Cyclooxygenase-2 is inhibited in prolonged luteal maintenance induced by intrauterine devices in mares

Maria Montserrat Rivera del Alamo[a*], Tiina Reilas[b1], António Galvão[c], Marc Yeste[d], Terttu Katila[e]

[a]*Animal Medicine and Surgery Department, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, 01893, Spain, mariamontserrat.rivera@uab.cat*

[b]*Animal Production Research, MTT Agrifood Research Finland, Opistontie 10 A 1, 32100 Ypäjä, Finland, tiina.reilas@luke.fi*

[c]*Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, 10–748 Olsztyn, Poland, a.galvao@pan.olsztyn.pl*

[d]*Department of Biology, Faculty of Sciences, University of Girona, Girona, 17071 Spain, marc.yeste@udg.edu*

[e]*Department of Production Animal Medicine, Faculty of Veterinary Medicine, Helsinki University, Paroninkuja 20, 04920 Saarentaus, Finland, terttu.katila@helsinki.fi*

[*]Corresponding author. Tel.: +34 93 581 1045; E-mail address: mariamontserrat.rivera@uab.cat

[1]Present address: Natural Resources Institute Finland (Luke), Myllytie 1, Jokioinen, Finland, *tiina.reilas@luke.fi*

ABSTRACT

Treatment with intrauterine devices (IUD) prolongs luteal phases in mares, but the mechanism for this has not been fully elucidated. The aims of the present study were to examine how IUDs affect the uterus to induce longer luteal phases, particularly the role of cyclooxygenase-2 (COX-2) in the maintenance of the corpus luteum (CL). Twenty-seven reproductively normal mares were included: 12 were inseminated (AI), and 15 were fitted with IUDs. Blood samples for progesterone were obtained on Days 0, 3, 5, 7, 9, 11, 13, 14, and 15 (relative to day of ovulation). The groups were further divided into non-pregnant (AI-N, $n = 4$), pregnant (AI-P, $n = 8$), normal (IUD-N, $n = 8$) and prolonged luteal phase (IUD-P, $n = 7$) based on ultrasonic examinations and serum progesterone concentrations on Days 14 and 15. Blood sampling to quantify the $PGF_{2\alpha}$ metabolite (PGFM) was performed through a catheter hourly from 15:00 to 20:00 hour on Day 14, and from 6:00 until 13:00 hour on Day 15. On Day 15, a low-volume uterine lavage followed by an endometrial biopsy was performed. Estradiol concentration in the Day 15 serum and lavage fluid was determined, while the abundance of COX-2 was evaluated in the biopsy specimens using western blotting (WB) and immunohistochemistry (IHC). All pregnant mares were negative for COX-2 in IHC samples and 5 of 8 were negative in WB samples while all mares of the IUD-N group were positive for COX-2. Of the seven mares in the IUD-P group, five and four were negative for COX-2 with the IHC and WB samples, respectively. The results from this study indicate that IUDs, when effective, suppress COX-2, leading to the inhibition of PGF2α release and maintenance of CL.

Keywords:

Cyclooxygenase-2;

PGF2α;

Progesterone;

Inflammation;

Intrauterine device;

Horse

1. Introduction

Treatment with intrauterine devices (IUDs) induces prolonged luteal phases in mares (Nie et al., 2003; Rivera et al., 2008). The biological mechanism that induces this effect has not been elucidated, but endometrial inflammation and embryo mimicking have been suggested as possible explanations.

Rivera del Alamo et al. (2008) reported that concentrations of prostaglandin F2α ($PGF_{2\alpha}$) metabolite are basal and progesterone (P_4) concentrations are relatively greater in mares in which the IUD induces luteostasis, but not in those in which the IUD fails to do so. No clear evidence for inflammation was observed in this previous study, and it remains unclear how the IUD causes the inhibition of $PGF_{2\alpha}$ actions.

During maternal recognition of pregnancy, the presence of an embryo in the uterus results in the inhibition of the synthesis and/or release of $PGF_{2\alpha}$ from the endometrium and, subsequently, leads to the maintenance of the corpus luteum (CL; Kindahl et al., 1982). The role of $PGF_{2\alpha}$ is essential, the inhibition of its release from the endometrium leads to a sustained luteal function (i.e., luteostasis), and inhibition by different mechanisms at the expected time of luteolysis may lead to prolonged luteal activity (Santos et al., 2013).

The enzyme cyclooxygenase-2 (COX-2) catalyzes the synthesis of prostaglandin H_2 from arachidonic acid. The synthesized PGH_2 is subsequently converted to prostaglandins, such as $PGF_{2\alpha}$. On Day 15 after ovulation, the presence of COX-2 in the endometrium was lower in pregnant mares than in cyclic mares (Boerboom et al., 2004). In addition to being key regulators of reproductive processes, COX-2 and $PGF_{2\alpha}$ are also involved in inflammation (reviewed by Jabbour and Sales, 2004).

The aim of the present study was to elucidate the mechanisms for the IUD-induced luteostasis by comparing mares with IUDs to pregnant and non-pregnant mares. The goal of this experiment was to study COX-2 (the immediate precursor for PG synthesis) in mares with IUDs and in inseminated mares. The hypothesis was that there would be a lesser abundance of COX-2 in mares with prolonged luteal function.

2. Materials and methods

2.1. Animals

Twenty-seven mares (Finnhorses, Standardbreds and warmbloods) from the Equine College and MTT Agrifood Research Finland in Ypäjä, Finland, were included in the experiment. The mares aged between four and 17 years old (mean 9.6 years), had no history of reproductive failure, and were clinically normal. They were age-matched and divided into two different groups: the inseminated (AI) group (n = 12; mean age 9 years, range 4–16) and the IUD group (n = 15; mean age 10.3 years, range 4–17 years). These two groups were further divided into two sub-groups, depending on the outcome of inseminations and insertion of IUD. The AI group was assigned to the AI-N (non-pregnant mares: n = 4; mean age 14.8 years, range 13–16 years) and AI-P (pregnant mares: n = 8; mean age 6.1 years, range 4–9 years) groups. Similarly, mares in the IUD group were assigned to the IUD-N (IUD resulted in normal length luteal phase; n = 8;

mean age 10.8 years, range 5–17 years) and IUD-P (IUD resulted in prolonged luteal phase; *n* = 7; mean age 9.3 years, range 4–16 years) groups.

