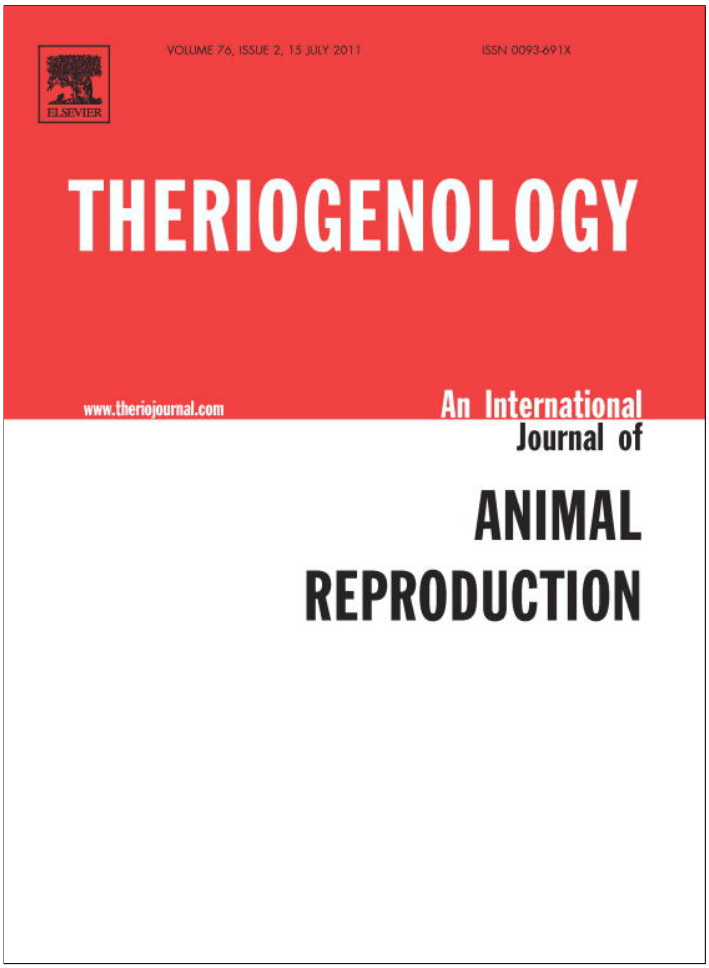

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Theriogenology 76 (2011) 361–373

Theriogenology

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Follicle and systemic hormone interrelationships during induction of luteinized unruptured follicles with a prostaglandin inhibitor in mares

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Received 16 November 2010; received in revised form 7 February 2011; accepted 10 February 2011

Abstract

The objective was to determine differences in follicle and reproductive hormone characteristics in mares with ovulatory and flunixin meglumine (FM)-induced anovulatory cycles. Estrous mares were given 1500 IU hCG when the follicle was ≥ 32 mm (0 h). In Experiment 1, control mares (n = 7) were not treated further. The remaining mares (n = 11) were given 1.7 mg/kg FM i.v. twice daily, from 0 to 36 h after hCG treatment. Blood samples and ultrasonographic examinations were performed every 12 h. All control mares ovulated normally between 36 and 48 h. In contrast, eight of 11 FM mares did not ovulate, but developed luteinized unruptured follicles (LUFs). Three FM-treated mares did not develop conventional LUFs. Plasma progesterone concentrations were lower ($P < 0.05$) in LUF mares at 96, 120, and 216 h than in controls, whereas plasma LH concentrations were higher ($P < 0.05$) between 108 and 120 h in LUF mares than in controls. Plasma concentrations of PGFM and estradiol did not differ significantly between groups. In Experiment 2, the three mares that did not develop LUFs were treated, during the consecutive cycle, with the same dose of FM but with increased frequency at zero, 12, 24, 30, 36, and 48 h after hCG. One mare formed a LUF, whereas the other two did not. These two mares had lower LH concentrations than LUF or control mares in the two consecutive cycles. In conclusion, systemic treatment with FM blocked ovulation in 73% of treated mares. Mares with LUFs had lower progesterone and higher LH concentrations than control mares.

Keywords: Mare; Luteinized unruptured follicle; Prostaglandin inhibitor; Progesterone

1. Introduction

Ovulation involves the collapse of a preovulatory follicle with follicular fluid evacuation and oocyte release into the oviductal infundibulum. The preovulatory surge of LH initiates ovulation by triggering a complex series of events involving various hormones and enzymes [1]. Prostaglandins (PGs) play an essential role during the process of follicular rupture [2–4]. In the follicle, PGs are produced by the inducible cyclo-oxygenase isoform-2, also known as prostaglandin G/H synthetase (PGHS-2) [5]. The preovulatory surge of LH induces the expression of PGHS-2 in granulosa cells of rats [6].

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doi:10.1016/j.theriogenology.2011.02.015

The obligatory role of PGs during the ovulatory process has been confirmed on numerous occasions in several species. Intrafollicular administration of indomethacin (a PG synthetase inhibitor) blocks ovulation in the mare [7], rabbit doe [8], rat [9] and ewe [10,11]. Furthermore, systemic or intra-ovarian treatment with indomethacin blocks ovulation by inducing luteinized unruptured follicles (LUFs) in women [12] and cows [13]. The role of PGs during the process of follicular rupture is not known, but the results of a recent study [14] linked the role of PGs to downstream regulation of matrix-metalloproteinases and plasmin, which are enzymes involved in degradation of extracellular matrix in the follicular wall.

In mares, treatment with human choronic gonadotrophin (hCG) results in an immediate decrease in estradiol and a more rapid increase in endogenous LH concentrations within 24 h after treatment [15]. Expression of PGHS-2 in granulosa cells occurs 30 h after hCG treatment [16]. Administration of hCG to estrous cyclic mares induces ovulation between 36 and 42 h after treatment [17,18].

In the mare, unlike in ruminants, the rise in peripheral progesterone from luteal origin occurs relatively soon after ovulation [18]. Within 24 h after ovulation, the microscopic appearance of the equine early corpus luteum shows folds of stromal tissue beginning to grow into the luteinizing tissue, accompanied by proliferating capillaries which provide the required nutrients and growth factors for continued development of luteal cells [19,20]. Nonetheless, follicular collapse is not essential for luteinization of follicular cells and progesterone production. This has been shown in several species by experimental induction of LUFs in guinea pigs [21], rats [22], ewes [10], cows [13] and women [12].

Mares develop a naturally-occurring anovulatory condition which involves hemorrhage into the antrum of a preovulatory-sized follicle, anovulation, and luteinization of the wall without previous rupture. This spontaneous form of anovulation has been referred to as a hemorrhagic anovulatory follicle (HAF) [23,24], persistent anovulatory follicle [25], and anovulatory hemorrhagic follicle [26]. In a recent study [27], LUFs were experimentally induced in mares by systemic administration of high doses of flunixin meglumine (FM), a PGHS inhibitor. The ultrasonographic characteristics of these induced LUFs resembled those of previously reported spontaneous HAFs [24]. However, it is unknown if a reduction in prostaglandins plays a role in the pathogenesis of spontaneous HAF syndrome.

