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Effects of follicular ablation and induced luteolysis on LH and follicular fluid factors during the periovulatory period in mares

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ABSTRACT

Haemorrhagic anovulatory follicles (HAFs) are the most common pathological anovulatory condition in the mare. To enhance understanding of the physiopathology of HAFs, the aim of the present study was to determine the effects of an induced-follicular wave on LH concentrations and follicular fluid factors relevant to the ovulatory process. Mares were allocated to treatment or control groups ($n = 7$/group) in a crossed over design during 14 oestrous cycles with a period of one cycle occurring when there were no treatments between the times when treatments were administered. In the treatment group, all antral follicles ≥ 8 mm were ablated on Day 10 after ovulation followed by administration of a luteolytic dose of $PGF_{2\alpha}$. All mares of both groups were treated with 1500 IU of hCG when a follicle ≥ 32 mm was detected (Hour 0), and follicular fluid was aspirated 35 h later. Blood samples were collected every 48 h from Day 10 until Hour 0 from all mares. Follicular fluid was assayed for PGE_2, estradiol and progesterone. Plasma was assayed for LH concentrations. A follicular wave followed follicle ablation in the treated mares. Concentrations of LH were greater ($P = 0.05$) in mares ot the treatment compared with control group. Concentrations of PGE_2, estradiol and progesterone in follicular fluid did not differ between groups ($P > 0.05$). Treatment resulted in an earlier increase in circulating LH, however, there was no effect on concentrations of intra-follicular PGE_2, estradiol or progesterone in hCG-stimulated preovulatory follicles.

1. Introduction

Haemorrhagic anovulatory follicles (HAFs) are the most prevalent cause of ovulatory failure in mares (Ginther et al., 2007; Cuervo-Arango and Newcombe, 2009). This anovulatory condition has also been reported in woman where it is termed as a luteinized

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un-ruptured follicle (LUF) and it is a common cause of infertility (Marik and Hulka, 1978; LeMaire, 1987; Qublan et al., 2006). The HAFs occur when ovulation from a preovulatory follicle does not occur and a hematoma forms in the follicular antrum, preventing release of the oocyte. Although the overall incidence of HAF in mares is small occurring in 5% to 8% of all estrous cycles (Ginther and Pierson, 1989; McCue and Squires, 2002; Cuervo-Arango and Newcombe, 2009), individual mares, so-called 'repeater mares', have an HAF incidence during estrous cycles of as great as 25% to 50% (Ginther et al., 2007; Cuervo-Arango and Newcombe, 2009), rendering these mares infertile during the estrous cycles when this condition prevails. This syndrome, during breeding season, is considered a serious economic problem in the horse industry. It, therefore, is important to investigate the mechanisms of development of HAFs.

The etiology of this syndrome is unknown. Greater knowledge about the physiopathology of HAFs can be applied to prevent or avoid occurrence of these abnormalities and thus to develop safe and effective treatments to optimize reproductive health. There are some risk factors that can affect the incidence such as the use of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or its synthetic analogues (Cuervo-Arango and Newcombe, 2010). This induces a release of LH directly (Jochle et al., 1987) and through the removal of the negative feed-back of progesterone at the hypothalamus resulting in an increase in release of GnRH and subsequently LH from the anterior pituitary (Gastal et al., 2000). The hypothesis has been put forward that the increased HAF incidence after $PGF_{2\alpha}$ treatment could be due to the greater concentrations of LH that occur after luteolysis and resulting lesser progesterone concentrations early during the follicular wave (Ginther et al., 2008a, 2008b). Perhaps, this effect is through the LH interference with intra-follicular metabolism of prostanoids and proteolytic enzymes in immature follicles (Cuervo-Arango and Newcombe, 2010). Treatment with equine LH (eLH) or $PGF_{2\alpha}$ did not induce HAF formation (Bashir et al., 2016). Consistent with this finding, Schauer et al. (2013) reported that treatment with LH or $PGF_{2\alpha}$ increased the concentration of circulating LH but did not affect follicle growth or ovulation. Follicles > 32 mm that are stimulated when there are increased LH concentrations during development contained relatively lesser concentrations of androstenedione and greater concentrations of Insulin-like growth factor as compared to follicles where there is not stimulation by the relatively greater concentrations of LH. Furthermore, the intra-follicular concentrations of PGE_2 tended to be less in LH-treated than in control mares (Schauer et al., 2013).