2.2. Experimental design

The Centre for Economic Development, Transport and Environment for Southern Finland granted permission for animal experimentation (permit number 1102101). The mares were estrous synchronized with one or two i.m. injections of 0.125 mg of cloprostenol (Estrumat vet®, Schering Plough A/S Farum, Denmark) and maintained in lots for easier management. The mares were examined by trans-rectal palpation and ultrasonography every other day while in early estrus. After a 35-mm diameter follicle was observed, the examinations were performed daily until ovulation was detected. The day of ovulation detection was assigned as Day 0. To induce ovulation, 1500 IU of hCG (Chorulon®, Intervet International B.V., Boxmeer, the Netherlands) was administered i.v. when the mare had a follicle of ≥35 mm. The mares of the AI group were inseminated approximately 24 h after the administration of hCG using semen from a stallion with normal fertility.

A blood sample was obtained from the jugular vein on Days 0, 3, 5, 7, 9, 11, 13, 14, and 15. The IUD was a water-filled polypropylene sphere with a diameter of 20 mm and an average weight of 3.6 g (Rivera del Alamo et al., 2008). It was inserted into the uterus on Day 3 using the double-glove technique (Portus et al., 2005).

On Day 14, the mares were examined by transrectal palpation and ultrasonography to evaluate the stage of the estrous cycle, the presence/absence of an embryo in AI mares, and the location of the sphere in mares with an IUD. After this, a 14-gauge, 5.25-inch polyurethane i.v. catheter-over-needle (Mila International Inc., Florence, KY, USA) was inserted into the jugular vein of all mares with an IUD and

four pregnant and four non-pregnant AI mares to obtain blood for PGFM analysis. Blood sampling was performed every hour from 15:00 to 20:00 hour on Day 14, and from 06:00 until 13:00 hour on Day 15.

In the afternoon of Day 15, transrectal palpation and ultrasonography examination were performed, followed by a low-volume uterine lavage as previously described by Reilas and Katila (2002). The lavage fluid was kept in ice until centrifuged at 4 °C. The supernatant was collected and stored in aliquots at -80 °C until analyses were performed.

The perineal area was washed again, and two endometrial biopsy specimens were obtained from the base of the uterine horn closest to the embryonic vesicle or the IUD, or from the right uterine horn in AI-N mares, as described by Kenney (1978). One of the specimens was frozen immediately in liquid nitrogen for western blotting analysis. The other specimen was processed for histology and for conducting IHC procedures.

After this, all the mares not in estrus got an i.m. injection of 0.125 mg of cloprostenol (Estrumat vet®, Schering Plough A/S Farum, Denmark). After the mare was diagnosed to be in estrus, a uterine swab was obtained using a sterile, double-guarded uterine culture swab (Equi-Vet®, Kruuse, Marslev, Denmark). The IUDs were removed by transrectal manipulation.

2.3. Hormone analysis

2.3.1. Analyses in blood

Blood samples for serum P_4 and estradiol-17β (E_2) analyses were collected using 10 mL vacuum tubes. The samples were centrifuged at 2,200 *g* for 10 min and aliquots of serum were kept frozen until analysis was performed.

The P_4 concentrations were determined with the Spectra P_4 radioimmunoassay (RIA) kit (Orion Diagnostica, Espoo, Finland), using the 1270 Rackgamma counter (Wallac Oy, Turku, Finland). The serum E_2 concentration on Day 15 was determined with a commercially available human radioimmunoassay (ultra-sensitive E_2 RIA, DSL4800, Immunotech a.s., Prague), according to the assay procedure of the manufacturer in the aliquots of the same serum samples used for P_4 measurement. All samples were determined in duplicate in a single assay. The intra-assay coefficient of variation (CV %) was 8.7 (13.2 pg/mL) and 6.1 (39.7 pg/mL) calculated from six repeated measurements of two serum samples with different E_2 concentrations.

For 15-Ketodihydro-$PGF_{2\alpha}$ (PGFM) analysis, blood was dispensed in 4 mL vacuum lithium heparin tubes and centrifuged at 2,200 *g* for 10 min. After centrifugation, 1 mL of plasma was dispensed in 1.5 mL Eppendorf tubes and stored at -80 °C until analysis.

The concentrations of PGFM (the main initial $PGF_{2\alpha}$ plasma metabolite) were quantified to monitor $PGF_{2\alpha}$ release, as described by Granström and Kindahl (1982). The assay was performed with unextracted plasma (0.2 mL plasma or dilutions of plasma in 0.25% bovine gamma globulin buffer). Before the addition of the antibody and radioactive tracer, 0.3 mL if 0.25% (w/v) bovine gamma globulin (Sigma Chemical Co., Poole, St. Louis, MO, USA) in buffer was added, and the tubes were heat-treated for 30 min at 45 °C. The antibody cross-reacts with 15-keto-$PGF_{2\alpha}$ (16.0%), 13,14-dihydro-$PGF_{2\alpha}$ (4.0%), and 15-ketodihydro-PGE_2 (1.7%). The extent of cross-reactivity with all other PGs tested is <0.1%. The detection limit of the assay is 60 pmol/L. The intra- and inter-assay coefficients of variation are 6.6% and 11.7%, respectively. Concentrations less than the detection limit of the assay were considered to be equal to the detection limit of the assay.

176

177 *2.3.2.* E_2 *in uterine lavage fluid*

178 The concentration of E_2 in fluid from uterine lavage was quantified with a
179 commercial kit (ultra-sensitive E_2 RIA, DSL4800, Immunotech a.s., Prague) using the
180 procedures previously described by Gastal et al. (1999), with minor modifications.
181 Initially, eight standard concentrations in the 0 to 108 pg/mL range were prepared from
182 the standard of the kit with the greatest quantity of E_2 by diluting with 0.1% gelatin in
183 PBS. Control samples were diluted at a 1:5 dilution with the same buffer. Then the
184 lavage fluids were concentrated five-fold. A 500 µL aliquot of each sample was
185 extracted with 2.5 mL of diethyl ether. The ether phase was evaporated in a vacuum
186 concentrator (RVC 2-25, Martin Christ, Osterode am Harz, Germany) and the dried
187 extracts re-suspended in 100 µL of 0.1% gelatin in PBS. Extractions and determinations
188 were all performed in duplicates.

