# Temporal Release of Growth Factors from Platelet-rich Fibrin (PRF) and Platelet-rich Rlasma (PRP) in the Horse: A Comparative *in vitro* Analysis

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## ABSTRACT

*Objectives:* To evaluate platelet-rich fibrin (PRF) in horses and compare temporal release of growth factors (GF) to platelet-rich plasma (PRP).

*Study Design:* *In vitro* study

Animals: Twelve healthy Thoroughbred geldings <5 years of age

*Methods:* PRF was created by centrifugation using a human protocol. PRP was processed using a double spin, tube method. Four groups were created for each horse: immediate analysis of PRP (1) and PRF (2); slow-release analysis of PRP (3) and PRF (4) over 5 days. Concentrations of transforming growth factor β1 (TGF β 1) and platelet derived growth factor BB (PDGF-BB) were analyzed using commercial sandwich ELISAs and significance determined using Wilcoxin signed rank and Kruskal-Wallis tests.

*Results:* PRP released more GF than PRF upon immediate quantification (p=0.05). TGFβ1 was increased at all subsequent time points in PRF compared to PRP (p<0.001), although both methods demonstrated an increased cumulative TGFβ1 yield compared to immediate analysis (p<0.001). PDGF-BB did not show a temporal increase in yield, but release kinetics differed between PRF and PRP, indicating PRF may trap PDGF-BB, leading to a slower release. PRP gel dissolved by day 5in contrast to PRF clots, which remained solid.

*Conclusions:* PRF can be easily procured in the horse using standard laboratory equipment. It may present advantages over PRP by providing a solid fibrin biomaterial allowing steady elusion (Author, pls check this word) of GF over time. This slow release of GF may represent a novel treatment modality for surgical conditions in the horse, and future studies should be undertaken to determine appropriate clinical applications.

## INTRODUCTION

The ability to deliver growth factors to an injured area presents a therapeutic advantage to the clinician, especially when the injury is chronic in nature[1] or the healing response is poor.[2] Recently, the use of platelet-rich plasma (PRP) has been proposed as a method for increasing growth factor delivery.[3-6] Compared to whole blood, PRP contains increased concentrations of platelets and, therefore, platelet-derived growth factors such as transforming growth factor β (TGFβ1),[3] platelet-derived growth factors (PDGFs), and vascular endothelial growth factor (VEGF),[5] which have been shown, in vitro, to promote cell migration, differentiation, and matrix synthesis.[7-9] For this reason, there is great optimism that PRP may enhance the ability of injured tissues to heal, although the optimal timing and dosage is unknown.[10]

Contradictory evidence exists, however, regarding the effect of PRP on cell lines *in vitro*[11, 12] and in clinical cases,[6, 13] perhaps because the precisely orchestrated continuum of events integral to wound healing cannot be reliably controlled simply by supplying a milieu of generic platelet derived growth factors at a single point in time.[13,14] With PRP, platelets degranulate mostly within an hour of application,16 releasing a deluge of growth factors which may overwhelm healing tissues or be consumed by proteases.[17] This has been cited as one reason why longitudinal PRP studies often show an initial positive response, but only weak mid- to long-term responses in injured tissue.[18]

One other disadvantage of PRP is the lack of a true-solid fibrin network. This is necessary to support the platelets; enhance cytokine function; and to act as a biologic scaffold along which repair cells may migrate.[19]

The role of fibrin in healing has been previously documented,[20, 21] and this molecule has been postulated to be as important as growth factors to the healing process.[22] Fibrin-rich-in-platelets may, therefore, be clinically superior to PRP, as it combines the benefits of growth factors and fibrin to create a structural framework, which elutes growth factors steadily over time.[19]

In an attempt to provide sustained release of growth factors from a fibrin network, platelet-rich fibrin products have been investigated and have demonstrated desirable continued slow release of growth factors.[8, 14] To date, most of these products have been produced using commercial kits; are activated with exogenous calcium salts or thrombin; do not form a true-solid biomaterial; and are leukocyte deprived.

