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An evaluation of the bacteriostatic effect of platelet-rich plasma

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Abstract

Chronic wounds are a considerable health burden with high morbidity and poor rates of healing. Colonisation of chronic wounds by bacteria can be a significant factor in their poor healing rate. These bacteria can develop antibiotic resistance over time and can lead to wound infections, systemic illness, and occasionally amputation. When a large number of micro-organisms colonise wounds, they can lead to biofilm formation, which are self-perpetuating colonies of bacteria closed within an extracellular matrix, which are poorly penetrated by antibiotics. Platelet-rich plasma (PRP) is an autologous blood product rich in growth factors and cytokines that are involved in an inflammatory response. PRP can be injected or applied to a wound as a topical gel, and there is some interest regarding its antimicrobial properties and whether this can improve wound healing. This study aimed to evaluate the in vitro bacteriostatic effect of PRP. PRP was collected from healthy volunteers and processed into two preparations: activated PRP—activated with calcium chloride and ethanol; inactivated PRP. The activity of each preparation against *Staphylococcus aureus* and *Staphylococcus epidermis* was evaluated against a control by three experiments: bacterial kill assay to assess planktonic bacterial growth; plate colony assay to assess bacterial colony growth; and colony biofilm assay to assess biofilm growth. Compared with control, both preparations of PRP significantly inhibited growth of planktonic *S aureus* and *S epidermis*. Activated PRP reduced planktonic bacterial concentration more than inactivated PRP in both bacteria. Both PRP preparations significantly reduced bacterial colony counts for both bacteria when compared with control; however, there was no difference between the two. There was no difference found between biofilm growth in either PRP against control or against the other preparation. This study demonstrates that PRP does have an inhibitory effect on the growth of common wound pathogens. Activation may be an important factor in increasing the antimicrobial effect of PRP. However, we did not find evidence of an effect against more complex bacterial colonies.

KEYWORDS

antimicrobial therapy, chronic wounds, platelet-rich plasma

1 | INTRODUCTION

Chronic wounds are often colonised by micro-organisms, which may reduce their potential to heal. In up to 90% of wounds, these micro-organisms develop into biofilms.[1,2] Biofilm formation occurs when micro-organisms create an extracellular polymeric substance (EPS) that traps floating bacteria in a three-dimensional aggregation, allowing the biofilm to firmly adhere to a wound surface. This protective arrangement makes the enclosed micro-organisms robust against attack[3] and antibiotic penetration.[4] Protection against antibiotics is enhanced by the slower metabolic rate of certain biofilm cells. Bacterial cells found at the centre of the biofilm arrangement tend to exist at a lower metabolic rate than more peripheral cells, which in turn pass on nutrients through the EPS to the central cells via diffusion. The central cells, known as "persister" cells, are in essence metabolically inactive, meaning that antibiotics have limited effect.[5,6] Furthermore, biofilm bacteria can develop direct antibiotic resistance,[7] probably due to the overexpression of resistant genes.[8]

Figure 1 illustrates the process of biofilm formation. Initially bacteria adhere to a surface that offers the optimum conditions for survival through attachment of membrane structures such as pili and interaction between cell surface molecules such as lectins.[9] Once stable, the microbe will undergo multiplication and the adhesion will become irreversible.[10-12] This is self-perpetuated by cell-to-cell chemical signalling between bacteria known as quorum sensing, which leads to an increase in cell concentration. Once a certain concentration of bacteria is reached, the cell signalling leads to the formation of EPS, which binds the cells together in a three-dimensional structure. This extracellular matrix provides protection as well as allows diffusion of nutrients.[13] When a biofilm colony is established, fragments of the colony as well as individual cells can detach and embolise to a new site elsewhere in the body.

Key Messages

- colonisation of chronic wounds can contribute to poor healing
- the antimicrobial properties of platelet-rich plasma (PRP) may help heal chronic wounds
- the aim of this article was to examine the bacteriostatic effect of activated and inactivated PRP
- we found that PRP in both forms reduces bacterial growth, and activated PRP reduces growth more in planktonic bacteria
- there is no effect of PRP against organised biofilm colonies