189 The range for quantitation was 1.69 to 108 pg/mL. Thus, whenever E_2
190 concentration exceeded 108 pg/mL, the samples were diluted and re-analyzed. The
191 concentration of E_2 in uterine lavage fluid was calculated by dividing the results of the
192 concentrated extracts by five. Due to the fivefold concentration, the detection limit was
193 0.34 pg/mL. The intra-assay coefficient of variation was calculated from sample
194 duplicates for two concentration ranges, 1.85 to 7.80 pg/mL, and 29.2 to 126.3 pg/mL.
195 The corresponding CV percentages were 21.3 (n = 33) and 6.7 (n = 6).

196

197 *2.4. Microbiology and cytology*

198 Culture and cytological slides from endometrial swabs and lavage fluid were
199 processed and evaluated as described by Reilas and Katila (2002). Microbial growth
200 was scored as none, +1 (1–10 colonies), +2 (11–100 colonies) or +3 (>100 colonies).

The number of neutrophils per ten fields was evaluated at a magnification of 400 and scored as none, +1 (1–10), +2 (>10, but mostly isolated neutrophils), or +3 (large clumps of neutrophils).

2.5. Western blotting (WB) protocol

Protein extracts were prepared from frozen samples as described by Sirois and Dore (1997). Briefly, endometrial samples were homogenized in cold protein extraction buffer containing 50 mM of Tris (pH 8.0), 10 mM of EDTA, 1 mM of diethyldithiocarbamic acid (DEDTC), and 2 mM of octyl glucoside by means of an Ultra-Turmax T25 basic homogenizer (IKA®-Werke, Staufen, Germany). Subsequently, these samples were centrifuged at 30,000 *g* for 1 h at 4 °C. The supernatants were removed and the pellets resuspended in a buffer containing 20 Mm of Tris (pH 8.0), 50 mM of EDTA, 0.1 mM of DEDTC, 32 mM of octyl glucoside, and protease inhibitors. Once resuspended, the pellets were sonicated (IKA®-Werke, Staufen, Germany) at 30% frequency. The samples were centrifuged at 16,000 *g* for 15 min at 4 °C. The protein content of the supernatants was subsequently quantified by the Bradford method (Bradford, 1976) using a commercial kit (Bio-Rad Protein Assay Dye Reagent, BioRad, Hercules, CA, USA).

Proteins were separated by SDS-PAGE and transferred to PVDF membranes, as described by Sirois and Dore (1997). The membranes were probed with specific antisera against COX-2 at a 1:1000 dilution (COX-2 polyclonal antibody, Cayman chemical, Ann Arbor, MI, USA). Detection was performed using an anti-rabbit polyclonal antibody at the concentration of 1:5000. The membranes were then incubated for 5 min with an Immobilon TM Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA) and exposed to a radiograph film.

After detection, membranes were immersed in a stripping buffer (1% (w:v) glycine, 0.1% (w:v) sodium dodecyl sulfate, 1% (w:v) Tween 20, pH 2.2) to remove the specific binding for COX-2, and then re-probed with an anti-mouse α-tubulin antibody (ABR Affinity BioReagents, CO, USA). Alpha-tubulin was used as an internal control to verify that the same amount of protein was loaded in all lanes. The integral density of the bands was subsequently analyzed by means of a computer-assisted image analysis system (Multi Gauge v 3.0 software system, Fujifilm, Tokyo, Japan). The software provides numerical values of intensity in arbitrary units. To transform these arbitrary units into percentages, intensity of binding for COX-2 in mares from the AI-N group was considered to be 100%, while the intensity of binding by the COX-2 probe in samples from mares of the AI-P group was considered to be 0%. From these values, an equation ($y = 0.4919 + 149.88$, being y the intensity of binding and x the percentage value) for abundance of COX-2 was obtained, and values for mares with an IUD were evaluated.

2.6. Immunohistochemistry (IHC) protocol

Biopsy specimens were fixed in 4% formalin for 24 h, embedded in paraffin according to standard protocols, and cut into 4 μm sections. Immunohistochemical detection was performed by the immunoperoxidase method using the same antibodies as in WB at the concentration of 1:500 and 1:1000 for primary and secondary antibodies, respectively. Staining was performed by a 10 min incubation in a 3,3'-diaminobenzidine (DAB) peroxidase substrate solution. Prior to mounting, the slides were counterstained with hematoxylin-eosin.

For analytical purposes, endometrial biopsies were histologically divided into different areas. These areas were the luminal epithelium, stratum compactum,

superficial stratum spongiosum (considering the outer one-third of the total depth of the stratum spongiosum) and deep stratum spongiosum (the inner two-thirds of the stratum spongiosum).

2.7. Statistical analyses

The data were analyzed with a statistical package (IBM SPSS for Windows, Ver. 22.0; Armonk, New York; USA) and were tested for normality and homogeneity of variances through Shapiro–Wilk and Levene tests. When needed, the data were transformed with arcsine √x or with √x. The effects of factors (IUD/AI and P/N) were evaluated with a two-way analysis of variance (ANOVA), and Sidak's test was used for pairwise comparisons. Scheirer–Ray–Hare ANOVA for ranked data was conducted for those variables that did not have a normal distribution and/or homogeneity of variances after transformation (Day 15_P_4, lavage fluid E_2 and COX_WB). In this case, calculation of the 'H' statistic occurred with use of the Mann–Whitney test. The data are shown as mean ± standard error of the mean (SEM). The level of significance was set at $P < 0.05$.

