Intra-Articular Use of a Platelet-Rich Product in Normal Horses: Clinical Signs and Cytologic Responses

Jamie A. Textor, DVM PhD Diplomate ACVS, and Fern Tablin, VMD PhD

Objective: (1) To report the clinical and synovial effects of a platelet-rich product (PRP) in normal equine joints, (2) to assess the persistence of platelets within synovial fluid after intra-articular injection, (3) to compare responses to different preparations of that product, and (4) to evaluate a gravity filtration system for PRP preparation in horses.

Study Design: Experimental.

Methods: A platelet-rich saline product (PRPr) was prepared from 7 normal horses using a proprietary preparation device and was divided into 3 treatments: resting, CaCl₂-activated (23 mM, final), and bovine thrombin-activated (10 U/mL, final). Each horse had 3 concurrent randomly assigned intra-articular PRPr treatments administered in their metacarpophalangeal/metatarsophalangeal joints; the fourth limb was injected with saline (0.9% NaCl) solution as a control. Clinical assessments, cytologic analysis of synovial fluid and hemograms were performed at 6, 24, 48, and 96 hours after injection. PRPr composition and growth factor content were analyzed.

Results: The gravity filtration system produced a moderately concentrated PRPr. At 6 and 24 hours, when compared to control values, all PRPr treatments caused a significant increase in synovial WBC concentration (P < .0059) and neutrophil percentage (P < .0005). Bovine thrombin-activated PRPr injection consistently caused increased effusion scores and periarticular signs. At all time points, the synovial WBC concentration after thrombin-activated PRPr was significantly greater (P < .001) than for the control, CaCl₂-activated or resting PRPr. Intact platelets could be observed in synovial fluid for up to 5 days after intra-articular PRPr injection.

Conclusions: Resting and CaCl₂-activated PRPr may be safely used to treat equine joints, but bovine thrombin activation is not recommended at 10 U/mL. A PRPr can be prepared using a gravity filtration system, eliminating the need for centrifugation.

Platelet-rich plasma (PRP) is an autologous biological product that is used to deliver high concentrations of growth factors to an injured site, thereby improving the quality of native tissue repair. PRP use in horses has expanded from the treatment of tendon and ligament lesions¹–² to include joints, where it is reported to relieve pain and reduce effusion³⁴ (Hammock PD, Meyer ST, Simpson T: personal communications, 2011–2012). In human sports medicine, the intra-articular use of PRP is widely reported,⁵–⁹ and positive responses have been observed in people with osteoarthritis⁹,¹⁰ In horses, however, we are unaware of controlled studies on the effects of PRP in either normal or diseased joints. The synovial response of normal joints to PRP injection has recently been reported in rabbits,¹¹ but does not yet exist for other species. This baseline information is important for our understanding of the effects of PRP on joint physiology.

Joint disease in the horse is a significant cause of lameness in horses leading to lost working days for performance horses.¹²,¹³ Articular pathologies such as synovitis,¹⁴ cartilage defects,⁹ meniscal injury,¹⁵ intra-articular fractures,¹⁶ and osteoarthritis¹⁷ have been successfully treated with intra-articular PRP in humans and experimental animals, and horses suffer from a similar spectrum of disease. PRP has the advantage of being a potentially regenerative therapy. The preparation is rapid and requires only simple blood collection. Traditionally the process of concentrating platelets has involved serial centrifugation. The ability for equine practitioners to prepare PRP in the field would represent an additional advantage to PRP use in horses. The Equine Platelet Enhancement Therapy™ (E-PET) (Pall Corporation, Port Washington, NY) system, a gravity filtration device, concentrates platelets without the need for a centrifuge and enables “stall-side” preparation of a platelet-rich product (PRPr). This system has been the subject of one previous report in horses.¹⁸ The product is actually a suspension of concentrated platelets in saline solution rather than in plasma. Our pilot investigations
of the product demonstrated that it also includes leukocytes, erythrocytes, and free hemoglobin.

