

STABILITY OF GROWTH FACTORS IN PLATELET RICH PLASMA

A Thesis

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Master of Science

by

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ABSTRACT

The use of platelet rich plasma (PRP) in sports medicine has increased dramatically recently but there are still doubts towards its efficacy that likely stem from the wide variety between PRP samples and products. To further define PRP, this study aimed to test whether its composition was altered if PRP or the blood used to produce PRP, remained out at room temperature before use. Another aim was to establish if a manual platelet count would adequately quantify platelet concentration compared to using automated methods. All types of PRP product tested remained stable, according to growth factor and cytokine measurements, for up to four hours when stored at room temperature. No significant differences were found in platelet concentrations obtained from automated or manual methods. The results found confirm the ease of verifying platelet concentration manually and that PRP can be used in orthopedic and surgical situations without worry of product degradation.

BIOGRAPHICAL SKETCH

Brooke Wilson graduated from the United States Military Academy in 2003 with a Bachelor's of Science degree in Chemistry and a minor in Mechanical Engineering. After serving in the Army for five years with two combat deployments, she began her career at Cornell University's College of Veterinary Medicine in 2009. In January 2012, Brooke took a year-long leave of absence to participate in the Veterinary Student in Biomedical Research and Training program through the NIH. She completed her degree in Veterinary Medicine in May 2014.

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LIST OF ABBREVIATIONS

ACD- Acid-citrate dextrose

CBC- complete blood count

L^{hi}PRP- leukocyte high platelet rich plasma

L^{lo}PRP- leukocyte low platelet rich plasma

MMP-9 - matrix metalloproteinase nine

PRP- platelet rich plasma

TGF- β 1- transforming growth factor beta one

WBC- white blood cell

CHAPTER 1

BACKGROUND

Platelet rich plasma (PRP) is primarily used to promote healing or as a bioscaffold in areas of medicine such as dental surgery [17, 20], soft tissue injuries [16, 18], orthopedic surgery [21, 23], wound healing [12], and veterinary medicine [4, 25]. Despite its extensive use, there are still doubts about the clinical efficacy of PRP [19, 22, 26]. One of the difficulties in interpreting clinical outcome data is variations in the type and consistency of PRP that is generated between patients due to biological variability [5, 14] and variations between PRP products made from the numerous manufacturing kits available [5, 6]. There are classification schemes to categorize the various types of PRP [2, 8, 9] which can broadly be categorized as low leukocyte PRP (L^{lo} PRP) or high leukocyte PRP (L^{hi} PRP).

PRP can be a point of care therapy, prepared and used immediately such as in cases to treat knee pain, or it can be used in surgical procedures. Each clinical setting and operating theater has its own policy for generation of PRP. In some instances, blood is drawn by a phlebotomists and processed in the hospital laboratory, and in others scenarios, the primary attending physician or their assistant would draw the blood and process it to generate PRP patient-side [10, 21]. These various situations could result in blood or the resultant PRP retained at room temperature for minutes to several hours prior to administration to the patient.

The primary aim of this study was to test if room temperature retention of blood or PRP for various time intervals affects platelet degranulation. Concentration

of platelet-derived growth factors were used as a surrogate marker of platelet degranulation in blood, L^{lo} PRP, and L^{hi} PRP retained at room temperature for up to 4 hours. A second aim was to determine if manual platelet counts were an accurate reflection of automated counts. The use of manual platelet counts would provide a cost effective alternative to automated platelet counting for research studies. The outcomes of these experiments should provide immediately relevant information for the clinical application of PRP.

