Elsevier Editorial System(tm) for Theriogenology
Manuscript Draft

Manuscript Number:

Title: Systemic treatment with high dose of flunixin-meglumine is able to block ovulation in mares by inducing haemorrhage and luteinization of follicles

Article Type: Original Research Article

Keywords: Mare; Luteinized haemorrhagic follicle; Prostaglandin inhibitor; Flunixin-meglumine

Corresponding Author: Mr Juan Cuervo-Arango, DVM MSc

Corresponding Author's Institution: Royal Veterinary College

First Author: Juan Cuervo-Arango, DVM MSc

Order of Authors: Juan Cuervo-Arango, DVM MSc; Rosana Domingo-Ortiz

Abstract: Prostaglandins play an obligatory role during the process of ovulation in mammals. Ovulation can be blocked by intrafollicular administration of non-steroidal anti-inflammatory drugs (NSAIDs) in several domestic species included the mare as well as by systemic administration of these drugs in women. In the mare, the effect of systemic NSAIDs treatment on ovulation has not been critically studied. The objectives of this study were two folds: a) to determine whether high dose of flunixin-meglumine (FM) administered systemically to mares during the peri-ovulatory period was able to block ovulation; and b) to study in detailed the follicular ultrasound characteristics of FM treated mares. Six mares were used in the study during two consecutive oestrous cycles. Each mare received 2 mg FM / kg i.v. twice a day at the time of treatment with hCG when the follicle reached a diameter of ≥ 32 mm and was continued until ovulation or beginning of follicular haemorrhage. During the consecutive control cycle (CON) the mares received the same dose of hCG but were not administered FM. During the FM cycles five of six mares failed to ovulate and collapse the preovulatory follicle but filled with echoic specks and continued to grow until a mean diameter of 55 mm, eventually the follicular contents organized and luteinized. All CON mares ovulated normally. In conclusion, cycles treated with FM had a higher incidence of ovulatory failure and development of luteinized unruptured follicles (83%, P = 0.015) than CON cycles.

Systemic treatment with high dose of flunixin-meglumine is able to block ovulation in mares by inducing haemorrhage and luteinization of follicles

J. Cuervo-Arango*, R. Domingo-Ortiz

Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, Moncada, Spain

Abstract

Prostaglandins play an obligatory role during the process of ovulation in mammals. Ovulation can be blocked by intrafollicular administration of non-steroidal anti-inflammatory drugs (NSAIDs) in several domestic species included the mare as well as by systemic administration of these drugs in women. In the mare, the effect of systemic NSAIDs treatment on ovulation has not been critically studied. The objectives of this study were two folds: a) to determine whether high dose of flunixin-meglumine (FM) administered systemically to mares during the peri-ovulatory period was able to block ovulation; and b) to study in detailed the follicular ultrasound characteristics of FM treated mares. Six mares were used in the study during two consecutive oestrous cycles. Each mare received 2 mg FM / kg i.v. twice a day at the time of treatment with hCG when the follicle reached a diameter of ≥ 32 mm and was continued until ovulation or beginning of follicular haemorrhage. During the consecutive control cycle (CON) the

* Corresponding author: (J. Cuervo-Arango). Email: juan.cuervo@uch.ceu.es

mares received the same dose of hCG but were not administered FM. During the FM cycles five of six mares failed to ovulate and collapse the preovulatory follicle but filled with echoic specks and continued to grow until a mean diameter of 55 mm, eventually the follicular contents organized and luteinized. All CON mares ovulated normally. In conclusion, cycles treated with FM had a higher incidence of ovulatory failure and development of luteinized unruptured follicles (83%, P = 0.015) than CON cycles.

Keywords: Mare; Luteinized haemorrhagic follicle; Prostaglandin inhibitor; Flunixin-meglumine

---

1. Introduction

The first step in female reproduction that allows a successful fertilization and subsequent pregnancy begins after the collapse of the preovulatory follicle with follicular fluid evacuation and eventually release of the oocyte into the fallopian tubes. Only after the completion of this stepwise process, fertilization is possible.

During the process of follicular collapse that precedes ovulation, the preovulatory LH surge initiates a cascade of events that leads to extracellular matrix (ECM) degradation and follicular wall breakdown. Amongst them, prostaglandins (PGE and PGF) play an essential role in the process of ovulation [1].

The enzyme cyclooxygenase-2 (COX-2) and not cyclooxygenase-1 (COX-1) is the responsible to synthesize prostanoids in the ovary at least in the rat [2]. In the mare COX-2, also known as prostaglandin G/H synthase-2 (PGH-2), is the enzyme involved in the production of PGF and PGE_2 in the follicle 10 to 12 h before ovulation [3]. The latter study showed a gradual increase in granulosa cell expression of PGH-2 from 24 h to 39 h after treatment with 2500 IU hCG. The time sequence in expression of this

COX-2 enzyme paralleled the concentration of its products in follicular fluid and so PGE and PGF increased from baseline levels at 30 h post-hCG treatment to peak concentrations at 36 h to decrease again by 39 h post-hCG treatment. The peak mean concentration of PGF was around 10 ng/ml of follicular fluid.

