Muscloskeletal disease is a common cause of poor performance, days lost from training, and retirement from competition in the equine athlete. Orthobiologics, including blood-derived products, such as platelet-rich plasma (PRP) and autologous protein solution (APS), have become a common choice for the management of various musculoskeletal injuries in horses from tendinopathies and desmopathies to osteoarthritis, with the goal of utilizing the horse’s own immunomodulatory proteins to improve the quality of healing, manage pain, and decrease the recurrence of injury. Blood-derived products concentrate blood components (platelets in PRP and both leukocytes and platelets in APS) that subsequently increase concentrations of growth factors and immunomodulatory cytokines. Therapeutic benefits and clinical efficacy have been reported for the treatment of various musculoskeletal injuries with PRP and APS in horses, dogs, and humans.

OBJECTIVE
To determine the effects of prolonged administration of the oral NSAIDs phenylbutazone and firocoxib on concentrations of cytokines and growth factors in platelet-rich plasma (PRP) and autologous protein solution (APS).

ANIMALS
6 adult University owned horses.

METHODS
Horses were randomized to receive phenylbutazone (1 g, orally, q 12 h) or firocoxib (57 mg, orally, q 24 h) for 6 days. Blood was obtained and processed for APS (Pro-Stride) and PRP (Restigen) before the administration of NSAIDs and at 7 days (1 day following cessation of NSAIDs). Horses underwent a two-week washout period, during which blood was obtained at 14 days and 21 days. The protocol was repeated with a crossover design. PRP and APS were analyzed for concentrations of platelets, leukocytes, and several cytokines (IL-1β, IL-10, IL-6, IL-8, and tumor necrosis factor-α) and growth factors (PDGF, FGF-2, and TGF-β1) using immunoassays. Plasma was evaluated for drug concentrations.

RESULTS
No significant differences existed in concentrations of growth factors and cytokines before or after prolonged administration of NSAIDs. There were significant differences in concentrations of leukocytes and platelets in PRP compared to APS, with higher concentrations of leukocytes at the day 7 time point (T) in APS (phenylbutazone) and in concentrations of platelets in APS at T0 (firocoxib) and in APS at T7 (phenylbutazone).

CLINICAL RELEVANCE
Veterinarians can recommend the administration of these oral NSAIDs prior to obtaining blood for PRP and APS provided a single-day washout period is instituted.

Keywords: cytokines, growth factors, nonsteroidal anti-inflammatory drugs, platelet-rich plasma, autologous protein solution
Horses with musculoskeletal injuries, in addition to more targeted treatments, are often administered systemic anti-inflammatories, such as NSAIDs to manage pain and inflammation. In humans, concerns have been raised regarding the administration of NSAIDs concurrently or prior to obtaining and processing blood for PRP and APS due to the possible effects of NSAIDs on the components of blood, such as platelets, which may then affect the cytokine and growth factor profiles of these products. There is particular concern regarding the administration of nonselective NSAIDs given the known effects of cyclooxygenase-1 (COX-1) on thromboxane and platelet aggregation. The administration of NSAIDs such as naproxen, aspirin, and acetaminophen have, in fact, been shown to alter the cytokine and growth factor profiles of PRP in humans.\textsuperscript{10–12} This research has led to physicians commonly recommending the cessation of NSAIDs 3 to 7 days prior to obtaining blood for PRP processing in humans.\textsuperscript{10–12} Until recently, there have been no discrete guidelines regarding the need for the cessation of NSAIDs in horses prior to blood processing for PRP and APS in horses given the paucity of literature regarding this subject. Previous research has demonstrated an alteration of leukocytes and platelets in PRP subsequent to the administration of the NSAID ketoprofen in horses, although the effects of the administration of NSAIDs on cytokine and growth factor profiles were not directly evaluated in this study.\textsuperscript{13} However, in vitro, incubation of blood with NSAIDs, such as phenylbutazone, firocoxib, ketoprofen, and flunixin meglumine, does not affect the concentration of interleukin-1 receptor antagonist protein (IL-1Ra) or IL-1β.\textsuperscript{14} In addition, it has recently been shown that phenylbutazone, firocoxib, ketoprofen, and flunixin meglumine in vivo does not significantly alter the cytokine and growth factor profiles of PRP or APS when blood was obtained 6 hours after a single administration of any of these NSAIDs.\textsuperscript{15} However, this study did not evaluate the effect of prolonged administration of NSAIDs on the cytokine and growth factor profiles of these products, an important topic given that prolonged administration of NSAIDs (particularly those administered orally) is a very common practice in horses. Therefore, the objective of this study was to evaluate the effects of prolonged administration of commonly utilized oral NSAIDs, phenylbutazone and firocoxib, at clinically relevant doses on the cytokine and growth factor profiles of PRP and APS preparations. We hypothesized that the prolonged (6 days) administration of these NSAIDs would not significantly alter the concentrations of clinically relevant cytokines and growth factors in PRP and APS when blood was obtained the day after cessation of administration.

