

In vitro evaluation of the anti-bacterial effect of two preparations of platelet rich plasma compared with cefazolin and whole blood

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Introduction

Recent literature has focused on the potential for platelet rich plasma (PRP) products to provide relief for various musculoskeletal diseases, conditions, and disorders¹. PRP refers to blood solutions with platelet concentrations above baseline values¹. Current evidence suggests that platelets contribute many functions in antimicrobial host defense, including their ability to release potent antimicrobial peptides from their alpha granules^{2,3}. These peptides have been shown to possess broad-spectrum antimicrobial activity against gram negative gram positive, and fungal pathogens^{4,5}.

In addition, PRP products have demonstrated an *in vitro* antibacterial effect against gram negative (*Escherichia coli*^{6,7}, *Klebsiella pneumoniae*⁸, and *Proteus mirabilis*⁷) and gram positive bacteria (*Staphylococcus aureus*^{6,9}, *Bacillus megaterium*⁷, and *Enterococcus faecalis*⁷). Other studies have found no *in vitro* activity against gram negative (*Enterobacter cloacae*⁸) and gram positive bacteria (*Enterococcus faecalis*⁶, *Bacillus subtilis*⁸, *Bacillus cereus*⁸, and *Staphylococcus epidermidis*⁸). Mixed results have been shown with *Pseudomonas aeruginosa*^{6,7}. In fact, Bielecki et al. demonstrated that platelet-rich gel may actually induce the *in vitro* growth of *P. aeruginosa*⁶. Tang et al.¹⁰ were the first to suggest a direct relationship between platelet concentration and antimicrobial effects. While no studies have directly com-

pared PRP antimicrobial activity to pharmaceuticals, one study did find similar inhibition as has been seen with gentamicin and oxacillin⁶.

Recent literature has also focused on the effect of leukocyte concentration in PRP preparations with regard to antimicrobial activity^{9,11-13}. Anitua et al.¹¹ and others have proposed that leukocytes play a key role in the activation of platelets, in regard to their antimicrobial activity, by releasing growth factors and cytokines which may serve as platelet activators. However, the literature is varied when examining the dose dependent response of leukocyte concentration in PRP preparations, and further study seems warranted^{9,11,13}.

The purpose of this study is to investigate the antibacterial properties of two different platelet concentration methods using common bacteria responsible for infections in arthroplastic surgery: *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epi*), *Propionibacterium acnes* (*P. acnes*) and methicillin-resistant *Staphylococcus aureus* (MRSA) using a standard time kill assay. The null-hypotheses are: (1) there will be no difference in bacterial growth between two different platelet concentration preparations [(low platelet PRP (PRP_{LP}) and high platelet PRP (PRP_{HP})] and (2) there will be no difference in bacterial growth in preparations with PRP when compared to bacteria treated with positive controls phosphate buffered saline (PBS) or whole blood and an antibiotic as a negative control: Cefazolin (Ancef®, GlaxoSmithKline, Research Triangle 46 Park, NC).

Methods

The methods described in this paper were considered best practice in regards to commonly considered standards for research protocols. Best efforts were made to make the following justifiable and appropriate for the procurement of the conclusions stated, and the results from which they were drawn¹⁴.

Experimental Groups

Five different experimental groups were formed including two positive controls, one negative control, PRP_{LP} and PRP_{HP}. These groups were then treated with standard inoculations of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Propionibacterium acnes*, and methicillin-resistant *Staphylococcus aureus* (MRSA). This yielded 20 tubes, each containing a different combination of experimental group to

species of bacterium. Each of these tubes were then plated in duplicate at two different dilutions for each group at five different time points (0,1,4,8 and 24 hours).

