Evaluation of two platelet-rich plasma processing methods and two platelet-activation techniques for use in llamas and alpacas

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OBJECTIVE
To evaluate 2 processing methods (commercial kit vs conical tube centrifugation) for preparing platelet rich plasma (PRP) for use in llamas and alpacas.

SAMPLES
Blood samples (30 mL each) aseptically collected from 6 healthy llamas and 6 healthy alpacas.

PROCEDURES
PRP was prepared from blood samples by use of a commercial kit and by double-step conical tube centrifugation. A CBC was performed for blood and PRP samples. Platelets in PRP samples were activated by means of a freeze-thaw method with or without 23mM CaCl₂, and concentrations of platelet-derived growth factor-BB and transforming growth factor-β₁ were measured. Values were compared between processing methods and camelid species.

RESULTS
Blood CBC values for llamas and alpacas were similar. The commercial kit yielded a significantly greater degree of platelet enrichment (mean increase, 8.5 fold vs 2.8 fold) and WBC enrichment (mean increase, 3.7 fold vs 1.9 fold) than did conical tube centrifugation. Llamas had a significantly greater degree of platelet enrichment than alpacas by either processing method. No difference in WBC enrichment was identified between species. Concentrations of both growth factors were significantly greater in PRP samples obtained by use of the commercial kit versus those obtained by conical tube centrifugation.

CONCLUSIONS AND CLINICAL RELEVANCE
For blood samples from camelids, the commercial kit yielded a PRP product with a higher platelet and WBC concentration than achieved by conical tube centrifugation. Optimal PRP platelet and WBC concentrations for various applications need to be determined for llamas and alpacas. (Am J Vet Res 2016;77:1288–1294)

Autologous PRP is a biological product that has become an important treatment to promote tissue healing, particularly for tendon, ligament, and joint injuries. The main advantage of autologous PRP preparations is that they involve simple blood collection from a patient, followed by on-site centrifugation and extraction of the PRP fraction. This can usually be accomplished within a short period, allowing outpatient treatment. Therapeutic benefits have been demonstrated in horses, humans, and research animals, particularly with respect to tendon injuries, but also for joint and bone injuries after intralesional PRP injection. Therapeutic effects are derived from growth factors and other cell mediators released from the alpha granules during degranulation caused by platelet activation. Of these cell mediators, PDGF and TGF-β₁ are present in the highest concentrations and are the mediators most commonly measured in studies.

Llamas and alpacas have many pathological conditions for which they could derive benefit from PRP administration, including periarticular and intra-articular ligament injuries, meniscal tears, suspensory ligament desmitis, and osteoarthritis. Treatment for these conditions has traditionally included surgical stabilization (eg, extracapsular tension sutures for joint ligament ruptures), rest, and palliative medical management. Platelet-rich plasma may also be used as an adjunctive or primary treatment, and this would be advantageous from an economic perspective, particularly when surgical intervention is not an option.

Processing methods have a considerable effect on the quality of the PRP product, including differ-
ences in platelet, WBC, and RBC counts as well as growth factor concentrations and release. Several studies have been conducted to evaluate PRP processing methods for horses, dogs, and humans; however, no research has been conducted to determine the optimal processing method for PRP preparation for llamas and alpacas.

Previous reports of hematologic findings in camelids have indicated several differences from other species. Camelids have higher leukocyte counts (reference limits, 8,000 to 22,000 cells/µL) than horses, cattle, and sheep. In addition, camelid erythrocytes have a small, elliptical, and flattened shape and are present in higher numbers, compared with erythrocytes of other species. Camelid platelets are also smaller in volume and have a higher mean blood count (475,000 platelets/µL) than human (200,000 platelets/µL) and equine (142,000 platelets/µL) platelets. Because of these hematologic differences, it is important to evaluate PRP processing for llamas and alpacas specifically rather than to merely presume that processing methods reported for other species will be equivalent and suitable for camelids.

The objective of the study reported here was to evaluate 2 methods for PRP preparation for llamas and alpacas. We hypothesized that PRP preparation techniques used for horses would provide similar results when used for llamas and alpacas. We further hypothesized that the PRP product obtained by use of a commercial kit would yield superior platelet counts, lower WBC counts, and higher concentrations of growth factors than would a conical tube centrifugation technique.