In some reports, progesterone secretory ability of LUFs in women was lower than that of corpora lutea that develop from ovulated follicles [21], whereas others did not find any difference [10,28]. One study found lower concentrations of progesterone in women with LUFs at 5 d but not at 9 d after hCG treatment, compared with contemporaneous ovulatory patients [12]. In mares with HAFs, progesterone concentration was lower (approached significance) 3 d after the beginning of follicular hemorrhage, as indicated by extensive echoic specks in the follicular fluid [23]. It appears that the discrepancy in progesterone concentrations in LUF cycles among studies may be explained by interspecies variation and different methods of LUF formation. If progesterone production is decreased in LUF cycles, then other reproductive hormones interrelated to progesterone are also likely to be affected. To date, there has been no report on the systemic interrelationships among reproductive hormones after experimental induction of LUFs with PGHS inhibitors in mares.

The objective of this study was to determine differences in follicle and reproductive hormones (estradiol, LH, progesterone, and PGF metabolite) characteristics in mares with ovulatory and FM-induced anovulatory cycles.

2. Materials and methods

2.1. Animals

Mares were mixed breeds of large ponies and apparent pony-horse crosses. The mares were weighed at the beginning of the study (300–460 kg). Mares selected had a docile temperament and no apparent abnormalities of the reproductive tract, as determined by ultrasonographic examinations [29]. The experiments were done during July to August 2010 (summer in the northern hemisphere: Wisconsin, USA). The mares were kept under natural light in an open shelter and outdoor paddock and were maintained by free access to a mixture of alfalfa and grass hay, water and trace-mineralized salt. All mares remained healthy and in good body condition throughout the study. Mares were handled according to the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

2.2. Experiment 1

A total of 23 mares was studied. Fourteen days after ovulation, mares were examined daily by transrectal B-mode ultrasonography of the internal genital tract

using an ultrasound scanner (Aloka SSD-900; Aloka America, Wallingford, CT, USA) with a linear array 7.5 MHz transducer. Follicular diameters were determined as described previously [29]. When a mare first showed an endometrial edema score of three to four (four = maximum degree of endometrial folding) and a follicle ≥ 32 mm in diameter (0 h), hCG (Chorulon; Intervet Inc., Millsboro, DE, USA) was given in a single intravenous dose of 1500 IU. Mares were allocated to a control group with no further treatment (n = 10 mares) or an FM group (n = 13 mares). The FM mares were treated with 1.7 mg/kg of FM (FluMeglumine; Phoenix Pharmaceutical Inc., St Joseph, MO, USA) i.v. at zero, 12, 24 and 36 h.

Beginning at 0 h (hour of hCG administration), mares from each group (control and FM) were scanned ultrasonographically twice daily at 12 h intervals until 132 h and again at 216 h (Day 9). Beginning at 30 h, mares were scanned every 2 h until detection of ovulation. If ovulation had not occurred by 42 h, the mare was not further examined until 48 h. Finally, mares were scanned daily beginning 14 d after hCG treatment and continued until the next ovulatory period. At each ultrasonographic examination, the antrum of the follicle was evaluated by ballottement of the ovary to detect floating echoic-particles. Real-time B-mode images were captured with an on-line digital video-taping system and stored for later analysis. A luteinized unruptured follicle (LUF) was diagnosed ultrasonographically when a follicle failed to rupture and the antrum gradually filled with increasing amounts of echoic particles. The amount of antral echoic particles was evaluated subjectively upon ballottement of the ovary. A moderate amount of specks was determined when the number of echoic particles was too numerous to count. Eventually, the follicular contents became organized and firm. Luteinization of follicular cells was assumed by an increase in echogenicity of the unruptured follicular wall and confirmed by an increase in plasma progesterone concentration. Ovulation was diagnosed when the follicle ruptured between 36 and 48 h after hCG administration, with subsequent evacuation of > 90% of follicular fluid. A collapsed LUF was diagnosed when the follicular antrum filled with echoic specks by 48 h and the follicular wall increased in echogenicity. By 60 h, the collapsed LUF had ruptured with evacuation of > 90% of follicular fluid and floating echoic specks. The term "follicular collapse" was used to refer to the exact moment of follicular rupture of an ovulation. Two FM-treated mares that developed LUFs were also scanned every 2 h from 42 to 48 h to determine the exact hour at which one or more echoic specks were first observed.

Endometrial edema was assessed according to the prominence and size of endometrial folds by ultrasonographic examination of the uterine horns and body and scored subjectively from one (no endometrial folding) to four (maximum endometrial folding) as described [29]. To compare the effect of ovulation versus LUF formation on the equivalent of the interovulatory interval, the interval from hCG treatment (0 h) to ovulation at the end of the next preovulatory period was used for FM and control mares. This was done because ovulation was not available as a reference point in the mares that formed an LUF. A diestrous ovulation was defined as an ovulation occurring during the luteal phase (progesterone concentration > 2 ng/mL).

Ovulation between 0 to 36 h in four mares that had been assigned to the control group (n = 2) and the FM group (n = 2) was assumed to have been triggered by an early spontaneous endogenous LH surge and not from administration of hCG [17]; these mares were excluded from the study. In addition, a control mare was removed from the study because the largest follicle at the hour of hCG treatment did not ovulate or show signs of follicular luteinization (increase in echogenicity of follicular wall). The follicle of this mare became gradually smaller and a new follicle grew and ovulated. Therefore only mares that ovulated between 36 and 48 h were used as controls, since mares ovulating > 48 h after hCG treatment are known to not have responded to the initial ovulatory induction treatment [17]. Thus, 18 mares (seven control and 11 FM treated mares) remained for data analyses. In the FM group, only mares that developed an LUF (n = 8) were used for comparisons of follicular and hormonal characteristics between control and LUF mares.

Blood samples were collected from the jugular vein every 12 h from 0 to 132 h, and again at 216 h (0 h = hour of hCG treatment). Luteinizing hormone was assayed for all samples (0 to 216 h), whereas estradiol and PGF metabolite (PGFM) were assayed only for samples from 0 to 60 h and 0 to 72 h, respectively. Progesterone concentration was determined daily from 0 to 136 h, and then at 216 h.

2.3. Experiment 2

This experiment used three FM-treated mares from Experiment 1 that ovulated (n = 1) or showed ultrasonographic signs of antral hemorrhage and luteinization of the follicular wall, but the structure collapsed between 48 and 60 h and formed a *corpus luteum*

(collapsed LUF; n = 2). These three mares were treated with the same dose of hCG at the same follicle diameter as in Experiment 1 during the next preovulatory period. However, FM was administered at a higher frequency: 1.7 mg/kg i.v. at zero, 12, 24, 30, 36 and 48 h after hCG. The modification of the FM protocol with an increase in overall dose was made under the assumption that these mares failed to form fully developed LUFs owing to insufficient inhibitory activity of FM on PGHS-2. Transrectal B-mode ultrasonography and blood sampling were performed at the same intervals as in Experiment 1, as well as every 2 h from 48 to 60 h if mares had not ovulated by 48 h. Plasma concentrations of progesterone and LH were determined at 24 h intervals from 0 to 132 h and at 216 h.