The PGE_2 and $PGF_{2\alpha}$ are essential for the ovulatory process to occur (Robker et al., 2000). Concentration of these eicosanoids in follicular fluid markedly increase between 33 and 36 h after hCG administration in preovulatory mares (Sirois and Dore, 1997). Furthermore, the inhibition of prostaglandin synthesis when there is treatment with flunixin meglumine (a COX-2 inhibitor) between 24 and 36 h after hCG administration, induced development of HAF in greater than 80% of estrous cycles (Cuervo-Arango and Domingo-Ortiz, 2011) or in another study in all estrous cycles (Bashir et al., 2016) during which administrations occurred. Follicular PGE_2 synthesis begins between 33 and 36 h after hCG administration in mares (Sirois and Dore, 1997). In the study by Schauer et al. (2013), PGE_2 concentrations in follicular fluid were not determined at times relevant to the timing of the preovulatory LH surge. This may be the reason why there were no differences in PGE_2 concentrations in that study.

To gain a greater understanding of the physiopathology of HAF, the aim of the present study was to determine the effect of follicular ablation and administration of $PGF_{2\alpha}$ on concentrations of progesterone, estradiol and PGE_2 in the preovulatory follicles of an induced new follicular wave at the time of expected peak concentrations of intrafollicular PGE_2 (35 h post hCG). Two hypotheses were tested: 1) Follicular ablation and $PGF_{2\alpha}$ administration will induce greater concentrations of LH during the induced follicular wave; and 2) the increased LH resulting from this treatment will reduce the follicular concentrations of PGE_2 at 35 h after hCG administration.

2. Materials and methods

2.1. Animals, ultrasonic examinations and follicle puncture

Seven mixed-breed estrous cyclic mares aged 9–20 years and weighing between 300 and 550 kg were used. The study was conducted between April and July 2013 in Valencia (Spain). Mares were housed in stables and were provided *ad libitum* access to water and fed alfalfa hay and cereal grain. Animal procedures were conducted in accordance with the Spanish Department of Agriculture Guide for Care and Use of Animals in Research and approved by the Local Committee for Animal Welfare and Research of the Universidad CEU Cardenal Herrera (Ref: UCHCEU 13/14).

Transrectal B-mode ultrasonography of the genital tract was performed using an ultrasonic device (Sonosite 180 Vet Plus) equipped with a linear array 8-MHz transducer. All mares had at least one ovulation before the beginning of the study. This was confirmed by the presence of a corpus luteum (CL). Ultrasonic examinations were performed every 2 days after Day 10 (Day 0 = Day of ovulation) and twice a day at 12 h intervals during the periovulatory period (Hour 0 = hour of hCG administration). Endometrial edema was scored in a 0 to 5 scale (0 = diestrus-like echotexture, with no endometrial folding; 5 = maximum degree of endometrial folding). Follicular diameter was obtained using the electronic calipers from the average of two linear measurements of the antrum taken at right angles.

The follicular ablation (Day 10) and follicular sampling (Hour 35) were performed using an ultrasonic device (Honda Electronics HS-1500 VET) equipped with a 9 MHz convex transvaginal transducer (HCV 3710 MV). All mares were sedated with detomidine hydrochloride (Domosedan 0.01 mg/ Kg iv; Esteve Veterinaria, Tarragona, Spain) and butorphanol tartrate (Torbugesic 0.02 mg/Kg iv; Fort Dodge Laboratories, Girona, Spain). Hyoscine N-butyl bromide and metamizol (Buscapina compositum,) were administrated to induce rectal relaxation. Caudal epidural anesthesia with 5 ml mepivacaine hydrochloride (Scandinibsa 2%; Inibsa laboratories S.A, Barcelona, Spain) was administrated to induce local analgesia. The convex transducer was placed in the vagina against the vaginal fornix ipsilateral to the target ovary. A 5-cm long, 22-gauge needle was used to puncture and aspirate follicles ≥ 8 mm (follicular ablation) or the preovulatory follicle (follicular fluid sampling). Follicular fluid was aspirated using a 20 ml syringe fitted

to the probe through a silicone tube.

