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The effect of systemic treatment with cloprostenol on ovulation in flunixin-meglumine treated mares

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Contents

Prostaglandins (PG) are essential to trigger the cascade of events that degrade the extracellular matrix of follicles leading to follicular collapse and ovulation. In mares, systemic administration of flunixin-meglumine (FM), a PG synthase inhibitor, blocks ovulation by inducing luteinized unruptured follicles (LUF). In the rat, the administration of PGF and PGE restored ovulation in indomethacin treated animals. Mares were treated with FM at 0, 12, 24 and 36 h after hCG administration to induce experimentally LUFs (n = 15) or were left untreated (controls, n = 5). In addition, 250 µg of cloprostenol were administered intravenously to mares at 33, 35 and 36 h after hCG treatment (CLO 33, n = 5) or at 48, 49 and 50 h (CLO 48, n = 5). One group was treated with FM but not with cloprostenol (FM-control, n = 5). The ovulation rate, follicular diameter and progesterone concentration was compared amongst groups. The ovulation rate at 48 h was higher ($P < 0.05$) in controls (100%) than in FM-control (0%), CLO 33 (0%) or CLO 48 (20%) mares. All but one FM treated mares developed LUFs by 48 h after hCG treatment. Two LUFs collapsed between 48 and 60 h and 72 and 84 h in a mare from FM-control and one from the CLO 33 group, respectively.

Progesterone concentration was significantly higher ($P < 0.05$) in control mares than in any of FM treated mares at 5, 9 and 13 days after hCG. In conclusion, FM administered during the peri-ovulatory period blocked ovulation in mares. In contrast, the administration of cloprostenol, a PGF analogue, in previously FM treated mares failed to restore ovulation.

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 different hormones and enzymes (Robker et al. 2000). Prostaglandins (PGs) play an essential role during the process of follicular rupture (Armstrong 1981; Priddy and Killick 1993; Murdoch et al. 1993). In the follicle, PGs are produced by the inducible cyclo-oxygenase isoform-2 (COX-2), also known as prostaglandin G/H synthase (PGHS; Hedin et al. 1987). The LH surge (Ginther et al. 2009) induces the expression of COX-2 in granulosa cells of rats (Huslig et al. 1987) and mares (Sirois and Dore 1997).

The obligatory role of PGs during the ovulatory process has been confirmed on numerous occasions across different species. In the mare, intrafollicular administration of indomethacin (a PG synthetase inhibitor) blocks ovulation (Watson and Sertich 1991). Furthermore, systemic treatment with indomethacin inhibited ovulation by inducing luteinized unruptured follicles (LUFs) in women (Killick and Elstein 1987). The role of PGs during the process of follicular rupture is not known, but the results of a recent study in cattle (Li et al. 2006) linked the role of PGs to downstream regulation of matrix-metalloproteinases and plasmin, which are enzymes (collagenases) involved in the degradation of extracellular matrix in the follicular wall.

The administration of an ovulatory dose of hCG to oestrous mares induces a rise in LH concentration (Ginther et al. 2009) within 12 h of treatment and an expression of COX-2 in granulosa cells 24 to 30 h after treatment (Sirois and Dore 1997). The COX-2 expression in equine granulosa cells induces a gradual increase in PGF and PGE concentration in follicular fluid from 33 h post-hCG treatment reaching the peak concentration at 39 h (Sirois and Dore 1997).

Twice daily systemic treatment of flunixin-meglumine (FM), a non-specific COX inhibitor, 0 to 48 h after hCG administration to oestrous mares blocked ovulation by inducing haemorrhage and luteinization of follicles in more than 80% of treated mares (Cuervo-Arango and Domingo-Ortiz 2010). Although not analyzed in the latter study, the follicular fluid concentrations of both PGF and PGE were likely to be decreased in mares with luteinized unruptured follicles. A similar study (Priddy et al. 1990) showed the reduction in follicular fluid concentrations of PGF and PGE of women treated with systemic indomethacin and hCG.

Several studies on rats and rabbits have shown the efficacy of PGF, PGE or combination of both to overcome the inhibitory effect of indomethacin and restore ovulation (Sogn et al. 1987; Holmes et al. 1983; Gaytan et al. 2002), but it has never been attempted in mares.

A naturally-occurring anovulatory condition in mares has been termed haemorrhagic anovulatory follicle (HAF) (Ginther et al. 2007; Cuervo-Arango and Newcombe 2009). This condition affects negatively the reproductive efficiency of mares (McCue and Squires 2002) since mares mated or inseminated to a single preovulatory follicle that later becomes a HAF will not conceive. These HAFs do not rupture and therefore the oocyte cannot be released and fertilization is not possible. Furthermore, mares with HAFs enter a dioestrous-like period, as a result of luteinization

of anovulatory follicles, of a similar length that ovulatory mares (Cuervo-Arango and Newcombe 2010). The prevalence of HAFs in a normal population of mares is relatively low: 4 to 5 % (Ginther and Pierson 1989; Cuervo-Arango and Newcombe 2009), but can be dramatically increased in some individuals so called “repeater mares” (Ginther et al. 2006; Cuervo-Arango and Newcombe 2010). The treatment options of mares with HAFs are limited. Only a preventative treatment in repeater mares has been successfully attempted (Carnevale 2004). This involves aspiration of oocyte by ovum pick-up before the preovulatory follicle haemorrhages with subsequent transfer into a recipient mare.