2.4. Blood samples and hormone assays

Blood samples were collected into heparinized tubes, immediately placed in ice-cold water for 10 min, and centrifuged (2000 × g for 10 min). The plasma was decanted and stored (−20 °C) until assayed. The plasma samples were assayed for PGFM by an enzyme immunoassay that was developed in our laboratory for use in bovine plasma and has been described in detail [30]. The assay was adapted and validated for use in equine plasma with the modification that plasma from flunixin meglumine-treated horses was used for preparing standards. Serial volumes of a pool of equine plasma (100 to 7.5 μL) from PGF-treated mares (containing high PGFM concentration) were processed as for the experimental samples and resulted in a displacement curve that was similar to the standard curve. The intra- and interassay CVs and sensitivity were 13.1%, 10.1%, and 8.0 pg/mL, respectively. Plasma samples were assayed by validated radioimmunoassay, as described for mare plasma in our laboratory for LH [31], progesterone [32], and estradiol [33]. The intra-assay CV and sensitivity for LH and progesterone were 7.1% and 0.09 ng/mL and 13.4% and 0.02 ng/mL, respectively. The intra- and interassay CVs and sensitivity for estradiol were 10.7%, 6.7% and 0.07 pg/mL, respectively.

2.5. Statistical analyses

Sequential data for hormone concentrations were analyzed by the SAS MIXED procedure with a repeated statement to account for autocorrelation between sequential observations (Version 9.2; SAS Institute, Cary NC, USA). If an effect of group (control and FM) or an interaction of group and hour was significant in Experiment 1, data were examined further by an unpaired Student's *t*-test within each hour, whereas a difference between hours within a group was examined by a Student's paired *t*-test. Frequency data were analyzed by a chi-square test or Fisher's exact test when a cell had less than five observations. Individual values of hormone profiles from mares of Experiment 2 were compared with the mean values and 95% confidence intervals (CI) of the same group in Experiment 1. A probability of $P \leq 0.05$ indicated that a difference was significant, whereas probabilities between $P > 0.05$ and $P \leq 0.1$ indicated that a difference approached significance. Data are given as mean ± SEM, unless stated otherwise.

3. Results

3.1. Experiment 1

All seven control mares were ovulatory with ovulations occurring between 36 and 48 h after hCG administration (Table 1). In contrast, eight of 11 FM mares in the FM group were anovulatory and developed an LUF. Ultrasonograms of an ovulatory follicle and early luteal development (Fig. 1) and a follicle that formed an LUF (Fig. 2) are shown. All unruptured follicles showed

Table 1
Results (Mean ± SEM) of treatment of mares with hCG when the preovulatory follicle (POF) was ≥ 32 mm (0 h) followed by flunixin meglumine (FM), an inhibitor of prostaglandin synthetase, at zero, 12, 24, and 36 h (Experiment 1).

End points	Ovulatory controls (n = 7)	Anovulatory FM-treated (n = 8)[a]	Probability
Diameter POF at 0 h (mm)[a]	34.2 ± 0.4	35.2 ± 0.6	NS
Interval from 0 h to 1st ovulation (h)[b]	39.1 ± 0.8	—	—
Interval from 0 h to 2nd ovulation (days)[b]	23.6 ± 0.7	24.5 ± 0.9	NS
Endometrial score at 36 h[c]	2.1 ± 0.4	2.1 ± 0.3	NS
Multiple outcomes (n)[d]	0/7	0/8	—
Diestrous ovulations (n)	0/7	2/8	NS
Maximum concentration of LH (ng/mL)	19.5 ± 5.2	23.7 ± 6.5	NS
Progesterone concentration at 216 h (ng/mL)	11.6 ± 1.6	5.7 ± 11.6	$P < 0.02$

[a] Anovulation was associated with formation of a luteinized unruptured follicle (LUF) in eight of 11 mares.
[b] The 1st ovulation is from the POF at the hour of hCG treatment, and the 2nd ovulation is from the POF of the next preovulatory period.
[c] Score for endometrial edema ranged from one (minimal) to four (maximal).
[d] An outcome is an ovulation or LUF.
NS, Not significant.