Preparation of PRP

Peripheral blood was drawn from two healthy male volunteers with an average age of 24 ± 1 years. Exclusion criteria comprised any form of anticoagulant, antibacterial or immunosuppressive therapy within last 6 months, any form of systemic illness and current or recent history of cancer¹⁵. Two different PRP preparations and a whole blood control were obtained from each volunteer. For the PRP_{LP} preparation, 50 mL of blood was drawn into a 60 mL syringe prefilled with 5 mL of acid citrate dextrose (ACD-A, noClot 50, Citra Pharmaceuticals, Braintree, MA). A 12 mL aliquot of the anticoagulated blood was transferred to Autologous Conditioned Plasma (ACP) Double Syringes (Arthrex Inc., Naples, FL), which were then loaded into a centrifuge and spun for 5 minutes at 1500 rpm. This yielded increased platelet concentration with a low concentration of white blood cells (PRP_{LP}).

The PRP_{HP} preparation utilized the GPS III Platelet Concentrate System (Biomet Biologics, Warsaw, IN). As before, 50 mL of blood was drawn into a 60 mL syringe prefilled with 5 mL of ACD-A. 27 mL aliquot of anticoagulated blood was injected into disposable tubes containing buoy shelves to separate the blood. The tubes were centrifuged for 15 minutes at 3200 rpm, yielding high platelet and white blood cell concentrations (PRP_{HP})¹⁶.

Each native blood specimen and each PRP preparation were analyzed by the University of Connecticut's blood laboratory. The platelet number, number of red blood cells, and WBC differentiation was determined by a complete blood counter (Gen-S System 2 Hematology Analyzer; Coulter Corp, Miami, Florida)¹⁷.

A recent article by DeLong et al. suggests using the PAW Classification System to help standardize PRP research¹⁸. For the purpose of this study PRP_{LP} receives a P2-B β classification while PRP_{HP} receives a P3-A α classification.

Preparation of Bacterial Cultures

Each bacterial strain was subcultured and incubated for 16 hours at 37° C, after which colonies were microscopically analyzed for purity. The initial bacterial concentration for each preparation was standardized for all experimental and control tubes using a nephelometer. Four mL of the standardized inoculum of each individual strain was added to each experimental and control tube along with 1mL of control (either PBS, whole blood, or cefazolin) or experimental preparation (PRP_{LP} and PRP_{HP}). *S. aureus*, *S. epi*, and *MRSA* samples were grown in lysogeny broth (LB) at an initial concentration of 5.0×10^5 colony forming units per mL (CFU/mL) in a 5mL volume. *P. acnes*

was grown in Wilkins-Chalgren broth (WC) with an initial concentration of 1.0×10^6 CFU/mL in a 5 mL volume due to its slower growth rate. The cefazolin concentration was set at 4 micrograms per mL, which has demonstrated to be an effective surgical wound concentration¹⁹.

Time Kill Assay

A time kill assay was performed on these preparations with time points of 0, 1, 4, 8 and 24 hours²⁰. At each time point, 100 μ L was removed from each individual reaction tube and serial dilutions were performed to yield dilutions of 1:100, 1:1000 and 1:10,000. 50 μ L of each dilution was then plated on trypticase soy agar plates with 5% sheep blood (Becton Dickinson and Company, Sparks MD) in duplicate. The plates were incubated at 37°C for 48 hours after which the colony counts were determined. The plates containing *P. acnes* were incubated under anaerobic conditions.

Statistical Analysis

Descriptive statistics to characterize the groups were reported using mean and standard deviation. The Kolmogorov-Smirnov test was performed for each continuous measure to identify non-normal distributions. Between group comparisons of colony growth at each time point was assessed with Kruskal-Wallis rank test. For these tests, a probability (p) value of 0.05 was considered significant. Post hoc comparisons were performed with the Wilcoxon rank-sum test and sign-rank test. Given the small group sizes (n=4), the lowest possible p value that could be reached with post hoc non-parametric testing was 0.0625 therefore the threshold for significance with post hoc testing was 0.1. Groups were condensed to include both PRP donors in a single treatment group for analysis to permit evaluation of potential trends among the different treatment groups as opposed to variations between PRP donors, which has previously been established¹⁶. All statistical analysis was performed using Stata 12 (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP).