PRP activation is a process by which platelets are specifically stimulated to release their alpha granule contents, which include the growth factors PDGF, TGFβ, IGF-1, VEGF, bFGF, EGF, and HGF. However, in both human and equine sports medicine, platelet activation is not usually performed, meaning that PRP is administered in its non-activated, resting state. Positive effects have been documented using resting PRP in many reports, including an equine tendonitis model, but the lack of PRP activation in many studies reflects the inconsistency in methods currently reported in the PRP literature. Research suggests that platelet activation, and therefore complete growth factor delivery, may not occur spontaneously after exposure to collagen alone. Exogenous platelet activation methods can be employed to guarantee growth factor release from the PRP; the main methods for doing so are the use of thrombin, calcium chloride, or a combination of both. A recent in vitro study in horses indicated that either bovine thrombin (10 U/mL, final) or calcium chloride (23 mM) caused effective growth factor release from equine PRP; however, the ideal activation protocol for clinical use remains to be determined. Although bovine thrombin and calcium chloride have been widely used in human PRP applications and are reported for intraligamentous and topical use in the horse, the safety of these substances for intra-articular use in horses has not been established.

The lifespan of platelets in circulation is 5–9 days, but once injected into a non-vascular site that timeframe may change substantially. This issue may have implications for treatment, such as the need to sustain growth factor release by the addition of substances such as hydrogels or to perform serial re-treatment. To our knowledge, the duration of the platelets' presence in tissue after PRP injection has not been specifically demonstrated, despite being the subject of much speculation in the literature. This question may be addressed by use of a synovial structure as an experimental model, from which serial samples can be consistently obtained.

Our purpose was to address several of the aforementioned issues related to intra-articular PRP use in horses. Firstly, we intended to determine the baseline clinical and cytologic response of the equine joint to intra-articular PRP injection. Secondly, we hoped to gain some information about the duration of platelet presence within tissue after PRP injection. Thirdly, we wanted to compare the effects of different types of PRP on joints (resting vs. activated vs. bovine thrombin-activated PRP) and to assess these activators for intra-articular safety in horses. Lastly, we wanted to provide an evaluation of a gravity filtration system for clinical PRP preparation. Our main hypotheses were that intra-articular PRP injection would induce a transient but significant cellular response in treated joints, and that these responses would differ between preparations of PRP. We further hypothesized that the E-PET system would consistently provide a concentrated platelet product for use in horses.

### MATERIALS AND METHODS

#### Horses

Seven mature, healthy horses (4 geldings, 3 mares; 1 Thoroughbred, 2 Arabians, 3 Quarter Horses, and 1 Hanoverian; mean [range] age = 12.7 [3–21] years, median = 14 years) were studied. Horses were housed in stalls with runs, at the same facility, for the duration of the experiment. Animal use was approved by the Institutional Animal Care and Use Committee. Before study inclusion, all horses were examined to ensure they were sound at the trot and had no palpable abnormalities of any fetlock joint.

#### Preparation of PRPs

Each horse had 55 mL blood collected into a 60-mL syringe containing 5-mL acid-citrate-dextrose A (ACD-A) solution. Autologous PRP was prepared using a gravity filtration system (E-PET™, Fig 1) in accordance with manufacturer instructions. Briefly, anticoagulated blood was injected into a large bag containing 9 mL sterile water, intended to induce hypotonic swelling of the platelets to aid in their retention in the filter. After mixing, the blood passed through a filter and into a waste collection bag. Platelets were retained in the filter, which was flushed with 2% saline (NaCl) solution to recover the platelet concentrate. Using sterile technique, the platelet concentrate was divided into 3 treatment doses of 2.3 mL; the remaining volume was retained for determination of platelet, WBC, RBC, and hemoglobin concentrations. A control syringe of the 2% saline solution was also prepared.

#### Preparation of Activators

On the day of the experiment, a known concentration of bovine thrombin (Sigma-Aldrich®, St. Louis, MO) was thawed on ice and diluted with sterile water to produce a concentration of 125 U/mL, which was passed through a 0.2-μm filter. This solution was kept on ice until use. For joint injection, 0.2 mL of thrombin was added to 2.3 mL of PRP to produce a final thrombin concentration of 10 U/mL. A solution of calcium chloride (CaCl₂) also was prepared (42.5 mg/mL), similarly filtered, and kept on ice until use. For injection, 0.2 mL CaCl₂ was added to 2.3 mL of PRP to produce a final CaCl₂ concentration of 3.4 mg/mL (23 mM).