CHAPTER 2

EVALUATION OF PRP AND GROWTH FACTORS

Methods and Materials

Blood collection and generation of PRP - Venous blood (100mls) was collected from healthy human volunteers (n=5) into acid citrate dextrose (ACD) anticoagulant to a final concentration of 1%. Three 15mL aliquots were placed into disposable syringes used to generate L^{lo} PRP (Autologous Conditioned Serum Double Syringe, Arthrex, Naples, FL) and three 20mL aliquots were placed into the disposable used to generate L^{hi} PRP (SmartPreP 2, Harvest Technologies, Plymouth, MA). To test the effects of retaining blood at room temperature before processing PRP, one sample from each L^{lo} PRP and L^{hi} PRP group was processed immediately, the next sample was processed after being retained at room temperature for two hours, and the final sample was processed after four hours at room temperature. At the end of each retention time, the samples were used to generate PRP according to the respective manufacturer's directions. Aliquots of resultant PRPs were immediately frozen at -80°C for future analyses. Remaining PRP samples were allowed to sit at room temperature in their disposable devices with aliquots removed at one, two and four hours and frozen for future analysis. No PRP sample was buffered or activated after processing. Figure 1 is provided as a schematic outlining sample collection and preparation.

Automated and manual counts - A complete blood count (CBC) was performed on the initial samples of venous blood, L^{lo} PRP, and L^{hi} PRP samples. A

manual platelet count, using a modified Giemsa stain, was performed on smears of all whole blood samples taken as well as every PRP sample made at each of the time points. All slides were microscopically examined at 10x objective (Figure 2a) to identify an ideal area of the smear to perform a platelet count. The magnification was then increased to the 100x oil immersion where the total number of platelets was counted in ten of these high-powered fields of view (Figure 2b). The average number of platelets per high-powered field was determined and multiplied by 15 to find the total number of platelets (thou/ul) for the sample [13]. This data was used to compare the reliability of manual platelet counts versus automated counts and to ensure that at each time point PRP was produced.

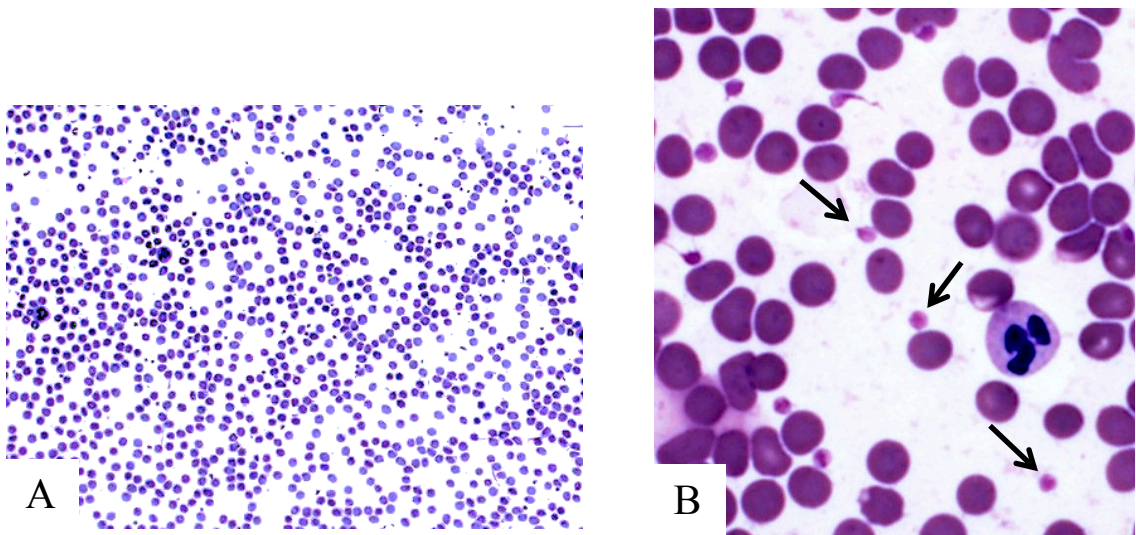


Figure 2: (A) Thin edge of a prepared blood smear under 10x objective where it is pertinent to look for platelet clumping or other abnormalities. This area is free of platelet clumps and the red blood cells are evenly spread out, indicating it is an ideal area to perform a platelet count. Once the blood smear is scanned at low power, (B) the area is observed at 100x oil immersion where the average number of platelets (arrows) per high-powered field is counted.