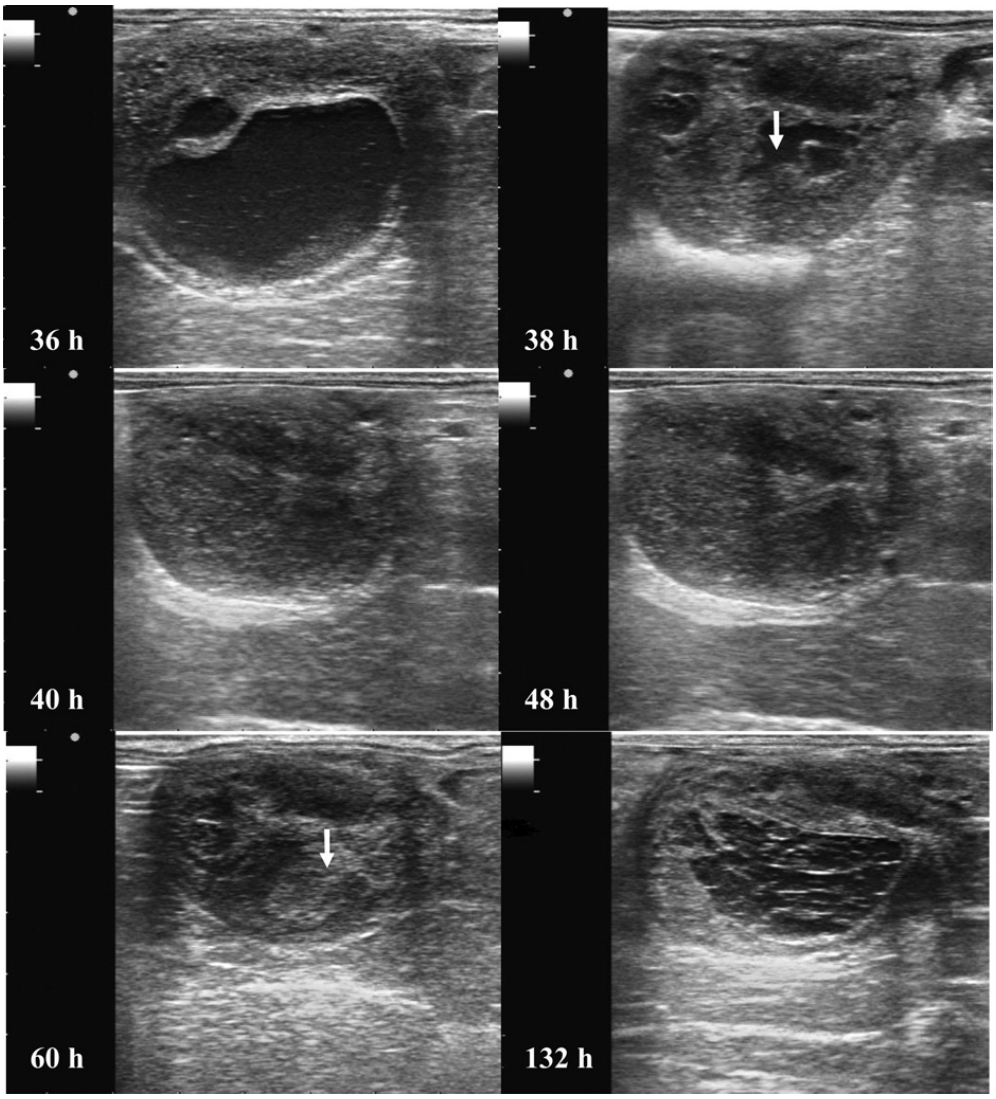

Fig. 1. Representative B-mode ultrasonograms of ovulation in a control mare showing changes relative to hCG administration (0 h) during the periovulatory period and early corpus luteum development. 36 h: first appearance of echoic particles within the follicular antrum (indicative of impending ovulation in some preovulatory follicles). 38 h: follicular collapse during the process of fluid evacuation; note the remaining follicular anechoic fluid in the centre of the image (white arrow). 40 h: end of follicular collapse (completion of fluid evacuation). 48 h: hypoechoic area of collapsed follicle. 60 h: early *corpus luteum* development; note the increased echogenicity of the folds of the collapsed follicle indicating formation of luteal tissue (white arrow). 132 h: well developed *corpus luteum* with a central blood clot surrounded by luteal tissue; between 60–132 h, the *corpus luteum* developed a blood clot with formation of echoic strands.

presence of some echoic specks floating freely in the antrum at 48 h. The amount of specks increased gradually at the following examination. By 60 h, the number of specks was too numerous to count. No FM-treated mare had any echoic speck within the follicular antrum by 42 h. In the two FM mares examined every 2 h from 42 to 48 h, the first evidence of antral hemorrhage (floating echoic specks) was observed at 44 h (Fig. 2), but the number of specks at this hour was low (< 10). The follicular contents of LUFs did not move freely upon ballottement after 115.5 ± 6.4 h.

The size of the LUFs increased gradually to a maximum mean diameter of 63.8 ± 1.4 mm (range 56 to 69 mm) at 105 ± 6.7 h. Diameter of LUFs decreased between 132 and 216 h. At 216 h, LUFs were larger ($P < 0.001$) than corpora lutea of control mares. Follicle diameter did not change in either group during zero to 36 h, whereas LUFs had a greater ($P < 0.05$) diameter at 48 h than at 36 h (Fig. 3).

Estrous cycle characteristics for the seven control and 8 FM-treated mares that developed LUFs are shown (Table 1). The scores for endometrial edema decreased in both groups from zero to 36 h and were not significantly different between groups at any hour. The interval from hCG treatment during the experimental preovulatory period to the ovulation of the next

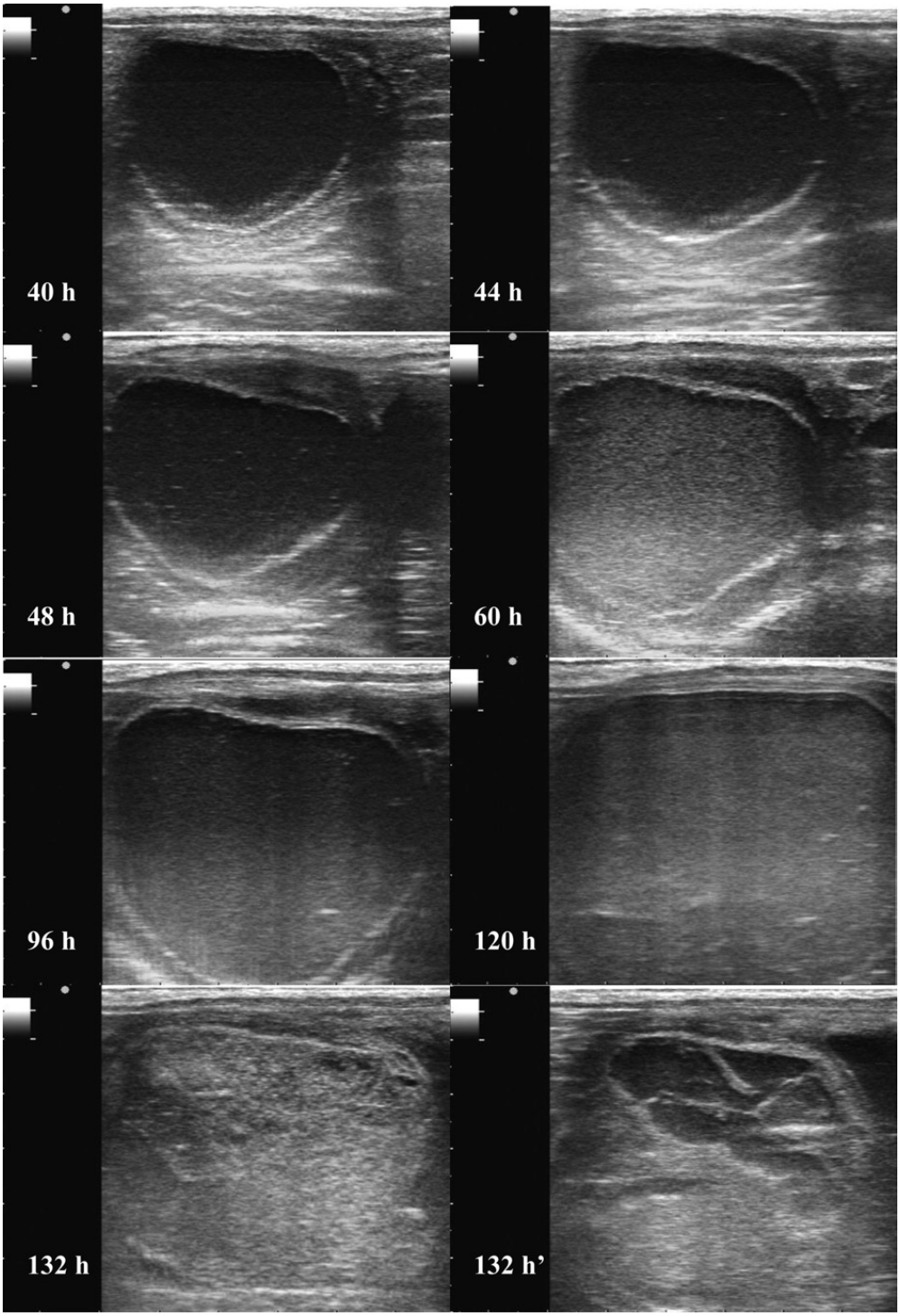

Fig. 2. Representative B-mode ultrasonograms of a mare from a flunixin meglumine (FM) treated cycle showing changes relative to hCG administration (0 h). 40 h: echoic-free follicular antrum. 44 h and 48 h: slight amount of echoic particles floating within the follicle and increased echogenicity of granulosa layer. 60 h: increase in apparent follicular hemorrhage and diameter from the previous image 12 h earlier. 96 and 120 h: gradual increase in follicular diameter. 132 h: follicular contents appear organized and firm upon ballottement; note the solid-like echoic mass; 132 h': opposite edge of same luteinized unruptured follicle with strands and specks within a cavity. The specks and strands floated and quivered, respectively, upon ballottement.

preovulatory period (second ovulation in controls) was not significantly different between the control and LUF groups. There were no cycles with multiple outcomes (outcomes = ovulations or LUFs). In two LUF mares (two out of eight mares, 25%), diestrous ovulations occurred between nine and 14 d after hCG treatment.