#### Treatment, Sampling, and Clinical Evaluation

One investigator performed all evaluations, arthrocenteses, and platelet preparations. Data were collected at 5 time points: T = 0 (just before injection), 6, 24, 48, and 96 hours post-injection. At each time point, the horse was examined and temperature, pulse, and respiration rate were recorded (normal reference intervals were considered 98.5–101.5°F, 28–44 beats/min, and 8–24 breaths/min, respectively). Blood was collected by jugular venipuncture for complete blood count, fibrinogen, blood urea nitrogen (BUN) measurement,
and PRPr preparation. Each fetlock was evaluated for effusion (scored 0–3: 0—absent, 1—mild, 2—moderate, 3—severe), response to flexion at rest (scored similarly, 0–3), and presence of peri-articular signs such as palpable swelling or heat (yes or no). The horse was then sedated with xylazine (0.05–1 mg/kg intravenously [IV]) or detomidine (0.008–0.02 mg/kg IV) as required. All fetlocks of each horse were used concurrently: 1 of 4 treatments was assigned by a random number generator for each leg and the investigator was blinded to treatment until study end. The 4 treatments were: resting PRPr (Group R), CaCl₂-activated PRPr (Group Ca), thrombin-activated PRPr (Group T), and a 2% saline solution control (Group S). For the resting treatment, a placebo activator (0.9% saline solution) was used to ensure blinding of the study. Arthrocentesis sites were aseptically prepared and each metacarpo/metatarsophalangeal joint was sampled using sterile technique. A 2-mL sample of synovial fluid was aspirated from each fetlock using a 21-gauge, 1.5”-needle, and 3-mL syringe, by inserting the needle through either the lateral or medial collateral sesamoidean ligament. As the sample was obtained, an assistant was instructed to mix the activator with the PRPr. With the needle in position, the sample syringe was passed to the assistant and exchanged for the treatment syringe, of which 2 mL was injected into the joint. The remaining 0.5 mL was retained for growth factor analysis. A light bandage was applied to each leg after arthrocentesis. Synovial sample sites were alternated between the lateral and medial collateral sesamoidean ligaments to avoid local effects of repeated sampling.

Sample Handling and Analysis

Synovial fluid was promptly transferred to a standard EDTA tube, a plain serum tube, and a plain polypropylene tube for cytologic assessment, BUN quantification, and platelet evaluation, respectively. Blood and synovial fluid samples were kept on ice in a Styrofoam cooler and were processed at the laboratory within 2 hours of collection. An aliquot of PRPr (without activator) was placed in a microcentrifuge tube and kept warm by placement in a Styrofoam container (to prevent platelet activation) until transfer to the laboratory. Complete cell counts of whole blood and synovial fluid were performed on an automated hematology analyzer (ADVIA® 120, Siemens Healthcare Diagnostics, Inc., Tarrytown, NY). Complete cell counts on PRPr were also performed on an automated hematology analyzer (COULTER® AcT Diff™ Hematology Analyzer, Beckman Coulter™, Miami, FL). PDGF and TGFβ concentrations in each treatment were determined by use of ELISAs (Human PDGF-BB and Human TGF-β1 Quantikine ELISAs, R&D Systems, Minneapolis, MN). Systemic fibrinogen levels were determined by heat precipitation. Before BUN or cytologic analysis, synovial fluid samples underwent hyaluronidase (from ovine testes, catalog number: 38594, Calbiochem, EMD Millipore, Billericia, MA) digestion (final concentration = 50 U/mL). BUN was measured using an automated chemistry analyzer (Cobas® c501, Roche Diagnostics Corp., Indianapolis, IN).

Synovial fluid slides were stained with hematoxylin and eosin. Microscopic evaluation of platelet number was performed by one author who was blinded to the treatment or time point for each slide. Platelets were counted in 20 fields at 60× magnification on a high-resolution microscope (Olympus BX61, Olympus America, Center Valley, PA). The criteria for identification of platelets were: discrete a nuclear cells that were pale and basophilic, smaller than erythrocytes, ovoid or irregular in shape, and containing either granules or discrete unstained spaces when granules were not present.