When wounds are treated with platelet-rich plasma (PRP), there may be an antimicrobial effect that contributes to enhanced wound healing. Platelets are known to have antimicrobial action against individual bacteria and biofilms. They accumulate immediately at the site of endothelial damage caused by microbial colonisation and play an essential natural defensive role in the body's fight against infection. Alpha granules are rich in growth factors (GF) and cytokines, which encourage an inflammatory response, recruiting immune cells to the site of injury to attack pathogens.[14] When platelets are exposed to bacteria, they participate in bacterial co-adhesion resulting in bacterial sequestration and phagocytosis of bacteria.[15] Platelets also support neutrophils in creating cell-to-cell interactions with endothelial cells and leucocytes.[16] A diabetic infected wound rat model demonstrated that platelets are able to shield immortalised human keratinocytes from damage caused by bacteria, stimulate anti-inflammatory cytokines, and cell proliferation, while at the same time inhibiting pro-inflammatory cytokines.[17]

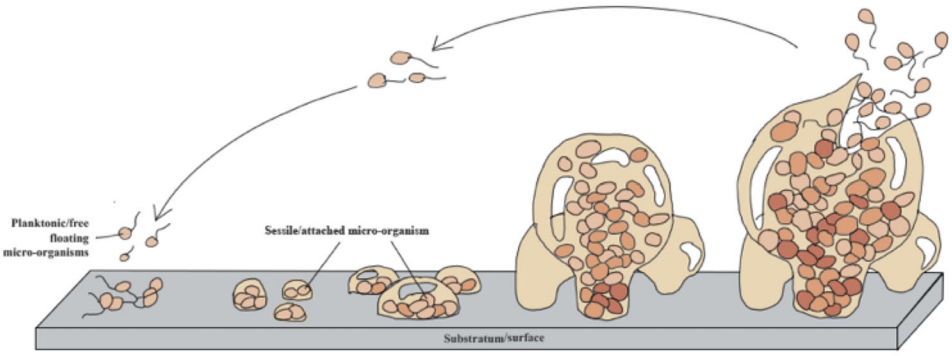

FIGURE 1 Diagram illustrating the developmental stages of biofilm formation: (a) adhesion/to a surface; (b) multiplication of microorganisms; (c) micro-colony formation; (d) maturation of biofilms; (e) dispersal and detachment leading to continuous repetition of the cycle

Alpha granules also contain molecules known as platelet microbicidal proteins/peptides (PMPs), which when released have an antimicrobial effect.[18] PMPs can act as chemokines, recruiting immune cells, and exaggerating a host response against pathogens. Certain PMPs, known as kinocidins, can also exert a direct antimicrobial effect, killing bacteria as well as coordinating the immune response.[19,20] Common PMPs are platelet factor-4 (PF-4)/CXCL-4, neutrophil-activating protein-2 (NAP-2)/CXCL-7, interleukin-8/CXCL-8, regulated upon activation normal T-cell expressed (RANTES)/CCL-5, and thymosin beta-4.

NAP-2/CXCL7 (thrombocidin-1 and thromocidin-2) is bactericidal against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis*, and *Lactococcus lactis*, and also fungicidal for *Cryptococcus neoformans*.[19] PF-4/CXCL-4 also exhibits bactericidal activity against *S aureus* and *Salmonella thyphimurium*.[20] Platelet alpha granules also secrete Fc receptor for immunoglobulin, complement factors C3a and C5a (C3a: increased vascular permeability; C5a: chemoattractant), as well as numerous chemokine toll-like receptors (TLRs), which are capable of provoking reactive oxygen species (ROS) and reactive nitrogen species (RNS). The host defence mechanism against infectious agents is also facilitated by ROS and RNS.[21] Furthermore, myeloperoxidase, which produces hypohalous acid, is found in alpha granules and has antioxidant and antimicrobial actions.[21]

PRP may work as an adjunct to conventional antibiotic therapy in wound infection or in some cases as an alternative due to the potentially lower risk of drug resistance.[22] PRP may also assist in reducing colonisation in uninfected chronic wounds. Several studies, summarised in Supplementary Table 1, have demonstrated that PRP may inhibit the growth of both gram-positive and gram-negative bacteria.[15,22-38] One study also demonstrated an inhibitory effect against *S aureus*, but not *E coli* or *Pseudomonas aureginosa*, in a diabetic rat model, suggesting that the effect may be carried over to diabetes.[17] PRP also has an inhibitory effect on the growth of the fungus *Candida albicans*[23] and the pathogenic yeast *C neoformans*.[18]

In addition to platelets, other cells found within PRP may also contribute to an antimicrobial effect. Leucocytes are found within PRP and the composition can be altered to include more or less during processing. Some studies in Supplementary Table 1 have found that leucocyte-rich PRP may have a more significant antibacterial response possibly due to leucocyte-platelet aggregation causing an enhanced inflammatory response although the evidence is not conclusive. Complement found within PRP may also contribute to the antibacterial response.[30]

1.1 | Study aims

Several studies have found that PRP has a direct bacteriostatic effect on common wound pathogens. However, the literature is limited, with few published studies and no evidence that has directly evaluated the effect of PRP on biofilms. Furthermore, no studies have compared the effect of activation on the antimicrobial effects of PRP against an inactivated control.