The role of prostaglandins in the process of ovulation seems essential across species since the intrafollicular injection of indomethacin, a COX inhibitor, was able to block ovulation in the mare [4], rabbit doe [5], cow [6], rat [7], and ewe [8]. Furthermore, systemic oral indomethacin blocked ovulation by induction of luteinized unruptured follicles in 100% of treated women [9].

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is widespread in both equine and human medicine. In the horse, one of the most used NSAIDs in veterinary medicine are flunixin-meglumine (FM) for colic pain relief and phenylbutazone for the management of laminitis and others lameness disorders. Recently, more specific COX-2 inhibitors such as vedaprofen has been advocated for its use in equine reproduction to mitigate the mating-induced endometritis [10] and the release of prostaglandins during the handling of the cervix in embryo transfer [11].

Women taking therapeutic doses of different NSAIDs have been reported anecdotally to suffer transient infertility due to the development of luteinized unruptured follicles (LUFs) and subsequent failure of oocyte release. This has been reviewed by Stone and co-workers [12].

In mares, only one study tested the hypothesis that NSAIDs would block ovulation [4]. The NSAD used was indomethacin (500 µg) administered intrafollicularly when the follicle reached 35 mm. Although the latter study did report a delay in the interval from indomethacin treatment to evidence of luteinization between treated and control mares,

74 the actual ultrasonographic changes in these treated follicles were not clearly
75 determined.
76 An anovulatory condition observed during the ovulatory season in mares has been
77 classically termed as haemorrhagic anovulatory follicles (HAFs) [7,14]. In this
78 anovulatory syndrome, follicles of mares during the ovulatory season failed to collapse,
79 instead fill with blood and luteinize. The detailed review of several papers on the
80 ultrasound characteristics of LUF in women [9,15], at least in the authors' opinion, lead
81 to the belief that this human anovulatory syndrome shares ultrasonographic
82 characteristics and follows a similar cascade of events to those observed in HAFs in
83 mares. These luteinized unruptured follicles, previously fluid-filled structures, fill with
84 blood then the granulosa layer becomes echoic and thickened and eventually the
85 follicular contents acquire a static appearance as the blood within the follicular antrum
86 clots and the network of fibrin organizes with or without previous formation of strands
87 [14,15] giving, in most occasions, the unruptured follicle a cobweb-like aspect. These
88 anovulatory structures function as luteal glands with normal production of progesterone
89 in the mare [7] and woman [16] since the granulosa layer luteinizes even though the
90 follicle never collapsed.
91 In addition, similar anovulatory conditions have been reported in the Genus camelids:
92 in the humped camel (*Camelus dromedarius*) [17] and in the llama (*Lama glama*) [18].
93 In both camelids, the anovulatory follicles presented echoic specks in the follicular
94 antrum which eventually formed fibrous strands and organized. These type of follicles
95 developed more frequently in non-mated cycles.
96 The objectives of this study were two folds: a) to determine whether systemic FM
97 administered to mares was able to block ovulation; and b) to study critically the
98 follicular ultrasonographic characteristics of treated mares during and after the expected

time of ovulation following hCG treatment. It was hypothesized that high doses of intravenous FM would be able to block ovulation by inducing haemorrhage and luteinization of unruptured follicles in a similar way to that observed in spontaneous HAFs of mares.

2. Materials and methods

2.1. Animals

Initially, a total of seven mares were used in this study. The mares were cross-breeds and pure Spanish bred mares with a body weight ranging from 280 to 450 kg and aged from 8 to 21. The trial was conducted between June and July 2010 (Northern Hemisphere). All mares were kept stabled and fed on alfalfa hay and a mixed of cereal grains. All animal procedures were handled in accordance with the Spanish Department of Agriculture Guide for Care and Use of Animals in Research.

2.2. Ultrasonography and clinical examination of the genital tract

Transrectal B-Mode ultrasonography was performed with an ultrasound scanner equipped with an 8 MHz lineal-array transducer. Ultrasound examinations were performed every day during early oestrus and twice a day at 12 h intervals during the peri-ovulatory period. Endometrial oedema was assessed subjectively according to the prominence of endometrial folds. Ovulation was detected as per the absence of the previously recorded follicle within a given ovary and confirmed by the later presence of an echoic corpus haemorrhagicum (CH) in the same ovary. The formation of a LUF was diagnosed as the absence of follicular collapse with increasing amounts of blood in the follicular fluid, seen ultrasonographically as echoic specks floating freely within the

follicular antrum. Luteinization of granulosa layer was assumed by ultrasonographic evidence of thickening and increased echogenicity of the follicular wall.

Follicular diameter was obtained from the average of 2 linear measurements of the antrum taken at right angles when the image of the follicle was maximum using the electronic callipers. The corpora lutea were measured following the same technique.