2.2. Experimental design

Each mare was randomly allocated to a control ($n = 7$) or treatment ($n = 7$) group during two different estrous cycles in a crossed over design. Mares were not treated during an estrous cycle between two experimental estrous cycles. In mares assigned to the treatment group, on Day 10 all follicles ≥ 8 mm were ablated using transvaginal ultrasonic-guided puncture and mares were treated with 10 mg of $PGF_{2\alpha}$ im (5 mg/ml dinoprost, Dinolytic; Pfizer España, Alcobendas, Spain). The rationale for follicle ablation was to synchronize timing among mares for a new follicular wave. All mares received an intravenous dose of 1500 IU hCG (Veterin Corion®, 750 UI/ml, Divasa Farmavic S.A, Spain) after a follicle developed that had a > 32 mm diameter and the uterus had mild to moderate endometrial edema (2 out of 5; Hour 0). The ovaries of all mares were ultrasonically assessed every 2 d from Day 10 to the time of hCG administration and then at Hour 0, 12 and 35. Blood samples were collected from Day 10, every 2 d, until the time of hCG administration and subsequently at Hour 12. Blood was collected into heparinized tubes from the jugular vein, immediately placed in ice and centrifuged (2,000 × *g* for 10 min). The plasma was decanted and stored at – 20 °C until assayed. At Hour 35, the pre-ovulatory follicle was puncture, and all follicular fluid was collected and stored at – 20 °C. The rationale for choosing Hour 35 was that a significant increase in PGE_2 in follicular fluid occurs between 33 and 36 h after hCG administration (Sirois and Dore, 1997). The increase in follicular fluid PGE_2 concentrations was considered to be indicative of an impending ovulation (within 10 h), while inhibition of prostaglandin synthesis results in a lack of ovulation occurrence (Sirois and Dore, 1997; Cuervo-Arango and Domingo-Ortiz, 2011).

2.3. Hormonal assays

The blood samples were assayed for concentrations of LH using a homologous double-antibody radioimmunoassay (RIA), with highly purified equine hormones as standard of comparison and with specific antiserum raised against this standard. Equine LH (AFP-5130A) and rabbit anti-eLH (AFP-240580), as primary antibodies for RIA were supplied by Dr. Parlow (National Hormone & Peptide Program, Torrance, CA 90502, USA). The intra- and inter-assay coefficients of variation (CV) and mean sensitivity for LH were 11.0%, 13.4% and 0.3 ng/mL, respectively.

Plasma PGE_2 concentrations were determined using a competitive enzyme immunoassay (Cayman Chemical Company, Michigan, USA), which included a purification step to dissociate PGE_2 from soluble receptors and interfering binding proteins. The sensitivity and intra-assay CV were 9.8 pg/ml and 16.7%, respectively.

A commercial ELISA kit was used to determine concentrations of estradiol (DRG Instruments GmbH, Marburg, Germany) as described (Schauer et al., 2013). Intra-assay CVs were 9.8%, 7.9% and 9.9% and assay sensitivities were 0.005 ng/ml, 0.040 ng/ml and 0.015 ng/ml, respectively. Progesterone concentrations in follicular fluid were quantified using a Coat-a-Count radio-immunoassay kit (Siemens Healthcare Diagnostics Inc.), as described (Schauer et al., 2013). The intra-assay CV was 4.3% and assay sensitivity was 0.01 ng/ml. All assays had been validated in the laboratory where the present study was conducted by assessing the parallelism between serial sample dilutions and the assay standard curve for which the reagents were provided by the company producing the radioimmunoassay kit. Sensitivity was in all cases calculated by subtracting two standard deviations from the mean optical density or counts per minute value at maximum percentage binding.

2.4. Statistical analysis

Data not normally distributed were ranked before analyses. All data were analyzed using a statistical software (Systat 13, Systat Software Inc., Chicago IL). Sequential data (LH concentrations over time) were analyzed using a general linear mixed model of variance including a repeated statement to account for autocorrelation between values from sequential samples from the same individuals taken every 2 d from Day 10 until the time of hCG administration. If an effect of group, day, or group by day interaction was detected, the data were further tested using the student *t*-test. The effects of the induced wave on concentrations of estradiol, progesterone and PGE_2 in follicular fluid were determined using the Mann-Whitney non-parametric tests. Fisher's exact test was used to determine the differences in the proportion of preovulatory secondary (F2) follicles between groups. The $P \leq 0.05$ values was considered to indicate there was a difference and the $P \leq 0.1$ value was considered to be indicative that a trend for a difference. Data are provided as mean ± S.E.M. (standard error of the mean), unless stated otherwise.