The ultrasound characteristics of experimentally-induced LUFs in mares by treatment with FM resembled those observed in naturally occurring HAFs (Cuervo-Arango and Domingo-Ortiz 2010). However, it is not known whether prostaglandins play a similar role in the pathogenic mechanisms leading to anovulation in mares with HAFs. Nevertheless, provided that the incidence of naturally-occurring HAFs is relatively low and unpredictable, the use of experimentally FM-induced LUFs could be used as a model to research different treatment options for anovulation in mares.

The objective of this study was to determine the effect of a systemic treatment of cloprostenol, a synthetic PGF analogue, administered at different times relative to hCG on restoring ovulation in FM-treated mares.

Materials and methods

Mares were mixed breeds of large ponies and apparent pony-horse crosses weighing 300 to 460 kg. Mares were selected with docile temperament and no apparent abnormalities of the reproductive tract, as determined by ultrasound examinations. The experiment was done in August 2010 (northern hemisphere). 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.

A total of 20 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. When a mare first showed an endometrial oedema score of 3 to 4 (4 = maximum degree of endometrial folding) and a follicle ≥ 32 mm in diameter (0 h), human chorionic gonadotropin (hCG; Chorulon; Intervet Inc., Millsboro, DE, USA) was administered in a single intravenous dose of 1500 IU. After that, mares were allocated to 4 different groups:

- Ovulatory-control group (n = 5). Mares had no further treatment.
- FM-control group (n = 5). Mares were administered 1.7 mg/kg of FM (FluMeglumine; Phoenix Pharmaceutical Inc., St Joseph, MO, USA) intravenously at 0, 12, 24, and 36 h.
- FM-CLO 33 (n = 5). Mares were treated with FM as in FM-control group in addition to three administrations of 250 µg of cloprostenol (Estrumate®, Intervet INC, Millsboro 19966 DE, USA) intravenously at 33, 35 and 36 h after hCG treatment.
- FM-CLO 48 (n = 5). Mares were treated with FM as above. If the mares had not ovulated by 48 h and showed signs of follicular haemorrhage (presence of echoic particles floating freely within the follicular fluid), they were given three administrations of 250 µg of cloprostenol intravenously at 48, 49 and 50 h after

hCG treatment. One mare allocated to this group ovulated between 36 and 48 h and therefore only 4 mares were administered cloprostenol.

All mares were examined transrectally by ultrasound every 12 h after hCG treatment until 132 h and again at 216 h after hCG treatment. Blood samples were taken at 2, 5, 9 and 13 days after hCG. Samples were collected into heparinized tubes from the jugular vein, immediately placed in ice-cold water for 5 min, and centrifuged (2000 x ***g*** for 10 min). The plasma was decanted and stored (–20 °C) until assayed. The plasma samples were assayed by validated radioimmunoassay, as described for mare plasma progesterone (Ginther et al. 2005). The intra-assay coefficient of variation and sensitivity were 13.4% and 0.02 ng/ml.

In all groups, at 48 h after hCG treatment, the preovulatory follicle of each mare was classified as ovulatory when the follicle had collapsed with loss of > 90% of follicular fluid or as LUF, described previously (Cuervo-Arango and Domingo-Ortiz 2010). The diagnosis of a LUF was confirmed by further increase in follicular diameter, thickness and vascularisation of the follicular wall (by colour-Doppler ultrasonography) at subsequent examinations and progesterone concentrations of > 1 ng/ml. If a LUF had presence of echoic particles (score ≥ 1) and increase in echogenicity of the follicular wall ≥ 48 h after hCG but collapsed thereafter with loss of > 90% of follicular fluid was not classified as an ovulation but as a "LUF collapse". The degree of follicular haemorrhage of LUFs was scored subjectively from 0 to 5 (Figure 1) according to the shape and amount of echoic particles within the follicular antrum. A score of:

- 0 was given to follicles with anechoic fluid (absence of echoic particles).
- 1 was given to follicles with the presence of a slight amount of echoic particles (easy to count).

- 2 was given to follicles with higher amounts of echoic particles, too numerous to count but which sill left some visible areas of anechoic fluid within the antrum, or to follicles with presence of echoic clots or aggregates of particles floating freely within the anechoic follicular fluid.
- 3 was given to follicles with massive haemorrhage, as evidenced by the presence of many echoic specks with no visible parts of anechoic fluid. These follicles did not show any solid strands or clots yet.
- 4 was given to follicles with massive haemorrhage that showed beginning of strand formation or gave the appearance of a solid sheet of hyperechoic particles. The follicular contents still remained movable upon ballottement of the ovaries.
- 5 was given to follicles whose contents had organized and either quivered or remained solid upon ballottement of the ovary. The appearance of the follicular contents appeared either as a solid echoic mass or as a network of fibrin strands with a cobweb-like appearance.

