How to Prepare Platelet-Rich Plasma for Use in Reproductive Practices with Mares

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1. Introduction

Infusion of platelet-rich plasma (PRP) has recently gained popularity in reproductive practice with mares1 to mitigate postbreeding inflammatory response and consequently improve the fertility of barren and embryo donor mares with persistent-breeding-induced endometritis (PBIE).1–6 The PRP consists of a plasma sample with high platelet concentrations; greater platelet concentrations result in the enrichment of growth factors (GFs, e.g., hepatocyte growth factor), cytokines (e.g., transforming growth factor β, CXL8, and IL1β) released from platelets after activation,7,8 and natural antimicrobial peptides (e.g., RANTES and platelet factor 4) that collectively modulate the uterine immune response and bacterial infections.9–11 Currently, there are various methods available to prepare PRP. Automatized commerciala,b and manual protocols have been described for intrauterine infusion in mares. Practitioners wanting to start using PRP or already using it in reproductive practice with mares may be confused with the numerous options available and not be able to understand all protocols available consistently. Commercial protocols offer the advantage of being standardized and less human dependent; however, the high cost of acquiring the machines and supplies may discourage practitioners to routinely use PRP. Conversely, manual methods do not require specialized equipment and can be an affordable alternative to produce PRP. In addition, the majority of protocols are able to produce a small volume of PRP (~2 to 5 mL) for intra-articular or intratendinous injection. However, for intrauterine infusion, a higher volume (~10 to 60 mL) is presumably needed to reach the entire endometrial surface. This manuscript is an overview of how to prepare PRP for intrauterine infusion in mares and how each available protocol can be useful for broodmare's practice. The most manual methods and automated systems to prepare PRP are discussed onward. In addition, the authors' clinical and research experiences preparing PRP are also incorporated in the review.

2. Methods

Regardless of the method used to prepare PRP, whole blood (WB) has to be aseptically collected by venipuncture of the jugular vein. The authors typically scrub the overlying skin with three rounds of povidone iodine or chlorhexidine scrub followed by three rounds with 70% isopropyl alcohol. The placement of an intravenous catheter is not mandatory but may be placed in needle-shy horses or in case the practitioner
is not comfortable drawing a large amount of blood off a needle. Also, the authors typically use 1.5-inch 18-gauge needles to avoid platelet damage and activation during the blood drawing. Following collection, the blood can be left at room temperature (~22°C) for 2-3 hours until processing with no apparent detrimental effects to PRP quality. Protocols to prepare PRP can be classified as manual or automated. The first type has the advantage of requiring minimal equipment and being low cost but is more time consuming, whereas the latter has the advantage of being standardized and requiring less labor but demands acquisition of expensive processing systems. The non-commercial methods are described herein, and the step-by-step of commercial systems can be found on the manufacturers’ webpages.

Noncommercial Methods

Single Centrifugation in Vacutainer Tubes

Blood is collected in 4.5-mL vacutainer tubes containing 3.2% sodium citrate and immediately gently homogenized. Blood tubes are then centrifuged at 120 × g for 10 minutes. In each tube after centrifugation, the top third layer of the plasma is discarded, while the remaining plasma adjacent to the buffy coat is recovered as PRP (Fig. 1).

Double Centrifugation in Vacutainer Tubes

Blood is harvested in 4.5-mL vacutainer tubes containing 3.2% sodium citrate. Citrated blood should be slowly homogenized and centrifuged at 120 × g for 10 minutes. After the first centrifugation, the upper 30% of the plasma can be discarded. The remaining plasma (70%) adjacent to the buffy coat should be gently recovered and transferred to 10-mL tubes without anticoagulant. This fraction is submitted to another centrifugation at 240 × g for 10 minutes. After the second centrifugation, ~30% of plasma at the bottom of each tube is considered as PRP (Fig. 2).

Double Centrifugation in Blood Transfusion Bag

Whole blood is collected in a 450-mL blood transfusion bag containing 63 mL of citrate-phosphate-dextrose solution with adenine as an anticoagulant (CPD-A). Four hundred milliliters of WB should be split into eight 50-mL tubes and centrifuged at 400 × g for 15 minutes. After the first centrifugation, the plasma fraction is recovered and transferred into 15-mL conical tubes. This fraction is submitted to centrifugation at 1000 × g for 10 minutes. After the second centrifugation, 2.5 mL of plasma at the bottom of each tube is preserved as PRP (Fig. 3).