3. Results

A group by day interaction ($P < 0.05$) resulted from a stimulatory effect of follicle ablation and $PGF_{2\alpha}$, compared to control mares, on circulating concentrations of LH (Fig. 1).

The diameter of the largest follicle (F1) was greater in mares of the control group ($P < 0.04$; Fig. 2) than the treatment group. The diameter of the second largest follicle (F2) was greater in mares from the treated than control group at Hour 12 and 36 subsequent to the hCG administration (Fig. 2; $P < 0.05$). There was a significant interaction of day and treatment for the F2 (second largest follicle) diameter as a result of a greater mean daily follicular growth after follicle ablation. The mean F2 diameter at the time of hCG treatment was greater ($P < 0.05$) in mares from the treatment group (26.9 ± 2.3) than in mares of the control group (21.2 ± 1.4). A larger number of F2 ($P = 0.07$) follicles developed to have a diameter of > 30 mm in the treatment group (4/7)

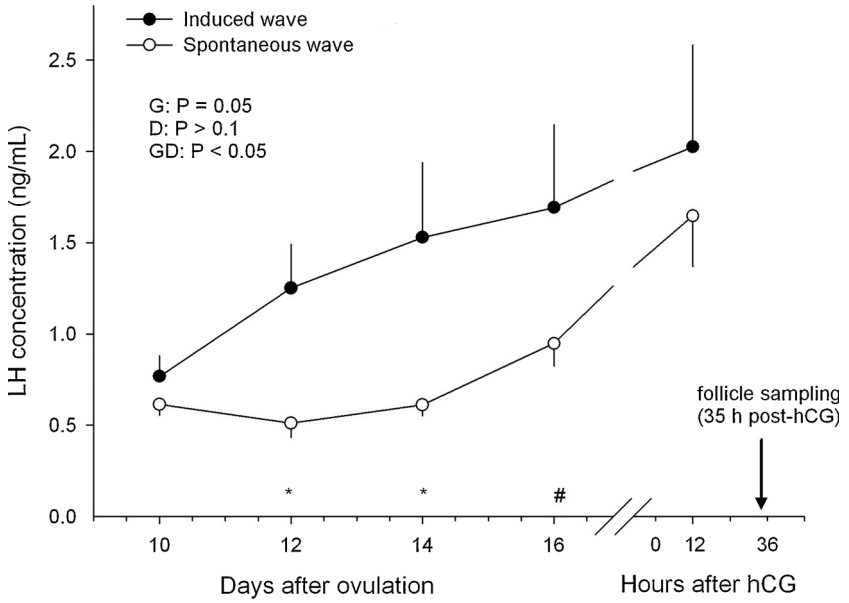

Fig. 1. Mean ± SEM plasma concentration of LH in mares treated with PGF2α and follicle ablation (induced wave, solid circles, $n = 7$) and control mares (spontaneous wave, open circles, $n = 7$); An asterisk (*) indicates a difference ($P < 0.05$) between groups of a given day; Symbol (#) indicates a tendency for a difference ($P < 0.1$); Time of follicle sampling (35 h after hCG administration) is indicated by an arrow.

compared with the control (0/7) group.

In the treatment group, one mare had an ovulation before sampling, between 24 and 35 h after hCG treatment. One aspirated follicle from a mare in the treatment group and three from mares in the control group were grossly contaminated with blood and the corresponding samples were discarded. There were no significant differences between the control ($n = 4$) and treatment ($n = 5$) groups for progesterone, estradiol and PGE_2 follicular fluid concentrations. The mean concentrations of progesterone were 136 ± 28 and 184.3 ± 81 ng/ml, estradiol 2127 ± 385 and 1490 ± 556 ng/ml and PGE_2 0.99 ± 0.3 and 2.31 ± 1.7 in the treatment and control groups, respectively.