The incidence of ovulatory follicles in each group was compared by Fisher's exact test. The difference in progesterone concentration at 2, 5, 9 and 13 days after hCG in all groups and the follicular diameter and score of follicular contents of groups with LUFs from 0 to 216 h were compared using a general linear model of ANOVA and Tukey's test. Data not normally distributed was transformed using ranks. All data was computed using a statistical software (Minitab15®, Minitab Inc. USA). A probability of $P \leq 0.05$ indicated that a difference was significant and probabilities between $P > 0.05$ and $P \leq 0.1$ indicated that a difference approached significance. Data are given as mean ± S.E.M., unless stated otherwise.

Results

All ovulatory control mares ovulated normally between 36 and 48 h (Fig. 2). The incidence of ovulatory follicles by 48 h after hCG treatment was 100%, 0% and 0% for ovulatory-control, LUF-control and LUF CLO 33 groups, respectively. By 48 h, the ovulatory rate of control mares was higher ($P < 0.05$) than that of LUF control or LUF CLO 33. One mare from the LUF-control group had a LUF collapse between 48 and 60 h. None of the LUF mares treated with cloprostenol at 48, 49 and 50 h had a LUF collapse (0/4). In contrast, one mare of LUF CLO 33 had a LUF collapse (Fig. 3) between 60 and 72 h (1/5, 20%). The number of ovulations by 48 h between LUF-control and LUF CLO 33 groups was not different: 1/5 and 0/5, respectively ($P > 0.05$) nor was the number of LUF collapses > 48 h after hCG amongst the three FM groups: 0/4, 1/5 and 0/4 for LUF-control, LUF CLO 33 and LUF CLO 48 groups, respectively ($P > 0.05$). The ovulation and LUF collapse rates for all groups are shown in Table 1.

The progesterone concentration of control mares was higher than the rest of groups at 5, 9 and 13 days after hCG treatment (Fig. 4). The progesterone concentration of FM mares did not differ at any of the time-points analyzed. The follicular diameter of mares from LUF CLO 33 was greater ($P < 0.05$) than LUF control and LUF CLO 48 groups at 216 h after hCG treatment (Fig. 4).

The degree of follicular haemorrhage as measured by the score of echoic particles within the follicular antrum of LUF CLO 33 (Fig. 5) was lower ($P < 0.01$) than the rest of LUF groups (Fig. 6). The fewer number of echoic particles resulted in the absence of a solid organization of follicular contents in LUF CLO 33 group by 9 days after hCG (Fig. 5) compared with a mean time of organization of follicular contents of 114 ± 4.5 h and 110 ± 9 h after hCG in LUF-control and LUF CLO 48 groups, respectively.

Discussion

The systemic treatment of mares with FM during the periovulatory period blocked ovulation in the majority of treated mares as reported previously (Cuervo-Arango and Domingo-Ortiz 2010). However the subsequent administration of cloprostenol, a synthetic PGF analogue, failed to overcome the anovulatory effect of FM regardless of the time of administration. Cloprostenol was chosen over native PGF (dinoprost) because of its longer half life and potency. This is 54 min in the rat (Bourne et al. 1979) compared with less than 1 min for native PGF (Pike 1971). The use of the intravenous route for cloprostenol had not been attempted previously in the mare. This route was used to favour a more rapid distribution of the drug within the peripheral circulation and hopefully within the intrafollicular fluid. The mares showed profound diarrhoea and sweating within 5 min of treatment, apparently more severe than in mares administered cloprstenol subcutaneously or intramuscularly. In addition the intravenous route induced a mild ataxia and locomotor incoordination within 5 min of treatment that lasted for about 20 min. This transient side effect was also described in mares after intramuscular or subcutaneous administration of very large doses (800 mg) of native PGF (Lauderdale and Miller 1975).

The timing of cloprostenol administration relative to hCG treatment (at 33, 35 and 36 h after hCG) intended to mimic the gradual increase in PGF concentration within the follicular fluid observed 33 to 39 h after the administration of an ovulatory dose of hCG (Sirois and Dore 1997). The second group of cloprostenol administered at 48 h after hCG was intended to evaluate whether the treatment of PGF would induce follicular collapse once the follicle already showed signs of haemorrhage and luteinization. In practice, the diagnosis of a HAF can be made only after the observation

of a gradual increase in echoic particles floating freely in the follicular antrum. Unfortunately, systemic treatment with cloprostenol did not increase significantly the incidence of ovulation or collapse of luteinized follicles.

There could be several reasons accounting for the apparent failure of cloprostenol to overcome the anovulatory effect of FM in this population of treated mares. The timing of administration seemed to be adequate since the first significant rise in concentration of PGF in follicular fluid occurs between 33 and 36 h after hCG. It is not known whether a sufficient amount of cloprostenol gained access to the follicular fluid to trigger the molecular mechanisms that lead to the process of ovulation. However, the dose of the current study, if compared with the minimum dose of cloprostenol required to induce luteolysis in the mare (Newcombe et al. 2008), seems far in excess. Furthermore, systemic treatment with FM at a 154 % of the recommended dose (manufaturer's data sheet information) appeared to be sufficient to gain access into the follicular fluid and block ovulation in most of treated mares. So it could be speculated that a similar dose of cloprostenol would also get into the follicular fluid. However, studies with follicular fluid sampling at different times after cloprostenol administration remain to be done to elucidate this statement.