Results

PRP Preparations

Native venous blood yielded a mean platelet concentration of $135.6 \pm 78.4 \times 10^3/\mu$ L and $5.4 \pm 1.9 \times 10^3/\mu$ L of white blood cells. After centrifugation, the platelet count of the produced PRP_{LP} was $386 \pm 65.5 \times 10^3/\mu$ L and $867 \pm 234.4 \times 10^3/\mu$ L for the PRP_{HP} (p=0.01). The white blood cell count of the produced PRP_{LP} was $0.62 \pm 0.265 \times 10^3/\mu$ L and $11.96 \pm 4.74 \times 10^3/\mu$ L for the PRP_{HP} (p=0.01).

Bacterial Growth

Both PRP_{LP} and PRP_{HP} showed a significant decrease ($p < 0.05$) in bacterial growth at 8 hours for all of the bacterial samples when compared to whole blood. The results for each individual bacterium are listed below.

Staphylococcus epidermidis (Fig. 1):

At 1, 4, 8 and 24 hours, both PRP_{LP} and PRP_{HP} limited *S. epidermidis* growth relative to the whole blood standard ($p < 0.03$). At 4 ($p < 0.02$) and 8 hours ($p < 0.01$), PRP_{HP} was found to significantly limit bacteria relative to PBS. The most significant difference between PRP_{HP} and PRP_{LP} was at 8 hours ($p < 0.01$). No significance in bacterial growth was found at any time point between cefazolin and the PRP preparations.

Staphylococcus aureus (Fig. 2):

At 1, 4, and 8 hours, PRP_{LP} limited *S. aureus* growth relative to whole blood ($p < 0.02$). At 1, 4, 8 and 24 hours PRP_{HP} limited *S. aureus* growth compared to whole blood ($p < 0.03$). The largest effect of PRP_{LP} was seen at 8 hours with a 3500% reduction in bacterial growth when compared to whole blood (2.00×10^5

CFU/mL for PRP_{LP} vs 7.05×10^6 CFU/mL for the blood control). PRP_{LP} and PRP_{HP} were found to significantly limit bacterial growth relative to PBS at 4 and 8 hours ($p < 0.01$). There was no significant difference in bacterial growth between cefazolin and the PRP preparations at any time point.

Propionibacterium acnes (Fig. 3):

At 4, 8, and 24 hours, both PRP_{LP} and PRP_{HP} were found to limit *P. acnes* growth relative to whole blood ($p < 0.03$). At 8 hours, PRP_{HP} was found to limit bacterial growth better than PRP_{LP} ($p < 0.05$). At 24 hours cefazolin appeared to limit bacterial growth relative to both PRP preparations, however significance at an alpha value of 0.05 was not found.

Methicillin Resistant Staphylococcus aureus (MRSA) (Fig. 4):

At 8 and 24 hours, both PRP_{LP} and PRP_{HP} were found to limit MRSA growth relative to whole blood ($p < 0.05$). At 1 and 4 hours, statistical significance was seen between PRP_{LP} and PRP_{HP} ($p < 0.03$). However, at 24 hours, no statistically significant difference was observed between either PRP_{LP} or PRP_{HP} and cefazolin.