Growth factor and catabolic cytokine measurements – Blood and PRP samples were thawed and centrifuged at 12000g for 15 minutes to pellet cell debris. Transforming growth factor-beta 1 (TGF- β 1) concentration was determined using the TGF- β 1 Emax ImmunoAssay System (Promega Corporation, Madison, Wisconsin). TGF- β 1 was chosen as an anabolic growth factor to measure in the PRP samples because of the multiple effects it has in healing. It has been shown to stimulate undifferentiated mesenchymal cell proliferation, regulate endothelial, fibroblastic and osteoblastic mitogenesis, regulate collagen synthesis, stimulate endothelial chemotaxis and angiogenesis, stimulate synthesis of extracellular matrix in cartilage, and decrease catabolic activity of interleukin-1 (IL-1) and matrix metalloproteinases (MMPs) [3, 7, 11]. It has also been shown to correlate to platelet concentration [24]. Matrix metalloproteinase nine (MMP-9) concentration was determined using the MMP-9 Biotrak Activity Assay (GE Healthcare Biosciences, Piscataway, New Jersey). MMP-9 was measured as an indicator of catabolic factors in PRP because it is a known collagenase [5, 27], shown to be linked to poor healing [28] and has been correlated to WBC levels [24]. TGF- β 1 and MMP-9 were evaluated in each sample to measure any increase or decrease in anabolic or catabolic factors due to the time samples remained at room temperature. Samples were measured in duplicate using a multiple detection plate reader (Tecan SAFIRE, Durham, North Carolina).

Statistical analyses - Platelet concentration values were normally distributed on a histogram so a paired t-test was performed comparing automated and manual methods of obtaining a platelet concentration in whole blood, L^{lo} PRP, and L^{hi} PRP. A paired t-test was also used to compare platelet, TGF- β 1, and MMP-9 concentrations

between L^{lo} PRP and L^{hi} PRP. TGF- β 1 concentrations were not normally distributed so a Kruskal-Wallis one-way ANOVA followed by a Dunn's all-pairwise comparison was used to compare the TGF- β 1 concentrations in L^{lo} PRP and L^{hi} PRP, separately, at each time point. A Kruskal-Wallis was also run on TGF- β 1 concentrations found in L^{lo} PRP and L^{hi} PRP produced from blood that was retained at room temperature. MMP-9 concentrations were normally distributed so a one-way ANOVA with a Tukey all-pairwise comparison was used to compare MMP-9 concentrations in L^{lo} PRP and L^{hi} PRP, separately, at each time point. A one-way ANOVA was also performed on MMP-9 concentrations found in L^{lo} PRP and L^{hi} PRP that had been produced from blood that was retained at room temperature. Statistical analyses were performed using Statistix 9 software (Analytical Software, Tallahassee, Florida). A p-value of <0.05 was considered significant.

Results

Validation of PRP generation – Both systems successfully generated PRP. Platelet concentration in L^{lo}PRP had an average of a 1.98 ± 0.14 fold increase and a 0.47 ± 0.07 fold change in WBC from baseline. L^{hi}PRP had an average of a 3.06 ± 0.24 fold increase in platelet concentration with a 1.05 ± 0.17 fold change in WBC from baseline. L^{hi}PRP had a significantly increased platelet concentration ($p = 0.001$) and WBC levels ($p = 0.020$) when compared to L^{lo}PRP. However, no difference was seen between TGF- β 1 ($p = 0.063$) or MMP-9 ($p = 0.365$) concentrations between the two PRP products.

Stability of platelets in PRP retained at room temperature – TGF β -1 concentrations were not significantly different in PRP products processed immediately or that were retained at room temperature for up to four hours (Figure 3). MMP-9 concentrations were also similar between immediately processed PRP and PRP retained at room temperature (Figure 4).