On the other hand, it is likely that PGF is not the only COX-2 product essential to trigger the complex process of ovulation. It has been shown than PGE_1 was more efficient than PGF to overcome the effect of a COX-2 inhibitor and restore ovulation in rats treated with indomethacin (Gaytan et al. 2002). In addition, this was a dose dependant effect. Further studies involving the administration of several types of prostaglandins at different doses are needed to determine the critical effect of prostaglandins upon ovulation in the mare. Unfortunately, an injectable form of PGE is nor readily available for commercial use in equine practice.

The absence of follicular collapse in mares with LUFs resulted in reduced production of progesterone by the luteal cells compared with mares with corpora lutea from ovulatory follicles. It is not surprising since during the development of the corpus luteum in ovulatory mares, within 24 h of ovulation, the microscopic appearance of the equine early corpus luteum shows folds of stromal tissue beginning to grow into the luteinizing cells accompanied by proliferating capillaries which provide the required nutrients and growth factors for continued development of luteal cells (Van Niekerk et al. 1975; Watson and Sertich 1990). Therefore, if the formation of new blood vessels within the body of the corpus luteum is impeded by the lack of follicular rupture in LUFs, then the development of luteal cells might not be complete and so their ability to secrete progesterone. Despite the repeated use of a luteolytic drug in mares with LUFs, they produced similar concentrations of progesterone than LUF mares without cloprostenol treatment. The treatment of cloprostenol stopped before the expected post-ovulatory rise in progesterone concentration. However, this is in contrast with a previous study that showed a reduction in progesterone concentrations in mares treated with 500 µg of cloprostenol within two days of ovulation (Troedsson et al. 2001).

Despite the failure of cloprostenol to induce ovulation in FM-treated mares, it appeared to interfere somehow with the normal development of LUFs. Treatment with cloprostenol at 33 to 36 h but not at > 48 h after hCG reduced the entry of blood in follicles of mares treated with FM. This resulted in the lack of clotting of blood and follicular contents of LUFs in mares from the CLO 33 group. The follicular fluid is rich in a heparin-like anticoagulant substance (Stangroom and Weevers 1962) that delays clotting of blood in the follicular fluid. When the amount of blood appears to exceed that of follicular fluid the contents clot and become solid. It is reasonable to think that if the degree of haemorrhage is minimal, the amount of follicular blood is not sufficient to

overcome the effect of the anticoagulant substance and therefore the follicular contents remain in a fluid state.

In conclusion, the administration of cloprostenol during the expected periovulatory period to mares treated with flunixin-meglumine failed to restore ovulation but influenced the appearance of LUF resultant from a milder entry of blood into the follicular antrum in mares treated with cloprostenol 33 to 36 h after hCG.

Conflicts of interest

The author has no conflict of interest to declare.