The point of maximum haemorrhage into the follicular fluid corresponded with the greatest presence of echoic specks before they formed strands or acquired a solid or jelly-like appearance (clotting of contents).

A dioestrous phase was confirmed by manual palpation of a tonic and tight cervix 10 days post ovulation / LUF formation and by the presence of a dioestrous-like echotexture of the uterus [19]. The length of dioestrus was estimated from the point when ovulation / LUF were first detected to the point when the uterus first acquired an estrous-like echotexture (presence of endometrial oedema) and semi relaxed and open cervix. For this purpose, mares were examined daily from day 12 post-ovulation / LUF-formation.

2.3. Experimental design

The experimental protocol followed a crossover design with all mares monitored during two consecutive oestrous cycles. In the first cycle (FM), after spontaneous return to oestrus, each mare received 2 mg FM (Fynadine®[†]) / kg of body weight twice daily starting at the day when the mare reached a follicle of ≥32 mm in diameter and showed mild to moderate endometrial oedema. The FM was continued until ovulation or beginning of follicular haemorrhage visualized ultrasonographicaly as moderate amount of echoic specks floating freely within the follicular antrum. During the consecutive

[†] Fynadine (50mg/ml), Schering-Plough España, Alcobendas, Spain

cycle, control cycle (CON), after spontaneous return to oestrus the mare was not administered FM. All mares during the two consecutive cycles, FM and CON, received a single intravenous dose of 2500 IU of hCG (Lepori-hCG®[‡]) irrespective of body weight just before the first administration of FM (FM cycles) or when the mares reached a follicular diameter of ≥32 mm (CON cycles), this point was set as time 0. In all FM cycles, the last administration of FM was administered at least 48 h after hCG treatment. If by 48 to 60 h the mare had not either ovulated or formed a LUF, she was removed from the study, since it was assumed that the mare did not respond to the ovulatory effect of hCG: most mares (89 %) are known to ovulate between 36 and 48 h after treatment with hCG (Ginther 1992).

The interval from hCG administration to ovulation / beginning of haemorrhage in addition to follicular diameters were recorded for each mare and cycle.

2.4. Statistical Analysis

The end points analysed were: a) the fate of the pre-ovulatory follicle, this could be either ovulatory or LUF. The LUF incidence at FM cycles was compared to that of CON cycles using Fisher's exact test. A two-tailed *P* value <0.05 was considered statistically significant. And b) the dioestrous length was compared between FM and CON cycles by paired t-test.

3. Results

Of the initial 7 mares, only 6 were included in the study. One mare was excluded due to the fact that during its first cycle (FM), she did not either ovulate or formed a LUF by 60 h (ovulated 5 days post-hCG administration). The remaining mares (n = 6) during the

[‡] Lepori-hCG (2500 IU hCG) Farma-Lepori, Barcelona, Spain

CON cycles exhibited normal follicle rupture between 36 and 48 h after hCG treatment. In contrast, during their FM cycles, the pre-ovulatory sized follicles of 5 out of 6 mares did not rupture but maintained their spherical shape and grew rapidly to a mean diameter of approximately 55 mm (**Fig. 1**). The contents of these unruptured follicles lost their echo-free appearance at the following examination after the expected ovulation time and became increasingly more echoic as a result of further haemorrhage (**Fig.2**). One mare during the FM cycle had a normal ovulation between 36 and 48 h after hCG in spite of FM treatment.

The LUF incidence (83%) of FM cycles was significantly higher than that (0%) of the CON cycles (P = 0.015; **Table 1**). The FM-induced LUFs were first detected at 48 h after the hCG treatment. At this point, the amount of echoic specks was still low. The maximum degree of haemorrhage into the LUF, indicated by the heavy presence of free-floating echoic specks, occurred 90.5 ± 8 h whereas the clotting of its contents developed at a mean of 102 ± 12 h after hCG treatment (**Fig.1**). The length of dioestrus in LUF cycles was unaltered (16.9 ± 1.8 days) compared with ovulatory cycles (16.1 ± 1.3 days).

The clotting of contents of LUFs gave place to different ultrasonic appearances visualized as either a solid echoic structure with (**Fig. 3a**) or without a central lacuna or a network of fibrin strands forming a cobweb-like image (**Fig. 3b)**. These ultrasound findings were different from those observed after normal follicular collapse and early CH formation.

In CON cycles, a total of 7 normal ovulations from the 6 mares took place. The interval from hCG treatment to ovulation was between 36 and 48 h in each of the 6 mares included in the study. The follicular diameters and rest of data for LUFs and ovulatory cycles are presented in **Table 1**.

4. Discussion

The purpose of this study was to determine the ability of FM, a prostaglandin synthetase inhibitor, administered intravenously to mares to block ovulation and to form LUFs. All five mares but one developed LUFs after FM treatment. That is a LUF incidence of 83%. The sample size of this study was relatively low, however because the spontaneous incidence of this anovulatory condition in the mare population is less than 10 % [13], it is significant that only during the FM cycles all but one mare developed LUFs.