Recently, a second generation leukocyte-platelet-rich fibrin (PRF) was developed for human use.[23] This is an entirely autologous fibrin clot with platelets and leukocytes enmeshed within it. It is a 3-dimensional, easily handled, biomaterial, which does not dissolve, but is destroyed by remodeling over time, similar to a natural blood clot.[24] The kinetics of growth factor release from PRF have been widely studied in humans but, to the authors' knowledge, PRF has not previously been objectively investigated in equine patients.

The aims of the current study were to document a procedure for procuring PRF in the horse; to quantify the immediate and temporal release of growth factors from this product; and to compare the concentration of growth factors to those from PRP procured from the same patient.

Our hypotheses were that PRF would be easy to produce in the horse without alteration of the human protocol; that immediately available growth factor concentrations would be similar between PRF and PRP; and that PRF would demonstrate a slow temporal release of growth factors in contrast to the rapid release from PRP.

## MATERIALS AND METHODS

Blood Collection and Sample Preparation

Twelve healthy Thoroughbred geldings <5 years of age were prepared for venipuncture from the left jugular vein. Blood was drawn through a 14-gauge needle directly into four

sterile glass 10 ml vacuum blood-collection tubes preloaded with acid-citrate dextrose (ACD) (BD Vacutainer, BD, Franklin Lakes, NJ, USA) which was then gently rocked to ensure thorough mixing of contents. Blood was also drawn directly into two sterile glass 10 ml vacuum blood-collection tubes without anticoagulant. Blood was concurrently collected for hematological analysis in a routine manner. Written owner compliance was provided for all horses and the blood collection procedure was performed according to current AVMA guidelines.

## PRP Preparation

PRP was processed using a modification of a technique previously reported in horses.[25] Briefly, blood tubes underwent a *soft* centrifugation at 120 g for 5 mins, resulting in four layers within each tube: the upper two layers (platelet-poor plasma and platelet-rich plasma) were removed by pipette, leaving the buffy coat and red cell layers in the tube. The decanted plasma was centrifuged at 240 g for 5 minutes and the bottom half (PRP) was removed and used for analysis. A total of 10 ml of PRP was obtained. Five ml was used for immediate analysis (group 1), and 5 ml was used for slow-release quantification over 5 days (group 3).

## PRF Preparation

PRF was prepared as previously described in the human literature.[19, 23] Immediately after collection, all four tubes were centrifuged for 12 minutes at 400g. After centrifugation, 3 distinct layers were formed: A base (bottom) layer of packed red cells; an upper layer of serum (supernatant); and, interspersed between these two layers, a solid PRF clot. This PRF clot comprised white cells and platelets enmeshed within a fibrin 3-dimensional structure. For each patient, one PRF clot was immediately processed to allow initial quantification of growth factors and one clot was used to quantify slow release of growth factor over the experimental time period.

## Sample Preparation

Four experimental groups were constructed for each patient: Immediate PRP (group 1); Immediate PRF quantification (group 2); PRP slow-release quantification (group 3); and PRF slow-release quantification (group 4).

## Immediate PRP (Group 1)

WBC and platelet concentrations were measured for each sample. PRP samples were subjected to platelet activation to release all available platelet-derived growth factors by the addition of 600 uL of a 10% calcium gluconate solution. Each sample was then incubated at 37°C (author, pls check change) for 1 hour to allow a platelet gel to form. The gel was homogenized using a commercial device (Polytron PT 1200E, Kinematica, Luzern, Switzerland) and the product centrifuged at 5,500 g for 8 minutes. The resulting supernatant was decanted into 1ml tubes (Eppendorf AG,New York, NY, USA), and stored at -80°C (Author, pls check change) until subsequent analysis.

## Immediate PRF Quantification (Group 2)

Each PRF clot was removed after 1 hour from the tube and placed in a sterile glass tube along with 1ml of sterile growth medium (Dulbecco's modified eagle's medium [DMEM], Invitrogen, Grand Island, NY, USA). A scalpel was used to macerate each sample prior to homogenization using a commercial device.e The resulting sample was centrifuged at 5,500 g for 8 minutes, and the supernatant decanted into 1ml tubes, and stored at -80°C (Pls change)until subsequent analysis.