Materials and Methods

Animals

Twelve healthy adult camelids (6 llamas and 6 alpacas) from the veterinary teaching herd at Oregon State University were used in the study. Llamas included 4 females and 2 gelded males, with a mean age of 5.5 years (range, 2 to 14 years). Alpacas included 3 females and 3 gelded males, with a mean age of 9.6 years (range, 6 to 17 years). The study protocol was approved by the Institutional Animal Care and Use Committee of Oregon State University.

Blood sample collection

Blood samples were aseptically collected from the right jugular vein of each camelid into 2 sterile 35-mL syringes containing 1 part acid citrate dextrose-A anticoagulant to 9 parts of blood (total volume collected, 70 mL). A 5-mL sample of blood was also drawn into EDTA collection tubes for performance of a CBC. Samples were gently rocked following collection to ensure proper mixing.

PRP processing

Thirty milliliters of citrated blood from each camelid was transferred into the blood chamber of a commercial kit per the manufacturer’s guidelines, and another 30 mL was transferred into a sterile polypropylene 50-mL conical centrifuge tube. The commercial kit method was performed in accordance with the manufacturer’s guidelines (centrifugation once for 14 minutes at a maximum force of 1,000 X g), resulting in retrieval of 3 mL of PRP from 30 mL of venous blood. The conical tube method involved centrifugation of blood samples for 15 minutes at 720 X g, as described elsewhere. Because the initial conical tube centrifugation resulted in poor separation of plasma and RBCs, a second centrifugation step was added. In this second step, the plasma and buffy coat layers were removed by use of a sterile pipette and transferred into a second sterile 50-mL conical tube. This second tube was centrifuged for 15 minutes at 720 X g, resulting in good separation of plasma and RBCs. The clear platelet-poor plasma fraction was then removed, leaving approximately 3 mL of plasma remaining above the buffy coat layer (PRP). Three milliliters of PRP was then harvested by use of a 6-mL sterile syringe and 18-gauge blunt-end needle.

Blood samples and 1 mL of each PRP sample from each camelid were submitted to the Veterinary Diagnostic Laboratory at Oregon State University for automated and manual CBCs. Platelets in the remaining 2 mL of each PRP sample were then activated by means of a freeze-thaw method, as described elsewhere, by freezing at −20°C until assays were performed. Frozen PRP samples were thawed at 37°C and centrifuged at 18,000 X g for 10 minutes. The resulting supernatants were frozen and stored at −80°C until growth factor assays were performed. Platelets in a portion of freeze-thawed PRP were additionally activated with 23 mM CaCl₂ (12 µL added to 510 µL of sample) for 30 minutes at 37°C, followed by centrifugation, harvesting of supernatant, and storage of supernatants as previously described.

Growth factor concentrations in PRP samples were measured by use of an ELISA kit for measurement of PDGF-BB and TGF-β, in accordance with the manufacturer’s guidelines. Validation of the ELISA was performed separately for llama and alpaca samples. Spike and recovery results and dilution linearity percentages were within the required range of 80% to 120%. In addition, standard curves obtained from analysis of diluted llama and alpaca platelet-poor plasma matrices were directly compared with standard curves obtained from analysis of kit diluents. An acid activation step was performed for the TGF-β1 ELISA. For TGF-β1 analysis, PRP samples were diluted to 1:100 and 1:50 for llama and alpaca samples, respectively. All PRP samples were diluted to 1:10 for PDGF-BB analysis.

Statistical analysis

The paired t test was used to compare normally distributed data (PCV, WBC, neutrophil, lymphocytes, and eosinophil data) between PRP methods, and the Wilcoxon signed rank test was used to com-
pare nonnormally distributed data (platelet, monocytes, and basophil data). The Mann-Whitney U test was used for fold-change comparisons of platelet and WBC values between llamas and alpacas. Values of $P < 0.05$ were considered significant.

**Results**

Baseline CBC values from analysis of blood samples were similar between llamas and alpacas, with combined mean counts of 313,330 platelets/µL and 11,745 WBCs/µL (Table 1). The commercial kit yielded a significantly ($P = 0.005$) greater degree of platelet enrichment for all camelids (combined mean for llamas and alpacas, 8.5-fold increase) than did double-step conical tube centrifugation (combined mean, 2.8-fold increase). Llamas had a significantly ($P < 0.05$) greater degree of platelet enrichment than did alpacas by either method. The commercial kit yielded a significantly ($P = 0.002$) greater degree of total WBC