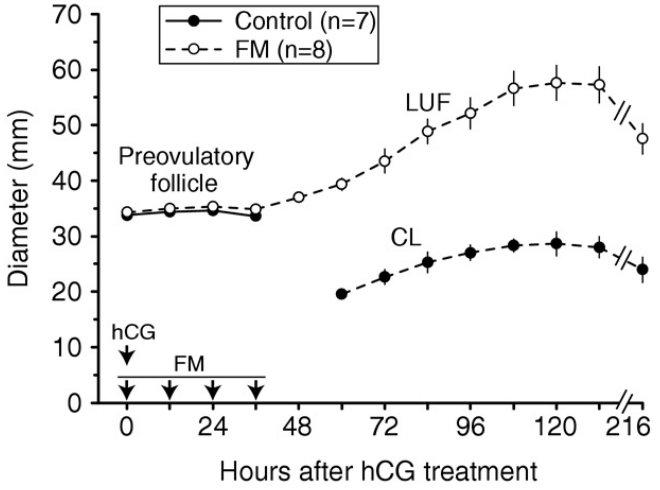

Fig. 3. Mean (± SEM) of diameter of ovarian structures in ovulatory and flunixin meglumine-induced (FM) luteinized unruptured follicle (LUF) cycles. Diameter of the follicle increased ($P < 0.05$) between 36 and 48 h in LUF cycles.

One returned to estrus and showed endometrial edema spontaneously 20 d after hCG treatment. The other did not and was excluded from the analysis of length of the interval from hCG treatment to the second ovulation. None of control interovulatory intervals had diestrous ovulations.

Comparisons of PGFM, LH, progesterone and estradiol concentrations were done only between the seven control mares and the 8 FM-treated mares that developed LUFs (Fig. 4). The effect of group on PGFM concentrations approached significance, but the effect of hour and the group-by-hour interaction were not significant (Fig. 4). The highest concentration of PGFM in the ovulatory group was low (maximum, 38 pg/mL). LUF mares had PGFM values near the assay sensitivity concentration (7 pg/mL) through 12 to 72 h. Control mares had higher ($P < 0.05$) PGFM concentrations than LUF mares at 36 h. The frequency of an increase in PGFM concentrations between 24 and 36 h was greater ($P < 0.04$) in the controls (increase in five of seven mares) than in the LUF group (one of eight mares). For LH concentration, the main effect of hour and the interaction between group (control and LUF) and hour were significant (Fig. 4). The group-by-hour interaction represented in part a decrease in LH concentration in the controls from 36 to 48 h, increase until 84 h, and decrease from 84 to 216 h. In contrast, LH concentration in LUF mares did not decrease between 36 and 48 h, but decreased between 108 to 216 h. The concentration of LH was greater in LUF mares than in the control group at 108 and 120 h.

The main effect of group and the group-by-hour

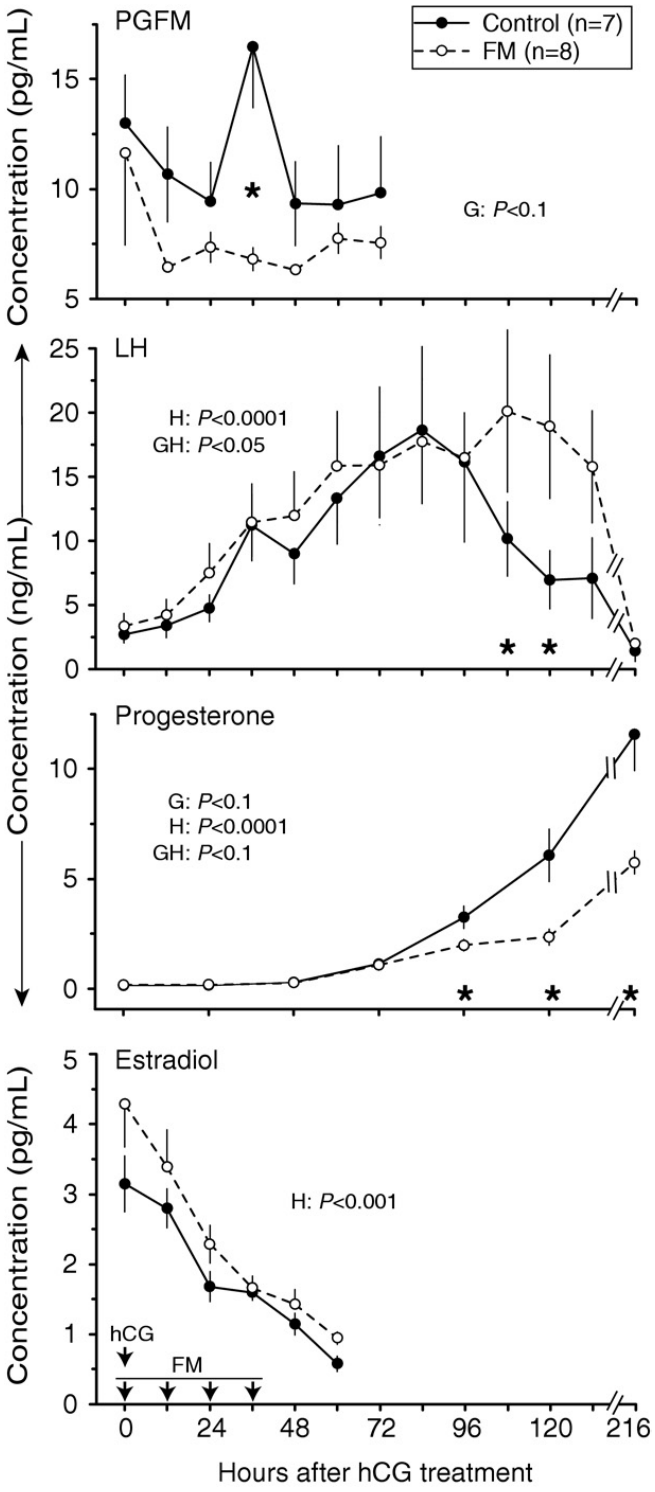

Fig. 4. Mean (± SEM) plasma concentrations of PGF metabolite (PGFM), LH, progesterone and estradiol in ovulatory control (n = 7) and flunixin meglumine-induced (FM) luteinized unruptured follicle (LUF) cycles (n = 8) from 0 h (hour of administration of an ovulatory dose of hCG). Probabilities for main effects of group (G) and hour (H) and the group-by-hour interaction (GH) are shown. Asterisks (*) denote a difference ($P < 0.05$) in PGFM, LH and progesterone concentrations between controls and LUF mares within an hour.