Acknowledgments

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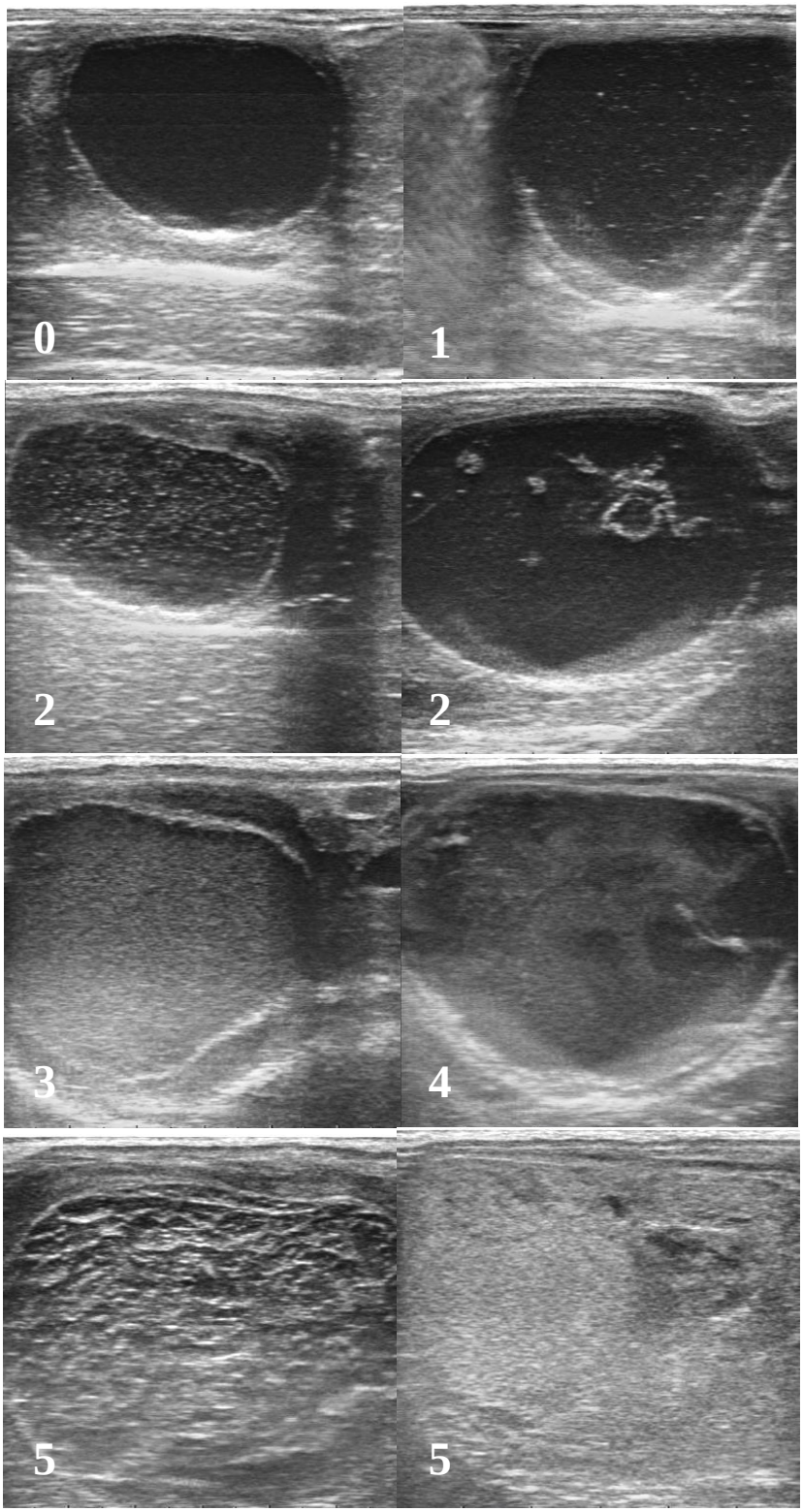

Fig. 1. Follicular contents score of luteinized unruptured follicles (LUFs). The score goes from 0 (anechoic fluid) to 5 (organization of contents).

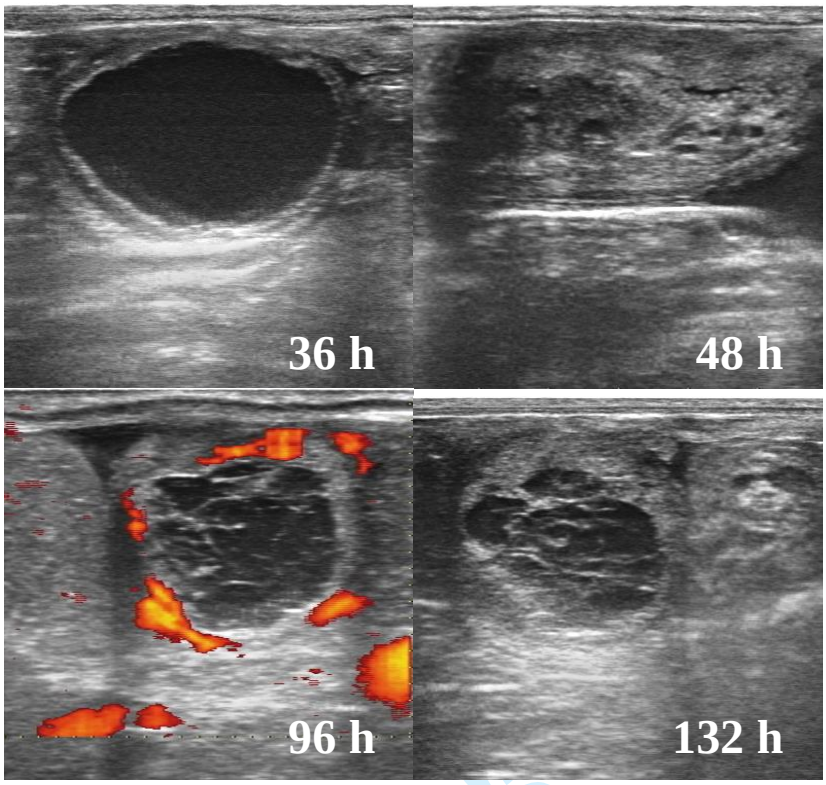

Fig. 2. B-Mode and colour-Doppler (96 h) sonograms of a mare from the ovulatory-control group. The mare was only treated with 1500 IU of hCG i.v. (0 h). 36 h: preovulatory follicle with echoic-free follicular fluid. 48 h: ovary with ovulatory area, note the remaining anechoic follicular fluid (small pocket of black fluid). 96 h: the ovulatory area developed into a corpus haemorrhagicum with a central blood clot; note the high intensity colour-Doppler signals around the corpus haemorrhagicum periphery. 132 h: the central lacuna of the CH has contracted slightly.