Synovial Fluid Cytology

To determine whether blood contamination of the synovial sample occurred during arthrocentesis, the percent blood
contamination was calculated as follows: synovial RBC/systemic RBC × 100%. Because statistically significant differences in data may not reflect differences of clinical consequence, clinically relevant values (i.e., those considered to suggest active inflammation within a joint) were determined \textit{a priori} as >5000/µL WBC, >30% neutrophils, and >2.5 g/dL total protein, based upon the lead author’s clinical experience. Strictly normal synovial fluid values are conventionally considered <1000 WBC/µL, <10% neutrophils, and <2.5 g/dL total protein.\textsuperscript{16}

\textbf{Statistical Analysis}

The cellular composition of E-PET\textsuperscript{TM} PRPr is presented using descriptive statistics. Initial mixed model analyses were run on all other datasets, to assess whether there was an effect of limb assignment on the outcome variables. Since none were detected, subsequent analyses were run irrespective of which joint received which treatment. They were then assessed for residual normality using Wilk-Shapiro tests; data were log transformed to achieve normality if necessary. If raw or log data were normally distributed, then repeated measures analyses were used. Otherwise, non-parametric testing was used. Statistical significance was set at \( P < .05 \). PDGF-BB and TGF-\( \beta \) contents of resting versus CaCl\(_2\)-activated versus thrombin-activated PRPr were compared by 1-way ANOVA, with Tukey’s HSD test post hoc. Platelet numbers in synovial fluid were analyzed by a Kruskal–Wallis test with Dunn’s post hoc analysis. Analyses were performed using software (Prism 5 for Mac OS X, Version 5.0d, GraphPad Software, San Diego, CA) and SAS, version 9.3 (SAS Institute, Cary, NC; copyright 2002–2010).

\textbf{RESULTS}

\textbf{Platelet-Rich Product Preparation.} The E-PET\textsuperscript{TM} system was easy to use for rapid, stall-side preparation of a PRPr. Preparation time was \text~10 minutes for each horse. Approximately 7.5-mL PRPr were retrieved for each horse. In 1 of 7 horses, partial platelet activation occurred during the preparation process as indicated by high growth factor concentrations in the resting sample, but further growth factor release occurred after exogenous activators (CaCl\(_2\) or thrombin) were added.

\textbf{Cellular Content}

Platelets were successfully concentrated by the E-PET\textsuperscript{TM} system (data in Fig 2). Mean (SD) platelet concentration in PRPr was 542 [196.3] $\times 10^3$/µL (range: 313–833 $\times 10^3$/µL), which represented a 3.2-fold increase over the mean systemic platelet concentration. Mean (SD) WBC concentration in the E-PET\textsuperscript{TM} product was 13.1 [3.46] $\times 10^3$/µL (range: 8.8–17.9 $\times 10^3$/µL), which represented a 1.9-fold increase over the systemic WBC concentration.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure2.png}
\caption{Composition of the platelet-rich product produced by gravity filtration. Platelets and WBC are $\times 10^3$/µL; RBC are $\times 10^6$/µL. Hgb is in g/dL. Inset: Whole blood values are displayed for reference.}
\end{figure}

\textbf{Growth Factor Content}

PDGF was significantly different between PRPr treatment groups; all treatment groups were significantly greater than the control (S; \( P < .001 \); Fig 3A). PDGF concentrations in the activated PRPr groups (Ca and T) were both significantly greater than in resting (R) PRPr (\( P < .001 \)). For TGFB, all treatment groups were again significantly greater than the saline control (\( P < .001 \)). Group T content was also significantly greater than Group R (\( P < .05 \)).

\textbf{Clinical Variables}

\textbf{Vital Signs.} Body temperature was slightly elevated at 6 and 24 hours, though still within the normal range (mean [SD]: baseline = 98.7°F [0.86]; 6 hours = 100.0°F [0.78]; 24 hours = 99.3°F [0.75]; \( P = .0001 \) and .0354, respectively). Heart rate and respiratory rate were elevated and were significantly different from baseline at 6 hours (baseline = 39 [6.7] and 20 [4.6] beats/min, respectively; 6 hours = 51 [13.4] and 29 [8.2] beats/min; \( P = .0009 \) and .0041, respectively).