3. Results

3.1. Hormones

3.1.1. Blood analyses

The P_4 concentrations and endometrial edema on Days 14 and 15 were used to assign mares with an IUD inserted to the IUD-P or IUD-N groups. Thus, mares that had a P_4 concentration of 1 to 6 nmol/L on Day 15 were assigned to the IUD-N group, while mares with P_4 concentrations of 10 to 33 nmol/L were assigned to the IUD-P group (Fig. 1, Table 1).Considering this endocrine milieu, 53.3% (eight of 15) had a normal

luteal phase, while 46.7% of the mares (seven of 15) had a prolonged luteal phase. The average P_4 concentration in mares of the IUD-N group was 9.4 ± 6.1 and 2.9 ± 2.0 nmol/L on Days 14 and 15 post-ovulation, respectively, while the values in mares of the IUD-P group were 21.1 ± 7.4 nmol/L and 19.0 ± 7.1 nmol/L, respectively (Fig. 2).

Regarding inseminated mares, the average P_4 concentrations of non-pregnant mares were 6.0 ± 4.9 nmol/L (range: 1–12 nmol/L) and 5.6 ± 6.0 nmol/L (range: 1–14 nmol/L) on Days 14 and 15, respectively, whereas in pregnant mares the values were 14.9 ± 5.9 nmol/L (range: 9–25 nmol/L) and 16.0 ± 7.9 nmol/L (range: 9–31 nmol/L). There were no significant differences in serum P_4 concentration between mares of the AI-P and IUD-P groups on Day 14 or on Day 15. The values for mares in the IUD-P group were greater ($P < 0.05$) than those of mares in the AI-N and IUD-N groups on both days (Fig. 2).

Non-pregnant mares had greatest serum E_2 concentrations, and mares in the IUD-P group had the lowest. There were differences only ($P < 0.05$) in serum E_2 concentrations between mares in the AI-N and IUD-P groups (Fig. 3).

Plasma concentrations of PGFM for individual mares during Days 14 and 15 are depicted in Figure 4. The mares from the IUD-N group had the greatest PG activity. There were values exceeding 200 pmol/L in one of four mares in the AI-N and AI-P groups, in all eight mares of the IUD-N group, and in two of the seven mares in the IUD-P group.

3.1.2. Uterine lavage fluid analyses

299 Pregnant mares had greater concentrations of E_2 in lavage fluid, with a mean
300 concentration of 52.22 pg/mL, compared with the other groups, which had
301 concentrations of <1 pg/mL ($P < 0.05$; Fig. 5).
302
303 *3.2. Microbiology and cytology*
304 Day 15 lavage fluid cultures had no detectable microbiological growth (0 or +1)
305 after 48 h of incubation, and all the cytological smears were negative for PMNs.
306 Endometrial swabs, taken 1 to 7 days after the uterine lavage and biopsies, were
307 positive for neutrophils in most of the mares. Neutrophils (scores +1 or +2) were
308 observed in all four mares of the AI-N, three of eight in the AI-P, six of eight in the
309 IUD-N, and six of seven in the IUD-P groups. Microbiological growth from
310 endometrial swabs was not significant (0 or +1).
311
312 *3.3. Endometrial cyclooxygenase-2*
313 Western blotting results for inseminated mares indicated COX-2 was detectable
314 in three of four mares in the AI-N and three of eight mares in the AI-P groups.
315 Regarding the mares treated with an IUD, COX-2 was detected in all of the mares in
316 IUD-N and three of seven mares in the IUD-P groups (Fig. 6, Table 1).
317 For the IHC samples, perinuclear COX-2 was primarily present in the luminal
318 epithelium and the portion of glands located in the stratum compactum. There was
319 sporadic detection of COX-2 in the glands located in the most superficial layer of the
320 stratum spongiosum. In addition to epithelial and glandular cells, COX-2 was also
321 present in several mares in the nucleus of leukocytes inside the vessels and in leukocytic
322 infiltrations in the stratum compactum and spongiosum.

In the two mares of the AI-N group with relatively lesser P_4 concentrations, there was COX-2 detected in the luminal epithelium and in the portion of glands present in the stratum compactum (Fig. 7A), while in the two mares with relatively greater P_4 concentrations there was no COX-2 detected in epithelia (Table 1). In the eight pregnant mares, COX-2 was not detectable in the luminal epithelium and in the most superficial glands (Fig. 7B, Table 1).

All eight mares of the IUD-N group had COX-2 in the luminal epithelium. In seven mares, COX-2 was also present in the superficial glands (Fig. 7C), and in five mares in the leukocytes (Table 1). In the IUD-P group, two of seven mares had a marked abundance of COX-2 in the luminal epithelium (Table 1). Additionally, in one of these mares COX-2 was present in the glandular cells located in the stratum compactum. Serum P_4 concentrations of these mares decreased by 50% from Day 14 to Day 15. In the remaining mares COX-2 was not detectable, neither in the luminal nor in the glandular epithelia (Fig. 7D, Table 1).

Cyclooxygenase-2 was also observed to be present in leukocytes inside the vessels, and in infiltrations of the stratum compactum and stratum spongiosum in four of the mares of IUD-P group (Fig. 7E) while five of eight pregnant mares had a marked abundance of COX-2 in leukocytes located in the vessels and in the stratum compactum (Fig. 7F).

4. Discussion

The aim of the present study was to clarify how intrauterine devices inhibits luteolysis in mares and compare the results with what occurs in pregnant and non-pregnant mares. The first challenge in the study was to establish which mares in the IUD group had a prolonged luteal phase. For that purpose, there was use of ultrasonic

348 examinations (uterine edema, CL), serum P_4, and, to some extent, plasma PGFM. On
349 Day 15, individual variations in the diestrus length made the classification of mares into
350 correct categories difficult. Based on the findings, it is believed that at least 13 of the 15
351 mares in IUD group were correctly categorized.

352 As expected, all the pregnant mares had relatively greater values of P_4 on
353 Days 14 and 15 than mares of the other groups, with similar concentrations to those
354 reported by Kindahl et al. (1982). Non-pregnant mares were expected to have lesser or
355 abruptly decreasing values for P_4 on days 14 and 15 (reviewed by Ginther, 2012);
356 however, two of the four mares had values of 10 nmol/L and 14 nmol/L on Day 15. This
357 may indicate these mares had spontaneously prolonged luteal phases (Neely et al.,
358 1979) or their diestrous phase was slightly longer than in the other mares (Ginther et al.,
359 2007). Day 15 was selected as the day for collecting samples in the present study,
360 however, Ginther et al. (2007) have reported that the luteolytic period is from Day 15 to
361 17 in mares. Consequently, only two mares in the AI-N group in present study were
362 categorized as estrous mares on Day 15.