Amongst the commercially available NSAIDs for horses, FM was chosen because it has the highest COX-2 inhibitory activity which doubles that of indomethacin and is about 20 times more potent than phenibutazone (PBZ) [20]. The FM half life is 2.4 h in the horse [21].

The ability of FM, and likely of other NSAIDs, on blocking ovulation in the mare is a relevant finding to equine practice. Studies testing the effect of therapeutic doses on follicular collapse and oocyte quality are warranted. This can be extremely important to equine reproduction because the use of these drugs in practice is widespread. In the case of FM, the recommended dose is 1.1 mg/kg once a day, however twice daily administration of the same dose is indicated when stronger analgesia and anti-endotoxic effects are sought [22].

Higher doses of FM than recommended by the manufacturer's datasheet were attempted since no data was available of the passage rate of FM from plasma to follicular fluid nor the mean residence time within the follicular fluid. A total daily dose of 4 mg/kg body weight was chosen as a result of a preliminary trial. These results obtained from this

same group (unpublished) indicated that 1.5 mg/kg administered twice a day blocked ovulation in 2 of 3 treated mares when started 12 to 24 h before hCG administration. A higher dose was attempted in the current study since it was thought that the lack of response in the 33 % of treated mares was due to insufficient dosage. However, this same mare did not respond again to the higher dose of 2 mg/kg.

The recommended dose of 1.1 mg/kg is substantially lower than the one used herein. Nevertheless, no side effects such as colic, diarrhoea or decreased appetite were observed during the trial. However, despite of the high dose one mare still did not respond to FM and ovulated within the expected interval after hCG. In one clinical trial carried out in women [9], 100 and 50% of treated patients with oral systemic indomethacin and azapropazone developed LUFs respectively. The authors of the latter study suggested that the differences in LUF incidences between both NSAIDs were attributable to weaker prostaglandin synthetase inhibitory activity of azapropazone. Whether the lack of response to FM from this mare is attributed to individual differences in pharmacokinetics or biological activity of FM or necessity of prostaglandins to initiate the ovulatory cascade in the whole population of mares is not know from the results of this study and therefore needs to be further investigated.

It is known that the LH-induced expression of COX-2 in the follicle is not initiated until 24 h post-hCG [3], therefore it could be hypothesized that the first FM administration might have been delayed until 24 h after hCG treatment. However, due to the lack of data on pharmacokinetics of FM in the follicular fluid and on the interval required between intravenous administration of FM and effective inhibition of COX-2 within the granulosa layer, further studies testing the effect of FM administered at different timings relative to the LH surge will be needed to confirm this hypothesis.

For other common NSAIDs used in equine practice, such as PBZ, which has a longer half life in the horse of 5.4 h [23] it could be speculated that one single dose at the right time could block the follicular production of prostaglandins E and F for sufficient time. Hence, this seems an interesting area of clinical research which is relevant to equine reproduction and therefore should be further investigated.

Other possible implication of the results of this study is the development of a method of contraception. Theoretically this could be used in a commercial programme of equine oocyte transfer. To date the recipient mare whom the oocyte from a valuable mare is transferred, needs to be removed its own oocyte by ovum-pick up before mating with the preferred spermatozoa [24]. Progesterone concentrations were not measured in the current study, which would be required to maintain a successful pregnancy. However the ultrasound characteristics of these FM-induced LUFs indicated presence of luteal tissue and all mares entered a dioestrous-like phase that lasted for a period similar to that of the consecutive ovulatory cycles. In addition, human gynaecologists have performed successfully embryo transfers into women who had LUF cycles indicating an adequate progesterone production by this type of unruptured follicles. One possible drawback of this protocol to block ovulation in mares is the fact that not all mares seem to respond (17 % in this small mare population did not respond).

The ultrasonographic characteristics of the LUFs induced by FM treatment appear to be comparable to those reported in both naturally occurring [15] and experimentally-induced [9] LUFs in women. In indomethacin treated women, the follicular diameter of future LUFs increased from approximately 20 mm at the time of hCG treatment to a maximum diameter of 35 mm (1.75 fold-increase) maintaining its spherical shape during the 5 days following hCG administration [9]. Similarly the follicular diameter of equine LUFs reported herein increased from 33 to 55 mm (1.67 fold-increase) during

the same time interval. Moreover, LUFs in both species filled with free-floating echoic specks suggesting intrafollicular haemorrhage.

A spontaneously occurring form of anovulation in the mare, termed as haemorrhagic anovulatory follicle (HAF), has been extensively described in the recent past few years [13,14,25]. In addition, the ultrasonographic and clinical characteristics of LUFs from the mares of the present study followed a similar time-point events. These are comparable to those observed in spontaneously occurring equine HAFs [14,25] which may indicate the involvement of similar pathogenic mechanisms in the formation of spontaneously occurring HAFs and FM-induced LUFs.

