Intrafollicular treatment with prostaglandins \( \text{PGE}_2 \) and \( \text{PGF}_{2\alpha} \) inhibits the formation of luteinised unruptured follicles and restores normal ovulation in mares treated with flunixin-meglumine

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Summary

Reasons for performing study: Haemorrhagic anovulatory follicle is the most common pathological anovulatory condition in the mare, but its cause remains unknown. An experimental model to induce luteinised unruptured follicles (LUF) with flunixin-meglumine (FM) has been developed. Luteinised unruptured follicles share similar morphological and hormonal characteristics with haemorrhagic anovulatory follicles.

Objectives: To test the effect of intrafollicular administration of prostaglandins \( \text{PGE}_2 \) and \( \text{PGF}_{2\alpha} \) during the periovulatory period on ovulation and pregnancy in FM-treated mares.

Study design: In vivo experiment in a crossover design.

Methods: Five mares were followed during 2 oestrous cycles each. All mares were given FM at 1.7 mg/kg bwt i.v. every 12 h from Hour 0 (Hour 0 = human chorionic gonadotrophin treatment) to Hour 36. In treatment cycles (n = 5), at Hour 32 the preovulatory follicle was punctured and 0.5 ml of a solution containing 500 \( \mu \text{g} \) of \( \text{PGE}_2 \) and 125 \( \mu \text{g} \) of \( \text{PGF}_{2\alpha} \) was deposited within the follicle. In control cycles, water for injection was administered into the follicle at the same time. In 3 control and 3 treatment cycles, mares were also inseminated at Hour 24. Diagnosis of ovulation/LUF formation and pregnancy was performed by ultrasound examination between Hours 36 and 72 and 14 days after ovulation/LUF formation, respectively.

Results: During the treatment cycles, all mares ovulated normally (100% ovulation rate) 36–48 h after human chorionic gonadotrophin, while in 4 of 5 control cycles the mares developed an LUF (80%, \( p<0.05 \)). All 3 inseminated mares became pregnant in the treatment cycles, but not in the control cycles.

Conclusions: Intrafollicular treatment with \( \text{PGE}_2 \) and \( \text{PGF}_{2\alpha} \) overcame the anovulatory effect of FM. This sheds new insights into the knowledge on the possible therapeutic options for ovulatory failure in the mare.

Keywords: horse; mare; luteinised unruptured follicle; prostaglandin; ovulation

Introduction

Haemorrhagic anovulatory follicle (HAF) is a cause of ovulatory failure in the mare [1–5]. During the development of an HAF, the preovulatory-sized follicle fails to ovulate despite typical secondary signs associated with ovulation, such as the preovulatory surge and luteinising hormone (LH) peak, an abrupt decrease in oestradiol and a gradual increase in progesterone concentrations [2,6], decreasing endometrial oedema score [4,5] and normal length of the subsequent dioestrous phase [2,4]. Equine HAF fails to rupture but increases in diameter. Subsequently, the HAF wall thickens and becomes highly echogenic, indicating active luteinisation [2]. Simultaneously, the follicular antrum fills with increasing amounts of echoic particles which move freely upon ballottement of the ovary. Eventually, the HAF contents organise. Although the overall incidence of HAF in the mare population is relatively low, 5–8% of all oestrous cycles [1,4,7], incidences can be as high as 25–50% of all cycles in individual mares, so-called ‘repeater mares’ [2,4] and is a cause of infertility in these mares.

The term luteinised unruptured follicle (LUF) syndrome has been used in human medicine to refer to a common cause of infertility in women who fail to rupture and ovulate their preovulatory follicle despite secondary ovulatory changes such as an LH peak, a rise in progesterone or secretory transformation of the endometrium [8–11]. Instead of ovulating and forming a typical corpus luteum (CL), the anovulatory follicle increases in diameter, fills with echoic particles and eventually luteinises with active production of progesterone [12,13]. As in equine spontaneous HAF, the natural occurrence of spontaneous LUF is difficult to predict. An experimental protocol that reliably allowed the induction of LUF in women consists of systemic administration of prostaglandin synthase inhibitors [14,15]. The ultrasonographic characteristics of both spontaneous and experimentally induced human LUF are similar [13,14], which might also indicate similar pathogenic mechanisms. Intrafollicular prostaglandins (\( \text{PGE}_2 \) and \( \text{PGF}_{2\alpha} \)) are essential key factors for the ovulatory process in mammals [16]. They are actively produced in the granulosa cells approximately 10 h before ovulation [17].