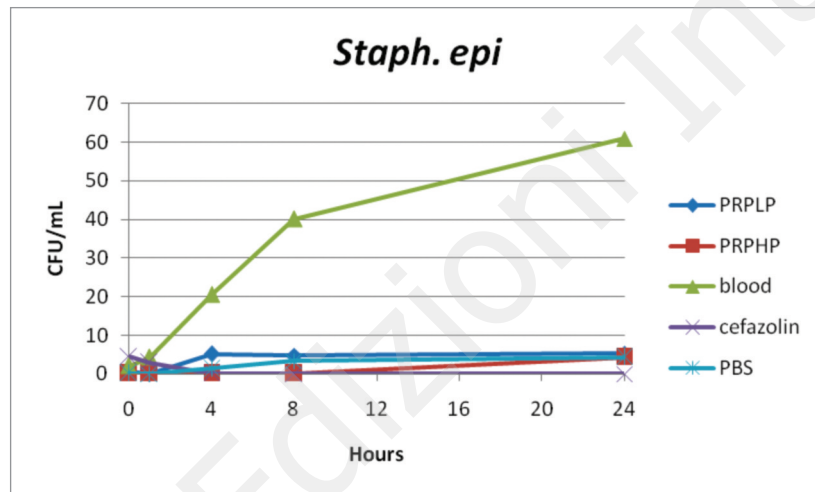


Figure 1. *Staphylococcus epidermidis* growth in experimental groups as measured by colony count; CFU: Colony forming units. Legend: PRPLP = platelet rich plasma-low platelets; PRPHP = platelet rich plasma-high platelets; PBS = phosphate buffered saline.

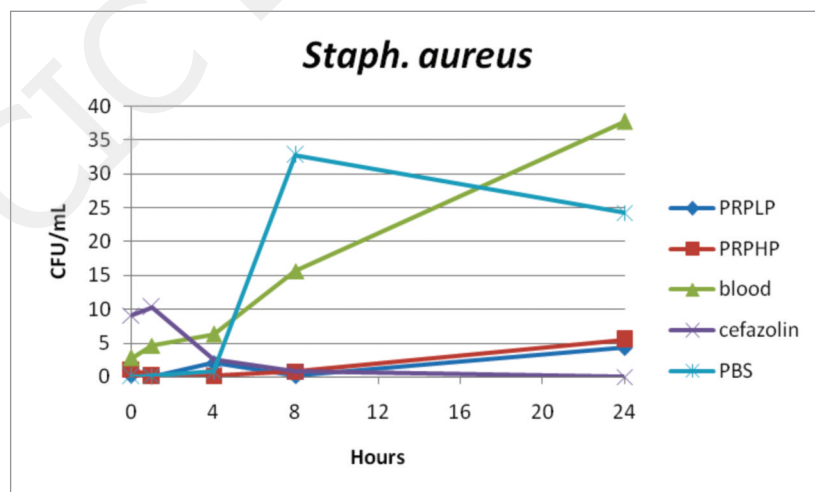


Figure 2. *Staphylococcus aureus* growth in experimental groups as measured by colony count. CFU: Colony forming units. Legend: PRPLP = platelet rich plasma-low platelets; PRPHP = platelet rich plasma-high platelets; PBS = phosphate buffered saline.

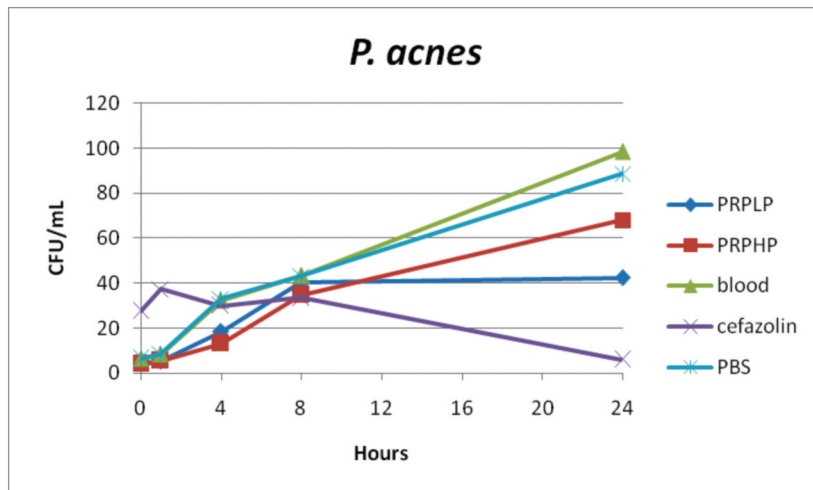


Figure 3. *Propionibacterium acnes* growth in experimental groups as measured by colony count. CFU: Colony forming units.