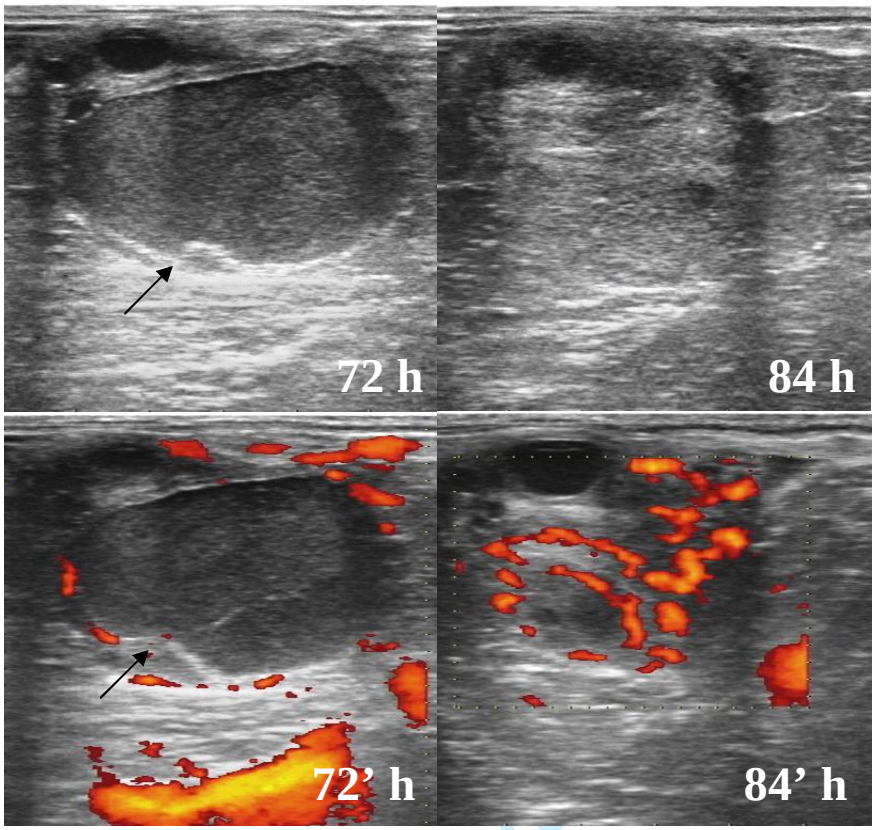

Fig. 3. B-Mode and Colour-Doppler sonograms of a mare from LUF CLO 33 group. The mare was treated with 1.7 mg/kg flunixin-meglumine i.v. every 12 h from 0 to 36 h after hCG (0 h) and 250 µg cloprostenol i.v. at 33, 35 and 36 h after hCG. The luteinized unruptured follicle (LUF) showed signs of follicular haemorrhage by 48 h. The LUF contents score increased gradually thereafter until 72 h. Between 72 and 84 h, the LUF collapsed. Note the disruption in the follicular wall integrity at the ventral left part of the LUF (black arrow: 72 h and 72’ h). The degree of vascularisation of the follicular wall is indicated by the yellow-red colour intensity in Doppler sonograms before (72’ h) and after (84’ h) LUF collapse.