\textbf{Synovial Effusion Scores.} Before intra-articular injection, effusion scores of the fetlocks of each treatment group were not significantly different; the overall mean (SD) of effusion scores was 0.3 (0.52). Mean (SD) effusion scores across all time points were 0.6 (0.66), 0.7 (0.73), 0.7 (0.68), and 1.4 (1.18) for S, R, C, and T groups, respectively. Throughout the 96-hour experiment, effusion scores after thrombin-activated PRPr
injection were significantly greater than at Time 0 ($P < .0079$ for all time points compared to baseline). Effusion scores for Groups R, Ca, and S were significantly increased at 6 hours only ($P < .001$). Thrombin-activated PRPr injection resulted in effusion which was significantly greater than the saline control ($P = .0011, .0001, and .0402$ at 6, 24, and 96 hours, respectively) and also Groups R and Ca at 6, 24, and 96 hours ($P < .0176, .0008, and .0403$, respectively). On 7 occasions after intra-articular treatment, periarticular swelling of the limb prevented the assessment of effusion (6 in Group T and 1 in Group R).

**Flexion Scores.** No differences were detected in baseline flexion scores between groups; overall mean (SD) was 0.2 (0.48). For Groups R and Ca, flexion scores were not significantly different from the saline control at any time point after treatment (across all time points, mean [SD] = 0.3 [0.49], 0.4 [0.54], and 0.5 [0.91] for R, Ca, and S, respectively). Horses were painful on flexion after injection with thrombin-activated PRPr; flexion scores for Group T were significantly greater than baseline ($P < .0001$) and also greater than all other groups ($P < .0003$) for the duration of the experiment (overall mean [SD] Group T flexion score = 1.7 [1.10]). At 6 hours, 5 of 7 horses were so lame in the thrombin-PRPr limb that they would not bear weight on it to allow flexion of the contralateral limb.

**Periarticular Effects.** At the start of the experiment, no horse had palpable heat or swelling of any fetlock. No instances of periarticular signs were observed at any time point after intra-articular treatment with saline solution. Heat and/or swelling were observed for Group R on 12 occasions out of 28 observations; these findings occurred in 4 horses. For Group Ca, periarticular effects were noted on only 3 of 28 observations, occurring in 2 horses. For Group T, all horses had periarticular symptoms at all time points, with the exception of a single observation at 6 hours. All clinical data are displayed in Fig 4.
**Hematology**

All hematology values remained within the normal reference intervals for our laboratory. Some statistically significant differences were observed, however: mean (SD) hematocrit was 33.9 (2.04)% at baseline and 30.7 (2.31)% at 6 hours ($P = .0439$). WBC concentration increased significantly at 24 hours (mean [SD] = 8913 [2315.0] $\times 10^3/\mu$L) in comparison to baseline (6869 [1749.0] $\times 10^3/\mu$L; $P = .0279$). The differential proportion of peripheral blood neutrophils also increased significantly at 24 hours (from 59 [6.6]% to 75 [9.4]%, $P = .0106$). Systemic fibrinogen concentration increased significantly by 48 hours and remained significantly elevated at 96 hours: baseline, 243 (53.5) mg/dL; 48 hours, 371 (95.1) mg/dL; 96 hours, 357 (161.8) mg/dL ($P = .0066$ and .0342, respectively).

**Synovial Fluid**

Synovial fluid samples could not be obtained on 8 occasions, which were fairly evenly distributed among treatment groups and observation times (1 sample each for S, R, Ca, T at Time 0; 1 for Ca at 6 hours, 1 for Ca at 96 hours, and 1 for R at 24 hours) and did not affect statistical analysis. Sampling was successful in 132/140 attempts (94% of the time). Blood contamination was minimal, with a mean (SD) of 2.2 (3.73)%.

Successful in 132/140 attempts (94% of the time). Blood contamination was minimal, with a mean (SD) of 2.2 (3.73)%. Platelets were observed in greater number in synovial fluid after intra-articular PRPr injection (Fig 6A).
found between subsequent time points and baseline (mean [SD] = 4 [1.5], 7 [5.7], 10 [1.7], 8 [7.6], 10 [1.7] × 10⁴ platelets/μL at 0, 6, 24, 48, and 96 hours, respectively). Significant differences between PRPr groups were apparent at 6 hours, at which point more platelets were seen in Group T than in Groups Ca (P < .05) and S (P < .001). Platelet aggregates were observed with surprising rarity, on only 3 occasions in the 136 slides evaluated.