## PRP and PRF Slow-release Quantification (Groups 3 and 4)

One PRP gel per patient was placed in a sterile glass tube along with 4 ml of growth medium. This was repeated for one PRF clot per patient and tubes were incubated at 37°C. (Pls check change) At each time point the PRP gel/ PRF clot was removed from the glass tube and placed in a fresh tube containing 4 ml of DMEM. Upon removal of the gel/ clot, the remaining solution was centrifuged at 5,500 g for 8 minutes. The supernatant was decanted and stored at -80°C(Pls check change) for analysis. Samples were taken at 24 hours, 48 hours, 72 hours, 96

**Table 1.** *Immediate growth factor release from PRP (group 1) and PRF (group 2) and temporal release from day 1-5 from PRP (group 3) and PRF (group 4). Cumulative yield from groups 3 and 4 over the 5 day study period is also presented. Significance <0.05*

| | TGF β1 ng/ ml (+/- 95% CI) | | | PDGF-BB ng/ ml (+/- 95 % CI) | | |
|---|---|---|---|---|---|---|
| | **PRF** | **PRP** | **significance** | **PRF** | **PRP** | **significance** |
| **immediate** | 20.18 (1.41) | 22.64 (1.96) | **p<0.01** | 31.45 (4.47) | 33.66 (4.26) | **p=0.05** |
| **1** | 27.2 (3.08) | 23.47 (2.86) | p>0.1 | 16.54 (1.93) | 21.59 (1.99) | **p<0.001** |
| **2** | 22.7 (3.19) | 6.4 (0.72) | **p<0.001** | 6.16 (0.33) | 3.67 (0.13) | **p<0.001** |
| **3** | 15.3 (1.57) | 6.33 (0.49) | **p<0.001** | 3.6 (0.4) | 2.48 (0.14) | **p<0.001** |
| **4** | 11.32 (1.37) | 3.65 (0.25) | **p<0.001** | 1.63 (0.2) | 1.5 (0.17) | p>0.1 |
| **5** | 7.3 (0.97) | 3.25 (0.57) | **p<0.001** | 1.38 (0.18) | 0.46 (0.15) | p>0.1 |
| **total yield** | 83.9 (5.25) | 43.1 (2.95) | **p<0.001** | 29.32 (1.63) | 27.69 (2.05) | p>0.05 |

hours, and 120 hours.

### Growth Factor (GF) Determination

For each sample, concentrations of TGFβ1 and PDGF (subtype 'BB') were measured using a commercially available sandwich ELISA designed for human use (Quanti-kine, R and D Systems, Minneapolis, MN, USA). Testing was performed according to manufacturer's instructions and absorbances were read on a microplate reader with a wavelength of 420 nm. Samples were diluted empirically as required. All results were reported as total weight of molecules (nanograms) per 1ml of supernatant volume. GF released at each time point was compared between PRF and PRF. In addition, in groups 3 and 4, growth factor released at each time point was totaled and the cumulative total compared.

### Statistical Analysis

Means, standard deviations, and confidence intervals were calculated for each molecule at each sample point. Correlations between hematological values (white blood cell count [WBC], and platelet concentration) and TGFβ1, and PDGF-BB release were analyzed using Spearman's rank correlation (rho). Numerical data was assessed for normality and homoscedasticity and failed to satisfy requirements for parametric testing. Kruskal-Wallis and Wilcoxin signed rank tests were used to calculate relationships between growth factors released from each experimental group. Groups 1 and 2 were compared to evaluate the initial concentrations of growth factors in PRF, relative to PRP. Groups 3 and 4 were compared to evaluate the slow temporal release of growth factors from PRF relative to PRP. Groups 1 and 3 and Groups 2 and 4 were compared to evaluate differences in cumulative yield of GF compared to immediately available levels. Significance for all tests was set at P=0.05 and analysis performed using commercially available software (Excel, Microsoft Corp, WA, USA).