Sedimentation

Blood is collected using an 18-gauge needle into a 60-mL syringe prefilled with 7 mL of anticoagulant (CPD-A). Right after collection, each syringe should be wrapped in aluminum foil and placed in an upright position at room temperature. Four hours later, the top 10 mL of plasma can be discarded as platelet-poor plasma (PPP), whereas the remaining sedimented plasma can be recovered as PRP (Fig. 4). For this, the syringe is kept in an upright position, a 21-gauge butterfly catheter can be connected to the syringe, and PRP and PPP are recovered by applying steady pressure to the syringe’s plunger.

3. Discussion

The cell composition, as well as soluble factors reported in the PRP produced by different methods, are highlighted in Table 1. All methods, but one, were reported to be able to improve platelet concentration in PRP compared with the WB. Although the cell composition cannot be compared among results from different studies, it is important to note that there is a discrepancy in the volume of blood harvested to produce PRP, the final volume of PRP, and the number of platelets and leukocytes in PRP from different studies, which can impact for intrauterine infusion in mares. Also, the concentration of erythrocytes and GFs are not available for all methods. Of interest, one system produces 10 mL of PRP; however, up to date, there is no report of the cell composition of the PRP produced by this system. The methods described in the present study, with the exception of the four commercially available products and sedimentation, have been tested in broodmare’s practice and used to mitigate postbreeding uterine inflammation (i.e., PMN counts in cytology or endometrial biopsy, intrauterine fluid accumulation, uterine inflammatory cytokines [IL1, IL6, IL8, IL10], endometrial inflammatory markers [COX-2]) and
improve fertility rates of mares susceptible to endometritis, barren mares, or mares inseminated with frozen semen that do not become pregnant in the first cycle (Table 2). Also, there were differences in the time and protocols of treatment and volume of PRP among studies. A summary of protocols used to obtain PRP, the respective platelet concentration, volume of plasma, time of uterine infusion, endometrial inflammatory markers, and fertility of mares is highlighted in Table 2. One of the first reports using PRP in mares described the postbreeding expression of inflammatory cytokines in the endometrium of barren mares treated with intrauterine PRP. The protocol used in that study to produce PRP applied a specialized blood fractionated machine. Although the cell composition of the PRP was not described and the PRP was mixed with PPP for intrauterine infusion, the treatment reduced endometrial expression of proinflammatory cytokines (IL1β, IL6, and CXCL8) in this group of mares. Later, another study using the same technique to produce PRP and treating barren mares showed that intrauterine PRP therapy response lowered intrauterine fluid accumulation and improved fertility when compared to the untreated cycle. Although in these studies the cell composition of PRP was not reported, another study using the same
method produced PRP with platelet concentration two-to three-folds greater than the whole blood. Later, one study using a semiautomated method to produce PRP by double centrifugation in vacutainer tubes also reported a reduction in postbreeding inflammatory reaction in mares with chronic degenerative endometritis after intrauterine PRP therapy. The mares were treated 4 hours after insemination with the intrauterine infusion of 20 mL of PRP and had lower uterine fluid and PMN counts in endometrial cytology postbreeding. In that study, 100 mL of WB was harvested in vacutainer tubes and then centrifuged at 120 \times g for 10 minutes, then after the first centrifugation, the lower 70% of the plasma was further centrifuged at 240 \times g for 10 minutes. This protocol yielded 20 mL of PRP containing \( \geq 250 \times 10^3 \) of platelets/\( \mu L \) and \( \sim 5 \times 10^9 \) platelets per treatment.4 Later, the same group compared the previously published treatment times by infusing PRP, either 24 hours before or 4 hours after insemination, and demonstrated that both time points reduced endometrial PMN counts and COX2 expression in mares susceptible to PBIE, as well as improved pregnancy rates of mares.5 In that study, PRP was produced by single centrifugation in vacutainer tubes. For this, 45 mL of WB was collected in 4.5-mL sodium citrate tubes and centrifuged once at 120 \times g for 10 minutes; the protocol yielded 20 mL of PRP with platelet concentration of \( 354 \pm 17 \times 10^9 \) platelets/\( \mu L \) and \( 7 \pm 0.3 \times 10^9 \) platelets per treatment.6 Most recently, PRP produced by another commercially available system was reported in a clinical trial to treat mares that did not become pregnant after insemination with frozen semen. Similar to the early studies, the authors did not provide the cell composition of PRP used for treatment; however, 61% of the mares treated with PRP became pregnant after insemination with frozen semen.1 In a most recent study, the effect of PRP and PPP was tested on postbreeding endometrial inflammation and fertility.6 PRP and PPP were obtained after the collection of WB (450 mL) in a blood transfusion bag and double centrifuged. The mean number of platelets infused in the uterus of mares was \( 24.9 \pm 1.2 \times 10^9 \) platelets in PRP and \( 1.4 \pm 0.2 \times 10^9 \) platelets in PPP. Embryo donor mares susceptible to PBIE were treated four times (two before and two after insemination) during the estrous cycle with 40 mL of one of the treatments. Interestingly, both plasma therapies reduced the postbreeding...
Table 1. Protocols for Preparation of Platelet-Rich Plasma for Use in Mare’s Reproductive Practice