**DISCUSSION**

Joint disease is widespread in horses and encompasses a spectrum of pathologies, ranging from relatively minor insults (e.g., synovitis, small osteochondral lesions) to more debilitating conditions (e.g., intra-articular fractures, severe osteoarthritis). Over the past 20 years, corticosteroids and hyaluronic acid have been the mainstays of intra-articular medication in horses; the intra-articular use of pentosan polysulfate has also been recently reported. All of these treatments are intended to achieve anti-inflammatory and chondroprotective effects, thereby mitigating the impact of traumatic and inflammatory arthritides. However, because of the minimal intrinsic repair capability of joints, these drugs may require frequent dosing to maintain their therapeutic effect or may be insufficient to restore functional joint health in many patients. As a result, the search for better intra-articular therapies is ongoing in horses as well as in other species. Recently, treatment by intra-articular injection of biological therapies has been described, including autologous conditioned serum, stem cells, and PRP products (Hammock PD, Meyer ST, Simpson T: personal communications 2011–2012). We described the response of normal fetlocks to injection of 3 different preparations of a PRPr, in order to define the baseline clinical and synovial cytologic responses that can be expected after intra-articular (IA) use of these products in horses.

Our results indicate that in normal joints, intra-articular PRPr induces a transient but significant cellular influx into the synovial fluid, including a prominent neutrophilia. A control limb was injected with saline and had significant increases in synovial leukocyte concentration, resulting from the process of fluid injection and/or serial arthrocenteses alone. Saline injection did not change effusion scores, flexion scores, paretic signs, or platelet concentration in comparison with baseline samples, but these variables did change after certain PRPr treatments. Compared to the saline control, injection of all PRPr treatments resulted in a statistically significant increase in total cell count and neutrophil percentage in synovial fluid for at least 24 hours. This effect was significantly greater in thrombin-PRPr-injected joints than in resting-PRPr or CaCl₂-PRPr-treated joints, and was accompanied by effusion, pain on flexion, reluctance to bear weight when the contralateral limb was elevated, and paretic heat and swelling in the thrombin-PRPr-injected limb. These clinical signs were worst at 6 hours post-injection and were accompanied by statistically significant increases in temperature, pulse, and respiration. Although these signs diminished each day,
they were not yet resolved 5 days after injection. Analysis of the 3 PRPr preparations indicated that thrombin activation resulted in the highest concentrations of PDGF and TGFβ, but because of the adverse reactions we observed, bovine thrombin as used in our study cannot be recommended for PRP treatment by intra-articular injection. It is possible that the response to bovine thrombin-activated PRP could be mitigated by anti-inflammatory therapy, or perhaps avoided by use of a lower thrombin concentration. In contrast, both resting and CaCl2-activated PRP appear safe for intra-articular use in horses as tested in this study.

We used a proprietary gravity filtration system to prepare the platelet-rich saline product. The process was rapid and self-contained, without the requirement for a centrifuge or laboratory facility. Platelet concentrating ability varied and the platelet concentrations we achieved were less than those reported by other investigators using this same system. Inter- and intra-donor variation is recognized as a major determinant of platelet concentrating ability for most PRP preparation systems and has been investigated for the E-PET™ system as well (Guercio V, Director of Cell Therapy, Pall Medical: personal communication 2011). We are unaware of any studies that directly compare a platelet-rich saline product to PRP, but some differences may exist. It is reasonable to assume that the relative paucity of plasma proteins would make the E-PET™ product less useful as a “provisional matrix” than true PRP, which, when activated, becomes a platelet-rich fibrin graft. Nonetheless, the product certainly does clot upon activation, indicating the presence of adequate fibrinogen to form a firm gel. In terms of volume, the dose of PRPr per joint (2.3 mL) was arbitrarily selected based on our anticipated ability to recover ~7.5 mL PRPr, less ~0.5 mL reserved for analyses, meaning that ~7 mL remained for the treatment of 3 joints. (By way of comparison, the volume of PRP injected into 1 osteoarthritic knee in people is 5–8 mL.) The concentration of thrombin (10 U/mL) was selected based on a range of concentrations reported in the literature, and the results of a recent in vitro study that indicated significantly greater PDGF release after activation with 10 U/mL when compared to 1 U/mL, and no significant difference in TGFβ output between 10 versus 143 U/mL (both of which were >1 U/mL). We were also concerned about the intra-articular effects of thrombin, a potent serine protease, if used at the high end of the dose range (i.e., 143 U/mL). For these reasons, 10 U/mL was selected as a reasonable intermediate concentration. The originally described thrombin concentration for PRP activation in oral surgery was 143 U/mL, and concentrations as high as 100 U/mL have been reported for intra-articular use in people. In horses, thrombin has been reported at final concentrations ranging from 1.7 U/mL to 20 U/mL as an activator for PRP. The CaCl2 concentration we used (23 mM) has been widely reported in the human literature and was also investigated in the same recent in vitro study. To evaluate whether any dilution of synovial fluid had occurred as part of a transudative process, BUN concentrations were evaluated in each synovial fluid sample and compared to the serum BUN. The normal ratio of synovial: serum BUN is 1:1, and measurement of this ratio can be used to assess synovial fluid volume. Had the ratio of synovial: serum BUN decreased after injection of PRPr, it would have indicated a dilutional effect on the variables we measured, and would have required that these values be adjusted for differences in volume. Since the normal ratio of 1:1 was maintained for all samples, we verified that all synovial fluid variables were directly comparable to baseline values.