Methods

Case selection

This prospective, crossover, randomized controlled clinical trial utilized 6 adult, systemically healthy research horses owned by the University of Pennsylvania, New Bolton Center. This study was performed in accordance with the guidelines set forth by the IACUC at New Bolton Center, University of Pennsylvania. Horses were kept on pasture turnout for the entirety of the study.

Blood collection, drug administration, and product preparation

Blood was obtained from the left jugular vein after aseptic preparation for the processing of PRP (Restigen; Zoetis) and APS (Pro-Stride; Zoetis) in addition to plasma collection for drug concentration evaluation at all time points described below. PRP and APS products were prepared in accordance with manufacturer instructions and as previously described.\textsuperscript{15} Blood (55 mL) was drawn from the jugular vein into 2 60-mL syringes with 5 mL of anticoagulant (acid citrate dextrose) and placed into separate PRP and APS separators. The PRP and APS separators were centrifuged at 3,200 × g for 15 minutes. After the removal of platelet-poor plasma from the device, the remaining PRP was placed into aliquots for characterization. For APS, the PRP was removed and transferred to the APS concentrator containing polyacrylamide beads and centrifuged at 2,000 × g for 2 minutes, removed from the device, and placed into aliquots for characterization.

For the analysis of drug concentrations, as previously described, blood (60 mL) was obtained from the jugular vein, placed into Na-fluoride/K-oxalate tubes and centrifuged (2,500 × g for 15 minutes) at each time point.\textsuperscript{15} Three aliquots of the resultant plasma (2 mL per aliquot) were then frozen at −80°C.

Horses were randomized to receive phenylbutazone (1 g, orally, q 12 for 6 days) or firocoxib (57 mg, orally, q 24 for 6 days). These doses were chosen as they were considered to be those most commonly used in the field. Blood was obtained before administering the first dose of oral NSAID (T0) in the morning and at 7 days (T7; the day following the final dose of NSAID). Horses then underwent a two-week washout period, during which blood was collected 1 week into the washout period (T14) and 2 weeks into washout period (T21). The protocol was then repeated in a crossover design, with horses receiving phenylbutazone first receiving firocoxib and those receiving firocoxib first receiving phenylbutazone. Blood was again collected at all time points as described above.

PRP and APS aliquots were analyzed for concentrations of platelets and leukocytes immediately after processing. Quantification of growth factors and cytokines in PRP and APS was performed at a later date after storage in a −20°C freezer.

Quantification of firocoxib and phenylbutazone

Firocoxib and phenylbutazone were quantified in equine plasma by LC-MS-MS. The phenylbutazone (Chemical Abstracts Service # 50-33-9) reference standard was purchased from Frontier BioPharm, whereas firocoxib (CAS # 189954-96-9) and phenylbutazone-δ\textsubscript{3} (CAS # 1189479-75-1) were obtained from Toronto Research Chemicals. Ketoprofen-δ\textsubscript{3} (CAS # 15490-55-8) was acquired
from Sigma-Aldrich. HPLC-grade methyl tert-butyl ether and American Chemical Society–grade phosphoric acid (85%) were purchased from EMD Millipore.