| Analyte Baseline Commercial kit Conical tube $P$ value* | Platelets ($X 10^3$ platelets/µL) | Llamas 267 ± 142 2,340 ± 784 928 ± 474 — | Alpacas 359 ± 69 2,179 ± 396 723 ± 335 — | All camelids 313 ± 117 2,260 ± 598 826 ± 416 — |
| | Fold change from baseline for all camelids | — 8.5 ± 4.4 2.8 ± 1.2 0.003 |
| | WBCs ($X 10^3$ cells/µL) | Llamas 12.6 ± 2.6 39.8 ± 16.0 22.3 ± 6.8 — | Alpacas 10.9 ± 1.6 44.0 ± 18.0 20.9 ± 11.1 — | All camelids 11.7 ± 2.2 41.9 ± 16.4 21.6 ± 8.8 — |
| | Fold change from baseline for all camelids | — 3.7 ± 1.5 1.9 ± 0.8 0.002 |
| | PCV (%) | Llamas 29.5 ± 3.1 3.2 ± 2.7 1.8 ± 1.2 — | Alpacas 30.8 ± 6.9 6.2 ± 4.7 3.7 ± 2.0 — | All camelids 30.2 ± 5.2 4.7 ± 4.0 2.7 ± 1.8 — |
| | Percentage of all WBCs for all camelids | — 0.14 ± 0.11 0.09 ± 0.06 0.09 |
| | Neutrophils (cells/µL) | Llamas 9,073 ± 2,630 22,024 ± 10,116 14,628 ± 4,605 — | Alpacas 6,902 ± 1,273 25,851 ± 10,727 14,024 ± 8,176 — | All camelids 7,988 ± 2,273 23,938 ± 10,140 14,326 ± 6,335 — |
| | Percentage of all WBCs for all camelids | 68 57 66 0.007 |
| | Lymphocytes (cells/µL) | Llamas 1,913 ± 689 10,609 ± 6,165 3,976 ± 1,247 — | Alpacas 1,787 ± 908 8,258 ± 4,634 2,897 ± 1,181 — | All camelids 1,850 ± 771 9,434 ± 5,343 3,437 ± 1,288 — |
| | Percentage of all WBCs for all camelids | 68 57 66 0.007 |
| | Monocytes (cells/µL) | Llamas 207 ± 193 1,322 ± 1,152 596 ± 677 — | Alpacas 324 ± 224 521 ± 497 91 ± 143 — | All camelids 266 ± 209 921 ± 944 344 ± 536 — |
| | Percentage of all WBCs for all camelids | 2 2 2 0.20 |
| | Eosinophils (cells/µL) | Llamas 1,364 ± 345 5,585 ± 4,049 3,064 ± 1,825 — | Alpacas 1,805 ± 1,131 9,024 ± 6,263 3,796 ± 3,137 — | All camelids 1,585 ± 830 7,304 ± 5,339 3,430 ± 2,476 — |
| | Percentage of all WBCs for all camelids | 16 23 16 0.002 |
| | Basophils (cells/µL) | Llamas 39 ± 60 230 ± 366 0 ± 0 — | Alpacas 74 ± 117 341 ± 390 102 ± 251 — | All camelids 57 ± 91 286 ± 365 51 ± 177 — |
| | Percentage of all WBCs for all camelids | 0.5 0.7 0.2 0.29 |

*Values represent comparisons between processing methods. Values of $P < 0.05$ were considered significant.

— = Not applicable.
enrichment for all camelids (combined mean, 3.7-fold increase) than did conical tube centrifugation (combined mean, 1.9-fold increase), but no significant ($P > 0.60$) species differences were identified.

Differential WBC counts revealed significantly greater numbers of neutrophils, lymphocytes, and eosinophils in PRP samples yielded by the commercial kit, compared with counts yielded by conical tube centrifugation. No significant ($P = 0.09$) difference between PRP preparation methods was identified for PCV.

Concentrations of PDGF-BB and TGF-$\beta_1$ in PRP samples following freeze-thaw platelet activation were significantly greater in samples obtained by use of the commercial kit than in those obtained by conical tube centrifugation (Figure 1; Table 2). However, when concentrations of these growth factors were normalized by platelet counts, no differences were identified. Also, no significant differences were identified between llamas and alpacas for either growth factor. Additional activation of platelets by use of $23\text{mM } \text{CaCl}_2$ following the freeze-thaw cycle resulted in clot formation and a significant ($P < 0.001$) decrease in supernatant PDGF-BB concentration, compared with the PDGF-BB concentration achieved with freeze-thaw activation alone (Figure 2). There was no significant ($P = 0.35$) effect of the added $\text{CaCl}_2$ step on supernatant TGF-$\beta_1$ concentration, compared with the concentration achieved with freeze-thaw activation alone.

