Theriogenology 104 (2017) 120–126

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Uterine clinical findings, fertility rate, leucocyte migration, and COX-2 protein levels in the endometrial tissue of susceptible mares treated with platelet-rich plasma before and after AI

Lorenzo G. Segabinazzi [a], Aime M. Friso [a], Sebastian B. Correal [d], André M. Crespilho [b, c], José Antonio Dell'Aqua Jr. [a], Jordi Miró [d], Frederico O. Papa [a], Marco Antonio Alvarenga [a, *]

[a] Department of Animal Reproduction and Veterinary Radiology, São Paulo State University – UNESP, Botucatu, Brazil
[b] Santo Amaro University, UNISA, São Paulo, Brazil
[c] Severino Sombra University, Vassouras, Rio de Janeiro, Brazil
[d] Equine Reproduction Service, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Spain

ARTICLE INFO

Article history:
Received 27 February 2017
Received in revised form
3 August 2017
Accepted 3 August 2017
Available online 12 August 2017

Keywords:
Endometritis
Equine
Embryo transfer
Platelet-rich plasma

ABSTRACT

Persistent mating-induced endometritis (PMIE) results in decreased fertility in horses, thereby causing a significant impact in the horse market. Platelet-rich plasma (PRP), a modulator of the inflammatory response, has been largely used in veterinary medicine. Here, we investigated the effects of PRP on uterine inflammation, conception rate, endometrial polymorphonuclear neutrophil (PMN) migration, and COX-2 protein levels in the endometrial tissue. Thirteen PMIE-susceptible mares were used for artificial insemination (AI). The mares were inseminated with fresh semen in three consecutive cycles in a cross-over study design. The following cycle classifications were used: control cycle, no pharmacological interference; pre-AI, 20 mL of PRP was infused 24 h before AI; and post-AI, 20 mL of PRP was infused four h after AI. Follicular dynamics were monitored daily by transrectal ultrasound. When a follicle larger than 35 mm was detected, ovulation was induced with deslorelin acetate (1 mg, im). AI was performed 24 h after ovulation induction. Intrauterine fluid (FLU) was evaluated by ultrasonography before and 24 h after AI. PMNs in uterine cytology (CYT) and biopsy (HIS) were also observed before and 24 h after AI. Pregnancy was determined within 14 days after ovulation. Number of COX-2 positive cells was evaluated by immunohistochemistry. Both PRP treatments resulted in a decrease of PMNs in the CYT after breeding when compared to controls. FLU did not differ between cycles; however, the conception rates were significantly higher in the PRP mares. Mares positive for endometritis decreased in both treatment groups, and a more intense positive COX-2 labeling was observed in the control group when compared to the two treatment groups. In conclusion, PRP beneficially reduces inflammatory response in PMIE mares independent of when treatments were administered, thus increasing chances of successful pregnancy.

1. Introduction

Platelet-rich plasma (PRP) is whole blood plasma with a high platelet concentration, and contains diverse growth factors that can act in injured tissue by mitogenic, neovascular, and anti-inflammatory effects [1–4]. After the platelets are activated, growth factors are released in the injured area, altering the chemotactic gradient and reducing leucocyte attraction to inflamed tissue [5,6]. Studies have also shown in mares with uterine inflammation treated with PRP a downregulation of intrauterine inflammatory [7,8].

Uterine inflammation is a physiological process that occurs after artificial insemination (AI) or natural breeding to clear excess semen and microorganisms from uterine lumen [9]. Mares that are considered resistant to persistent mating-induced endometritis (PMIE) are able to reduce this inflammatory process within 12–24 h [10] However, mares that are unable to do it are classified

* Corresponding author. Departamento de Reprodução Animal e Radiologia Veterinária, FMVZ, UNESP, Distrito de Rubião Junior, s/nº, 18618-970 Botucatu, SP, Brazil.
E-mail address: malvarenga@fmvz.unesp.br (M.A. Alvarenga).

http://dx.doi.org/10.1016/j.theriogenology.2017.08.007
0093-691X/

as susceptible to PMIE. Susceptible mares shows excessive amount of polymorphonuclear neutrophil (PMNs) and fluid accumulation inside the uterus [11–13]. Additionally, the endometrium of susceptible mares expresses more pro-inflammatory cytokines and less anti-inflammatory cytokines to regulate acute inflammation when compared to resistant mares [14–17].

Furthermore, the acute inflammatory process begins after bacterial or semen recognition by the Toll-like receptors (TLRs) in the endometrial cells [14,15,18–23]. After activation of the TLRs, nuclear factor-kappa B (NF-κB) is expressed, thereby activating pro-inflammatory cytokines, chemokines, and cyclooxygenase-2 (COX-2) [24,25]. These molecules regulate the inflammatory signals to the immune cells [15,26].

Finally, usually used endometritis therapies could be consider supporting treatments since they aim to reduce the predisposing factors instead of acting in the inflammatory process directly. Additionally, immunomodulators have been used to modulate the uterine inflammatory process [13,14,27,28]. The PRP acts by inhibiting NF-κB, and is capable of downregulating pro-inflammatory cytokines in mares endometrium [29,30]. Therefore, here we investigated the effects of PRP on uterine inflammation, conception rate, endometrial PMN migration, and COX-2 protein levels in the endometrial tissue. Furthermore, we either assessed for the optimal time to use the PRP treatment, either pre- or post-AI.