4. Discussion

Ablation of all follicles greater than 8 mm in diameter on day 10 after ovulation followed by intramuscular administration of 10 mg of $PGF_{2\alpha}$ resulted in an increase in LH concentrations thus supporting Hypothesis 1. The greater LH concentrations in the treatment group may have accounted for the greater F2 mean diameter in mares of the treatment group (Ginther et al., 2008b). The increase in LH was likely a result of the removal of progesterone feedback after luteolysis (Ginther et al., 2008b; Schauer et al., 2013). Increased LH concentrations during early follicle development has been proposed as a possible cause of ovulatory failure in the mare (Ginther et al., 2008a). Results of the present study, however, indicated the two-fold increase in LH concentrations between Day 12 and 18 did not affect the concentrations of PGE_2, estradiol or progesterone in the subsequent periovulatory follicle. Thus, Hypothesis 2 was not supported by findings in the present study. These results, however, are consistent with those after administration of recombinant eLH or following PGF-induced luteolysis in mares (Schauer et al., 2013). In that previous study hCG was, however, not administrated before follicular fluid collection. In mares, the enzyme prostaglandin G/H synthase-2 (PGHS-2), also known as COX-2, is in greater abundance in granulosa cells 30 h after the beginning of the preovulatory LH surge, or administration of hCG. The PGHS-2 enzyme is the first rate-limiting enzyme in the biosynthesis of prostanoids from arachidonic acid. Parallel increases in the abundance of PGHS-2 in granulosa cells and the concentrations of PGE_2 and $PGF_{2\alpha}$ in follicular fluid occur 33 to 36 h after hCG administration in mares (Sirois and Dore, 1997). For this reason, in the present study there was administration of hCG to characterize the time course of increase in COX-2 abundance and, simultaneously, the changes in PGE_2 and $PGF_{2\alpha}$ in preovulatory follicles. Nevertheless, there were no differences in follicular fluid PGE_2 concentrations in the present study.

Ginther et al. (2008) reported that there was a greater incidence of HAFs during follicular waves following follicle ablation (24% compared with 0% of ablation-induced compared with spontaneous waves) (Ginther et al., 2008b). These results indicate that the use of the ablation-induced ovulatory wave was a potentially useful model to study the mechanisms that underlie HAF formation. Alternatively, COX-2 inhibitors, such as flunixin meglumine (FM), have been administered to inhibit ovulation and induce LUF formation (Bashir et al., 2016; Martínez-Boví and Cuervo-Arango, 2016a; Cuervo-Arango and Domingo-Ortiz, 2011). In this context, the term LUF was used to refer to haemorrhagic anovulatory follicles induced experimentally as opposed to spontaneously induced HAFs. Bashir et al. (2016) reported that intra-follicular $PGF_{2\alpha}$ and PGE_2 concentrations decreased in response to systemic FM administration. Although in the present study the increased LH concentrations did not affect the concentrations of PGE_2 in the largest follicle, further studies with larger groups of mares will be required to more conclusively establish the effects of greater LH