Analytes were extracted from samples by liquid-liquid extraction using methyl tert-butyl ether. For phenylbutazone analysis, samples were acidified with 1 M H₃PO₄ prior to extraction. The extracted analytes were separated on a reverse-phase ACE C18 column (75 X 2.1-mm inside diameter, 5-µm particle size; Mac-Mod Analytical) using a Shimadzu Nexera LC-30AD chromatography system (Shimadzu USA Manufacturing Inc). The mobile phases consisted of 5 mM ammonium formate and acetonitrile. The total elution time was 5 minutes.

The eluted analytes were detected and quantified on a triple quadrupole mass spectrometer (Sciex 7500; AB Sciex LLC). The mass spectrometer was operated in both positive and negative mode with multiple reaction monitoring. Ketoprofen-d₃ and phenylbutazone-d₉ were used as internal standards for quantifying firocoxib and phenylbutazone, respectively. The multiple reaction monitoring transitions were m/z 337 → m/z 283 for firocoxib, m/z 258 → m/z 212 for ketoprofen-d₃, m/z 307 → m/z 131 for phenylbutazone, and m/z 316 → m/z 288 for phenylbutazone-d₉. Linear regression was employed for quantification. The standard dynamic range was 1 to 250 ng/mL for firocoxib and 10 to 1,000 ng/mL for phenylbutazone. The coefficient of determination for all calibration curves was ≥0.99. Sample concentrations that were above the linear dynamic range were diluted and reanalyzed. During analysis, quality-control samples were included alongside the test samples. The accuracy and precision of these control samples met the in-study recommendations outlined in the US FDA guidance for bioanalytical method validation.

PRP and APS characterization

Concentration of platelets, leukocytes, cytokines, including IL-1β, IL-10, IL-6, IL-8, and tumor necrosis factor-α; and growth factors, including FGF-2, TGF-β1, and PDGF-BB, were evaluated at all time points (T0, T7, T14, and T21) for horses receiving both phenylbutazone and firocoxib as previously described. Platelet and leukocyte concentrations were quantified with a veterinary hematology analyzer (Element HT5 Veterinary Hematology Analyzer; Heska) with a combination of flow cytometry, impedance, and colorimetry after inverting the product several times. A fluorescent bead–based multiplex assay (Milliplex; Millipore) using the Luminex 200 Instrument (Luminex) was used to quantify concentrations of FGF-2, IL-1β, IL-10, IL-6, IL-8, and tumor necrosis factor-α in PRP and APS. A fluorescent bead–based single-plex immunoassay (Milliplex, MilliporeSigma) using the Luminex 200 Instrument (Luminex) was used to quantify TGF-β1 in PRP and APS. Twenty-five microliters of standard, control, or sample were added to the appropriate well with the addition of 25 µL of detection antibody to each well. The plate was then incubated at room temperature on a shaker for 1 hour. Then, 25 µL of streptavidin–phycoerythrin was added to each well and incubated for 30 minutes on a shaker at room temperature. The plate was washed 3 times, and then 150 µL of drive fluid was added prior to analysis. The parameters for analysis on the Luminex 200 with xPONENT software were set at 50 events per bead and a sample size of 100 µL. Concentrations of PDGF-BB were quantified using a commercial ELISA kit (R&D Systems) per the manufacturer’s instructions. All antibodies were equine specific except for TGF-β1, a human antibody that has been validated for use in horses.

