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*Published Version:*

Effect of platelet lysate on uterine response of mares susceptible to persistent mating-induced endometritis / Colombo I.; Mislei B.; Mari G.; Iacono E.; Merlo B.. - In: THERIOGENOLOGY. - ISSN 0093-691X. - ELETTRONICO. - 179:(2022), pp. 204-210. [10.1016/j.theriogenology.2021.12.001]

*Availability:*

This version is available at: <https://hdl.handle.net/11585/850051> since: 2022-01-31

*Published:*

DOI: <http://doi.org/10.1016/j.theriogenology.2021.12.001>

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Colombo, I., Mislei, B., Mari, G., Iacono, E., Merlo, B. Effect of platelet lysate on uterine response of mares susceptible to persistent mating-induced endometritis. *Theriogenology*, 2022, 179, pp. 204–210

The final published version is available online at: 10.1016/j.theriogenology.2021.12.001

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## **Effect of platelet lysate on uterine response of mares susceptible to persistent mating-induced endometritis**

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## Abstract

Many mares are susceptible to persistent mating-induced endometritis (PMIE), an important cause of reduced fertility. Platelet lysate (PL) derives from freeze-thawing platelets after concentration, so that growth factors are released from the platelets. Among the advantages of PL compared to platelet-rich plasma (PRP), it can be frozen stored and allogenic use for PL might also be conceivable. Platelet-rich plasma beneficially reduced inflammatory response in PMIE mares when administered 24 h pre- or 4 h post-AI. The aim of this study was to test the effect of PL on inflammatory uterine response in mares susceptible to PMIE. A total of 14 mares susceptible to PMIE (based on presence of fluid or inflammatory cells 24 h after AI) underwent an untreated (Ctr) cycle followed by a treated (PL) cycle. From each mare, 100 mL of citrated whole blood was obtained for PRP production by centrifugation. The resultant PRP was brought to a final volume of 10 mL with platelet poor plasma and frozen at  $-80^{\circ}\text{C}$  to obtain PL. On untreated cycles, mares were inseminated with frozen-thawed semen 36 h after ovulation induction. On treated cycles, PL was thawed, infused into the uterus 12 h after ovulation induction, and AIs were performed 24 h later. The number of neutrophils in uterine cytology (score 1(normal)-3(severe inflammation)) evaluated by optical microscopy, uterine fluid accumulation (height x width) and uterine edema (score 0-3) observed in ultrasonography, were analysed. Pregnancy was evaluated by ultrasonography 14 days after ovulation. A significant decrease ( $P<0.05$ ) was observed on cytology score (PL  $1.3\pm0.1$  vs Ctr  $2.0\pm0.1$ ), fluid accumulation (PL  $79.5\pm30.1\text{ mm}^2$  vs Ctr  $342.7\pm52.9\text{ mm}^2$ ) and edema score (PL  $1.8\pm0.2$  vs Ctr  $2.3\pm0.2$ ) in treated mares. Pregnancy rate in PL-treated cycles (3/12) and control cycles (2/14), were not significantly different ( $P>0.05$ ). According to the results, we conclude that treatment with PL in mares classified as susceptible to PMIE appears to reduce the inflammatory response after breeding, based on clinical signs of uterine edema, IUF accumulation and PMNs migration.

## Keywords

Platelet lysate; Equine; Persistent mating-induced endometritis; Susceptible mare; Frozen semen

## 1. Introduction

Persistent mating-induced endometritis (PMIE) is a frequent inflammation of the inner layer of uterine wall, which occurs after natural breeding or artificial insemination (AI) and affects broodmares of all breeds [1,2]. It is worth noting that a transient endometrial inflammation is a physiological response of the equine uterus to semen, necessary for the effective removal of excess spermatozoa and microorganisms introduced into the lumen [3], whereas failure to clear uterine inflammation reduces fertility, by diminishing the viability of an embryo under this condition [4–6]. Therefore, mares that show inflammatory signs, such as abnormal endometrial edema [7,8], excessive amount of polymorphonuclear neutrophils (PMNs) [9,10] and intrauterine fluid (IUF) accumulation [11], for more than 24 h after insemination are considered susceptible to PMIE and need particular cares in their management [11–13].