Recently, the experimental protocol to induce LUF in women with prostaglandin synthase inhibitors [14] was successfully attempted in mares [18]. Flunixin-meglumine (FM), a prostaglandin synthase inhibitor, blocked ovulation during the expected periovulatory period in over 80% of treated mares. The resultant FM-induced anovulatory follicles underwent ultrasonographic signs of luteinisation and therefore were termed LUF [18]. Flunixin-meglumine-induced equine LUF share a similar profile of reproductive hormones [19] and ultrasound characteristics [20] to that reported for spontaneous HAF.

The treatment options for mares with HAF are limited [21]. The objectives of this study were to characterise the effect of \( \text{PGE}_2 \) and \( \text{PGF}_{2\alpha} \) administered intrafollicularly in mares treated with FM on ovulation and pregnancy rate.

Materials and methods

Animals and ultrasound examinations

Five mixed-breed cyclic mares from the institute’s research herd aged 5–18 years and weighing 350–550 kg were studied during 10 oestrous cycles (2 cycles each mare) in April–June 2014. Mares were kept stabilised with ad libitum access to water and fed alfalfa hay and cereal grain.

Transrectal B-mode ultrasonography of the genital tract was performed using an ultrasound scanner (Sonosite nanomaxx®) equipped with a linear array 8 MHz probe. All mares had had at least one ovulation by the time of the beginning of the study. This was confirmed by the presence of a corpus luteum. Ultrasound examinations were performed every day from Day 15 (Day 0 = day of ovulation) and twice a day at 12 h intervals.
during the periovulatory period. Endometrial oedema was scored on a 0–3 scale (0 = dioestrus-like echotexture, with no endometrial folding; 3 = maximum degree of endometrial folding). The follicular diameter was obtained using electronic calipers from the average of 2 linear measurements of the antrum taken at right angles when the size of the follicle was maximal. An ovulation was diagnosed when the previous preovulatory follicle had disappeared with at least the loss of ≥90% of follicular fluid. The formation of an LUF was diagnosed when the follicle did not collapse but filled gradually with an increasing amount of echoic specks that moved freely during ballottement of the ovary. Finally, the LUF contents organised, forming a network of fibrin, as described previously [18].

**Experimental design and intrafollicular puncture**

For each mare, 2 oestrous cycles, with a wash-out period of one cycle in between, were allocated randomly to a control (n = 5) or to a treatment (n = 5) group in a crossover design. The first cycle of 3 mares was a control cycle while the remaining 2 were treatment cycles. After Day 15 (Day 0 = day of previous ovulation), when the mares reached a follicle diameter ≥32 mm and showed mild to moderate endometrial oedema (score of 1–3), they were given 1500 iu of human chorionic gonadotrophin (hCG) i.v. (Veterin Corion)\(^1\) to induce the preovulatory surge of LH. The time of hCG treatment was set as Hour 0. In all cycles (control and treatment), mares received flunixin-meglumine (Finaudyne)\(^1\) 1.7 mg/kg b.wt. i.v. every 12 h from Hour 0 to Hour 36 or until the diagnosis of ovulation/beginning of LUF formation, whichever came first. Three mares, in their treatment (n = 3) and control (n = 3) cycles, were inseminated with 1 × 10\(^3\) progressively motile sperm from a stallion of proven fertility at Hour 24. The remaining 2 mares were not inseminated because of cervical lacerations and a history of post mating intrauterine fluid accumulation.