### RESULTS

The procedure was well tolerated in all patients. No significant differences were found in baseline hematological comparison of patients that had a mean WBC concentration of 7.4x103/ml (+/- 0.8 CI 95%) (p=0.24) and a mean platelet count of 166x103/ml (+/- 14.4 CI 95%) (p=0.15). No correlations were identified between baseline hematologic values and growth factor concentration in any of the four groups. A significant positive correlation between platelet numbers and release of both TGFβ1 (P=0.005) and PDGF-BB (P=0.04) was present, however, in Groups 1 and 3. It was not possible to quantify platelet or WBC concentrations within the PRF clot in Groups 2 or 4. The mean (+/- CI 95%) weight of PDGF-BB and TGFβ1 recovered at each sample point is illustrated in table 1 along with the mean cumulative totals for Groups 3 and 4.

Significantly greater quantities of both

*Figure 1:TGFβ1 and PDGF-BB recovered from immediate analysis of PRP and PRF. For each growth factor, matching superscript symbols indicate significant differences. PRP released greater quantities of both growth factors upon immediate quantification. Box plot and whiskers represent maximum/ minimum values and 95% confidence interval*
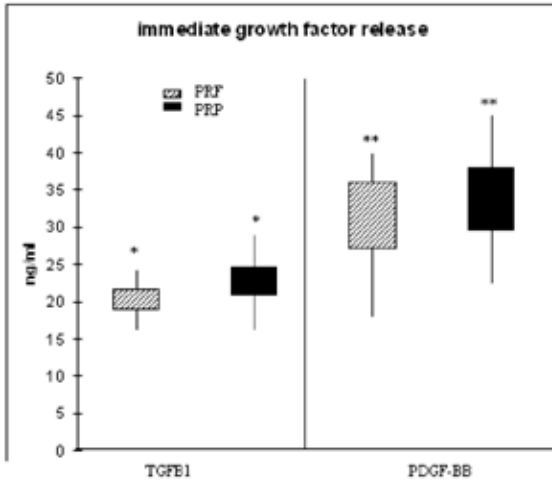


GF were immediately released from the PRP (Group 1) samples than from PRF samples (Group 2) (Figure 1). In Group 3, significantly greater values of PDGF-BB were obtained immediately (p<0.001), but then rapidly reduced within 48 hours. By days 2-3, significantly greater yields of PDGF-BB were observed in Group 4 (p<0.001) (Figure 2). The cumulative total of PDGF-BB was no different, however, between PRP and PRF samples (p>0.05), and no significant difference was observed between total temporal release and immediate release of PDGF-BB from PRP or PRF. Significantly greater quantities of TGFβ1 were immediately recovered from group 1 than 2 (P<0.01). When Groups 3 and 4 were compared, from days 2-5, significantly higher levels of TGFβ1 were recovered from PRF (Group 4) than PRP (group3) (p<0.001) (figure 3). PRF samples yielded twice as much TGFβ1 than PRP samples over 5 days (p<0.001). Cumulative TGFβ1 was greater in temporal release Groups 3 and 4 compared to the initial quantification Groups 1 and 2 (p<0.001), with a 4-fold increase in

TGFβ1 yield in Group 4 compared to Group 2.

By day 5, 10 of 12 PRP gel samples had dissolved into the DMEM. All 12 PRF clots remained intact throughout the study period.

## DISCUSSION

This study demonstrates for the first time that equine-derived PRF provides both an immediate and sustained source of growth factors available for use in healing tissue. The technique is technically easy to perform, has a low cost, and does not require specialized equipment. Equine PRF can be processed using a standard table-top centrifuge with no significant alteration of the technique described for human PRF.23

We hypothesized that PRF should contain similar quantities of GF to PRP immediately after activation. TGFβ1 (p<0.01) and PDGF-BB (p=0.05) concentrations were, however, significantly lower in Group 2 than Group1, presumably indicating a lack of efficiency in the release of growth factor in the macerated PRF sample. Exogenous platelet activation and the lack of a strong fibrin network, in the PRP samples, probably permits greater release of growth factors into the medium than maceration and centrifugation ,4 thus it is proposed that the difference in yield between Groups 1 and 2 is most likely due to experimental design.