**Discussion**

Results of the present study indicated that use of a commercial kit to prepare PRP from blood samples from camelids yielded a greater degree of platelet and total WBC enrichment than did use of the double-step method for conical tube centrifugation. Single-step centrifugation failed to adequately separate RBCs in camelid blood samples, contrary to a report of its use for horses. Camelids also had greater blood and PRP platelet and WBC counts than are typical for horses or humans.

Single- and double-step tube centrifugation methods for PRP preparation have been used successfully for horses and dogs. However, in the study reported here, continued suspension of RBCs in the plasma portion of PRP samples following single-step centrifugation resulted in the need for a second centrifugation step. The smaller cell volume and higher numbers of erythrocytes in blood samples from camelids versus other species may have caused the initial inadequate erythrocyte separation.

The observed difference between llamas and alpacas in platelet concentrations in PRP samples in the present study was unexpected. This finding is difficult to explain, particularly given that CBC values for blood samples were similar. For 2 of the 6 llamas used, platelet clumping was noticed when the blood CBC was performed, which may have resulted in

![Figure 1](image_url)  
**Figure 1**—Mean concentrations of PDGF-BB and TGF-$\beta_1$ in PRP samples prepared from blood samples obtained from 12 camelids (6 llamas and 6 alpacas) by use of a commercial kit (white bars) or double-step conical tube centrifugation (black bars). Error bars represent SD. *Value differs significantly ($P < 0.05$) between methods.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Commercial kit</th>
<th>Conical tube</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-BB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Llamas</td>
<td>7.612 ± 2.565</td>
<td>2.690 ± 1.766</td>
<td>—</td>
</tr>
<tr>
<td>Alpacas</td>
<td>6.047 ± 1.884</td>
<td>2.415 ± 1.107</td>
<td>—</td>
</tr>
<tr>
<td>All camelids</td>
<td>6.830 ± 2.296</td>
<td>2.553 ± 1.413</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Concentration for all camelids normalized to platelet count</td>
<td>3.14 ± 1.10</td>
<td>3.13 ± 0.86</td>
<td>0.97</td>
</tr>
</tbody>
</table>

| TGF-$\beta_1$   |                |              |           |
| Llamas          | 17.101 ± 7.155 | 8.860 ± 5.636| —         |
| Alpacas         | 10.470 ± 8.434 | 4.639 ± 3.494| —         |
| All camelids    | 13.786 ± 8.222 | 6.749 ± 4.985| 0.02      |
| Concentration for all camelids normalized to platelet count | 6.59 ± 4.37 | 7.65 ± 4.26 | 0.60 |

See Table 1 for key.
in lower initial platelet counts and a relatively higher fold change after PRP preparation. However, when these llamas were omitted from analysis, the significant species difference in mean fold-change platelet concentration remained.

Whether differences exist between llamas and alpacas in platelet volume or mass that might affect centrifugation separation is unknown. Differences between horses and humans in PRP platelet enrichment yield have been identified when different centrifugation techniques are used. Breed differences in hematologic values for PRP have also been identified for horses. Platelet aggregation may also play a role in the observed differences between llamas and alpacas, given that camelids differ from other species in platelet aggregation and variations may also exist between alpacas and llamas.

Obtaining optimal platelet and WBC counts in PRP products has been the focus of recent research. Platelet counts are positively correlated with PRP growth factor concentrations, similar to the findings of the present study that normalization of growth factor concentrations by platelet count eliminated the observed differences between PRP preparation methods with respect to supernatant PDGF-BB and TGF-β1 concentrations. In humans, a 3- to 5-fold increase in PRP platelet concentration from blood values may be necessary to achieve therapeutic effects. Platelet concentrations 3 to 9 times that in blood have been used with beneficial effects in horses with experimentally induced tendon and ligament injuries. However, another study involving horses revealed that excessively high platelet concentrations in leukocyte-reduced PRP samples may have detrimental effects on tissue explants and that only moderate (2-fold) increases may be needed for clinical effect. Indeed, a plateau effect has been detected in humans and horses with PRP stimulation of tendon healing when platelet concentrations > 1.5 x 10⁶ to 2 x 10⁶ platelets/µL of PRP are used. Those findings suggest that for llamas and alpacas, platelet enrichment may be optimal at approximately 5- to 6-fold, given the mean blood platelet concentrations identified in the present study. However, additional research is necessary to determine species differences regarding optimal platelet concentration for tissue healing in camelids.