Statistical analysis

A power analysis was performed using data from a previous study evaluating concentrations of TGF-β1 in PRP and assuming means of 15% and 10% (assumed 5% decrease in the means) and a standard deviation of 2.5. Power analysis revealed an estimated required sample size to be n = 6 for 80% power to be achieved. A mixed-effects model was used to compare continuous variables, including cytokine, growth factor, leukocytes, and platelet concentrations, in PRP and APS. Horse was considered a

Figure 1—Leukocyte concentrations before (T0) and 7, 14, and 21 days (T7, T14, and T21) after administration of firocoxib and phenylbutazone. APS = Autologous protein solution. F = Firocoxib. P = Phenylbutazone. PRP = Platelet-rich plasma. *P < .05; n = 6.

Figure 2—Platelet concentrations of firocoxib and phenylbutazone at T0, T7, T14, and T21. *P < .05; n = 6.
random effect. Treatment and time were considered fixed effects with the interaction of treatment and time probed. Post hoc pairwise comparisons were made with the Tukey method adjustment for pairwise comparisons. All analysis was performed using statistical software (JMP, version 14; JMP Statistical Software). Statistical significance was set at $P < .05$.

**Results**

**Animals**

All 6 horses completed the study without adverse events. The median age of horses included in this study was 3.5 years (range, 3 to 12 years), 4 of which were geldings, and 2 were mares. Breeds represented included 4 Thoroughbreds, 1 Appaloosa, and 1 Standardbred.

**Leukocyte and platelet concentrations**

Concentrations of platelets and leukocytes at T0, T7, T14, and T21 for both phenylbutazone and firocoxib are shown (Figures 1 and 2). With respect to leukocytes, there were significant elevations in concentrations of leukocytes in APS at T7 (phenylbutazone group) when compared to PRP at T0 (firocoxib group) and PRP at T21 (phenylbutazone group).

![Figure 3](image-url)—Cytokine concentrations of firocoxib and phenylbutazone at T0, T7, T14, and T21 for tumor necrosis factor-α (TNF-α), IL-6, IL-1β, IL-10, and IL-8. No significant differences were found; $n = 6$. 
However, there were no significant elevations in leu-
kocyte concentration within NSAID groups subse-
quent to the administration of either NSAID. With
respect to platelet concentrations, there were sig-
nificant elevations in concentrations of platelets
in APS at T0 (firocoxib group) compared to PRP at
T21 (firocoxib group) and in APS at T7 (phenylbuta-
zone group) when compared to PRP at T0 (phenyl-
butazone group) and T21 (firocoxib group). Similar
to leukocytes, there were no significant elevations
in concentrations of platelets within NSAID groups
subsequent to the administration of either NSAID
when compared to baseline.

Cytokine and growth factor concentrations
When evaluating cytokines, there were no sig-
nificant differences in concentrations between
products or time points (Figure 3). Cytokine
concentrations were often higher in APS than in PRP,
but these differences did not reach statistical sig-
nificance. No significant differences in growth factor
concentrations were identified between products or
time points (Figure 4).

Plasma concentration of NSAIDs
Concentrations of phenylbutazone and firo-
coxib were present in detectable quantities in all
horses at T7 and in some horses at T14 and T21.
Phenylbutazone concentrations were below the half-
maximal inhibitor concentration for COX-1 (1,900
ng/mL) and COX-2 (1,200 ng/mL) in all horses at
all time points (Figure 5). Firocoxib was above the
half-maximal inhibitor concentration for COX-2
(30 ng/mL) at T7 in most horses (4/6) but not at
T14 or T21 (Figure 6). No drug was present in any
sample at T0.

Figure 4—Growth factor concentrations of firocoxib and
phenylbutazone at T0, T7, T14, and T21 for PDGF, FGF-2,
and TGF-β1. No significant differences were found; n = 6.

Figure 5—Detectable plasma concentrations of phenyl-
butazone (ng/mL) at T7, T14, and T21 (168, 336,
and 504 hours) with reference to half-maximal inhibi-
tory concentration (IC₅₀) for cyclooxygenase-1 and -2
(COX-1 and -2).