<table>
<thead>
<tr>
<th>Method</th>
<th>Amount of Blood Anticoagulant</th>
<th>Equipment Necessary</th>
<th>Volume of PRP Recovered</th>
<th>Platelet Concentration (10^6/mL)</th>
<th>Leukocyte Concentration (10^3/mL)</th>
<th>Platelet-rich Plasma Concentration (PA/PDGF and TGF-b)</th>
<th>Growth factors (ng/mL)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angle®</td>
<td>180 mL ACD-A</td>
<td>None</td>
<td>320 ± 118.1</td>
<td>320 ± 24.0</td>
<td>185 ± 22.9</td>
<td>141 ± 7</td>
<td>2.6</td>
<td>Hessel et al., 2015</td>
</tr>
<tr>
<td>Regenx PRP®</td>
<td>60 mL ACD-A</td>
<td>Angel spinsystem</td>
<td>760 ± 240</td>
<td>760 ± 24.0</td>
<td>460 ± 29.6</td>
<td>142 ± 4</td>
<td>2.3</td>
<td>Hessel et al., 2015</td>
</tr>
<tr>
<td>Arthrex ACP®</td>
<td>4 mL 320</td>
<td>Angel spinsystem</td>
<td>189.2 ± 30.7</td>
<td>189 ± 23.7</td>
<td>1.9 ± 0.9</td>
<td>1.6 ± 0.4</td>
<td>0.3</td>
<td>Hessel et al., 2015</td>
</tr>
<tr>
<td>Carmona et al., 2007</td>
<td>60 mL Sodium citrate Centrifuge</td>
<td>None</td>
<td>20 mL &gt;250;</td>
<td>159.8 ± 64.2</td>
<td>64.2 ± 1.9</td>
<td>1.7 ± 0.1</td>
<td>0.027</td>
<td>Segabinazzi et al., 2014</td>
</tr>
<tr>
<td>CLINICAL PERSPECTIVES ON MANAGING EQUINE UTERINE HEALTH</td>
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In addition, the results of the study suggested that PRP may have antimicrobial properties and may be useful in the treatment of endometriosis. However, further studies are needed to determine whether this new approach has clinical relevance. 

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<table>
<thead>
<tr>
<th>Method</th>
<th>Volume of PRP per Treatment</th>
<th>Amount of Platelet per Treatment</th>
<th>Time of Treatment</th>
<th>Type of AI</th>
<th>Mares</th>
<th>Inflammatory Markers</th>
<th>Fertility</th>
<th>Reference</th>
</tr>
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<tr>
<td>Angel®</td>
<td>10 mL (PRP+ PPP)</td>
<td>Not available</td>
<td>24–36 h before AI</td>
<td>Not available</td>
<td>Barren mares</td>
<td>PRP reduced IUF and expression of IL1β, IL6, CXCL8, and iNOS</td>
<td>Control: 19% (3/19); PRP: 67% (16/24)</td>
<td>Metcalfe et al., 2012²; Metcalfe, 2014³</td>
</tr>
<tr>
<td>Restigen PRP®</td>
<td>15 mL (6 mL PRP + 9 mL PPP)</td>
<td>Not available</td>
<td>44 h before AI</td>
<td>Frozen semen</td>
<td>No difference in IUF</td>
<td>Control: 0% (0/18); PRP: 61% (11/18)</td>
<td>Control: 31% (3/13); PRP 24 before AI: 69% (9/13); PRP 4 h after AI: 58% (8/13)</td>
<td>Pasch et al., 2021¹</td>
</tr>
<tr>
<td>Single centrifugation in vacu-tainer tubes</td>
<td>20 mL PRP 7 ± 0.3 × 10⁹ platelets</td>
<td>24 h before or 4 h after AI</td>
<td>Fresh semen</td>
<td>Susceptible to PBIE</td>
<td>PRP reduced PMN counts and endometrial expression of COX2</td>
<td></td>
<td></td>
<td>Segabinazzi et al., 2017⁵</td>
</tr>
<tr>
<td>Double centrifugation in vacu-tainer tubes</td>
<td>20 mL PRP −5 × 10⁹ platelets</td>
<td>4 h after AI</td>
<td>Fresh semen</td>
<td>Mares with CDE</td>
<td>PRP reduced PMN counts and IUF</td>
<td>Not available</td>
<td></td>
<td>Reghini et al., 2016⁴</td>
</tr>
<tr>
<td>Double centrifugation in blood transfusion bag</td>
<td>40 mL PRP 24.9 ± 1.2 × 10⁹ platelets</td>
<td>48 and 24 h before, and 6 and 24 h after AI</td>
<td>Fresh semen</td>
<td>Susceptible to PBIE</td>
<td>PRP reduced PMN counts, IUF, and IL1β and CXCL8. Plasma P4 concentrations were increased</td>
<td>Control: 33% (4/12); PRP: 83% (10/12)</td>
<td></td>
<td>Segabinazzi et al., 2020⁶</td>
</tr>
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</table>