Ideal WBC concentrations in PRP preparations have also been investigated, given that leukocytes have both positive and negative effects in tissues. Leukocytes release proinflammatory cytokines that can have detrimental effects on tissue healing, but these cells may also enhance growth factor activity and provide protection against infection. In general, PRP preparations with a high leukocyte content induce cytokine production and proinflammatory conditions. Target leukocyte concentrations < 3,000 cells/µL were recommended in a report of an in vitro study involving horses; however, species, as well as tissue injury types, differ in their response to high and low leukocyte concentrations in PRP products.

Intra-articular administration of PRP with leukocyte concentrations greater than those in the peripheral blood results in an increase in the amount of synoviocyte death and inflammation in humans but no inflammatory effect in dogs. In rabbits with experimentally induced tendon injury, a greater inflammatory reaction was initially identified in those that received intra-lesional leukocyte-rich (vs leukocyte-poor) PRP, but this effect was not appreciable 2 weeks following administration. In sheep with experimentally induced bone injury, no difference in effects was identified between administration of PRP preparations into bone defects with lower leukocyte concentrations than that in blood and administration of platelet-poor plasma with no detectable leukocytes. Differences in response to leukocyte concentrations in administered PRP preparations by various tissue types and species highlight the need for additional research for optimization of PRP use for camelids. Whether the high leukocyte counts yielded by use of the commercial kit method (approx 42,000 cells/µL of PRP) in the present study would be detrimental to tissue healing is unknown. In vivo studies will be necessary to determine optimal leukocyte and platelet concentrations in PRP products used for tendon or ligament healing in camelids.

Activation methods for maximizing growth factor release from platelets in PRP preparations, including addition of CaCl₂ or bovine or autologous thrombin and freeze-thaw activation cycles, have been investigated in species other than camelids. These techniques differ greatly in their ability to stimulate growth factor release by platelets in PRP preparations, and species, differences in response to leukocyte and platelet concentrations in PRP products used for tendon or ligament healing in camelids. 

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fective and inexpensive activator of platelets in PRP preparations, particularly of platelet PDGF production. Freeze-thaw techniques can result in good PRP TGF-β concentrations but low PDGF concentrations in equine PRP preparations.

In contrast, superior release of PDGF-BB was identified in the present study following freeze-thaw platelet activation without versus with CaCl₂. This difference may have been attributable to the lack of use of a sonification step, causing growth factors to remain within the clot rather than to be released into the supernatant. This step was omitted to imitate the expected release in vivo, given that sonification would not typically be performed in a clinical setting. Volume dilution of PRP samples when CaCl₂ was added may also have had an effect on growth factor concentrations, although the total volume of CaCl₂ added was negligible, compared with the total sample volume (12 µL added to 510 µL of PRP sample). Contact of platelets with tissue collagen can also activate platelet degranulation, and stimulation of platelet aggregation by collagen reportedly occurs in llamas. Investigators in a study involving horses concluded that PRP activation prior to intra-articular administration may not be necessary because of growth factor release by platelet contact with synovial fluid. On the basis of our findings, we recommend freeze-thaw activation (without CaCl₂) of PRP preparations prior to injection into camels.

A limitation of the present study was the low number of camels in each group, which prevented evaluation of the effect of sex and age on the findings. Studies with larger numbers of subjects are needed to confirm the species differences that we detected. Research into the effect of sex and age on PRP preparation in camels is also necessary, given that these factors affect PRP preparations for horses. Another limitation was the use of different anticoagulants for blood (EDTA) and PRP (acid citrate dextrose-A) samples, which may have affected platelet counts.

Additional studies are needed to determine optimal platelet and WBC concentrations in PRP preparations as well as the clinical efficacy of these preparations for use in camels. Species differences in PRP platelet concentrations between llamas and alpacas may mean that clinicians will need to select different PRP methods to yield similar platelet concentrations for these species. Findings of the present study provide a basis for beginning this process, both in PRP preparation and platelet-activation methods. Before clinical efficacy studies can be conducted, the effects of various PRP platelet and WBC concentrations on matrix and cytokine expression in camelid tissue explants also need to be determined.

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Footnotes


b. VWR, Radnor, Pa.

c. Quantikine human PDGF-BB and TGF-β ELISA kits, R&D Systems, Minneapolis, Minn.


References