Recently there has been found a clear association between increased LH concentration from early stages of follicular development and the later development of HAFs in mares [26]. The elevated LH concentration of these mares appeared to be endogenous (in spontaneously occurring HAFs) or originated from exogenously PGF-induced luteolysis as a result of the fall in progesterone concentration and subsequent removal of the negative feedback that progesterone exerted on LH [26]. In this regard, an association between the use of PGF to induce oestrus and increased HAF incidence were consistently found [25,27]. Furthermore, mares with high HAF recurrence rates have been shown to have intrinsically high LH concentrations even in non PGF-induced oestruses [26]. Interestingly, the incidence of LUFs in women treated with clomiphene citrate is substantially higher (25%) than that of spontaneous non-treated cycles (0%) [28]. The LH concentrations of these women during the follicular phase were higher in clomiphene citrate treated cycles than in non-treated cycles. An attractive hypothesis to link the lack of prostaglandins to these anovulatory syndromes would be that prolonged stimulation of elevated LH during early stages of follicular development could

somehow down regulate the later expression of COX-2 in the granulosa cells during the pre-ovulatory surge.

In conclusion, systemic intravenous administration of high dose of flunixin-meglumine to mares during the peri-ovulatory period was able to block ovulation and induce luteinized unruptured follicles in 83% of treated mares. This protocol appears to be reliable in inducing consistently HAFs which in turn could be used as a model to research these pathological anovulatory conditions encountered in equine and human medicine. Finally, this FM protocol treatment can be used as a method of contraception in the mare. The main limitations of the present study were the relatively small sample size of treated animals and the lack of determination of reproductive hormones' concentration.

References

1. Robker RL, Russell DL, Yoshioka S, Sharma SC, Lydon JP, O'Malley BW, Espey LL, Richards JS. Ovulation: a multi-gene, multistep process. Steroids 2000; 65: 559–70.
2. Sirois J, Simmons DL, Richards JS. Hormonal regulation of messenger ribonucleic acid encoding a novel isoform of prostaglandin endoperoxide H synthase in rat preovulatory follicles. Induction in vivo and in vitro. J Biol Chem 1992; 267: 11586–92.
3. Sirois J, Dore M. The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process. Endocrinol 1997; 138: 4427–34.

4. Watson ED, Sertich PL. Concentration of arachidonate metabolites, steroids and histamine in preovulatory horse follicles after administration of hCG and the effect of intrafollicular indomethacin. J Endocrinol 1991; 129: 131-9.
5. O'Grady JP, Caldwell BV, Auletta FJ, Speroff L. The effects of an inhibitor of prostaglandin synthesis (indomethacin) on ovulation, pregnancy and pseudo-pregnancy in the rabbit. Prostaglandins 1972; 1: 97.
6. De Silva M, Reeves JJ. Indomethacin inhibition of ovulation in the cow. J Reprod Fertil 1985; 75: 547–9.
7. Sogn JH, Curry TE, Brannstrom M, Lemaire WJ, Koos RD, Papkoff H, Janson PO. Inhibition of follicle-stimulating hormoneinduced ovulation by indomethacin in the perfused rat ovary. Biol Reprod 1987; 36: 536–42.
8. Murdoch WJ, Peterson TA, Van Kirk EA, Vincent DL, Inskeep EK. Interactive roles of progesterone, prostaglandins, and collagenase in the ovulatory mechanism of the ewe. Biol Reprod 1986; 35: 1187–94.
9. Killick S, Elstein M. Pharmacologic production of luteinized unruptured follicles by prostaglandin synthetase inhibitors. Fertil Steril 1987; 47: 773-7.
10. Rojer H, Aurich C. 2010. Treatment of Persistent Mating-Induced Endometritis in Mares with the Non-Steroid Anti-Inflammatory Drug Vedaprofen. Reprod Domest Anim 2010; In press.
11. Koblischke P, Budik S, Müller J, Aurich C. 2009. Practical Experience with the Treatment of Recipient Mares with a Non-Steroidal Anti-Inflammatory Drug in an Equine Embryo Transfer Programme. Reprod Domest Anim 2009; In press.
12. Stone S, Khamashta MA, Nelson-Piercy C. Nonsteroidal anti-inflammatory drugs and reversible female infertility: is there a link? Drug Saf 2002; 25:545-51.