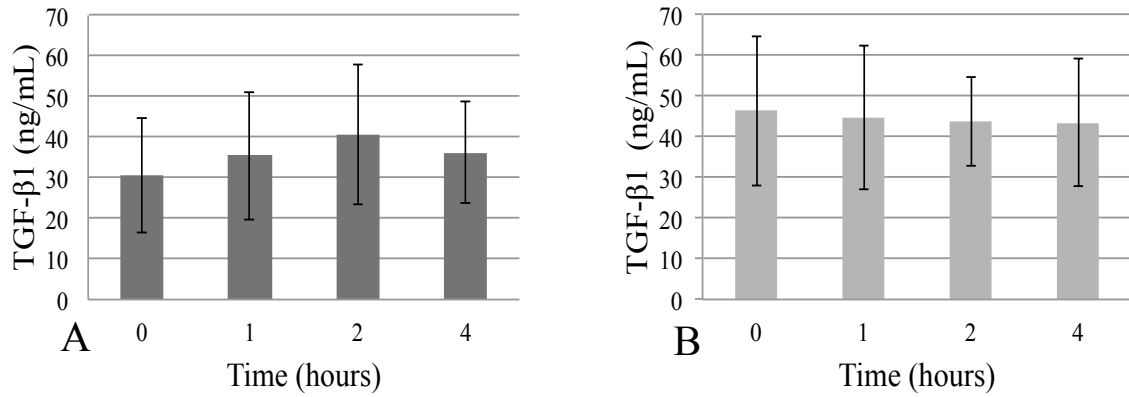


Figure 3: Concentration of TGF-β1 in (A) L^{lo}PRP and (B) L^{hi}PRP processed immediately or retained at room temperature for 1, 2, or 4 hours after processing. Bars represent mean (N=5) ± SE. Significance was determined using a Kruskal-Wallis one-way ANOVA. L^{lo}PRP p = 0.966, L^{hi}PRP p = 0.986.

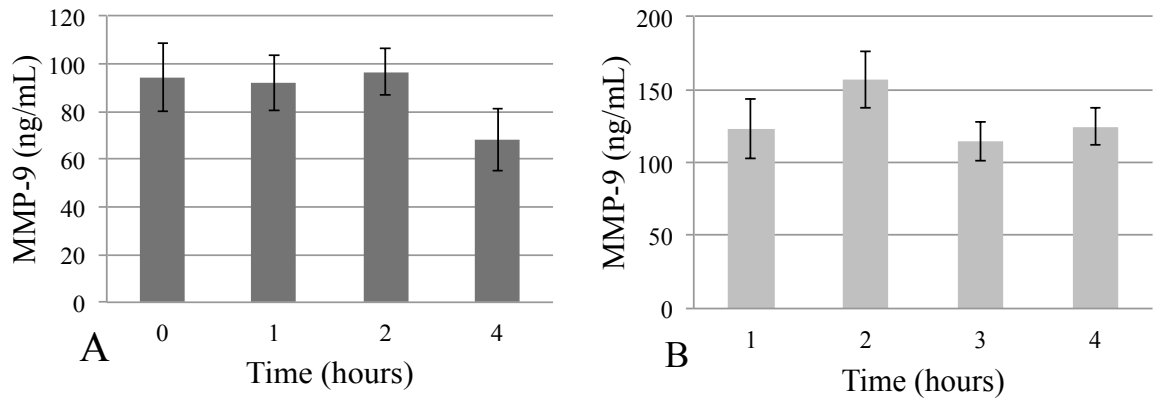


Figure 4: Concentration of MMP-9 in (A) L^{lo}PRP and (B) L^{hi}PRP processed immediately or retained at room temperature for 1, 2, or 4 hours after processing. Bars represent mean (N=5) ± SE. Significance was determined using a one-way ANOVA. L^{lo}PRP p = 0.187, L^{hi}PRP p = 0.490.

Stability of platelets in blood retained at room temperature – Blood samples were retained at room temperature for up to 4 hours prior to producing PRP. Neither TGF β -1 (Figure 5) nor MMP-9 concentrations (Figure 6) changed significantly over time when blood was retained at room temperature prior to processing the PRP.