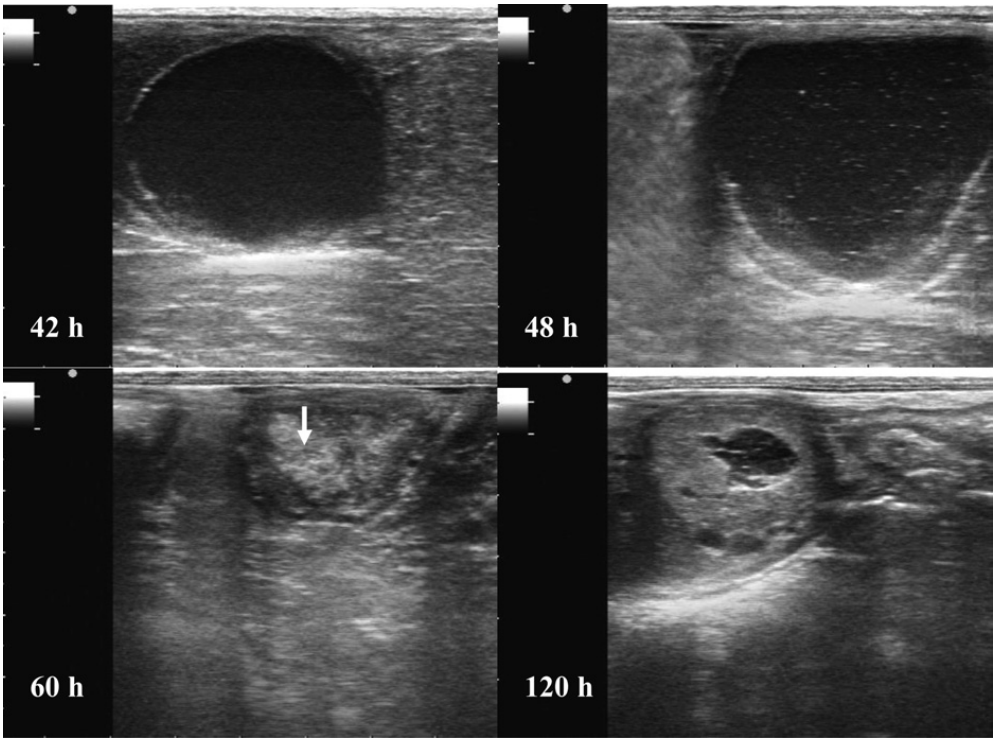

Fig. 5. Representative B-mode ultrasonograms of one mare treated with flunixin meglumine (FM) (Mare C, *Experiment 1*) of changes relative to hCG administration (0 h). 42 h: echoic-free follicular antrum. 48 h: presence of slight amount of echoic particles. 60 h: ovary with hyperechoic area (white arrow) of collapsed luteinized unruptured follicle (LUF). 120 h: corpus luteum with central cavity. Plasma progesterone concentration of Mare C at 48 and 120 h was 0.6 and 4.6 ng/mL respectively.

interaction for progesterone concentration approached significance (Fig. 4). The interaction represented lower ($P < 0.05$) progesterone concentration in LUF mares than in control mares at 96, 120 and 216 h. The rate of increase in progesterone concentration from 120 to 216 h was not significantly different between groups. The effect of hour on progesterone concentration resulted from a significant increase between each set of sequential hours beginning at 24 h. For estradiol, there was an hour effect from a gradual decrease between zero and 60 h averaged over groups, but no group effect nor group-by-hour interaction (Fig. 4).

For the three mares in the FM group that did not form a conventional LUF, one mare ovulated (Mare A) at 42 h. In Mares B and C, the follicle appeared to initially form an LUF, but collapsed at 60 h. The follicular wall was hyperechoic within 48 h and the antrum contained many fine echoic particles that floated upon ballottement (Fig. 5). In Mares B and C, 60 h (hour of LUF collapse) was beyond the 95% CI for the hour of ovulation in the controls (Table 2).

The PGFM concentrations for each of Mares A, B, and C during the first experimental period are shown (Fig. 6). The PGFM concentration in the three FM mares that ovulated or did not form a conventional LUF remained near assay sensitivity concentrations throughout the sampling period in two of the three mares (Mares A and B; Fig 6). Mare C had a transient increase in PGFM concentration at 12 h and began to increase again after 36 h. None of the mares had a transient increase in PGFM at 36 h. The LH and progesterone concentrations for each of the three mares are shown (Fig. 7). The relationships of individual follicular and hormonal values for Mares A, B and C within each experiment to the 95% CI in the control and LUF groups of Experiment 1 are shown (Table 2).

3.2. Experiment 2

Two of the 3 FM mares that ovulated or did not form a conventional LUF in Experiment 1 (Mares A and B) had the same outcome after administration of FM at increased frequency during the next preovulatory or experimental period (Experiment 2). During the second experimental period, Mare A ovulated again at 42 h without previous development of echoic particles within the follicular antrum. Mare B developed echoic particles and increased echogenicity of the follicular wall by 48 h, maintained the same follicular appearance

Table 2
Relationships of individual follicular and hormonal values for Mares A, B, and C within Experiments 1 and 2 to the 95% confidence intervals (CI) of the control and LUF groups of Experiment 1.

End points	Experiment 1					Experiment 2		
	95% CI[a]		Individuals			Individuals		
	Controls	LUF group	A	B	C	A	B	C
0 h to ovulation (h)[b]	37.1–46.2	Anov	42	60	60	42	54	Anov
Maximum LH (ng/mL)	7.2–31.8	8.3–39.1	1.4	3.0	22.5	1.1	3.2	NA
Progesterone at 216 h (ng/mL)	7.4–15.8	4.5–7.0	5.9	11.5	9.9	4.3	10.8	5.2

[a] The 95% CIs are for the control mares (n = 7) and for the flunixin meglumine (FM) mares (n = 8) with a luteinized unruptured follicle (LUF). Mares A, B, and C were in FM group of Experiment 1 but did not form an LUF, and were repeated in Experiment 2 during the next preovulatory period, using greater frequency of FM treatment.
[b] 0 h is hour of hCG treatment when the largest follicle was ≥ 32 mm.
Anov, anovulatory (LUF); NA, not available.

until 50 h, had a collapsed LUF at 54 h, and eventually formed a solid *corpus luteum*. The interval from hCG treatment to collapse of the LUF for Mare B was above the 95% CI for the control group, but the interval was within the 95% CI for Mare A (Table 2). In contrast, Mare C developed an LUF which remained unruptured throughout the cycle.

The concentrations of LH and progesterone for each of the three mares during the second experimental period (Experiment 2) are shown (Fig. 7). Maximum concentrations of LH for Mares A and B in Experiment 2 were below the 95% CI for control and the LUF groups of Experiment 1 (Table 2). The concentration of LH was not available for Mare C. The concentrations of progesterone at 216 h for Mares A and C were below the 95% CI for the controls and within the CI for the other LUF mares. For Mare B, the progesterone concentration at 216 h was within the 95% CI for the controls and above the 95% CI for the LUF mares (Table 2).