13. Ginther OJ, Gastal EL, Gastal MO, Beg MA. Incidence, endocrinology, vascularity, and morphology of haemorrhagic anovulatory follicles in mares. J Equine Vet Sci 2007; 27: 130-9.
14. Cuervo-Arango J, Newcombe J.R. Risk factors for the development of haemorrhagic anovulatory follicles in the mare. Reprod Domest Anim 2010; 45: 473-80.
15. Coulam CB, Hill LM, Breckle R. Ultrasonic evidence for luteinization of unruptured preovulatory follicles. Fertil Steril 1982; 37: 524-9.
16. Wang L, Qiao J, Liu P, Lian Y. Effect of luteinized unruptured follicle cycles on clinical outcomes of frozen thawed embryo transfer in Chinese women. J Assist Reprod Genet 2008; 25: 229–33.
17. Skidmore JA, Billah M, Allen WR. The ovarian follicular wave pattern in the mated and non-mated dromedary camel (Camelus dromedarius). J Reprod Fertil Supl. 1996; 49: 545-8.
18. Adams GP, Sumar J, Ginther OJ. Haemorrhagic ovarian follicles in llamas. Theriogenology 1991; 35: 557-68.
19. Ginther OJ. Reproductive biology of the mare. Basic and Applied Aspects, 2nd ed. 1992; Cross Plains, WI: Equiservices Publishing.
20. Brideau C, Van Staden C, Chung Chan C. In vitro effects of cyclooxygenase inhibitors in whole blood of horses, dogs and cats. AJVR 2001; 62: 1755-60.
21. Pellegrini-Masini A, Poppenga RH, Sweeney RW. Disposition of flunixin meglumine injectable preparation administered orally to healthy horses. J Vet Pharmacol Therap 2004; 27: 183–6.
22. Cook VL, Jones Shults J, McDowell MR, Campbell NB, Davis JL, Marshall JF, Blikslager AT. Anti-inflammatory effects of intravenously administered

lidocaine hydrochloride on ischemia-injured jejunum in horses.. Am J Vet Res. 2009; 70: 1259-68.

23. Lees P, Taylor JBO, Higgins AJ, Sharma SC. Phenylbutazone and oxyphenylbutazone distribution into tissue fluids in the horse. J Vet Pharmacol Therap 1986; 9: 204–12.
24. Carnevale EM. Oocyte transfer and gamete intra-fallopian transfer in the mare. Anim Reprod Sci 2004; 82-83: 617-24.
25. Cuervo-Arango J, Newcombe J.R. The effect of hormone treatments (hCG and cloprostenol) and season on the incidence of haemorrhagic anovulatory follicles in the mare: a field study. Theriogenology 2009; 72: 1262-7.
26. Ginther OJ, Gastal EL, Gastal MO, Jacob JC, Beg MA. Induction of haemorrhagic anovulatory follicles in mares. Reprod Fertil Dev 2008; 20: 947-54.
27. Ginther OJ, Al-Mamun, M. Increased frequency of double ovulations after induction of luteolysis with exogenous prostaglandin $F_{2\alpha}$. J Equine Vet Sci 2009; 29: 581-3.
28. Randall JM, Templeton A. The effects of clomiphene citrate upon ovulation and endocrinology when administered to patients with unexplained infertility. Human Reprod 1991; 6: 659-64.

Tables

Table 1

Follicular data of 6 mares during two consecutive cycles

Cycle	Mares (n)	POF (n)	FD (mm)	Ov (n)	LUFs (n)	Dioestrus length
FM	6	6	33.6±2.3	1	5[b]	16.9±1.8[a]
CON	6	7	33.5±2.1	7	0[a]	16.1±1.3[a]

A crossover design with 6 mares monitored ultrasonographically during two consecutive cycles; FM: intravenous treatment with 2 mg flunixin-meglumine/kg body weight and 2500 IU hCG when the follicle was ≥ 32 mm; CON: control cycles, mares were administered 2500 IU hCG but no FM when a follicle ≥ 32 mm; POF: number of follicles > 30 mm during at the time of hCG treatment; FD: maximum mean follicular diameter (mm) reached before hCG administration; Ov: number of follicles that totally collapsed and formed a corpus luteum; LUFs: number of luteinized unruptured follicles that never collapsed. All means are expressed ± SD. Within columns different letters indicate statistical difference ($P<0.05$).