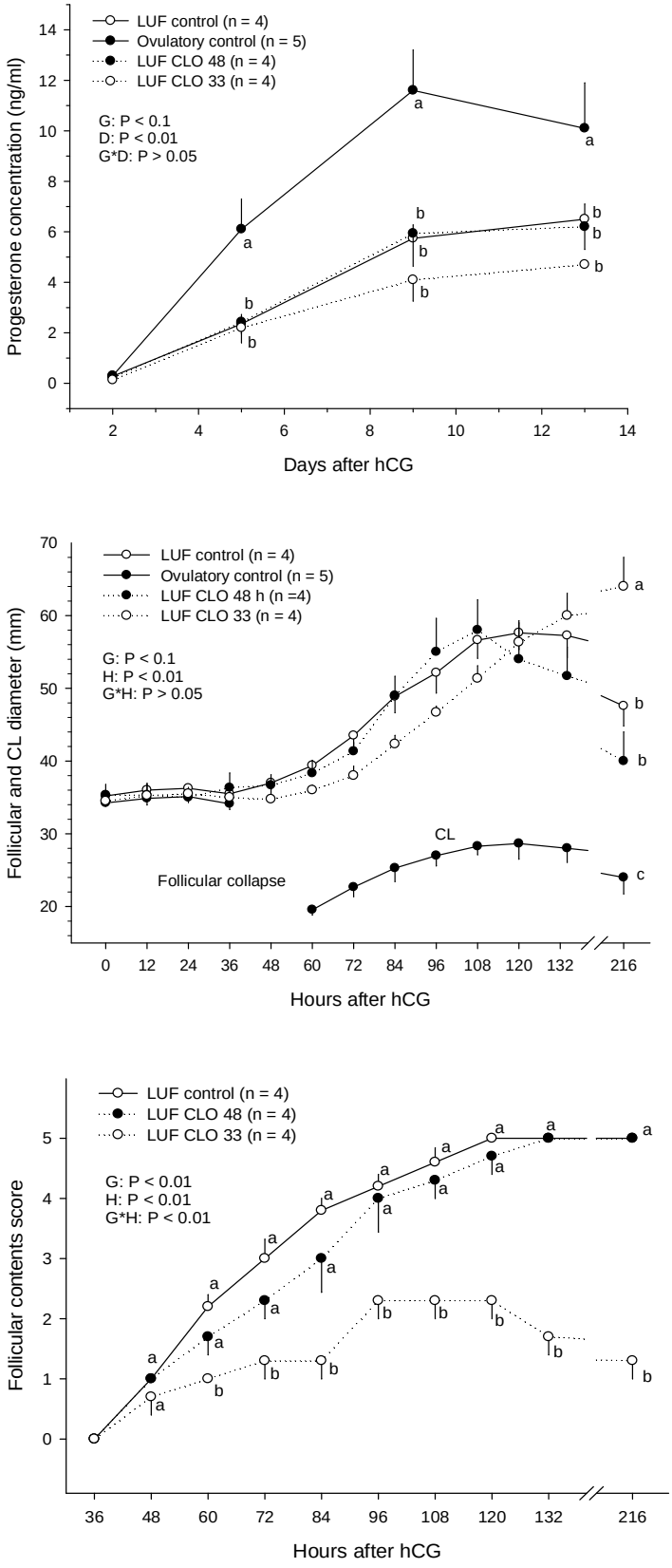

Fig. 4. Mean ± S.E.M. of progesterone concentration, follicular diameter and follicular contents score of ovulatory control, FM-treated (LUF-control) and FM and cloprostenol treated mares (LUF CLO 33 and LUF CLO 48). The effect of group (G), hour or day (H, D) and group by day or hour interaction (G*H, G*D) on progesterone concentration, follicular diameter and follicular contents score are shown. Within each time point, different letters indicate significant difference ($P < 0.05$).