Figure 6—Detectable plasma concentrations of firo-
coxib (ng/mL) at T7, T14, and T21 (168, 336, and 504
hours) with reference to half-maximal inhibitory con-
centration (IC₅₀) for cyclooxygenase-1 and -2 (COX-1
and -2).
Discussion

This is the first in vivo study evaluating the effects of prolonged administration of oral NSAIDs, phenylbutazone and firocoxib, on the cytokine and growth factor profiles of PRP and APS in horses. Similar to a previous study evaluating the effect of single-dose administration of NSAIDs in horses on PRP and APS and in support of our hypothesis, no significant differences in concentrations of cytokines or growth factors were identified when these blood-derived products as a result of prolonged administration of either oral NSAID, although there were significant alterations in the concentrations of leukocytes and platelets.

There were significant differences in leukocyte concentrations between time point and product, with APS at T7 in the phenylbutazone group having higher concentrations than PRP in the firocoxib group at T0 and PRP at T21 in the phenylbutazone group. This is in accordance with the previously obtained results of a study evaluating the effect of a single administration of NSAIDs on PRP and APS, in which higher concentrations of leukocytes were identified in APS when compared to PRP, which is likely due to similar causes. Higher concentrations of leukocytes in APS when compared to PRP products can be explained by the effect of the second centrifugation step in APS, which results in a higher concentration of many blood components in the end product. This is more likely to account for this elevation in the concentration of leukocytes than an effect of the administration of phenylbutazone as there were no significant differences in the concentrations of leukocytes at T0 and at T7 within the NSAID treatment groups. It is possible that the elevation in leukocyte concentrations in APS at T7 after phenylbutazone administration was compounded by the inhibition of leukocyte chemotaxis as an effect of NSAID administration, resulting in an increase in leukocyte concentrations in the vasculature and therefore in these blood-derived products similar to in the previous study.

Interestingly, in the previously performed study on the effects of a single administration of NSAIDs on PRP and APS, in which blood was drawn 6 hours after the administration of NSAIDs, significant elevations in leukocytes were noted in APS subsequent to the administration of NSAID within treatment groups. This could be accounted for by the difference in blood collection time after administration of NSAIDs, which was more prolonged in the current study, potentially allowing for a return of leukocyte concentrations to baseline levels.

Like leukocytes, there were significant alterations in concentrations of platelets with respect to time point and product, with higher concentrations of platelets present in APS at T0 (firocoxib) than in PRP at day 21 (firocoxib) and in PRP at T7 (phenylbutazone) than in PRP at T0 (phenylbutazone) and T21 (firocoxib). This reflects previous data demonstrating that prolonged administration of ketoprofen intravenously to horses results in an increase in platelet concentrations in PRP and that the administration of single doses of phenylbutazone, flunixin meglumine or firocoxib to horses results in elevated platelet concentrations in APS. Again, the effect of second centrifugation likely accounts for some of the elevation in the concentrations of platelets noted in APS when compared to PRP. It is also possible that the elevation in the concentrations of platelets in PRP at T7 after phenylbutazone administration compared to PRP prior to administration of phenylbutazone are compounded by the known systemic effects of non-selective NSAIDs on COX-1, resulting in decreased platelet aggregation and an increase in platelet concentration in the end product.