Platelets are known to contain multiple growth factors in their alpha granules. We selected PDGF and TGFβ for analysis because they are involved in every stage of tissue healing and are the most widely studied growth factors in the PRP literature. In addition, PRP is often combined with mesenchymal stem cells (MSCs) in clinical applications and PDGF and TGFβ have been identified as 2 of the 3 factors crucial to MSC differentiation. The ELISAs used in this study are designed for detection of human PDGF and TGFβ, but are considered valid for equine samples. One additional technical note relates to the ambient temperature when using the E-PET™ preparation system. The main advantage of this system is that it literally allows for PRPr preparation in the barn, but it must be borne in mind that platelets are temperature-sensitive. The average ambient temperature during this study was 71°F. When we attempted to use the system on a cold day (35°F—after the completion of this study), no platelets were present in the final product. We suspect this was an effect of temperature, and that the platelets were activated by the cold and therefore not retrievable from the filter. The system could presumably still be used on a cold day, but we recommend doing so in a heated room (i.e., at least 68°F) and using blood that has been kept warm before processing. Platelet counts should be performed on a sample of PRP or PRPr whenever possible, to ensure that the patient receives the intended therapeutic product. Many authors believe that higher platelet concentrations are more therapeutically desirable.

Thrombin is a potent serine protease most notable for its cleavage of fibrinogen into fibrin. It is also the most potent naturally occurring agonist for platelet activation, and is produced on the platelet surface after the “tenase” complex (coagulation factors VIIIa and IXa) activates Factor X, which in turn converts prothrombin to thrombin. Interestingly, bovine thrombin is widely used in PRP treatment in people with few reported adverse effects. Subclinical immunologic responses to bovine thrombin are well-documented and relatively frequent in people. We can suggest 3 possible explanations for the adverse thrombin reactions we observed: (1) an immunologic response (i.e., delayed-type hypersensitivity) to a xenogeneic protein; (2) direct proteolytic effects on the joint by residual active thrombin; and (3) a primary inflammatory response to platelet contents released en masse by thrombin activation, such as the pro-inflammatory chemokines IL-1 and serotonin or even the pleiotropic and sometimes pro-inflammatory growth factor, TGFβ. Bovine thrombin has been used in horses previously without reported adverse effect, thus we suspect explanations 2 and 3 are more likely. In the report by Ortved et al., bovine thrombin use was intra-articular and applied at a higher concentration than that reported here. However, in that study the thrombin...
was applied surgically in a confined lesion site, under gas arthroscopy, and the clotting process was allowed to progress to completion before reintroduction of fluid. This is in contrast to injection of thrombin and PRP into a fluid-filled space immediately after mixing, as we performed. The PRP product we used does not contain plasma, and is instead a suspension of concentrated platelets in saline. Plasma contains antithrombin substances (Antithrombin III, heparin, heparin sulfate)\(^6\) that may limit the adverse effects of thrombin in instances of true PRP. Autologous thrombin has been investigated as an alternative to bovine thrombin use in horses,\(^32\) but was found to be wholly inferior to bovine thrombin and CaCl\(_2\) (23 mM) in terms of growth factor release.