A mixed solution of naturally occurring PGE\(_2\) (dinoprostone 10 mg/ml, PGF\(_2\alpha\); Pfizer\(\text{\textregistered}\)) and PGF\(_2\alpha\) (5 mg/ml dinoprostone, Dinolyst\(\text{\textregistered}\)) was prepared. This solution was used for each treatment cycle and contained 500 μg PGE\(_2\) and 125 μg of PGF\(_2\alpha\) diluted in 0.5 ml of water for injection. Each mare received a single injection in the largest preovulatory follicle at Hour 32 of 0.5 ml of the treatment solution (treatment cycles) or placebo (0.5 ml of water for injection) during the control cycles by transvaginal ultrasound-guided follicle puncture. The follicular puncture was performed with the mare restrained in the stocks, previously sedated with detomidine hydrochloride 0.01 mg/kg b.wt i.v. (Domosedan\(\text{\textregistered}\)) and butorphanol tartrate 0.02 mg/kg b.wt i.v. (Torbugsic\(\text{\textregistered}\)). The perineum was thoroughly cleansed.

Ultrasound-guided follicular puncture was performed using an ultrasound scanner (Honda Electronics HS-1500 VET\(\text{\textregistered}\)) equipped with a 9 MHz convex transvaginal transducer (HCV 3710MV). The transducer was introduced into the vagina and placed next to the side of the vaginal fornix ipsilateral to the ovary containing the target follicle. A 5 cm, 21 gauge needle was fitted to the end of the working channel of the ultrasound probe. The needle was connected through a silicone tube to a 1 ml-syringe containing the treatment or placebo solution. During the transvaginal ultrasound-guided follicle puncture, the needle was introduced into the follicular antrum while the ovary was held manually per rectum. Care was taken to avoid any excessive movement of the ovary during follicle puncture so that the wall was minimally damaged. The whole procedure was performed easily in <5 min. Mares were scanned transrectally just before and 15 min after follicle puncture to measure follicle diameter and to assess any loss of fluid due to follicle puncture.

Thereafter, mares were scanned at least at Hours 36, 48, 60 and 72. In addition, 2 mares in the treatment cycles were scanned every 2 h from Hour 36 to Hour 48 to determine more precisely the exact time of ovulation. A final ultrasound examination was performed 9 days after ovulation/beginning of LUF formation to evaluate and measure the corpus luteum/LUF. Pregnancy diagnosis was performed 14 days post ovulation/LUF formation. If a mare was diagnosed as pregnant in their first cycle, 5 mg of dinoprost was administered subcutaneously to induce luteolysis and the vesicle was crushed manually. This occurred in one of the 3 mares that conceived. The remaining 2 mares conceived in their last cycle.

**Data analyses**

Sequential data (follicle diameter) was tested for normality using the Anderson-Darling test. Data not normally distributed were ranked. Differences in follicle diameter between the control and treatment cycles were analysed by unpaired t test. Differences in endometrial score between the control and the treatment cycles were analysed by Mann–Whitney nonparametric test. Frequency data (ovulation/LUF formation rate) were analysed by Fisher’s exact test (Minitab15\(\text{\textregistered}\)). Statistical significance was set at Ps0.05. All images obtained during the ultrasound examinations were frozen, labelled and saved for further detailed analyses and creation of figures.

**Results**

All 5 mares in the treatment cycles had a normal ovulation 36–48 h after hCG treatment (Fig 1). The ovulation rate (100%) in the treatment cycles was higher (P <0.05) than that of the control cycles (20%; 1/5). In the 2 treatment cycles monitored at 2 h intervals between Hour 36 and 48, the mares ovulated by Hour 40 (Fig 2) and 42 (8 and 10 h after intrafollicular treatment, respectively). In 4 control cycles, the preovulatory follicle did not collapse but developed into an LUF. The LUF showed a gradual increase in diameter from Hour 36 onwards (Fig 1) and follicle haemorrhage evidenced by the presence of echoic specks that moved freely upon ballottement of the ovary. Luteinisation of the follicle was evidenced by the increase in the thickness and echogenicity of the granulosa layer. Finally, the LUF contents organised, forming a network of echoic strands (Fig 3). In the control cycle that did not form an LUF, the follicle showed a gradual decrease in diameter between Hour 32 (34 mm) and Hour 48 (25.5 mm). The follicle of this mare collapsed at Hour 60 (Fig 4). Only one follicle from the treatment cycles developed signs of follicular haemorrhage before ovulation. In this cycle, the mare had a follicle with moderate signs of haemorrhage (echoic specks) at Hour 32 (before treatment), but then ovulated normally by Hour 48 (Fig 5).