Legend: PRPLP = platelet rich plasma-low platelets; PRPHP = platelet rich plasma-high platelets; PBS = phosphate buffered saline.

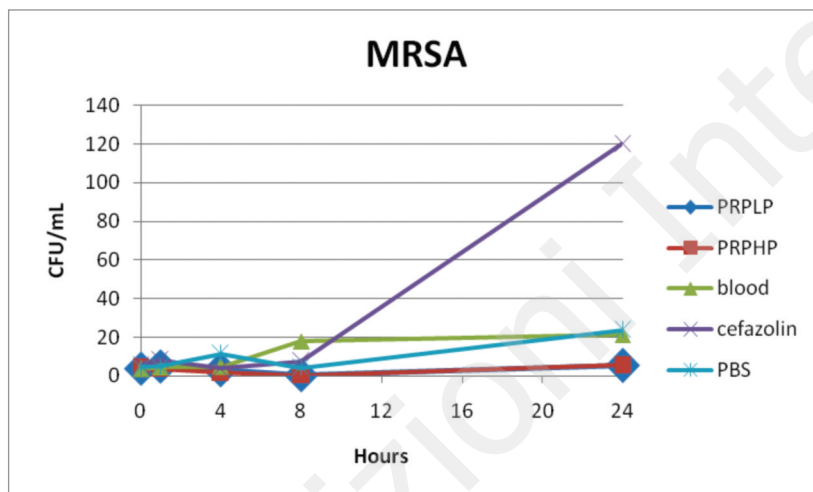


Figure 4. *Methicillin Resistant Staphylococcus aureus* growth in experimental groups as measured by colony count. CFU: Colony forming units.

Legend: PRPLP = platelet rich plasma-low platelets; PRPHP = platelet rich plasma-high platelets; PBS = phosphate buffered saline.

Discussion

This study was designed to evaluate two PRP preparations with regard to antimicrobial activity against *S. aureus*, *S. epidermidis*, *P. acnes*, and *MRSA*. Two commercially available preparations of PRP were used in this study in an effort to compare low platelet and WBC concentration to high platelet and WBC concentration.

The observed anti-bacterial properties of PRP are consistent with past *in vitro* studies of *S. aureus* and *S. epi*⁴⁻⁹. Little evidence exists documenting PRP's inhibitory effect on *P. acnes* and *MRSA*. Although a statistically significant effect was found with each bacteria, the effect of PRP_L and PRP_H on *P. acnes* and *MRSA* was minimal and may not be clinically significant. This decreased effect may be explained by the characteristics of the bacteria studied: *P. acnes* is a gram positive anaerobic rod bacteria and *MRSA* differs from *S. aureus* in its altered Penicillin Binding Protein (PBP). Likewise, the decreased effect of cefazolin is also explained by the microbiology of these bacterium. Furthermore, the pharmacokinetic and

pharmacodynamic properties of PRP and other antibiotics may prove a more meaningful prediction of *in vivo* efficacy than the strength of the agent *in vitro*²¹. PRP's maximum inhibition was consistently seen around 8 hours with inhibitory effects diminishing after 24 hours. A clinical effect could manifest in the first few hours following surgery – a crucial time to counteract any intra-operative bacterial exposure. Knobben et al. found intra-operative contamination in as many as 30% of operations²². Likewise, Davis et al. showed that the vast majority of contaminated operating room equipment was positive for coagulase negative staphylococci²³, an organism that PRP successfully inhibited.

In this study, whole blood was chosen as a physiologically relevant positive control. Hemarthrosis and blood clots frequently form in the joint following a surgical procedure. Thus, the potential antibacterial properties of PRP compared to whole blood are clinically relevant.