The investigation of any new biomedical therapeutic agent (i.e., drug, surgical technique, device) traditionally begins with an evaluation of its effects on a normal biological system. This conventional approach establishes the baseline physiologic response that can be expected in the patient, and also determines the safety of the intended treatment. Although these methods are logical, it may be difficult to extrapolate the response of a healthy system to one of disease. This is especially true when the treatment product contains the patient’s own cells, which may be pleiotropic in their response. Platelets behave differently according to the tissue and cytokine environment to which they are exposed, and are now recognized to perform a host of physiologic functions in addition to their primary hemostatic role.\(^23,70\) These diverse capabilities of platelets are partially the result of differential release of alpha granule subpopulations.\(^71\) Alpha granules contain over 300 proteins,\(^72\) many of which have distinctly opposite actions\(^23\) and are released under different circumstances.\(^35\) In a recent paper on the response of normal tissue to PRP injection,\(^11\) inflammatory responses were observed in all treated tissues and lasted 2–6 weeks after injection. Our results also suggest an inflammatory response to intra-articular platelet-rich injection in normal joints. This finding is not particularly surprising, since synovial fluid is normally of very low cellularity and since whole blood is known to elicit an inflammatory response in joints.\(^74,75\)

A few limitations of the study should be discussed. To minimize the number of animals and costs associated with these experiments, the study was conducted by a repeated measures design in which 4 treatments were applied simultaneously, in different limbs. The repeated measures format allows a robust form of ANOVA data analysis while minimizing the confounding effect of inter-individual variation. However, the use of all 4 limbs concurrently meant that lameness could not be accurately assessed as an outcome variable. Hemogram changes (transient increase in leukocyte number and neutrophil percentage) indicated that the injection of all 4 limbs did induce a mild systemic inflammatory response in our horses, although all variables remained within normal reference intervals. Ideally, we would also have tested the intra-articular effects of the PRPr activators alone (CaCl\(_2\) and bovine thrombin) but were constrained by our available funds and the number of available limbs per horse. We are therefore unable to differentiate whether the observed responses to thrombin-PRPr injection were because of the thrombin itself or because of thrombin-induced changes in the PRPr. Financial constraints also prevented radiographic examination of each fetlock before study inclusion. To maintain a blinded approach to treatment, activators were added immediately before joint injection, but in most clinical and \textit{in vitro} reports, CaCl\(_2\) is allowed to incubate with PRP for 30–60 minutes before use. Nonetheless, a recent clinical study in people also applied PRP immediately after mixing with CaCl\(_2\),\(^9\) and reported a significant therapeutic effect which lasted at least 6 months. Lastly, we evaluated platelet concentration in synovial fluid after injection and found increased numbers of platelets for up to 5 days after PRPr injection. Although these platelets are likely to represent PRPr-origin platelets, we did not label the PRPr platelets and therefore cannot be certain of their origin. An alternative explanation for the observed increase is that platelets entered the joint from the systemic circulation as a component of the inflammatory responses we observed after PRPr injection.

Our hypotheses were confirmed: intra-articular effects differed between different types of a PRPr, and the E-PET™ system did reliably concentrate platelets. Based on a 5-day evaluation period, resting or CaCl\(_2\)-activated PRP appears to be safe for IA injection in horses. Bovine thrombin use, as described here, is not recommended for activation of PRP as an intra-articular injection, but it is possible that the observed inflammatory response may be manageable with lower concentrations of thrombin or with concurrent non-steroidal anti-inflammatory therapy. In normal joints, intra-articular PRPr induces a mild to moderate inflammatory response in synovial fluid, which lasts \(~1\) day. Increased numbers of platelets can be recovered from synovial fluid for up to 5 days after intra-articular injection of a platelet-rich saline product, which may have implications for the kinetics of growth factor release \textit{in vivo}. Future research is needed to assess the response of diseased joints to intra-articular PRP injection, the levels of platelet-derived growth factors and cytokines in synovial fluid and tissues after intra-articular PRP injection, and also the activation status, lifespan, and clearance of injected platelets. In addition, the appropriate clinical use of resting versus activated PRP remains to be determined, and may differ according to the specific lesion type or site.

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**DISCLOSURE**

The authors received several E-PET™ preparation kits free of charge from the manufacturer, Pall Biomedical, but declare no other conflicts of interest related to this product, nor are
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