363 In the present study, serum P_4 concentrations greater than 10 nmol/L on Day 15
364 were considered to indicate a prolonged luteal phase in the mares of IUD group
365 (46.7%), while P_4 concentrations less than 6 nmol/L were considered to be
366 representative of mares having a normal luteal phase even when treated with the IUD
367 (53.3%). Reports from earlier studies, however, indicate that some mares with
368 prolonged diestrus have relatively lesser P_4 concentrations at the time of the expected
369 luteolysis (Rivera del Alamo et al., 2008; Ginther et al., 2015, 2016). These decreased
370 P_4 concentrations occurred concurrent with the day when there was the maximal
371 diameter of a dominant follicle (Santos et al., 2015a) or may have resulted as a
372 consequence of partial luteolysis (Ginther et al., 2016).

373 Mares in the IUD-N group had distinctive PGFM pulse releases, whereas in
374 mares of the IUD-P group, the values were near basal. Only two mares in the IUD-P
375 group had one or two PGFM pulse releases (200–350 pmol/L) but this did not lead to
376 luteolysis. These results are consistent with those of a previous study (Rivera del Alamo
377 et al., 2008). Two pulse releases of PGFM and a 50% decrease in P_4 may imply that one
378 of the two mares in the IUD-P group had a normal cycle.
379 It was assumed that pregnant mares would have no pulse releases of PGFM, but
380 one mare had a single sample with a value that was markedly greater than basal values.
381 Inconsistent with expectations in the present study, only one mare of the AI-N group
382 had PGFM concentrations that were greater than basal. It is possible that some pulsatile
383 releases of PGFM had occurred before Day 14 of during the 8-hour period when no
384 samples were collected between Days 14 and 15. Two mares with the relative greater P_4
385 concentrations and no pulse releases of PGFM may have had prolonged luteal phases. If
386 these mares were cycling normally, sampling on Day 15 was too early (Ginther et al.,
387 2007) for detection of PGM pulse releases. One mare had already had an ovulation
388 before Day 0 (P_4 was 8 nmol/L on Day 0), and the P_4 concentration had already
389 decreased to the basal on Day 13. The detection that this mare had an ovulation was
390 only confirmed after analyzing the P_4 values. Hence, the mares of the AI-N group in the
391 present study differed from what it was expected.
392 There were no differences in serum concentrations of E_2 among groups, with the
393 exception of mares in the AI-N and IUD-P groups. Sato et al. (1977) reported that on
394 Day 15 after ovulation, cycling mares had relatively greater concentrations of serum E_2
395 than early pregnant mares. Argo and Turnbull (2010) reported that there were no
396 differences in plasma E_2 concentrations between mares in a control and IUD-treated
397 group. There is some evidence that in idiopathic luteal persistence, peripheral E_2

concentrations are elevated on Day 12 compared to concentrations in diestrous mares (Santos et al., 2015b). Sampling days in the present study were later, and besides, the mechanisms for idiopathic persistence of CL are likely different from that induced by an IUD.

Interestingly, the presence of the IUD was concomitant with time period during which there were relatively lesser of serum E_2 concentrations. Results from previous studies in other species, such as sheep, rats and heifers (Battaglia et al., 1999; Kaneko et al., 2004; Faccio et al., 2013), indicate endometritis induces a decrease in serum E_2 concentration, either by suppressing the secretion of GnRH (Peter et al., 1989; Battaglia et al., 1999), or by interfering with the ovarian follicular synthesis of E_2 (Battaglia et al., 1999). Thus, these E_2 results suggest that the device may induce endometrial inflammation.

The E_2 concentration in uterine lavage fluid from pregnant mares, however, was significantly greater than in the other groups. This result was expected because equine embryos start to synthetize estrogens from Day 6 of pregnancy (Paulo and Tischner, 1985), and large amounts of estrogens are detected in the intrauterine fluid from Day 12 to 20 of pregnancy (Zavy et al., 1984). In one mare in the present study, one of the twin conceptuses ruptured during the lavage, and the E_2 concentration was twice that of the other mares. Although estrogen concentrations are greater in the yolk sac and uterine fluids of pregnant mares from Day 12 to 20, this is not discernible in the systemic circulation (Zavy et al., 1984).

In the IHC samples, COX-2 was detected in perinuclear in cells of the luminal epithelium and the most superficial glands. These results are consistent with those from a previous study by Boerboom et al. (2004). Non-pregnant mares were expected to have large abundances of COX-2. The lack of detection of COX-2 in two mares of the AI-N

group with the relatively greater serum P_4 can be explained by a spontaneous prolonged luteal phase or a delayed return to estrus (Ginther et al., 2007).

Expectedly, in pregnant mares there was no COX-2 detected in IHC samples of the endometrium, but three samples were positive for COX-2 in WB samples. This inconsistency between IHC and WB results is difficult to understand. All pregnant mares received an injection of PG on Day 15, so it is unknown if the pregnancies would have been prolonged. It is unlikely that three young mares would have had abnormal pregnancies. Samples for IHC from these three mares, however, were positive for COX-2 in leukocytes. Because with the WB technique there is not the capacity to differentiate between epithelia, stroma or leukocytes, the positive staining for COX-2 could be due to its presence inside the leukocytes. Six mares in the present study, however, were negative for COX-2 in the WB samples and were positive for COX-2 in the IHC samples. In addition, samples from two mares were positive for COX-2 in WB samples, however, were negative for COX-2 in the IHC samples. It should be noted that WB and IHC are very different techniques. Thus, the processing of samples for WB can allow for ligands to bind to some epitopes that cannot be detected with IHC techniques.

