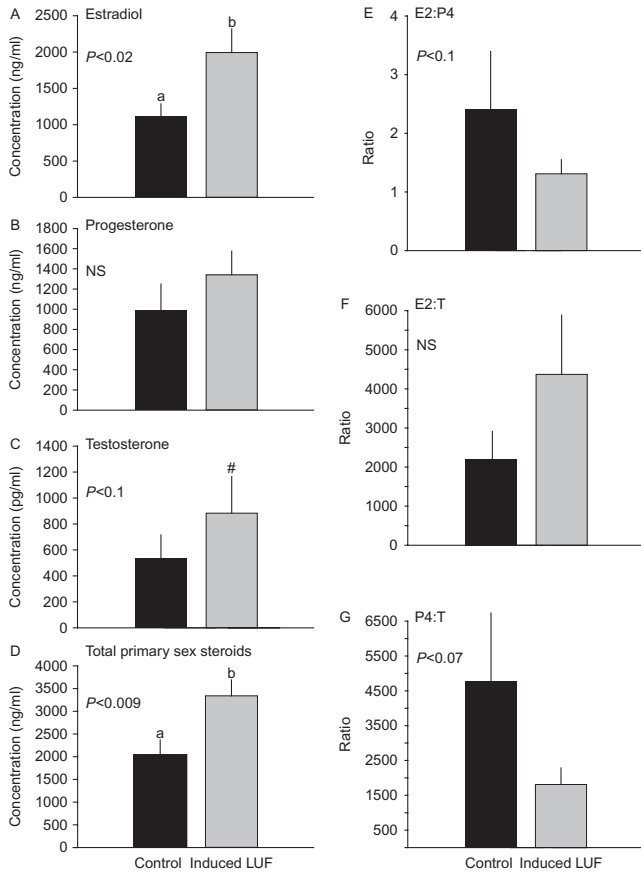
and P4:T tended ( $P < 0.1$ ) to be lower in the induced LUF group (Fig. 7E and G); however, the E2:T ratio was not different between groups (Fig. 7F).

The follicular fluid LH, PRL, VEGF-A, and NO concentrations did not differ between the control and induced LUF groups (Fig. 8A, B, E, and F). However, the



**Figure 6** Mean ( $\pm$ S.E.M.) follicular fluid concentrations of PGF<sub>2</sub> $\alpha$  metabolite (PGFM), PGF<sub>2</sub> $\alpha$ , PGE<sub>2</sub>, and PGE<sub>2</sub>:PGF<sub>2</sub> $\alpha$  ratio for the induced LUF group (flunixin meglumine treatment) vs the control group (saline). Bars with different superscripts within an end point are different ( $P < 0.05$ ).





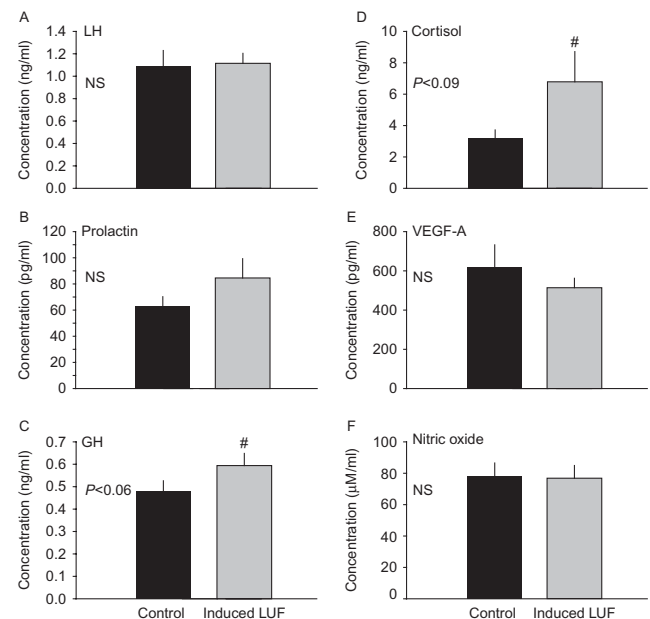
**Figure 7** Mean ( $\pm$  S.E.M.) follicular fluid concentrations of E2, P4, T, total primary sex steroids and ratios of E2:P4, E2:T, and P4:T for the induced LUF group (flunixin meglumine treatment) vs the control group (saline). Bars with different superscripts within an end point are different ( $P < 0.05$ ), and a pound mark (#) indicates a difference that approached significance ( $P < 0.1$ ) between groups. NS, not significant.

concentration of cortisol tended ( $P < 0.09$ ) to be higher in the induced LUF group ( $6.8 \pm 1.9$  ng/ml) compared to the control group ( $3.2 \pm 0.5$  ng/ml; Fig. 8D). Also, the follicular GH levels tended ( $P < 0.07$ ) to be higher in the induced LUF ( $0.59 \pm 0.05$  ng/ml) vs the control group ( $0.48 \pm 0.05$  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 and 125  $\mu$ g, 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



**Figure 8** Mean ( $\pm$  S.E.M.) follicular fluid concentrations of LH, prolactin, GH, cortisol, VEGF-A, and NO for the induced LUF group (flunixin meglumine treatment) vs the control group (saline). Bars with a pound mark (#) indicate a difference that approached significance ( $P < 0.1$ ) between groups. NS, not significant.

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<sub>2</sub> synthesis in preovulatory follicles in mice (Toda *et al.* 2012) may explain the increased PGE<sub>2</sub>:PGF<sub>2</sub>α 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 (Espesy & 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 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 and 3 mg 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 vs 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<sub>2</sub>α concentrations, since PGF<sub>2</sub>α 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 Day 7 and Day 15 with or without treating with PGF<sub>2</sub>α 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<sub>2</sub>α 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.* 1999a, 2000). Daily injections of eLH one day after a follicle ablation-induced follicular wave, followed by hCG when the preovulatory follicle was 32 mm, 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<sub>2</sub>:PGF<sub>2</sub>α 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 vs 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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