In the temporal release comparison (Groups 3 and 4), no difference existed between cumulative PDGF-BB in either group, although the release kinetics at each sample point differed: almost the entire cumulative yield was released within 24 hours in Group 3, whereas a slower, steadier release was witnessed in Group 4. It is known that PDGF-BB is almost entirely contained in α granules and is released upon activation 25. This explains why the net yield was not greater in Groups 3 and 4 than Groups 1 and 2. As only 5% of temporal release is attributable to continued production of

**Figure 2:** *Temporal release of PDGF-BB over 5 days from PRP (group 3) and PRF (group 4). Matching superscript symbols indicate significant differences. Group 3 initially released greater quantities of PDGF-BB; by days 2-3 significantly greater quantities were recovered from PRF; by days 4-5 no difference in yield was detected between groups. Cumulative yield of PDGF-BB was not different between groups 3 and 4. Box plot and whiskers represent maximum/ minimum values and 95% confidence interval*
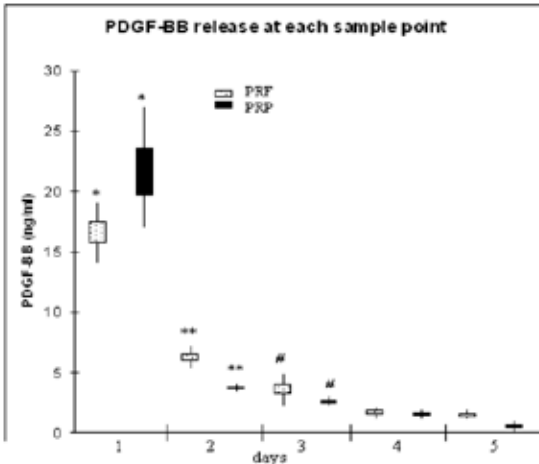


PDGF-BB,[16] it is likely that the slow release kinetics from Group 4 were due to trapping of the initially release PDGF-BB within the 3-dimensional fibrin framework.

In contrast, cumulative yield of TGFβ1 was increased over initial levels in the slow-release Groups 3 and 4. Slow- release PRF, however, demonstrated a significantly greater increase than PRP. As most of the growth factors present in the α granules are released upon degranulation, the marked increase in TGFβ1 yield observed in Groups 3 and 4 must be from a source other than the platelets. It has been reported that WBC trapped in the PRF matrix represent the major source of this additional TGFβ1 release, and that WBC continue to produce and secrete this growth factor from within the PRF clot for several days.[19]  The process of creating PRF purposefully captures most of the leukocytes within the fibrin matrix.

In contrast, the technique chosen for

procurement of PRP is specifically designed to limit WBC inclusion. [26] TGFβ1 concentration correlates well with WBC concentration within PRP,[3] which would imply that the subjective manual technique used to create PRP in the current study does not adequately prevent WBC inclusion in the final product. It is unknown if the increase in TGFβ1 release in Group 4 over Group 3 simply represents greater numbers of WBC within the PRF than PRP, or if fibrin-entrapment of WBC further stimulates TGFβ1 production from WBC. The different release kinetics between Groups 3 and 4 would appear to suggest that the fibrin network influences the manufacture of TGFβ1:  if TGFβ1 was merely trapped within the matrix and released slowly over time, the cumulative yield would not be twice as high in PRF than in PRP.

The inclusion of WBC is, however, controversial in platelet-derived therapies,[27] with some authors arguing against inclusion, citing the pro-inflammatory effect of these cells. [28] Other authors recommend inclusion of the cells to increase the yield of growth factor [29] and, perhaps, to mediate immunological processes.[30]

PRP was used as a positive control in this study to permit the direct comparison of temporal growth factor release from PRF with a previously validated product.[3, 25] Prior investigations have documented increased growth factor concentrations in PRP over whole blood,[3, 4, 26] and for this reason, the authors considered PRP to be the *gold standard* for evaluating growth factor release from novel products. This is the first time, to the author's knowledge, that growth factor release from PRP and PRF has been compared in the horse.