All mares in the IUD-N group were positive for COX-2 both in IHC and WB samples, indicating that luteolysis occurred in these mares. Two mares in the IUD-P group were positive for COX-2 in IHC samples and three other mares of this group were positive in WB samples, although this occurrence was unexpected. Remarkably, the two mares of the IUD-P group that were positive for COX-2 in the epithelial cells were negative in WB. The P_4 concentrations in the mares that were positive for COX-2 in IHC samples were 11 nmol/L and 12 nmol/L (i.e., greater than the concentration that was considered to be indicative of the presence of an active CL on Day 15). Thus, the expectation was that these mares would be negative for COX-2 in IHC samples. A

448 possible explanation could be that these mares were nearing the time of initiation of a
449 subsequent estrous cycle but had yet to do so (Ginther et al., 2007). The P_4
450 concentrations were decreasing during this time period and were somewhat less than
451 those of the mares that were COX-2 negative in this group. Another explanation could
452 be fluctuating P_4 concentrations at the time of expected luteolysis, since P_4
453 concentrations decrease in both IUD-induced and idiopathic luteal persistence (Rivera
454 del Alamo et al., 2008; Ginther et al., 2015, 2016). Partial luteolysis (Ginther et al.,
455 2016) could occur as a result of increased COX-2.

456 In addition to endometrial cells, COX-2 was also present in the marginal
457 leukocytes inside the vessels and in leukocytes from the stratum compactum. Boerboom
458 et al. (2004) reported that COX-2 was present in the endothelium of vessels. There is
459 COX-2 present in leukocytes - specifically in macrophages, monocytes, neutrophils and
460 lymphocytes - as a response to an inflammatory stimulus (Glew, 2006), and this
461 subsequently leads to $PGF_{2\alpha}$ synthesis (Jabbour and Sales, 2004). The presence of
462 COX-2 in endometrial cells is a reliable and sensitive marker of endometrial
463 inflammation in mares (Palm et al., 2008; Melkus et al. 2013). Thus, the presence of
464 COX-2 in WB and IHC endometrial samples of some mares might be due to
465 inflammation. Perhaps the inflammation was not extensive enough to cause the release
466 of $PGF_{2\alpha}$, yet intensive enough to induce an increase of COX-2.

467 A question that requires discussion is why some mares respond to the presence
468 of the IUD while others do not. Two possible factors have been suggested: the age of
469 the mares, and the presence of angiopathies in the endometrial vessels (Klein et al.,
470 2016). Thus, younger mares and mares with normal endometrial vascularization have a
471 greater incidence of prolonged luteal phases as a response to the IUD. The mean age of
472 mares in the IUD-N group in the present study was 10.8 years (range: 5-17 years), while

for mares in the IUD-P group it was 8.3 years (range: 4–16 years). It, therefore, is expected that the efficacy of IUDs in suppressing estrous behavior in mares used for racing is greater than for those used in experiments with IUDs. Mares that are used in racing are young and maiden, unlike experimental mares, which usually have a wide range of ages. The efficacy of IUDs, however, continues to be less than desirable. In addition, serious complications have been reported for glass ball IUDs (Morris et al., 2017), and for these reasons the use of these types of IUDs in estrous suppression is not recommended. There, however, is not breakage of polypropylene IUD devices, and apparently these devices do not induce significant histological changes in the endometrium after remaining in the uterine lumen for 2 months (Rivera del Alamo at al., 2008).

Although the COX-2 results from the present study were not as consistent as expected, inflammation, partial luteolysis or varying times of luteolysis explain the inconsistent results (Ginther et al. 2007 and 2016). It seems reasonable, to conclude that the effect of the IUD is mediated by the inhibition of COX-2 synthesis and release, which leads to inhibition of $PGF_{2\alpha}$ and subsequent luteostasis. A similar sequence of events occurred in mares in which luteal maintenance was prolonged by repeated oxytocin injections during diestrus (Keith et al., 2013).

Inflammation seems to be a logical explanation for the effect of IUDs, but the evidence for this is not consistent. After 1 year of IUD use in wild horses, all mares had chronic endometritis at the time of IUD removal (Daels and Hughes, 1995). In a previous experiment, six of nine mares in an IUD-P treatment group had non-echogenic intrauterine fluid in one to three ultrasonic examinations, although swabs indicated acute inflammation in only one mare, and inflammatory scores of biopsies did not change (Rivera del Alamo et al., 2008). Similarly, Nie et al. (2003) detected uterine

fluid in ultrasonography in a few mares in the first few days, but no inflammation was detected to be present when biopsy samples were assessed. Leukocytic infiltration within the endometrial stroma did not increase in biopsy samples taken after IUD implantation as compared to pre-treatment samples (Argo and Turnbull, 2010). In the present study, all lavage fluids were negative for leukocyte infiltration, but most swabs obtained 1 to 7 days after the lavage (when the mares returned to estrus) were positive in all groups of mares. This probably is a consequence of uterine manipulations, lavage, and particularly from conducting biopsies, more than inflammation.

In endometrial biopsies of mares in the IUD-P group, increased lactoferrin concentrations indicate inflammation, however, there were no significant differences in any of the inflammatory cells (T-lymphocytes, B-lymphocytes, plasma cells, macrophages, neutrophils, eosinophils, mast cells) in the biopsy samples (Klein et al., 2016). The presence of COX-2 in leukocytes and in mares that were not supposed to be positive for COX-2, and decreased serum E_2 concentrations also indicate IUD-induced inflammation. Lavage fluids were negative for bacteria and PMNs, and biopsy histology assessments did not indicate that there were any differences between the groups for inflammatory cells. It is possible that the mares had previously had inflammation but that it was resolved by Day 15. Twelve days had elapsed from the insertion of the IUD and during this time acute inflammation may have been resolved and become chronic. It is also possible that the diagnostic methods used in the present study to detect bacteria and PMNs were not sensitive enough.

In conclusion, abundance of COX-2 was less in mares in which an IUD was effective and, therefore, no $PGF_{2\alpha}$ was synthetized, leading to the maintenance of the CL. In the present study, however, there was not an elucidation of how an IUD induces a decrease in COX-2. In the prostaglandin synthesis pathway, the product produced

before COX-2 is phospholipase 2 (PLA2) which is required to release arachidonic acid from the membranes of cells. It seems logical to assume that the IUD could inhibit PLA2, but this has yet to be proven. The role of inflammation remains obscure and there is need for further study with the use of more sensitive indicators of inflammation than those used in the present and previous studies.

Funding

The Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) financed Dr. Rivera del Alamo's stay in Finland to perform the present study.

The Finnish Veterinary Foundation financed the analyses of the samples.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

All authors have contributed equally to the development of the experiment and the preparation of the manuscript.