The follicle puncture induced an immediate (within 15 min) reduction in diameter of 2.1 ± 1.2 mm (range 0–5.5 mm) and 3.7 ± 1.7 mm (range 0.5–10.5 mm) in the treatment and the control cycles, respectively. The preovulatory follicle diameter at Hour 36 (h after follicle puncture) was reduced by 6.7 ± 2.4 (range 0.5–14 mm) and 4.8 ± 1.9 mm (range...
0–11 mm) compared with the prepuncture diameter for the treatment and the control cycles, respectively (Fig 1). The degree of reduction was not different between groups ($P > 0.05$) at either 15 min or 4 h after follicle puncture. The endometrial oedema score did not differ between groups ($P > 0.05$) at any time-point analysed.

All 3 mares inseminated in their treatment cycles conceived (100% pregnancy rate). The same mares mated in their control cycles did not conceive (0% pregnancy rate). These 3 control cycles formed an LUF. The 3 embryonic vesicles identified 14 days after ovulation in the treatment cycles measured 13.0, 13.5 and 6.0 mm. The small for age vesicle corresponded to the mare that showed follicular haemorrhage before the follicle puncture at Hour 32 (Fig 5). This embryonic vesicle was measured again one day later (15 days after ovulation) for confirmation of pregnancy before manual reduction and measured 9.5 mm.

**Discussion**

Prostaglandins are key factors in the process of ovulation [16]. The exact role of prostaglandins during the ovulatory process of mammals is not completely understood but it is believed that they activate several matrix metalloproteinases and plasminogen activator families [22]. These matrix metalloproteinases and other proteolytic enzymes produce an extensive tissue remodelling at the apex of the follicle that culminates in the rupture of the follicular wall and ovulation [16]. In the mare, the enzyme prostaglandin G/H synthase-2 (PGHS-2), also called COX-2, is expressed in granulosa cells 30 h after the beginning of the preovulatory LH surge, whether it is spontaneous or induced by hCG. The PGHS-2 is the first rate-limiting enzyme in the biosynthesis of prostanoids from arachidonic acid [17]. There is a parallel increase in the expression of PGHS-2 in...
Fig 3: Representative B-Mode ultrasonograms of a mare during the control cycle at follicle puncture and development of a luteinised unruptured follicle (LUF) from 32 h after human chorionic gonadotrophin (hCG) until 9 days after beginning of LUF formation. Note the partial reduction in the follicle diameter after puncture (32 h POST and 36 h) presumably by loss of follicular fluid, and subsequent increase in diameter by entry of blood (echoic specks within the antrum) during the development of the LUF (48, 60 and 72 h). The LUF contents organised, forming a network of echoic strands 96 h after hCG (96 h). Hour 0 = time of hCG treatment.
granulosa cells and in the concentration of PGE2 and PGF2α in follicular fluid 33–36 h after hCG administration at a mean concentration of 40 and 10 ng/ml of PGE2 and PGF2α, respectively [17]. These data underline the rationale for designing a treatment protocol that delivers exogenous PGE2 and PGF2α into the follicle at the expected time of physiological appearance (Hour 32) and at the same PGE2/PGF2α concentration ratio of 4:1. The overall concentration used in the present study (500/125 μg) exceeded the physiological concentrations expected in follicular fluid at the same time for the average volume of fluid of 20–30 ml in a follicle of 35 mm in diameter [17]. However, this higher concentration was chosen to account for any possible loss of treatment solution during the follicle puncture due to leakage of follicular fluid.

The chosen solution of PGE2 and PGF2α used in the present study seemed to induce ovulation consistently 4–16 h after treatment in mares with pharmacological inhibition of endogenous production of prostaglandins.