2. Material and methods

This study was approved by the Animal Care and Use Committee of São Paulo State University.

Thirteen crossbred mares from the Department of Animal Reproduction and Veterinary Radiology of São Paulo State University, with ages ranging from 8 to 20 years old were chosen based on reproductive histories. These animals exhibited following characteristics: presence of fluid accumulation 24 h after AI (>10 mm of fluid column), exacerbated number of neutrophil cells (>20%) 48 h after AI as determined via uterine cytology, and poor embryo recovery rates (<30%). The mares were maintained in similar handling and pasture, and they received 10 kg of silage, water, and mineral salt *ad libitum*.

Before starting the study, a breeding soundness examination was performed to confirm that all of the experimental mares had a negative uterine culture, negative cytology (<5% of PMNs) [31], and no free fluid into the uterine lumen.

2.1. Detecting the estrous cycle and treatment protocol

Mares were examined by transrectal palpation and ultrasonography (Sono Scape A5V, Domed, SP, Brazil) daily. When a follicle measuring ≥35 mm and an endometrial edema of at least grade 2 was diagnosed (min. grade 0; max. grade 4) [32], an intramuscular injection of 1 mg of deslorelin acetate (Sincrorelin, Ouro Fino, Brazil) was performed to induce ovulation. Artificial inseminations with fresh semen (800×10^6 total spermatozoa) were performed 24 h after ovulation induction.

Three cycles of each mare were used and randomly assigned to control and treated groups in a crossover study. In the control cycle group, the mares had no intrauterine infusion. In the treated group, 20 mL of PRP was infused with an insemination pipette (pipette to mares, Provar Comercial LTDA, SP, Brazil) inserted into the uterine body. The treatments differed by the time of infusion: pre-AI treatment consisted of 20 mL of PRP infused 24 h before AI at the same time ovulation was induced, and post-AI treatment consisted of 20 mL of PRP infused 4 h after AI.

The perineum was cleaned using a mild detergent, rinsed with clean water, and dried with paper towels. For the intrauterine procedures, sterilized materials were used to minimize contaminations. Mares received only one treatment per estrous cycle.

2.2. Semen collection

Semen from only one stallion was used for all inseminations. An artificial vagina Botucatu Model (Botupharma, Botucatu/SP, Brazil) was used to semen collections. Gel fractions were removed using a nylon filter. The sperm concentration was measured using a Neubauer chamber, and the sperm motility was measured by computerized analysis (Hamilton Thorne Research, Danvers, USA). The semen was diluted to a concentration of 50 million sperms per mL using a skimmed milk-based extender (Botu-semen, Botupharma, Botucatu, SP, Brazil). An AI dose of total 1 billion sperm was used.

2.3. PRP preparation

PRP was prepared by a single centrifugation. In brief, 45 mL of blood samples were collected from each animal through a puncture of the external jugular vein and conditioned into tubes containing 3.2% sodium citrate (Vacutainer, Labor Import, SP, Brazil). Blood samples were homogenized and accommodated in an isothermal box for 1 h. Subsequently, samples were centrifuged at 120 xg for 10 min. From each centrifuged tube, the top half layer of the plasma was discarded and the remaining fraction was used as PRP. The tubes with PRP were placed in an isothermal box (Botuflex, Botupharma, Botucatu/SP, Brazil) to be maintained at a controlled temperature between 20 °C and 25 °C for 1 h until uterine infusion.

The platelet concentration was measured using a hemocytometer chamber. The minimum platelet concentration used for the treatment was 250,000 platelets/mL, and the PRP was used without activation as previously described [33].

2.4. Sampling strategy

2.4.1. Intrauterine fluid evaluation

Transrectal ultrasound evaluations were performed 24 h before and after AI, and the presence or absence of fluid was recorded. If fluid was present, the height and width of the intrauterine fluid column was measured in the bifurcation region of the uterine horn. The amount of uterine fluid was quantified using the height and width multiplication (mm^2).

2.4.2. Endometrial exfoliative cytology

Endometrial exfoliative cytology was performed 24 h before and after AI. The samples were obtained using a disposable cytobrush (cytological disposable collector for mares, Provar Commercial LTDA, SP, Brazil) as previously described [34]. After the collection, the slides were air-dried and stained by Dip Quick (Instant Prov; NewProv, Brazil). The samples were then microscopically examined under a 1000X oil immersion objective, and the percentage of PMNs was randomly determined. Cytological samples were classified as either lacking inflammation (<5%), mild inflammation (5 – <15%); moderate inflammation (≥15 – <30%); and severe inflammation (≥30%) as described previously [31].

2.4.3. Conception rates

Ultrasound examinations were performed 14 days after ovulation was determined. Mares that did not ovulated 24 h after AI were excluded and the next cycle was used. After the examination, 5 mg (im) of dinoprost tromethamine (Lutalyse®, Zoetis, SP, Brazil) was applied to interrupt the pregnancy.