Acknowledgements

The authors would like to thank Merja Pöytäkangas and Satu Sankari from the Clinical Research Laboratory of the Faculty of Veterinary Medicine at the University of Helsinki in Viikki, Finland, for performing the E_2 analyses; technician Marja Rauvola from Ypäjä for the P_4 analyses; and Marjatta Lehtisaari for the bacteriological and cytological examinations. The authors would also like to thank the stablemasters and

students at Ypäjä for their help in examining the mares and taking blood samples for the PGFM analysis.

References

Argo, C.M., Turnbull, E.B., 2010. The effect of intra-uterine devices on the reproductive physiology and behaviour of pony mares. Vet. J. 186, 39-46.

Battaglia, D.F., Beaver, A.B., Harris, T.G., Tanhehco, E., Viguie, C., Karsch, F.J., 1999. Endotoxin disrupts the estradiol-induced luteinizing hormone surge: Interference with estradiol signal reading, not surge release. Endocrinology 140, 2471-2479.

Boerboom, D., Brown, K. A., Vaillancourt, D., Poitras, P., Goff, A. K., Watanabe, K., Doré, M., Sirois, J., 2004. Expression of key prostaglandin synthases in equine endometrium during late diestrus and early pregnancy. Biol. Reprod. 70, 391-399.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-256.

Daels, P.F., Hughes, J.P., 1995. Fertility control using intrauterine devices: an alternative for population control in wild horses. Theriogenology 44, 629-639.

Faccio, L., Da Silva, A.S., Tonin, A.T., França, R.T., Gressler, L.T., Copetti, M.M., Oliveira, C.A., Sangoi, M.B., Moresco, R.N., Bottari, N.B., Duarte, M.M.M.F., Monteiro, S.G., 2013. Serum levels of LH, FSH, estradiol and progesterone in female rats experimentally infected by *Trypanosoma evansi*. Exp. Parasitol. 135, 110-115.

571 Gastal, E.L., Gastal, M.O., Wiltbank, M.C., Ginther, O.J., 1999. Follicle deviation and
572 intrafollicular and systemic estradiol concentrations in mares. Biol. Reprod. 61,
573 31-39.
574 Ginther, O.J., 2012. The end of the tour de force of the corpus luteum in mares.
575 Theriogenology 77, 1042-1049.
576 Ginther, O.J., Gastal, E.L., Gastal, M.O., Utt, M.D., Beg, M.A., 2007. Luteal blood flow
577 and progesterone production in mares. Anim. Reprod. Sci. 99, 213-220.
578 Ginther, O.J., Wolf, C.A., Baldrighi, J.M., Greene, J.M., 2015. Relationships among
579 nitric oxide metabolites and pulses of a PGF2α metabolite during and after
580 luteolysis in mares. Theriogenology 84, 193-199.
581 Ginther, O.J., Castro, T., Baldrighi, J.M., Wolf, C.A., Santos, V.G., 2016. Defective
582 secretion of Prostaglandin F2α during development of idiopathic persistent
583 corpus luteum in mares. Domest. Anim. Endocrinol. 55, 60-65.
584 Glew, R., 2006. Lipid Metabolism. Pathways of metabolism of special lipids: Devlin T.
585 Textbook of Biochemistry: With Clinical Correlations. Wiely Liss. 142, 730-
586 733.
587 Granström, E., Kindahl, H., 1982. Radioimmunoassay of the major plasma metabolite
588 of $PGF_{2\alpha}$, 15−keto-13,14-dihydro-$PGF_{2\alpha}$. Meth. Enzymol. 86, 320-339.
589 Jabbour, H.N., Sales, K.J., 2004. Prostaglandin receptor signalling and function in
590 human endometrial pathology. Trends Endocrinol. Metab. 15, 398-404.
591 Kaneko, K., Aoki, H., Furuichi, T., Hatori, S., Tanimoto, H., Kawakami, S., 2004.
592 Influence of uterine inflammation on the estrous cycle in rats. J. Reprod. Dev.
593 50, 361-367.
594 Keith, L., Ball, B.A., Scoggin, K., Esteller-Vico, A., Woodward, E.M., Troedsson,
595 M.H.T., Squires, E.L., 2013. Diestrus administration of oxytocin prolongs luteal

maintenance and reduces plasma PGFM concentrations and endometrial COX-2 expression in mares. Theriogenology 79, 616-624.

Kenney, R.M., 1978. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. J. Am. Vet. Med. Assoc. 172, 241-262.

Kindahl H., Knudsen O., Madej A., Edqvist L.-E., 1982. Progesterone, prostaglandin F-2α, PMSG and oestrone sulphate during early pregnancy in the mare. J. Reprod. Fertil., Suppl. 32, 353-359.

Klein, V., Müller, K., Schoon, H.A., Reilas, T., Rivera del Alamo, M.M., Katila, T., 2016. Effects of intrauterine devices in mares: A histomorphological and immunohistochemical evaluation of the endometrium. Reprod. Dom. Anim. 51, 98-104.

Melkus, E., Witte, T., Walter, I., Heuwieser, W., Aurich, C., 2013. Investigations on the endometrial response to intrauterine administration of N-acetylcysteine in oestrus mares. Reprod. Dom. Anim. 48, 591-597.

Morris, L.H.A., Fraser, B.S.L., Cantley, C., Wilsher, S., 2017. The hazards associated with the use of intrauterine glass balls to suppress oestrus in mares. Equine Vet. Educ. 29, 125-130.

Neely, D.P., Kindahl, H., Stabenfeldt, G.H., Edqvist, L.-E., Hughes, J.P., 1979. Prostaglandin release patterns in the mare: physiological, pathophysiological, and therapeutic responses. J. Reprod. Fertil., Suppl. 27, 181-189.

Nie, G.J., Johnson, K.E., Braden, T.D., Wenzel, J.G.W., 2003. Use of an intra-uterine glass ball protocol to extend luteal function in mares. J. Equine Vet. Sci. 23, 266-273.