Abbreviations: PRP, platelet-rich plasma; PPP, platelet-poor plasma; IUF, intrauterine fluid; PMN, polymorphonuclear neutrophil; COX2, cyclooxygenase-2; IL1β, interleukin-1β; IL6, interleukin-6; CXCL8, chemokine ligand 8 (interleukin-8); iNOS, nitric oxide synthase; AI, artificial insemination; PBIE, post-breeding-induced endometritis; CDE, chronic degenerative endometritis; P4, progesterone.
as PRP becomes sticky and adheres to the skin. Another factor that has been discussed in PRP practice is the amount of leukocytes in PRP samples. Some studies suggest that leukocytes are undesirable cells in the PRP for treating joints and tendons. High amounts of leukocytes can intensify the undesirable effects of inflammatory response, induce cellular catabolism, decrease extracellular matrix synthesis in tissues, and increase the release of proinflammatory cytokines, which may cause tissue damage. Neutrophils are the body's first line of defense and the most critical cell in uterine defense. These cells are already in the uterus about 30 minutes after semen contact with the endometrium and have a peak inflammatory reaction between 6 and 12 hours, being eliminated up to 48 hours in mares with a competent immune system. Postbreeding therapies such as uterine lavage aimed to prevent an excess of neutrophils and inflammatory molecules in the uterus of mares susceptible to PBIE as this is thought to be detrimental for a controlled immune response. However, one study suggested that intrauterine infusion of white blood cells (WBCs) could faster eliminate bacterial contamination from the uterus of mares susceptible to PBIE. In addition, removal of red blood cells seems beneficial as an excess of blood can be detrimental to sperm, limiting its use prebreeding. Controversy exists on whether fresh WBCs and red blood cells from blood could affect the uterine inflammatory response and if including WBCs from WB in PRP can be beneficial for the treatment of PBIE in mares.

Other methods to prepare PRP (sedimentation) that have not been tested in mare's practice are presented in this manuscript. The authors chose to include these methods since all of them have been tested for cell composition and have produced satisfactory platelet concentration after processing, and they are available for use in clinical practice. In addition, one product and sedimentation protocol can be performed in field situations when there is no equipment available for processing PRP. One commercially available system may fit some practitioner prospects; however, this last one should be tested for cell composition before being incorporated in equine practice. In the sedimentation method, the red blood fraction is separated by gravity. This method takes a bit longer to be processed (~4 hours) and produces PRP with higher WBC concentration than other methods. V-PET consists of two blood bags attached to a filter. Blood is added to the blood bag and passes through the filter under gravity. The platelets are retained into the filter by a complex interaction of size exclusion and adsorption. When the filtration is completed, the filter is isolated, and a harvest solution is back flushed through the filter to recover the PRP. This protocol also has been described to yield PRP with great platelet concentration and higher WBCs than baseline. Therefore, in vivo studies are warranted to assess the clinical efficacy of PRP obtained by these three methods in mitigating PBIE in mares. Of interest, the anti-coagulants reported for processing PRP differ among protocols. The acid citrate dextrose solution (ACD-A) is the most cited in the experiments, followed by sodium citrate and citrate-phosphate-dextrose with adenosine (CPD-A). Some protocols described in the present study have the kit provided by the manufacturer and use ACD-A as an anticoagulant. There are two studies that suggested that ACD-A has an inferior capacity to maintain platelet viability compared to CPD-A and sodium citrate. However, it should be clarified in the methods presented in the current study if changing the anticoagulant in these protocols can affect platelet viability and PRP composition. In conclusion, the methods described here are available to practitioners to produce PRP for intrauterine infusion in mares. It is important to note that PRP is not a standardized medical product. Therefore, variations can be observed between practitioners, methods, and animals. Some of the techniques presented in this manuscript require additional clinical trials to prove their efficacy. PRP is a safe and easy product for use in broodmare’s practice.

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Declaration of Ethics

The Authors have adhered to the Principles of Veterinary Medical Ethics of the AVMA.

Conflict of Interest

The Authors have no conflicts of interest.

References and Footnotes