Susceptibility to PMIE is related, among others, with both impaired uterine immune response and alteration in mechanical clearance [3,14,15]. In the last decade, much progress has been made towards the understanding of the innate immune mechanisms involved in PMIE [16–18]. Interestingly, several studies indicated that susceptible mares had an inappropriate balance between pro- and anti-inflammatory molecules [19–21]. Specifically, in resistant mares the inflammatory cascade was rapidly triggered by the semen, with the expression and activation of the pro-inflammatory cytokines and the influx of PMNs into the uterus within 0.5 h [22,23]. Up-regulation of the cytokine response has been reported at 2 h and peaked between 2 and 12 h, while PMNs migration to the uterine lumen peaked between 6 and 12 h [24], then decreased to baseline levels due to an increase in anti-inflammatory cytokines at 6 h [23]. In contrast, in susceptible mares, pro-inflammatory cytokines remained elevated up to 24 h and this was related to a defective anti-inflammatory cytokine production [20,21,23]. Furthermore, susceptible mares have higher basal levels of pro-inflammatory cytokines [25], therefore, early treatment to reduce the uterine inflammatory response could positively affect fertility in mares suffering from PMIE [26]. Hence, the use of treatments targeting the immunological response pathway is commonly used, alongside traditional treatments which support mechanical clearance (for instance uterine lavages and administration of ecbolics). [27,28].

Recently, platelet-rich plasma (PRP) has been frequently used in equine veterinary medicine for various conditions [29–33], including treatment of inflamed tissue, as it was found out to have an immune-modulating effect [34–36]. In particular, PRP is a volume of autologous plasma with a platelet

concentration above baseline, about a three/five-fold increase over whole blood [37]. The therapeutic effect of PRP is attributed to the presence of various growth factors released by platelets into the administration site [38]. Specifically, different studies indicated that growth factors had anti-inflammatory activity and were involved in mechanisms of downregulating pro-inflammatory cytokines [34,39–41]. These effects were also observed in the endometrium of susceptible mares when PRP was administered via intrauterine infusion [42–44].

Interest in using platelet lysate (PL) instead of PRP has been increasing in equine clinical practice [45,46], since, among the advantages, it can be long-term stored [47,48]. Indeed, PL is a plasmatic solution obtained by the cryogenic disruption of the platelets produced from PRP [49]. Cryopreservation of PRP is considered a safe procedure and does not affect the final properties of platelet concentrate [50]. Hence freeze-thawing may be the basis for a more flexible application of platelet-rich products also in the management of endometrial diseases.

To the best of our knowledge, the use of platelet lysate as an immunomodulatory agent in susceptible mares has never been reported. Thus, the aim of the present study was to test the efficiency of PL treatment on uterine inflammatory response of mares susceptible to PMIE.

## **2. Material and methods**

All procedures performed on animals in this study were approved by the Animal Care Committee of the University of Bologna (Animal Utilization Protocol number 1250), in accordance with the Italian Council on Animal Care guidelines.

### *2.1. Selection of mares*

Fourteen Standardbred mares with ages from 3 to 16 years old, housed in a commercial setting at the AUB-National Institute of Artificial Insemination, University of Bologna, were chosen based on reproductive histories. These broodmares failed to become pregnant in the previous breeding season or after at least three attempts of insemination in the same year. Additionally, all animals exhibited clinical signs of PMIE: persistence of >2 cm depth of intrauterine fluid, abnormal endometrial edema, and exacerbated number of neutrophil cells on endometrial cytology 24 h after AI [8,51,52].

All animals were kept in paddocks during the day and housed individually in boxes overnight. Mares remained healthy and in good body condition throughout the study, were fed with 10 kg/day good quality hay and 2 kg/day fodder, with water *ad libitum*.

A breeding soundness examination, including endometrial bacteriology, was performed before starting the study, which confirmed that all mares had no intrauterine fluid accumulation and a negative uterine culture.

## 2.2. PL preparation

Platelet lysate was prepared from each animal. A total of 100 mL whole blood was collected from the jugular vein using blood bag system (Compoflex<sup>®</sup>, Fresenius Kabi, Bad Homburg, Germany) containing citratephosphate-dextrose-adenine (CPDA-1) as anticoagulant. To avoid contamination, the collection area was previously clipped, cleaned with 70% ethanol and allowed to air dry. The bags were transported to the Laboratory of Reproduction and Biotechnology, Department of Veterinary Medical Sciences, University of Bologna, in an isothermal box to be maintained at a controlled temperature between 20°C and 25°C. Within 1 h of collection, whole blood was processed; to maintain aseptic conditions, all separation steps were performed under a laminar flow hood. First, blood samples were transferred into 50 mL sterile Falcon<sup>®</sup> tubes. The tubes were centrifuged at 535 x g for 20 min at 20°C to obtain separation of the blood into three layers. Both the upper and middle layers, rich in platelets and consisting of plasma and “buffy coat” respectively, were carefully aspirated avoiding to collect red blood cells at the lowest level. Then, the upper and middle layers were distributed into new 50 mL tubes. Subsequently, to use plasma containing a more concentrated amount of platelets, a second centrifugation at 2275 x g for 15 min at 20°C was performed. This caused separation into platelet poor plasma (PPP) on the upper layer and leukocytes in the bottom, which were discarded, whereas PRP in the middle layer was collected. In order to achieve a suitable volume for an intrauterine infusion, the resultant PRP was brought to a final volume of 10 mL with PPP of the same donor.