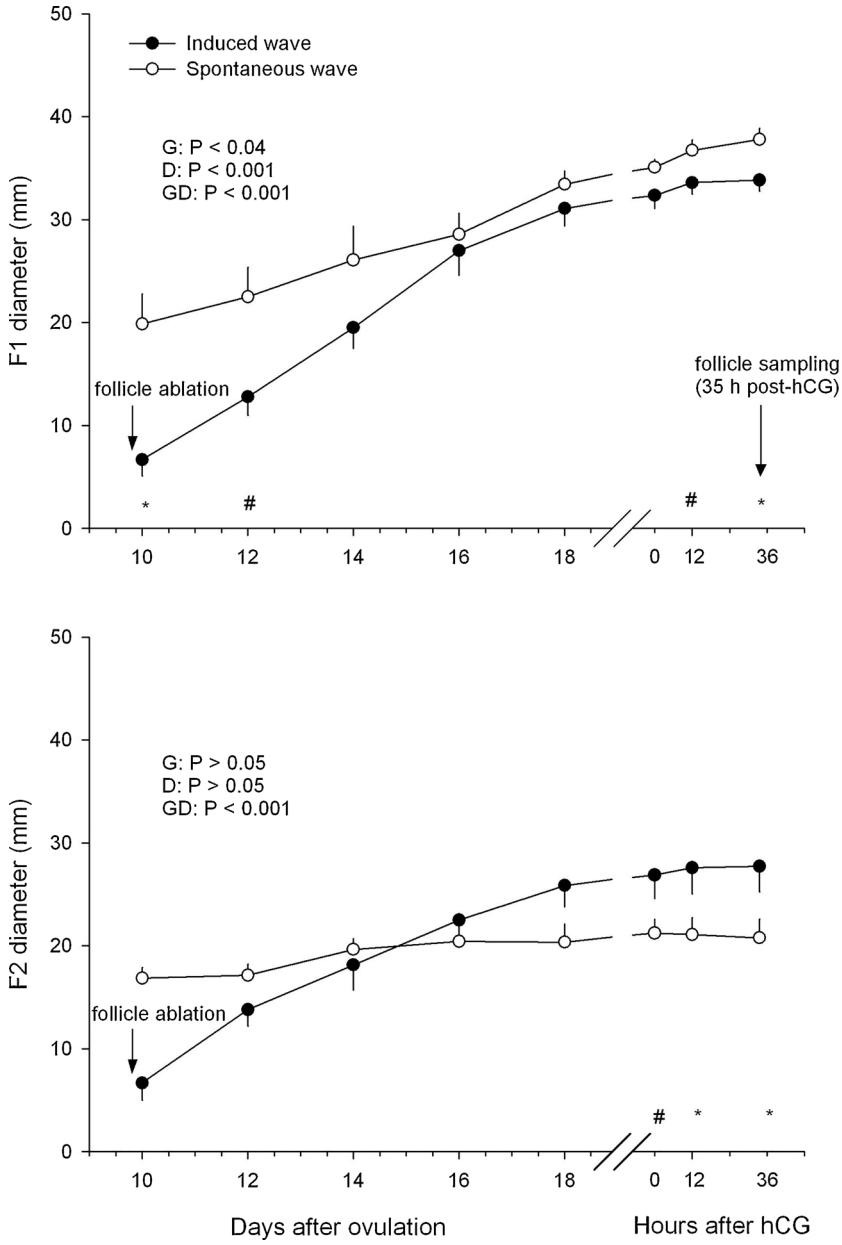

Fig. 2. Mean ± SEM follicle diameter for F1 and F2 (second largest follicle) in mares with an induced follicular wave (induced follicular wave, solid circles, $n = 7$) and control mares (spontaneous follicular wave, open circles, $n = 7$); Follicle ablation (induced wave group) and follicle sampling (F1) are indicated with a black arrow at Day 10 and 35 h post hCG administration, respectively; An asterisk (*) indicates a difference ($P < 0.05$) between groups within a given day; Symbol (#) indicates there was a tendency for a difference ($P < 0.1$).

concentrations on follicular factors and granulosa cells, specifically to determine the effects on abundance or activity of PGHS-2.

There have been no previous reports that relatively lesser intra-follicular prostaglandin concentrations than those typically present in ovulatory follicles lead to a lack of occurrence of ovulation from spontaneously occurring HAFs. Intra-follicular administration of PGE_2 and $PGF_{2\alpha}$ was used to successfully induced ovulation in preovulatory mares that had been treated with FM to inhibit prostaglandin production (Martínez-Boví and Cuervo-Arango, 2016a). Furthermore, intra-follicular prostaglandin treatment was able to induce normal ovulation in mares during early to mid- estrus (Martínez-Boví and Cuervo-Arango, 2016b; Aguilar et al., 2018). The results of these studies indicate prostaglandin concentrations in the preovulatory follicle may be important in determining ovulation outcomes and as a pre-condition leading to development of HAFs. These results from these previous studies and the present study indicate that prostaglandin treatments might be a valuable approach for preventing development of HAFs.

In conclusion, ablation of follicles ≥8 mm together combined with administration of $PGF_{2\alpha}$ 10 days after ovulation in mares resulted in an induced follicular wave and an earlier increase in circulating LH concentrations as compared with what would typically

occur in a spontaneous estrous cycle. The earlier LH increase did not have any marked effects on the concentrations of intra-follicular PGE_2, estradiol or progesterone 35 h after hCG administration.

Author contributions

R. Martínez-Boví performed the collection, processing of data, figure creation and editing, statistical analyses and wrote the manuscript. A. Zagrajczuk determined LH concentration by RIA. F.X. Donadeu assisted in estradiol and progesterone assays and revised the manuscript. D. Skarzynsky and K. Piotrowska-Tomala helped with the determination of PGE_2 in follicular fluid. J. Cuervo-Arango designed the experimental protocol, helped with the collection of data and revised critically the manuscript.

Declaration of Competing Interest

None of the authors have any conflict of interest to declare.

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