The positive effect upon ovulation can be attributed to the prostaglandins themselves rather than to the puncture of the follicular wall. It is true that the follicular collapse could have been precipitated by damage to the follicle wall during the process of follicle puncture. However, the majority of control mares (80%) did not ovulate but developed an LUF as expected, despite the follicle puncture to deliver the placebo. Similarly, the results of 2 previous studies in which mares were treated with the same FM protocol but in which the follicles were not punctured, showed that 27% [19] and 17% [18] of the mares did ovulate despite FM treatment. However, it is not known whether both types of prostaglandin (PGE2 and PGF2α) are necessary to induce normal ovulation successfully. In this regard, a previous study compared the effect of PGE1, PGE2 and PGF2α on the ovulation rate in rats treated with indomethacin, another PGHS-2 inhibitor [23]. The PGE series were superior to PGF2α in inducing normal follicle rupture. Although PGF2α was only partially effective in inducing ovulation, it...
was superior to rats from the control group treated with indomethacin only. A recent study in mares tried to reverse the inhibitory effect of FM on ovulation by systemic administration of cloprostenol (a PGF synthetic analogue), with disappointing results [24]. Whether the lack of ovulatory effect was due to the type, dose or route of administration of the prostaglandin is not known. Nevertheless, it is disappointing, since PGF$_2\alpha$ and its analogues are drugs readily available to equine practitioners. Furthermore, a systemic route would be more practical, rapid and inexpensive than intrafollicular delivery. Further research should be carried out to determine the exact roles that the route of administration, dose and type of prostaglandin play in the induction of ovulation.

During their treatment cycles, all mares ovulated within the expected time window of 36–48 h after hCG, which is the common period reported for induction of ovulation after hCG administration [25]. The timing of ovulation depends on the time at which PGHS-2 is expressed in granulosa cells and the prostaglandins are secreted into the follicular fluid. This timing is approximately 10 h prior to ovulation in several mammals, including the mare [17]. The current results are in agreement with the latter statement since all mares ovulated 4–16 h after prostaglandin treatment, and in the 2 mares scanned more frequently, at exactly 8 and 10 h after treatment.

The treatment protocol used in this study not only induced follicle wall rupture and ovulation but also allowed normal release of the oocyte into the oviduct, as all 3 inseminated mares conceived. As in previous studies on the pregnancy rate of mares with spontaneous HAF [1], the 3 mares that developed an LUF after insemination in their control cycles, did not conceive (0% pregnancy rate). Histological studies in rabbits have shown that the oocyte remains trapped within the LUF’s network of organised contents and therefore oocyte release into the oviduct and fertilisation are not possible [26]. One mare showed signs of LUF development (moderate to heavy presence of echoic specks) before follicle treatment (before Hour 32) that may indicate that she had already initiated the preovulatory surge of LH before hCG treatment. It has been shown that the first appearance of echoic specks in FM-induced LUF occurs 40–44 h after hCG [19].

The pathogenesis of spontaneous HAF is largely unknown. To date there has not been evidence of any link between the development of HAF and the lack of intrafollicular prostaglandins as the mechanism to inhibit follicular wall rupture and ovulation in this anovulatory condition. Therefore, the fact that the treatment protocol in the present study was able to reverse the inhibitory effect of FM on prostaglandin production and ovulation does not imply that it would work in mares with spontaneous HAF. Nonetheless, the results of the current study are encouraging and it has been shown that the ultrasound and hormonal characteristics of spontaneous HAF and FM-induced LUF are similar [20].

In conclusion, intrafollicular administration of 500 μg of PGE$_2$ and 125 μg of PGF$_2\alpha$ 32 h after hCG induces ovulation and pregnancy in mares treated with flunixin-meglumine during the periovulatory period.

Authors’ declaration of interests
No competing interests have been declared.

Ethical animal research
Animal procedures were approved by the local animal welfare committee of the Universidad CEU Cardenal Herrera (ref: PRCEU-UCH 13/14).

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Authorship
J. Cuervo-Arango contributed to study design, study execution, data analysis and interpretation, preparation of the manuscript and final approval of the manuscript. R. Martínez-Bovi contributed to study execution, data analysis, figure creation and final approval of the manuscript.

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Fig 5: B-mode ultrasonograms of a mare during the treatment cycle. Note the moderate follicular haemorrhage evidenced by presence of numerous echoic specks within the antrum 32 h after hCG treatment but before follicle puncture (32 h PRE). The follicle collapsed 42–44 h after hCG (12 h after puncture and the delivery of 500 μg PGE$_2$ and 125 μg of PGF$_2\alpha$). This mare became pregnant. However, the embryonic vesicle was small for age and measured only 6.0 mm 14 days after ovulation. Hour 0 = time of hCG treatment.