Finally, PRP+PPP was frozen and stored at -80°C to disrupt the platelet membranes, and PL, containing platelet-released growth factors, was obtained [53]. Prior to intrauterine infusion, PL was thawed at room temperature (20-25°C).

In order to verify platelet concentration, samples of peripheral whole blood and diluted PRP were sent to the clinical analysis laboratory of the Department of Veterinary Medical Sciences for platelet counts.

### *2.3. Study design and treatments*

The progression of mares into each estrous cycle was monitored by transrectal palpation and ultrasound examination (Sonosite Micromaxx™; Fujifilm Sonosite, WA, USA). When one follicle with a diameter greater than or equal to 30-35 mm and no corpus luteum (CL) were detected in the presence of endometrial edema, a relaxed cervix and a positive response to a teaser stallion, ovulation was induced with intravenous injection of 2500 IU of hCG (Vetecor®, Farma Mediterranea, San Just Desvern, Spain). Artificial inseminations with frozen-thawed semen ( $800 \times 10^6$  sperm cells) from stallions with proven fertility, determined by owner preference, were performed 36 h after ovulation induction, deep in the uterine horn near the utero-tubal junction ipsilateral to the preovulatory follicle. Insemination was performed with a 75 cm flexible, smooth tipped pipette (Minitube, Germany, ref. 89 17207/1275) that was guided by manipulation per rectum, and a flexible stylet (Minitube, Germany, ref. 17209/1075). Ovulation was ultrasonographically detected between 10 and 12 h after AI, mares that failed to ovulate were excluded and the next cycle was used.

Mares were first assigned to control cycle (Ctr; n=14) and then to platelet lysate-treated cycle (PL; n=12). Each mare served as its own control, and the commercial practice conditions determined the choice of the cycles order. Indeed, mares that were pregnant in the control cycle were excluded from the PL-treated cycle. Whereas mares that were not pregnant in the control cycle were subsequent assigned to the PL-treated cycle. Before starting the second cycle (PL-treated), clinical and bacteriological examinations were performed in all mares to confirm that they were free from inflammation and/or infection.

Pregnancy was evaluated by ultrasonography 14 days after ovulation.

In the control cycle, mares had no intrauterine infusion and were treated with 20 IU oxytocin im (Izossitocina, Izo s.r.l., Brescia, Italy) 12 h after AI. In the PL-treated cycle, 10 mL of PL was infused 24 h before AI with an insemination pipette (Profbovi catheter, Professionalvet, San Martino in Strada, Italy) inserted into the uterine body, then mares were treated with 20 IU oxytocin im 12 h after AI. Before every transvaginal manipulation, the vulva and perineum were cleaned at least three times using clean warm water and povidone-iodine diluted at 2% (LH iodo, Lombarda H S.r.l., Albairate, Italy) and dried with paper towels. Furthermore, to minimize the potential for contamination of the uterus, all intrauterine procedures were performed using sterile materials.

#### *2.4. Intrauterine fluid and endometrial edema evaluation*

Transrectal ultrasound evaluations were performed 24 h and 12 h before AI, at the time of AI and 24 h after AI, using a linear probe working at a frequency of 5-10 MHz.

Absence or presence of intrauterine fluid was recorded. If present, the IUF was measured in the uterine body and quantified using the height and width multiplication (mm<sup>2</sup>).

A four tier edema score system (E0-E3), modified from the various score systems described in literature, was used to evaluate endometrial edema [17,54,55]. The absence of any uterine edema is represented by E0, all the physiological edema changes (including the normal peak at 24-48 h prior to ovulation and the decrease towards it) that occur during estrus are represented by E1, whereas pathological inflammatory edema is represented by E2 (mild) and E3 (severe).

#### *2.5. Endometrial exfoliative cytology*

Endometrial samples were collected for cytology 24 h after AI by manually guiding a double-guarded cytobrush (Minitube; Minitüb GmbH, Tiefenbach, Germany) through the vulva, vagina and cervix into the uterine body, using a long sterile sleeve and sterile gel. The brush was rolled at least three times in the uterine lumen and then retracted into the guarding sheath before removal from the uterus.