The effect of increased concentrations of leukocytes and platelets on the efficacy of PRP and APS is outside of the scope of this study, and the conclusions of current literature surrounding this subject are variable. Some studies suggest a positive effect of higher concentrations of leukocytes given a positive correlation with leukocyte concentrations and the growth factors PDGF and VEGF in PRP in humans. However, increased concentrations of leukocytes also correlate with increased concentrations of catabolic enzymes, such as matrix metalloproteinase-9, leading to concerns regarding potential deleterious effects of higher concentrations of leukocytes in these products on orthopedic tissues due to proteolysis. Similarly, high concentrations of platelets (5 X 10⁶ platelets/μL) have been shown to induce an increased release of matrix metalloproteinases. Concentrations of catabolic enzymes were not evaluated in the current study, and therefore it is unknown whether similar increases in proteolytic enzymes would be identified in PRP and APS with higher concentrations of platelets or leukocytes. An in vitro study of leukocyte-rich PRP cultured with equine tenocytes has indicated a decrease in gene expression of type 1 and type 2 collagen with increasing platelet concentrations up to 850 X 10⁶ platelets/μL. It is important to note, however, that in comparison to the median platelet concentrations reached in the current study (approx 500 X 10⁶ platelets/μL), the concentrations reached in these studies were typically much higher. In addition, these in vitro results have not necessarily been reflected in vivo, with leukocyte-rich PRP being equally as effective as leukocyte-poor PRP for the management of knee osteoarthritis in people and leukocyte-rich PRP noted to improve the production of type 1 collagen in a rabbit model of Achilles tendinopathy when compared to leukocyte-poor PRP.

While studies in people have previously demonstrated an effect of prolonged administration of the NSAIDs aspirin and naproxen on cytokine and growth factor concentrations in PRP (specifically for PDGF and IL-6), this result was not reflected in the results of the current study. No significant differences in cytokine or growth factor concentrations were noted by time point or product, although concentrations of cytokines were often higher in APS than those in PRP. The reason for this dissimilarity is currently unknown and may be due to differences in the metabolism of NSAIDs between species, the...
different structure of equine platelets, or different NSAIDs and protocols used.

There are limitations of this study that should be noted. While the effects of prolonged administration of oral NSAIDs at commonly used doses were evaluated, administration was discontinued the day prior to blood collection for processing APS and PRP. The study was designed in this way to account for a commonly recommended practice, to discontinue the administration of NSAIDs approximately 24 hours prior to lameness evaluation and collection of blood-derived products, and therefore provides clinically relevant information with respect to this protocol. However, given that NSAIDs were discontinued the day prior to blood collection, the results of this study cannot be applied to situations in which horses are evaluated and blood collected without this length of washout period after a prolonged dosing protocol. The use of primarily young Thoroughbreds may additionally be considered a limitation as the results may not apply to other breeds and ages of horses. In addition, due to volume limitations associated with APS and the high volumes of substance required for the analysis of leukocyte and platelet concentrations, only selected cytokines and growth factors were evaluated in this study. Therefore, the results of this study may not apply to other clinically relevant cytokines and growth factors in APS and PRP, such as IL-1Ra, which is not available as a multiplex assay. IL-1Ra is often touted as one of the most critical cytokines for the efficacy of substances such as APS, and therefore its quantification associated with NSAID administration would have been ideal. However, given that leukocyte concentrations were not decreased secondary to the administration of NSAIDs, and the positive correlation between IL-1Ra and leukocyte concentrations noted in previous studies, we would not expect this cytokine to be drastically decreased using this NSAID administration protocol. Also, given these volume limitations, the concentrations of firocoxib and phenylbutazone were not evaluated for APS and PRP. Given that in a previous study, the concentrations of these medications in PRP and the platelet-poor plasma from APS were not significantly different than that in plasma 6 hours after the administration of these NSAIDs, we would not expect for the concentrations of these drugs in APS and PRP to be different than that identified in plasma in this study.

In conclusion, the results of this study will aid practitioners in formulating appropriate protocols for withdrawal of the commonly used oral NSAIDs phenylbutazone and firocoxib when administered for a prolonged dosing interval. A recommendation to cease administration of these NSAIDs 24 hours prior to blood collection for processing PRP and APS should be sufficient to avoid significant alterations in cytokine and growth factor profiles in these blood-derived products. The clinical significance of alterations in leukocyte and platelet concentrations subsequent to administration of these NSAIDs, and the effects of prolonged administration of these NSAIDs on cytokine and growth factor profiles when a withdrawal period of 24 hours is not instituted, are areas deserving of further exploration.

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References


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