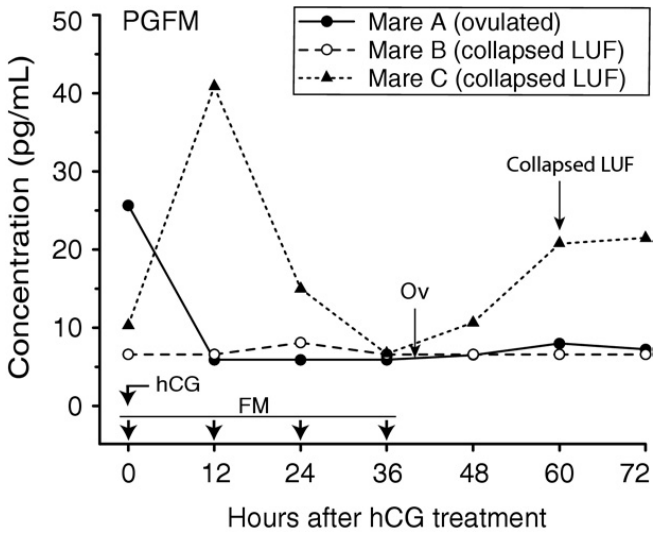

Fig. 6. Mean (± SEM) plasma PGF metabolite (PGFM) concentrations in the three flunixin meglumine-treated (FM) mares that ovulated or had a collapsed luteinized unruptured follicle (LUF) from 0 h (hour of hCG administration) to 72 h. Mare A ovulated (42 h) within the expected interval of ovulatory control mares; the ovulation (ov) is indicated by an arrow. Mares B and C had not ovulated by 48 h, but had a slight amount of antral echoic specks; the LUFs collapsed between 48 and 60 h; the time of LUF collapse is indicated by an arrow (Collapsed LUF).

4. Discussion

The administration regime and dose of FM prevented the mean transient PGFM increase 36 h after hCG treatment and blocked ovulation, with formation of an LUF in eight of 11 mares. The starting dose was based on a preliminary study in which 2 mg/kg inhibited ovulation in a small number of mares [27]. All previous studies on the production of LUFs by administration of PGHS inhibitors in several species involved treatment into the follicular antrum [7,11,14]. One report [13] showed that only intra-ovarian administration of indomethacin and not intramuscular or intrauterine infusion inhibited ovulation in cows even after intramuscular administration of high and repeated doses. The authors concluded that systemic indomethacin treatment did not provide sufficient inhibition of local production of follicular PGHS. Each sequential dose of FM in the current study was given i.v. at 154% of the manufacturer's recommended clinical dose (datasheet information FluMeglumine®). It appeared that systemically treated FM gained access to the granulosa cells, and blocked the activity of PGHS-2. This enzyme is expressed within equine granulosa cells between 30 and

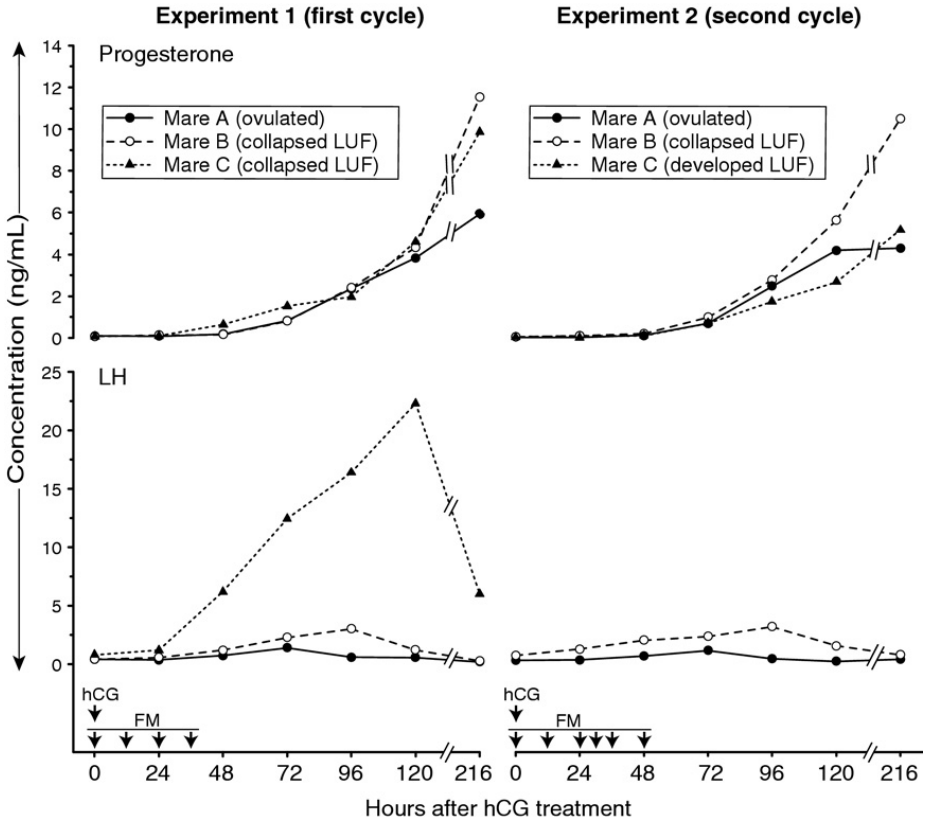

Fig. 7. Mean (± SEM) plasma concentrations of LH and progesterone in mares A, B, and C in their first cycle of *Experiment 1,* and in their consecutive cycle of *Experiment 2*. The mares were given 1.7 mg/kg flunixin meglumine (FM) at zero, 12, 24, and 36 h (*Experiment 1*) and at zero, 12, 24, 30, 36, and 48 h (*Experiment 2*) after administration of hCG (0 h). Concentration of LH was not available for Mare C in *Experiment 2*. Mare A and B ovulated and had a luteinized unruptured follicle (LUF) collapse in both consecutive cycles, respectively. Mare C had an LUF collapse in Cycle 1, but developed an LUF which remained unruptured throughout the consecutive cycle (*Experiment 2*).

39 h after hCG administration [16]. The inhibition of PGHS-2 activity during the preovulatory LH surge resulted in failure of the follicular wall's breakdown and collapse. Luteinization of the follicular cells occurred with active production of progesterone, entry of ultrasonographically echoic particles into the antrum apparently from hemorrhage, and a gradual increase in follicular diameter. This cascade of events also occurs in women after inhibition of ovulation by blockage of production of prostaglandins with oral treatment of indomethacin [12].

There was a delay of a few hours between the time of ovulation at a mean of 39 h after hCG treatment in control mares and the time when the follicular fluid of FM-treated mares contained a moderate amount of echoic specks. The follicular entry of echoic particles occurred between 42 h (last examination at 2 h intervals) and the next examination at 48 h. This was no earlier than 44 h in the two mares examined more frequently. A previous report on HAFs [23] concluded that the day of ovulation (day of disappearance of the preovulatory follicle) corresponded to the day of the beginning of HAF formation, as indicated by amounts of echoic specks greater than would normally expected before ovulation (too numerous to count). The amount of echoic particles observed in each LUF at 48 h was small and was not apparently different from that observed in some ovulatory mares a few hours before ovulation [34]. In addition, the apparent entry of a moderate amount of blood into the antrum did not occur until 60 to 72 h in most mares (specks too numerous to count). It appeared that the mechanisms by which blood vessels release blood into the follicular antrum during development of LUFs did not commence until several hours after the expected time of follicular collapse in ovulatory cycles. The preovulatory follicle did not increase in diameter in either group during zero to 36 h, consistent with a previous report on preovulatory follicles in mares [35]. In the controls, ovulation occurred between 36 and 48 h, and in the LUF group the follicle diameter increased between 36 and 48 h. These diameter results were consistent with the conclusion that development of an LUF begins between 36 and 48 h after hCG treatment.