Subsequently, the brush was rolled onto glass microscope slide, allowed to air-dry and stained with Wright's stain (Microscopy Wright's solution, Merck KGaA, Darmstadt, Germany).

The slide was then evaluated at 400× magnification, and the degree of inflammation was categorized with a three tier score system (C1-C3) counting the number of PMNs in 10 high-power fields (hpf) as previously described [56,57]. Mares with 0-2 PMNs/hpf were classified as normal (C1), 3 to 5 PMNs were classified as having moderate inflammation (C2), while mares with more than 5 PMNs/hpf were classified as having severe inflammation (C3).

#### *2.6. Statistical analyses*

Data, presented as mean ± standard error (SE), were analysed for normality using a Shapiro test, then were compared using Wilcoxon test. Pregnancy rates were compared using Chi Square test. All the

analysis were performed using the software IBM SPSS Statistics 26 (IBM Corporation, Milan, Italy). Differences were considered significant at  $P < 0.05$ .

### 3. Results

Mean platelet count in PRPs diluted with PPPs was 239000 platelets/ $\mu$ L (55000-576000 platelets/ $\mu$ L), thus increased 2.8-fold when compared to whole blood count (85000 platelets/ $\mu$ L) ( $P < 0.05$ ).

Intrauterine fluid was not present in any mare before AI and at the time of AI. There was significantly less IUF accumulation at 24 h after AI ( $P < 0.05$ ) in PL-treated cycles compared with control cycles (PL  $79.5 \pm 30.1$  mm<sup>2</sup> vs Ctr  $342.7 \pm 52.9$  mm<sup>2</sup>) (Figure 1).

Endometrial edema results are visualized in Figure 2. The score was 3 in all mares 24 h before AI, besides it was not significantly different ( $P > 0.05$ ) between PL-treated cycles and control cycles 12 h before AI (PL  $2.8 \pm 0.1$  vs Ctr  $2.9 \pm 0.1$ ). A significant decrease ( $P < 0.05$ ) in the endometrial edema score was observed in the PL-treated cycles compared with the control cycles at the time of AI (PL  $2.2 \pm 0.2$  vs Ctr  $2.8 \pm 0.1$ ) and 24 h after AI (PL  $1.8 \pm 0.2$  vs Ctr  $2.3 \pm 0.2$ ).

Cytology score significantly decreased ( $P < 0.05$ ) in PL-treated mares 24 h after AI (PL  $1.3 \pm 0.1$  vs Ctr  $2.0 \pm 0.1$ ) (Figure 3).

No significant difference was found ( $P > 0.05$ ) in pregnancy rates between PL-treated cycles (3/12) and control cycles (2/14).

### 4. Discussion

In the current study, intrauterine treatment with platelet lysate improved clinical signs in mares susceptible to PMIE, including IUF retention. Metcalf et al. [52], Reghini et al. [58] and Segabinazzi et al. [44] also showed a reduction in IUF accumulation using intrauterine PRP treatment in susceptible mares, whereas Segabinazzi et al. [43] observed no differences related to this parameter. This discrepancy may be due to multitude of factors, such as variability between mares and their deficiencies in uterine mechanical clearance, which leads to a decreased removal of IUF. Important reasons for delayed removal of fluid are decreased myometrial activity and failure in cervical relaxation [59–61]. In the present study, the selected mares had an adequate cervical dilation during estrus and, to stimulate uterine contractions, oxytocin was administered 12 h after AI. Consequently, in our study the variation

of IUF accumulation 24 h after AI between control cycle and PL-treated cycle could be more likely associated to the modulating effect of PL in the uterine inflammatory response to semen.

The biological mechanisms of platelet-rich products on the inflammatory process are not yet well elucidated [16]. However, different studies have demonstrated their ability to inhibit nuclear factor kappa beta (NF- $\kappa$ B) [34,62], a protein complex that activates genes coding for pro-inflammatory cytokines, including chemokines and cyclooxygenase-2 (COX-2) [63]. This leads to a downregulation of the inflammatory reaction and a decrease in PMNs migration. Actually, several studies [43,44,58] reported a lower number of PMNs present in the uterine lumen 24 h after AI in susceptible mares treated with PRP. Better effects in modulating uterine inflammation were detected when PRP was administered before AI, probably since inflammatory reaction was not already triggered [43]. Thus, in the present study we administered PL 24 h before AI and observed an effective reduction of PMNs count in endometrial exfoliative cytology after AI. Our results are consistent with a reduction in the inflammatory response, since in the control cycles we observed at least 3 PMNs/hpf at 24 h after AI, denoting the presence of an acute inflammation.