Figure

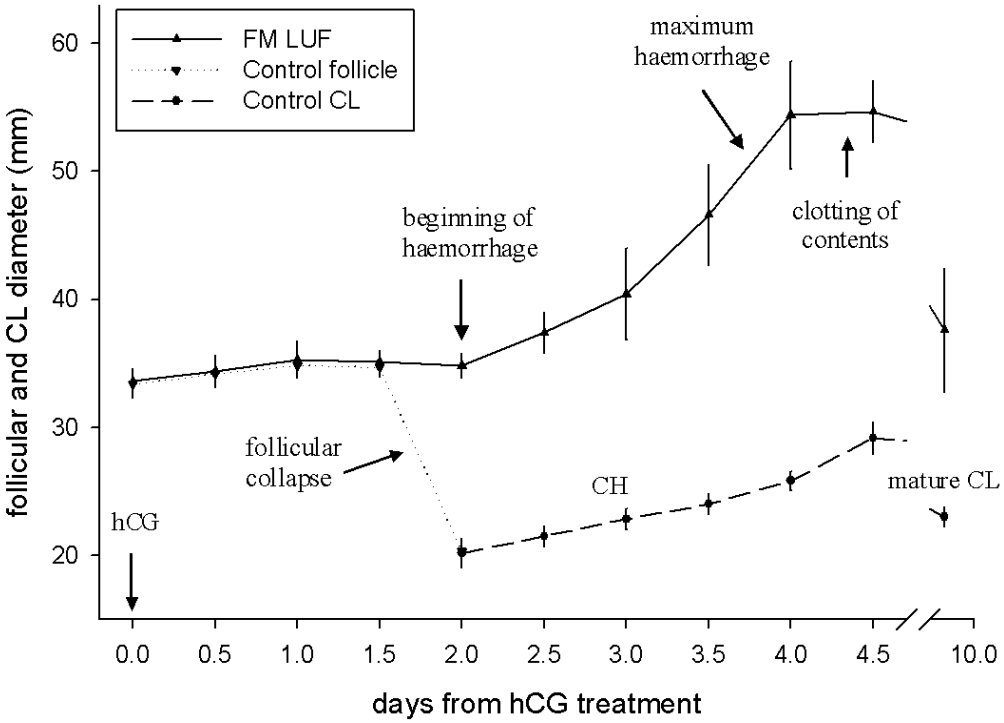

Fig. 1. Mean ± S.E.M. of luteinized unruptured follicles (LUFs) diameter of mares from cycle flunixin-meglumine (FM, n = 5) and follicular and CL diameters from mares of the control cycle (n = 6). Time 0 indicates the point when the mares were administered 2500 IU hCG. The increase in LUF diameter begins after follicular haemorrhage. The haemorrhage was greatest when the LUF showed the highest number of echoic specks before the contents acquired a firm appearance (clotting). CH: corpus haemorrhagicum; CL: corpus luteum.