It should be noted that quantification of growth factor from PRP varies widely between studies due to various factors such as instrumentation, and patient factors such as

age, sex, and breed.[4] The concentration of growth factors liberated from PRP in the current study was not intended to be directly comparable to other PRP-related studies; it was intended only to serve as a reference for evaluation of PRF in this study.

Growth factor concentration was positively correlated with platelet concentration in the PRP samples as reported elsewhere,[3,4] but it was not possible, in this study, to determine the relationship between platelet numbers and growth factor concentration in Groups 2 and 4 due to the inability to quantify the number of platelets trapped within the PRF. Previous attempts to quantify platelet counts within a PRF clot have been unsuccessful, although it has been demonstrated, using immuno-histochemical staining, that platelets are concentrated within the PRF clot.[8]

In the present study, no correlation was identified between whole blood parameters and subsequent growth factor concentrations in any group. This is in contrast to previous investigations regarding equine-PRP which observed a positive correlation between growth factor release and WBC and platelet concentrations in circulating whole blood.[3] In the PRP samples, the subjectivity of PRP collection may have contributed to these findings by inconsistently including WBC. It is possible that hematologic values cannot be used to predict either the immediate or the temporal release of growth factor from PRF.

PDGF-BB and TGFβ1 were chosen as representative growth factors in this study. Both have been examined extensively in the horse using similar analytical methods as employed in the current study.[3-5, 26] TGFβ1 is considered representative of the kinetics of multiple growth factors,[3] with an increase in TGFβ1 corresponding to increases in multiple other factors. PDGF-BB is one of the principal growth factors present in equine platelets.[4]

*Figure 3: Temporal release of TGFβ1 over 5 days from PRP (group 3) and PRF (group 4). Matching superscript symbols indicate significant differences. From day 2-5, group 4 released significantly greater quantities of TGFβ1 than group 3. Cumulative yield of TGFβ1 was greater in group 4 than group 3, and both groups demonstrated an increased yield compared to immediate analysis (groups 1 and 2). Box plot and whiskers represent maximum/ minimum values and 95% confidence interval*
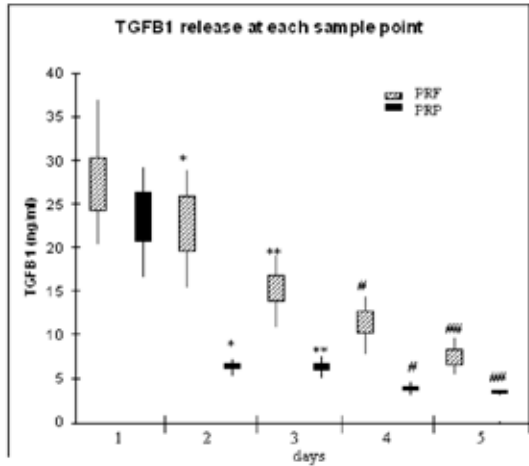


Fibrin is an important adjunct molecule in PRF. The binding of growth factors to fibrin prolongs the biological activity of these cytokines, which may represent a therapeutic advantage of fibrin therapies over traditional PRP.[31] It seems intuitive, therefore, that PRF would be an ideal delivery vehicle, especially as it is a biomaterial with the properties of a solid.[19] In the current study, PRP gels dissolved by day 5 in all but two samples. In contrast, all [12] PRF samples remained intact, further demonstrating the solid biomaterial nature of PRF. The current study was limited to 5 days of duration as a result of PRP gel degradation. Previous studies have documented release of growth factor from PRF up to day 7.[19]

Although there is evidence establishing the potential benefits for the use of PRF, the existence of contradictory studies demonstrates the need for further investigation into

the application of PRF in clinical cases. It has been shown, for example, that fibrin-rich-in platelets fail to increase the rate of healing in acutely severed canine tendons, and may even promote excessive fibroplasias.[14] An excessive fibroblastic response resulted in a tendon cross-sectional area 2 to 5 times that of the sham treated control tendons in that study. This is in contrast to human studies, which reported increased rates of healing in both chronic lower limb ulcers [1] and naturally injured tendons. [32]