Endometrial edema at the time of ovulation could be used as additional clinical sign of inflammation [51]. It is worth noting that edema of endometrial folds is normally present at estrus and reflects oestrogen dominance, then decreases towards ovulation; whereas strong and persistent edema, or its increment towards ovulation, has been related with uterine inflammation and lower pregnancy rate [7,8]. All mares involved in our study had a strong edema at the time of ovulation induction, which indicated their susceptibility to PMIE. In PL-treated cycles edema was reduced 24 h after AI, and interestingly in six mares it was diminished already at the time of AI, therefore 24 h after intrauterine administration. This may be due to a pre-existent mild inflammation that could have been modulated by the growth factors released from the platelets, resulting in an improved uterine environment. Our findings are in contrast with the only other study that considered endometrial edema scores at different timing after intrauterine infusion of PRP and PPP. Actually, Segabinazzi et al. [44] did not find difference among treated and untreated cycles. This discrepancy could be explained by the small numbers of mares used in both studies, thus further studies are needed on this topic.

The percentage of the pregnancy rate increased in PL-treated cycles compared to control cycles, even though the difference was not statistically significant. This data can be affected by the major limitation of using only mares that did not get pregnant following the control cycle for the PL-treated group. Actually, first cycle pregnancy rate is typically higher than second insemination cycle [64]. Therefore,

our results on PL-treated cycles have been potentially influenced by the less fertile rate that characterize the second insemination cycle. Moreover, the relatively small number of cycles included in the study may have affected the results, which need to be confirmed by at least doubling the number of treated cases, as it was also observed in a similar study [43]. Nevertheless, our study is the first using a platelet-rich product in mares with PMIE in a commercial artificial insemination program with frozen-thawed semen. In fact, in the only research reporting the effect on fertility of intrauterine PRP infusion in susceptible mares [43], fresh semen was used, leading to obviously higher pregnancy rates than those we obtained with cryopreserved spermatozoa.

As already mentioned, the interest around the use of treatments that target the immunological response pathway in PMIE mares is increasing. Compared to other immunomodulatory therapies, such as glucocorticoids [65,66], mycobacterium cell wall extract [25], and *Propionibacterium acnes* [67], PL has, at least, two advantages: the preparation is easy and low cost, requiring minimal equipment, and the administration is safe and minimally invasive. Moreover, PL is advantageous over PRP as it can be frozen stored to have it available for immediate patient use.

The acellular nature of PL is important because it has also the potential to be used in an allogeneic manner, contributing to reducing donor-to-donor variability [49]. Indeed, in the present study, the average concentration of platelets in the PRP diluted with PPP was close to the benchmark in PRP (250000 platelets/ $\mu$ L) commonly used in equine reproduction, whereas the range of variation was wide. This is explained by differences between mares, actually individual factors play a role in platelets concentration [68]. In addition, in the circumstances in which the animal suffers from a medical condition for which blood collection is not advised, or simply are more difficult to obtain, it could be considered advantageous treating the susceptible mare with the allogeneic methodology over the autologous.

In conclusion, our findings indicate that treatment with PL is a valuable alternative to PRP in reducing the clinical signs of uterine inflammatory response in PMIE mares after insemination. This study provides clinicians with a novel and easy handling treatment option in the management of mares susceptible to persistent mating-induced endometritis.

#### **Declaration of interest**

None.

**Acknowledgments**

The Authors thank Prof. Francesco Dondi, manager of the CLINLAB, for hematologic analysis.

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## Figures legend

Figure 1. Mean intrauterine fluid accumulation ( $\pm$  SE) in mares susceptible to persistent mating-induced endometritis during control cycle ( $\square$ ) and platelet lysate-treated cycle ( $\blacksquare$ ) at 24 h after artificial insemination (AI). Different superscripts (a,b) represent significant differences ( $p < 0.05$ ).

Figure 2. Mean endometrial edema score ( $\pm$  SE) in mares susceptible to persistent mating-induced endometritis during control cycle ( $\square$ ) and platelet lysate-treated cycle ( $\blacksquare$ ) at 24 and 12 h before artificial insemination (AI), at the time of AI, and 24 h after AI. Different superscripts (a,b) represent significant differences ( $p < 0.05$ ).

Figure 3. Mean endometrial exfoliative cytology score ( $\square$  SE) in mares susceptible to persistent mating-induced endometritis during control cycle ( $\square$ ) and platelet lysate-treated cycle ( $\blacksquare$ ) at 24 after artificial insemination (AI). Different superscripts (a,b) represent significant differences ( $p < 0.05$ ).