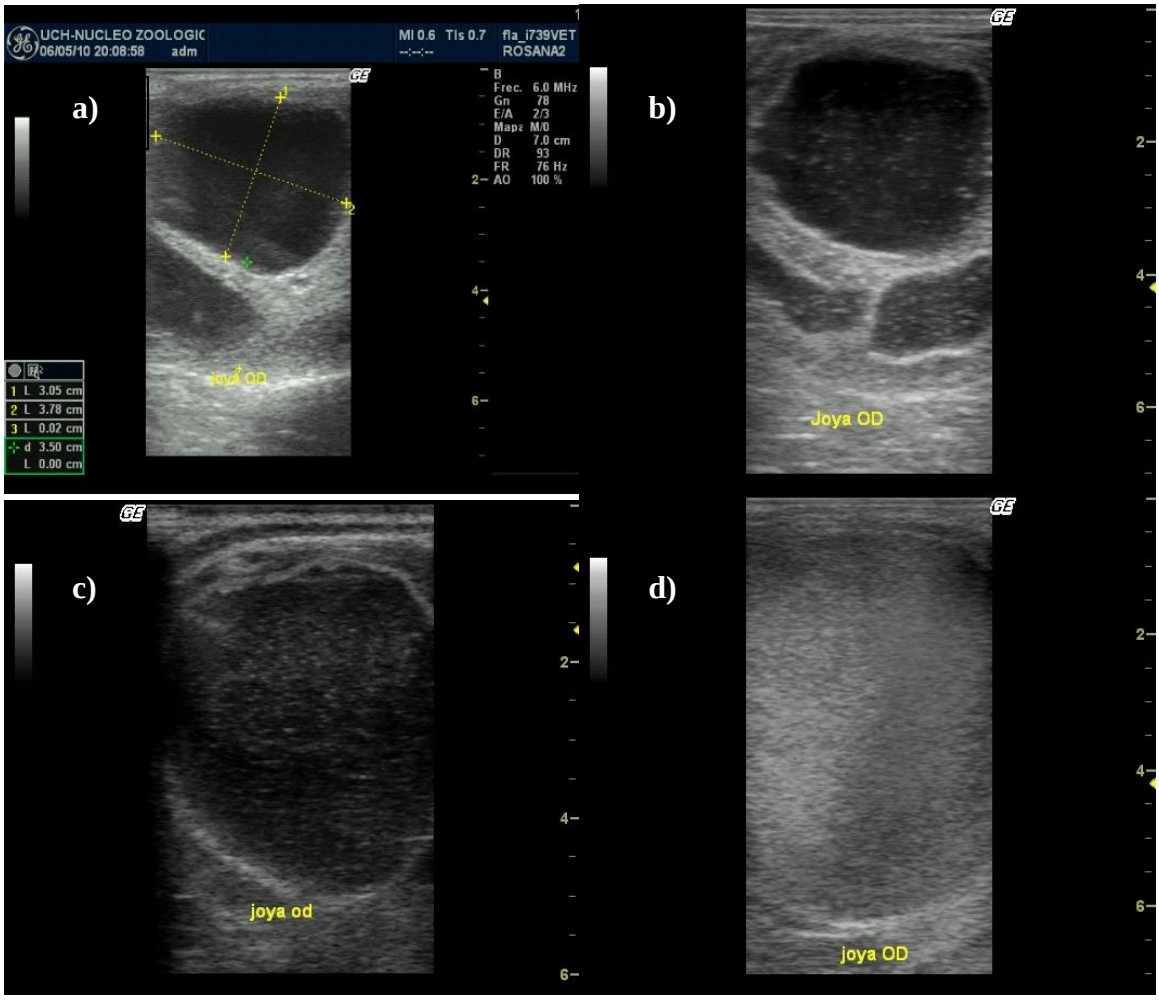

Fig. 2. Sonogram series of development of a LUF from a FM cycle mare. Hour 0 (hCG administration): a) + 36h, echo-free follicular antrum; b) + 48h, beginning of follicular haemorrhage; c) + 72h, d) + 108h, note the large amount of echoic specks within the antrum indicative of heavy haemorrhage.

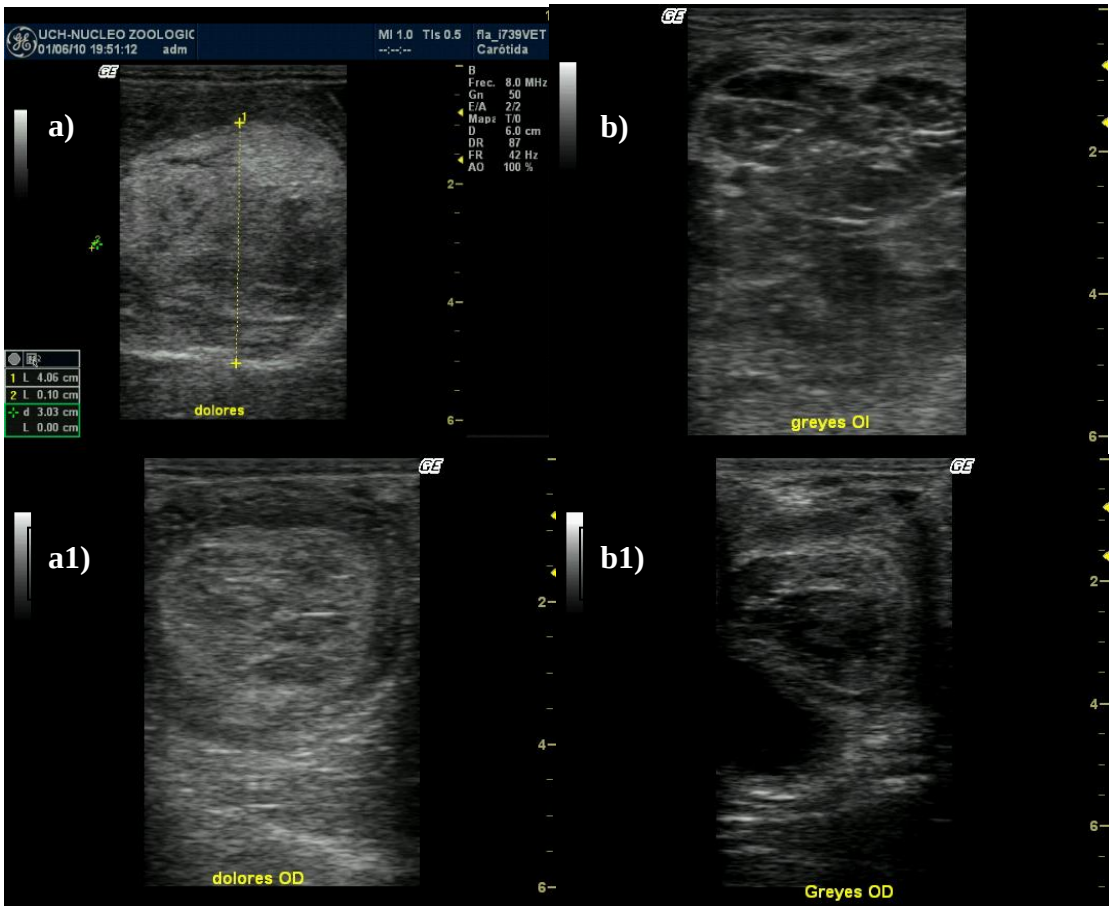

Fig. 3. Sonograms of two mares from FM cycles at two different times after hCG treatment (day 0); a) day +5: first evidence of clotting of contents, 12 h earlier there was a heavy haemorrhage but the echoic specks were still moving freely; a1) same mare as in a) on day +15, the solid LUF has contracted and decreased substantially in diameter but it is much larger than a normal same age CL; b) day +4: a cobweb-like LUF with a network of fibrin strands that quiver if balloted, first evidence of organization of LUF contents, 12 h earlier there was heavy haemorrhage seen as freely-moving echoic specks; b1) same mare as in b) on day +19: a remnant old LUF notably larger than a same age corpus albicans, but with no functional luteal tissue since at this point the mare had already returned to oestrus spontaneously (presence of endometrial edema).

Supplementary files

This piece of the submission is being sent via mail.