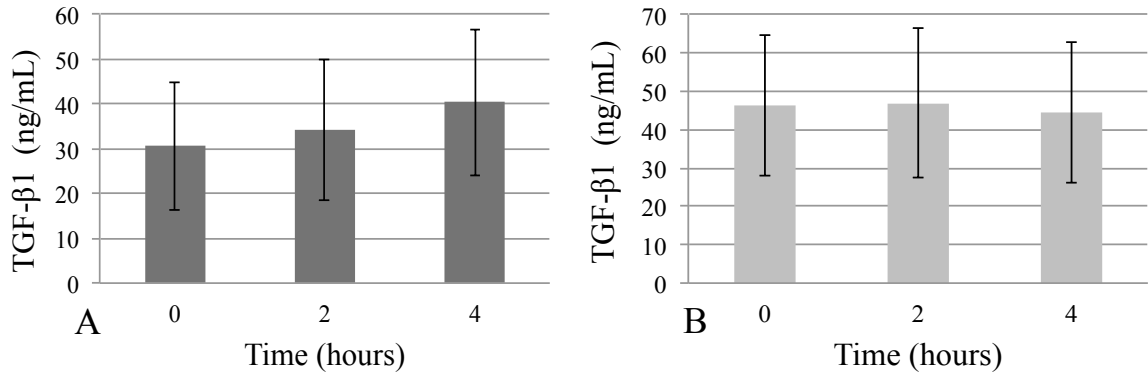


Figure 5: Concentration of TGF- β 1 in (A) L^{lo}PRP and (B) L^{hi}PRP processed immediately or that was processed with blood retained at room temperature for 2 or 4 hours. Bars represent mean (N=5) \pm SE. Significance was determined using a Kruskal-Wallis one-way ANOVA. L^{lo}PRP p = 0.833, L^{hi}PRP p = 0.894

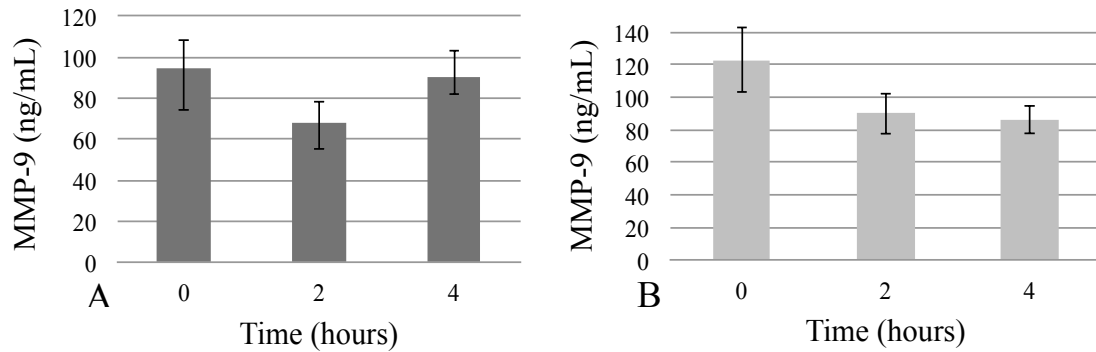


Figure 6: Concentration of MMP-9 in (A) L^{lo}PRP and (B) L^{hi}PRP processed immediately or that was processed with blood that was retained at room temperature for 2 or 4 hours. Bars represent mean (N=5) \pm SE. Significance was determined using a one-way ANOVA. L^{lo}PRP p = 0.362, L^{hi}PRP p = 0.516

Automatic vs. manual platelet count – A question this study posed was whether a manual platelet count was adequate to qualify a sample as PRP when compared to an automated platelet count. Our results show a significant difference between the automated and manual platelet counts ($p = 0.05$) for whole blood samples ($n=5$). However, we believe that the manual count is more accurate as platelet clumping was seen on two samples in the automated count, which contributed to falsely lower reported platelet concentrations. Manual platelet counts for both L^{lo} PRP and L^{hi} PRP were adequate ($p = 0.61$ and $p = 0.36$ respectively) when compared to the automated platelet counts performed (Table 1).