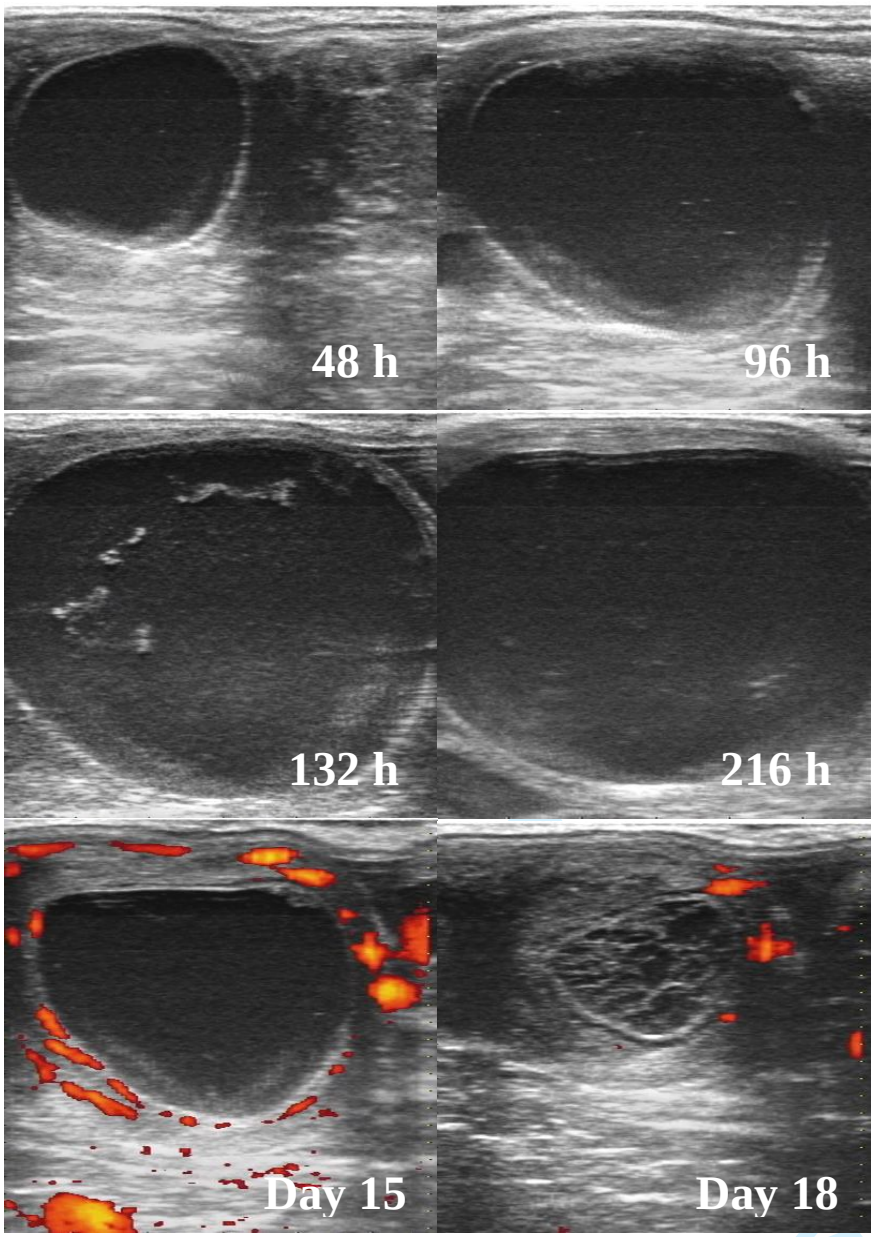

Fig. 5. B-Mode and colour-Doppler (Day 15 and Day 18) sonograms of a mare from LUF CLO 33. The mare was treated with 1.7 mg/kg flunixin-meglumine i.v. every 12 h from 0 to 36 h after hCG (0 h) and 250 µg cloprostenol i.v. at 33, 35 and 36 h after hCG. Note the low score of follicular contents (< 2) resultant from low number of echoic particles floating within the antrum throughout the LUF lifespan (48 h to 15 days). At one point (132 h) the scant follicular haemorrhage formed small clots of blood decanted at the bottom of the LUF which floated freely upon ballottement of the ovary. The active production of progesterone by the luteinized follicular wall is indicated by the presence of high-intensity colour-Doppler signals around most of the LUF wall circumference (Day 15). The LUF remnant (Day 18) has lost most of its Doppler signals since at this point the luteal tissue had regressed (the mare showed endometrial oedema 18 days post-hCG treatment.

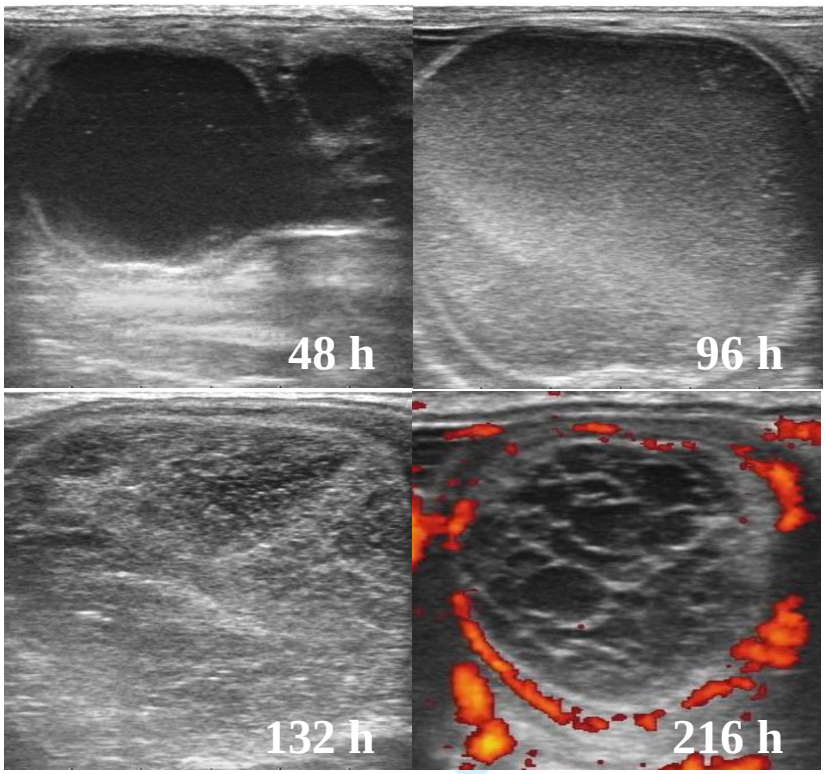

Fig.6. B-Mode and colour-Doppler (216 h) sonograms of a mare from LUF-control group. The mare was administered 1.7 mg/kg flunixin-megluimine i.v. every 12 h 0 to 36 h after hCG treatment (0 h). Note the high degree of follicular haemorrhage at 96 h. By 132 h the follicular contents had organized and did not move upon ballottement of the ovary. At 216 h after hCG (day 9) the LUF wall is highly vascularised indicated by colour-Doppler signals around most of the wall circumference of the LUF. The peripheral progesterone concentration at 216 h was 3.96 ng/ml.

Table 1. Effect of cloprostenol on ovulation rate and progesterone concentration in FM treated mares

Group	n	Ovulations by 48 h	LUF collapses > 48 h	P4 day 13 (ng/ml)	Maximum LUF diameter (mm)	LUF contents clotting time (h)
Ovulatory-control	5	5^{a}	-	10.1 ± 1.8^{a}	-	-
LUF-control	5	1^{b}	0^{a}	6.5 ± 0.6^{b}	62.1 ± 1.3^{a}	114 ± 4.5^{a}
LUF CLO 33	5	0^{b}	1^{a}	4.7 ± 0.1^{b}	65.9 ± 4.7^{a}	$> 216^{*\ b}$
LUF CLO 48	5	1^{b}	0^{a}	6.1 ± 0.9^{b}	59.3 ± 3.5^{a}	110 ± 9^{a}

Within column, different letters indicate significant difference ($P < 0.05$). The asterisk (*) indicates that the follicular contents of LUFs from mares treated with cloprostenol at 33, 35 and 36 h after hCG was still fluid at 216 h after hCG.