Such conflicting reports also exist regarding the in vivo success of conventional PRP. It has been shown, in one manuscript, to aid healing in chronic lower extremity wounds in horses, [6] whereas a contradictory study reported minimal positive effects on healing and an increase in undesirable granulation tissue in acutely created wounds in the same area. [13] It appears, therefore, that PRP and PRF share a similar contradictory evidentiary base regarding clinical usefulness, and that this is most likely due to the influence of the released growth factors upon healing tissues. It has been documented, for example, that endogenous growth factor release may be controlled through negative feedback and self-regulating pathways, which may be negatively influenced by the application of exogenous growth factor.[2, 14, 33] The application of PDGF-BB, for example, may down regulate the transcription of host PDGF. [2]

It has also been proposed that exogenous TGFβ1 may promote fibroplasia: it is naturally released in sufficient quantities to promote healing in injured tissue within the first several weeks of injury, and it is present for up to 30 days post-injury.[15] Application of exogenous TGFβ1 during this period, in the form of PRF or PRP may, therefore, create an excessive fibroplastic response [13, 14].

Many of the contradictory reports on the appropriate use of platelet therapies appear to relate to the timing of the application, with favorable outcomes more often noted when these biologic products are used in chronic or non-healing environments.[1,6,13,14]

PRF may be most appropriately used, therefore, in chronic, slow healing injuries,[1] large defects which cannot otherwise be filled,[14] or tissues which demonstrate a constitutively poor response to healing. [2] It may also provide tremendous advantages to hemostasis, by physically aiding clot formation and providing increased concentrations of specific factors essential to the clotting cascade.[19] These potential clinical applications require further investigation.

One of the great potential benefits of PRF over other platelet products is its ability to be handled as a true solid biomaterial.[19] This presents multiple therapeutic opportunities such as tendon repair, packing of bone defects, repair of cartilage, and repair of skin wounds, [32, 34] but such applications would require further investigation in the horse. PRF may also not be appropriate in all situations, such as where an injectable liquid product is desired.

PRF has been documented to be well tolerated by patients with no significant negative sequale reported.[14, 34] This is in contrast to some investigations regarding PRP that reported the potential for therapy to increase pain and inflammation in acute injuries. [26]

One potential complication of platelet products procured by an *open* method, such as PRF, is the potential for contamination of the product.[25] Great care should be taken when preparing PRF to ensure sterility throughout the collection, processing, and handling of the product.

One other technical complication of PRF production is the requirement for immediate centrifugation of fresh whole blood to ensure optimal PRF matrices.[19] A centrifuge and the ability to ensure sterile technique must, therefore, be present at the site of whole blood collection. This may present technical limitations in an ambulatory environment.

The size of each group in the current study is small, which can make interpretation of results difficult. It is possible that insignificant trends observed in the current

study may have reached significance had a larger study been undertaken and this is a limitation of the current investigation. By using a smaller population size, however, this study excluded many potentially confounding variables such as age; sex; and breed, and permitted investigation of our initial hypotheses. Future larger studies are now, therefore, indicated to evaluate if operator and inter-patient factors influence PRF parameters in a similar way to PRP parameters.[4]

The purpose of this study was to evaluate the preparation of PRF and to quantify growth factor release in an equine model. Results of the current study highlight the ease of procurement and the predictable slow-release of growth factor which may be of benefit to healing tissues. Our hypothesis that PRF could be processed from the horse with no significant alterations from the human protocol was confirmed; as was the hypothesis that growth factor would be steadily released from PRF over time. The sustained and increased release of TGFβ1 observed may be of benefit to healing tissues when appropriately applied. Second generation platelet therapies, such as the PRF examined in the current study, may have the potential to provide additional benefits over conventional PRP therapy. Future in vivo studies are, therefore, warranted to evaluate the suitability of PRF for clinical use in the horse.

## DISCLOSURE STATEMENT

The authors report no conflict of interest in this manuscript

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