620 Paulo, E., Tischner, M., 1985. Activity of delta(5)3beta-hydroxysteroid dehydrogenase
621 and steroid hormones content in early preimplantation horse embryos. Folia
622 Histochem. Cytobiol. 23, 81-84.
623 Palm, F., Walter, I., Budik, S., Kolodziejek, J., Nowotny, N., Aurich, C., 2008.
624 Influence of different semen extenders and seminal plasma on PMN migration
625 and on expression of IL-1beta, IL-6, TNF-alpha and COX-2 mRNA in the
626 equine endometrium. Theriogenology 70, 843-851.
627 Peter, A.T., Bosu, W.T.K., De Decker, R.J. 1989. Supression of preovulatory
628 luteinizing hormone surges in heifers after intrauterine infusions of *Escherichia*
629 *coli* endotoxin. Am. J. Vet. Res. 50, 368-373.
630 Portus, B.J., Reilas, T., Katila, T., 2005. Effect of seminal plasma on uterine
631 inflammation, contractility and pregnancy rates in mares. Equine Vet. J. 37, 515-
632 519.
633 Reilas, T., Katila, T., 2002. Proteins and enzymes in uterine lavage fluid of postpartum
634 and nonparturient mares. Reprod. Dom. Anim. 37, 261-268.
635 Rivera del Alamo, M.M., Reilas, T., Kindahl, H., Katila, T., 2008. Mechanisms behind
636 intrauterine device-induced luteal persistence in mares. Anim. Reprod. Sci. 107,
637 94-106.
638 Santos, V.G., Beg, M.A., Bettencourt, E.M., Ginther, O.J., 2013. Role of PGF2α in
639 luteolysis based on inhibition of PGF2α synthesis in the mare. Theriogenology
640 80, 812-820.
641 Santos, V.G., Bettencourt, E.M.V., Ginther, O.J., 2015a. Long-term characteristics of
642 idiopathic persistent corpus luteum in the mare. Theriogenology 84, 242-251.

643 Santos, V.G., Bettencourt, E.M., Ginther, O.J., 2015b. Hormonal, luteal, and follicular
644 changes during initiation of persistent corpus luteum in mares. Theriogenology
645 83, 757-765.
646 Sato, K., Miyake, M., Yoshikawa, T., Kambegawa, A., 1977. Studies on serum
647 oestrogen and progesterone levels during oestrous cycle and early pregnancy in
648 mares. Equine Vet. J. 9, 57-60.
649 Sirois, J., Dore, M., 1997. The late induction of prostaglandin G/H synthase-2 in equine
650 preovulatory follicles supports its role as a determinant of the ovulatory process.
651 Endocrinology 138, 4427-4434.
652 Zavy, M.T., Vernon, M.W., Sharp, D.C., Bazer, F.W., 1984. Endocrine aspects of early
653 pregnancy in pony mares: A comparison of uterine luminal and peripheral
654 plasma levels of steroids during the estrous cycle and early pregnancy.
655 Endocrinology 115, 214-219.

656

657 **Figure captions**

658 **Fig. 1**. Serum progesterone (P_4) profiles for individual mares. AI-N: inseminated, non-
659 pregnant mares; AI-P: inseminated, pregnant mares; IUD-N: mares with intrauterine
660 device and normal luteal phase; IUD-P: mares with intrauterine device and prolonged
661 luteal phase.

662

663 **Fig. 2.** Serum progesterone (P_4) concentrations (mean ± SEM) on Days 14 and 15 post-
664 ovulation. AI-N: inseminated, non-pregnant; AI-P: inseminated, pregnant; IUD-N:
665 intrauterine device, normal luteal phase; IUD-P: intrauterine device, prolonged luteal

phase. Significant differences ($P < 0.05$) between groups on Day 14 are depicted with different letters. Differences ($P < 0.05$) between groups on Day 15 are depicted with different symbols (§ ‡ †).

Fig. 3. Serum concentration of estradiol-17β (mean ± SEM) on Day 15 after ovulation. AI-N: inseminated, non-pregnant; AI-P: inseminated, pregnant; IUD-N: intrauterine device, normal luteal phase; IUD-P: intrauterine device, prolonged luteal phase. Different letters above the bars indicate differences between groups ($P < 0.05$).

Fig. 4. Individual values for $PGF_{2\alpha}$ metabolite (PGFM) on Days 14 and 15 post ovulation. AI-N: inseminated, non-pregnant ($n = 4$); AI-P: inseminated, pregnant ($n = 4$); IUD-N: intrauterine device, normal luteal phase ($n = 8$); IUD-P: intrauterine device, prolonged luteal phase ($n = 7$).

Fig. 5. Estradiol concentration (mean ± SEM) in uterine lavage fluid. AI-N: inseminated, non-pregnant; AI-P: inseminated, pregnant; IUD-N: intrauterine device, normal luteal phase; IUD-P: intrauterine device, prolonged luteal phase. Different letters above the bars indicate differences between groups ($P < 0.05$).

Fig. 6. Western blotting image for COX-2. AI-N(-): non-pregnant mare, negative for COX-2; AI-N (+): non-pregnant mare, positive for COX-2; AI-P(-): pregnant mare, negative for COX-2; AI-P(+): pregnant mare, positive for COX-2; IUD-N(+): device

mare with normal luteal phase and positive for COX-2; IUD-P(+): device mare with prolonged luteal phase and positive for COX-2; IUD-P(-): device mare with prolonged luteal phase and negative for COX-2. Non-pregnant mares were used as positive controls, while pregnant mares were used as negative controls.

Fig. 7. COX-2 presence evaluated by immunohistochemistry technique. **A:** AI-N mare (inseminated, non-pregnant). Blue arrows indicate positive staining cells of luminal and glandular epithelia. Non-pregnant mares were used as positive controls in the study. **B:** AI-P mare (inseminated, pregnant). Luminal and glandular epithelia were negative for COX-2. Pregnant mares were used as negative controls in the study. **C:** IUD-N mare (intrauterine device, normal luteal phase). Blue arrows indicate cells of luminal and glandular epithelia that were positive. **D:** IUD-P mare (intrauterine device, prolonged luteal phase). Luminal and glandular epithelia were negative for COX-2. **E:** Blue arrows indicate positive staining in infiltrated leukocytes. **F:** Blue arrows indicate positive staining of marginal leukocytes in vessels.