Significance between the two PRP preparations was found at select time points, as seen with *S. epidermidis*

and *P. acnes* at 8 hours and *MRSA* at 1 and 4 hours. In each of these circumstances, PRP_{HP} showed a significant decrease over PRP_{LP}. However, at 24 hours there were no observed statistically significant differences between the two preparations.

The platelet concentrations found in PRP_{LP} and PRP_{HP} were significantly different. This is consistent with past publications that have demonstrated a statistically significant difference in platelet counts between similar PRP_{LP} and PRP_{HP} systems¹⁶. Likewise, these same studies have documented a wide intra-individual variation in platelets, white blood cells, and growth factors¹⁶.

In 2002, Tang et al. identified seven thrombin-releasable antimicrobial peptides from human platelets: platelet factor 4 (PF-4), RANTES, connective tissue activating peptide 3 (CTAP-3), platelet basic protein, thymosin β -4 (T β -4), fibrinopeptide B (FP-B) and fibrinopeptide A (FP-A). All except FP-B and FP-A could also be purified from acid extracts of nonstimulated platelets. When testing these peptides against *E. coli*, *S. aureus*, *Candida albicans* and *Cryptococcus neoformans*, antimicrobial activities were found to be dose dependent. Tang et al.'s findings are consistent with the dose dependent relationship found in this study against *S. epi*, *P. acnes* and *MRSA*¹⁰.

The role leukocytes play in antimicrobial properties of PRP concentrations remains a debated topic¹⁵. Conflicting data has shown both a dose independent and dose dependent response of antimicrobial activity to increasing leukocyte concentration in PRP preparations¹⁵. In fact, leukocyte presence in PRP has been proposed as an additional source of growth factors, antimicrobial cytokines, and myeloperoxidase activity²⁴. However, Moorjen et al.⁹ found no correlation between myeloperoxidase activity and bactericidal properties of PRP preparations against *Staphylococcus aureus*. In addition, no difference in antibacterial activity was seen between PRP preparations with high-leukocyte concentration, and no leukocytes⁹.

This study was not designed to differentiate the antimicrobial activity of platelets relative to leukocytes. However, our results suggest that in the commercial setting, PRP preparations with low leukocyte concentration (PRP_{LP}) may produce equal antibacterial effects relative to preparations which produce higher leukocyte counts (PRP_{HP}).

Limitations

There are several limitations of this study. First, the *in vitro* behavior of bacterial plates may not mimic the *in vivo* environment surrounding the joint. Second, the clinical benefits are variable and unable to be correlated to a fixed reduction in bacterial number. The appropriate concentration of PRP remains to be further explored and could have a substantial effect on PRP's antibacterial properties. The final limitation was due to the methods requiring a cumbersome number of plate assays that were performed to evaluate four bacterial strains, including two experimental

PRP groups, two positive controls, and one negative control, with additional technical replicates (n=4) within groups. Therefore, the study was only able to use PRP preparations from two individuals, yielding only two biological replicates. Therefore, this study cannot exclude the possibility of inter-donor variability.

A power analysis was not performed on this *in vitro* study. Since we are unable to quantify how an *in vitro* reduction in bacterial colonies correlates to a clinical effect, we did not feel that a power analysis would be justified. Rather, the goal of this study was to determine trends amongst the PRP donors as evident by our statistical analysis which pooled all donors together.

Conclusion

PRP, regardless of preparation, has shown *in vitro* bacteriostatic properties against *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epi*), *Propionibacterium acnes* (*P. acnes*) and methicillin-resistant *Staphylococcus aureus* (*MRSA*). The application of PRP_{LP} and PRP_{HP} showed a significant decrease in bacterial growth after 8 hours for *S. aureus*, *S. epi*, *MRSA* and *P. acnes* compared to the whole blood control group. *S. epi*, *MRSA*, and *P. acnes* also showed a significant decrease in bacterial growth after 24 hours. Despite differences in platelet concentration and WBC concentration, no difference in antibacterial activity was seen between the two preparations.

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