The endometrial edema score decreased between zero to 36 h in each group and followed a similar pattern in control and LUF mares. The lack of difference between groups was not surprising, since the estradiol and progesterone profiles were also similar. The degree of endometrial folding depends upon the concentration of systemic estradiol [36,37] and progesterone [36]. Furthermore, luteal tissue of LUFs responded to endogenous release of PGF in that all LUF mares, except one, ovulated within a period similar in length to that in control mares. In two mares with a LUF, diestrous ovulations occurred between nine and 14 d after hCG administration. Although the 25% incidence of diestrous ovulation in LUF mares was not significantly greater than the 0% of the controls, diestrous ovulations are rare in pony mares (< 1% of slaughterhouse specimens with *corpora lutea*) [38]. The apparent propensity for diestrous ovulations in LUF mares likely reflected the greater LH concentration in LUF cycles than in control mares during the early luteal phase (96 to 132 h after hCG).

The differences in systemic reproductive hormones between ovulatory and LUF mares involved progesterone and LH. The lower concentrations of progesterone in LUF mares may be explained by the reduced vascularisation of developing luteal tissue in unruptured follicles compared with that of *corpora lutea* which originate from collapsed follicles. The increased LH concentration in LUF mares can be attributed to a slower increase in progesterone concentration during luteinization of the follicular cells. The lower progesterone concentration likely resulted in a reduced negative feedback on LH [39], which in turn allowed the gradual increase in LH to continue for longer and to greater values in LUF mares than in ovulatory mares. By 216 h, the LH mean concentrations of both groups had decreased to similar values. At this time (Day 9), although the progesterone concentration in LUF mares was lower than that of ovulated controls, the negative feed back of progesterone on LH may have been sufficient to reduce LH release. In each group, estradiol decreased during the 12 h after administration of hCG. The timing of the decrease in estradiol relative to hCG treatment in the mare was in agreement with other studies [37], and has been reviewed [40].

In a previous study [23], the concentrations of LH, estradiol, and progesterone in naturally occurring HAF cycles were compared daily with those of ovulatory cycles between Day -4 and Day 7 (Day zero = day of ovulation or anovulation). The progesterone concentration was lower (approached significance) in HAF cycles on Day 3, and LH concentrations were significantly greater from Days 3 to 5. The concentration of estradiol was only significantly greater in HAF cycles on Day −3. The progesterone and LH profiles of HAF mares from the reported study coincided largely with those of LUF mares from the current study in LH, but not in progesterone. However, there was a marked reduction in progesterone in the LUF mares in the current study. Two factors make comparisons difficult between studies of spontaneous HAFs and the present study of FM-induced LUFs. Firstly, the day of beginning of HAF formation (Day zero) of the reported study was assigned retrospectively and subjectively according to the day at which follicular echoic specks were too numerous to count, or alternatively the day before echoic-strands formation were first detected. In this regard, the LUFs of the current study did not show echoic-strands formation until a mean of 115 h after hCG treatment (range of 84 to 132 h). If the designation of Day zero for LUFs according to the day of specks formation or the day before beginning of strand formation and ovulatory cycles (day of ovulation) had been performed in this study according to the same criteria as in the HAF study [23], Day zero of LUF mares would have been equivalent to 60 to 108 h instead of 48 h (0 h = administration of hCG). Secondly, mares with HAFs were examined once a day instead of twice a day. The use of FM to induce LUFs in mares can be a useful model to research the spontaneous anovulatory condition of HAF, since both structures share ultrasonographic and hormonal characteristics. However, it is important to bear in mind that the pathogenic mechanisms behind the ovulatory failure in mares with spontaneous HAF syndrome may be totally different from those responsible for the lack in follicular rupture in FM-induced LUFs. Prostaglandins are only one factor of many others involved in the cascade of events that lead to the process of follicular breakdown and ovulation [1]. Nevertheless, provided that the incidence of naturally-occurring HAFs is relatively low and unpredictable, the use of experimentally FM-induced LUFs could be used for future studies to evaluate different treatment options for anovulation in mares.

The PGF metabolite (PGFM) in FM-treated mares was reduced to assay sensitivity concentrations within 12 h of the first administration of FM. The increase in PGFM from 24 to 36 h, and a decrease from 36 to 48 h in the ovulatory mares may have resulted from passage of PGF from the follicular fluid to the peripheral circulation with metabolism of PGF. The PGF concentration in follicular fluid in the

mare increases from undetectable values at 30 h to 10 ng/ml at 36 h after hCG treatment [16]. The highest concentration of plasma PGFM was relatively low (< 40 pg/mL) in the control mares compared with the concentration of systemic PGFM pulses resulting from endometrial PGF secretion during equine luteolysis (> 300 pg/mL) [41]. One of the FM mares (Mare C) with an LUF collapse between 48 to 60 h in Experiment 1 had a gradual increase in PGFM between 48 to 72 h. The clinical response of horses to FM at the recommended dose of 1.1 mg/kg lasted for approximately 10 h [42] and its half-life in plasma ranges from 1.6 to 2.5 h [43]. Mare C had an LUF collapse during the first experimental period (Experiment 1), but developed an LUF during the next cycle when FM was given at a greater frequency which remained unruptured throughout the cycle. Therefore, it could be speculated that Mare C did not form a fully developed LUF because of an insufficient inhibitory effect of the last administration of FM on PGHS activity. However, published data on pharmacodynamics and pharmacokinetics of FM in equine follicular fluid are not available to confirm this speculation.

The FM-treated mares in Experiment 2 that ovulated (Mare A) or had a collapsed LUF (Mare B) in spite of increased overall dose and frequency of FM, had a consistently lower LH concentration in each of the two consecutive cycles than the LH concentrations in ovulatory or LUF mares in Experiment 1. It is known that some mares are able to ovulate with minimal LH peripheral concentrations, based on the ability of hCG to induce ovulation in mares treated with a GnRH antagonist [44,45]. Perhaps, prostaglandins are not essential to trigger the cascade of events that lead to follicular wall degradation and ovulation in mares with minimal LH concentrations. Furthermore, there appeared to be only a minimal LH requirement for luteinization for follicular cells and subsequent progesterone secretion by the CL in the current study.

In conclusion, after treatment of mares with an ovulatory dose of hCG, systemic treatment with a PGHS inhibitor (*flunixin meglumine*) reduced the circulating concentrations of PGFM by 12 h after treatment, blocked ovulation in eight of 11 mares, and resulted in development of luteinized unruptured follicles (LUFs). The differences in systemic hormone concentration between LUF and ovulatory mares were lower progesterone in mares that developed LUFs than in controls, and greater LH concentrations by 96 h after hCG treatment. Systemic administration of FM from zero to 36 h after hCG treatment may be useful research model for study of the mechanisms of ovulation and the relationships between changes in progesterone and LH concentrations.

Acknowledgements

This study was supported by Eutheria Foundation (study n° E1-JCA-10) and by the Grant "Beca de Movilidad CEU-Santander" awarded to Dr Juan Cuervo-Arango by the "Universidad CEU Cardenal Herrera" in collaboration with "Banco Santander."

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