Platelet concentration (thou/uL)				
	Automated Count		Manual Count	
	Mean \pm SD	Range	Mean \pm SD	Range
Venous blood	111.8 \pm 59.47 ^a	54-202	210.9 \pm 59.36 ^b	144-297
L^{lo} PRP	421.4 \pm 132.8 ^c	319-620	410.1 \pm 94.18 ^c	318-543
L^{hi} PRP	634.4 \pm 88.77 ^d	517-766	635.4 \pm 176.61 ^d	491-933

Table 1: Platelet concentrations in whole blood, L^{lo} PRP, and L^{hi} PRP ($N=5$). A paired t-test was performed to compare results obtained from an automated platelet count to those obtained from a manual count. Superscript letters indicate significant difference between automated or manual platelet concentration within each category.

CHAPTER 3

DISCUSSION

The purpose of this study was to better define both L^{lo} PRP and L^{hi} PRP by characterizing any changes that may occur to PRP due to extended preparation times. This level of understanding is necessary due to the lack of a consistent definition for PRP and the multiple methods available for producing PRP. By better understanding the stability of growth factors in different types of PRP, it will be possible to provide more dependable treatment of lesions with PRP. This knowledge will also help to streamline future studies of PRP by eliminating one of the potential areas of biological variability found in PRP.

The main limitation of this study is the small sample size. However, the increased variability that occurs when producing PRP, as seen by the large range in growth factor concentration in this study, has also been shown in multiple other studies despite having used a larger sample size [2, 5, 14]. Another potential limitation of the study could be that only one growth factor, TGF- β 1, and one catabolic cytokine, MMP-9, were used as surrogate measures to represent overall growth factor stability. For the sake of time and cost, we decided to use only one type ELISA to measure growth factors, choosing TGF- β 1 because it has been shown to correlate to platelet concentrations [6, 14, 24]. MMP-9 was chosen to be measured as an indicator of catabolic factors in PRP because it is a known collagenase [5, 27] linked to poor healing [28] and has been correlated to WBC levels [24].

This study indicates that performing a manual platelet count on whole blood and PRP samples is adequate for classifying PRP production. Our study showed that manual platelet counts may be more beneficial than automated counts as the automated counts can be artificially low due to platelet clumping which has been shown to occur as early as one hour after blood collection in spite of what type of anticoagulant is used when collecting venous blood [15]. It is necessary to verify that PRP has been produced as multiple studies have shown that platelet concentration is highly variable, changing between people, PRP production systems, and even between multiple blood draws on the same patient [5, 14]. Being able to use a manual platelet count to verify PRP is more readily available to clinicians, less expensive, and more quickly performed than automated counts.

This study also found that growth factors remain stable in both L^{lo}PRP and L^{hi}PRP when either the blood is retained at room temperature for up to four hours before production of PRP or if the PRP itself is retained at room temperature for up to four hours before being utilized. To the authors knowledge, this is the first such type of study performed on PRP. The results are important to note because this eliminates one area of possible variability in PRP. Patients receiving PRP treatments that have had a delay in processing; e.g. when used during surgery or when placed in bioscaffolds [1, 10], are receiving a product with similar qualities as PRP that has not been delayed in production. Knowing that growth factors remain stable in PRP for up to four hours helps standardize and define PRP for future clinical studies, removing a potential area of variability seen between basic science studies and clinical studies.

This study illustrated that growth factors in both L^{lo} PRP and L^{hi} PRP are stable for up to four hours and it is acceptable to use those PRP products without worrying about potential variability in the product. This becomes most important when PRP is used in surgical situations and there may be a delay in the processing or use of prepared PRP. However, it is still important to ensure that the product made is actually PRP as this study and many others have shown a great deal of variability between patients and PRP systems [5, 14]. The fact that growth factors in PRP are stable for four hours takes away some of the variability between clinical studies and allows for clinicians to focus on other areas of difference between PRP products. The standardization and better definition of PRP will only lead to better studies and more comprehensive results.

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