Therefore, the aim of this study was to evaluate the antimicrobial effect of PRP on bacterial colonies of common wound pathogens and to assess whether activation of the PRP affected this activity.

2 | MATERIALS AND METHODS

Institutional ethical approval was obtained for the study and all experiments were carried out in compliance with the Declaration of Helsinki. Blood was collected from three male volunteers. No volunteer had any significant past medical history, had platelet disorders, or was taking anticoagulants.

2.1 | PRP preparation

To produce PRP, whole blood (52 mL) was obtained via peripheral venipuncture and was combined with 8 mL of adenosine citrate dextrose acid (ACD-A). PRP with a haematocrit of 8% was produced using the Angel PRP processing device (Arthrex, Naples, Florida), which utilised an automated two-step centrifugation method. Following centrifugation, an automated 3-sensor ultraviolet flow cytometry method sensed cell-specific wavelengths of light for platelets, red blood cells, and platelet poor plasma (PPP), allowing their separation into sterile compartments.

10 mL of PRP from each participant was divided into two 5 mL aliquots. One aliquot underwent activation with calcium chloride ($CaCl_2$) and ethanol using an ActiVat device (Arthrex, Naples, Florida). This device combined 12 mL of PPP with 0.13 mL of 10% $CaCl_2$ and 2.4 mL of 10% ethanol. The mixture was shaken with the reaction taking place on glass resin beads found within the device to encourage activation of thrombin. The mixture was left for 15 minutes and was then combined with the PRP at a 1:3 ratio (1.67 mL additive: 5 mL PRP). One aliquot remained inactivated. The activated and inactivated PRP samples were tested against a phosphate-buffered saline (PBS) control as outlined in Table 1. Supernatant from activated and inactivated PRP was also tested against a PBS control using the plate colony assay.

After collection, PRP samples were used immediately in the experiments. Samples were centrifuged using a microcentrifuge (PicoFuge, Stratagene, California) and vortexed to ensure proper mixing prior to use. For the supernatant experiment, separate PRP samples were collected and stored in an incubator with humidified air at 37°C for 1 hour. Supernatant was then extracted, placed in Eppendorf tubes, and stored in a freezer at −80°C until ready for experimentation. All samples were thawed and used in experiments within 2 weeks of storage. Supernatant samples were thawed only once to prevent excess growth factor activation with repeat freeze–thaw cycles and were centrifuged and vortexed prior to use.

2.2 | Bacteria preparation

The strains used in this study were *S aureus* NCTC 8235-4 and *Staphylococcus epidermidis* RP62A strain (ATCC 35984), both of which have been used extensively in previous laboratory studies and have been shown to form biofilms.[39,40] Bacterial stock vials were stored at −80°C and once ready for use were thawed at room temperature and then applied to Brain Heart Infusion (BHI) agar plates to grow bacterial cultures. Once bacteria were streaked onto plates, they were kept in a shaking incubator at 37°C for 24 hours under continuous rotation at 200 rpm (SciQuip, Newtown, UK). Gram stains were then performed on the plates to assess for colonial morphology and purity. Cultures were then kept in an incubator until ready for use.

For each experiment, fresh bacterial cultures were prepared as follows. A single colony of *S aureus* and *S epidermidis* were added to two sterile 50 mL screw cap tubes containing 15 mL of BHI broth. A third tube containing BHI broth-only acted as a control. The tubes were transferred to the shaking incubator at 200 rpm at 37°C for 16 to 18 hours. Bacteria were then removed and pipetted into cuvettes in a 1:10 ratio with BHI broth (900 μL of BHI broth: 100 μL of bacterial inoculum) and were mixed 10 times with a pipette. 1 mL of the BHI control broth was added to a cuvette to act as a control. The samples were then passed through a spectrophotometer to obtain a measure of concentration of bacteria—colony-forming units/ml (CFU/mL). The samples were then diluted (based on the optical density readout) with BHI broth to gain the desired concentration prior to use.

TABLE 1 A summary of the test variables

	Test variable
1	Phosphate-buffered solution (PBS)
2	Activated PRP
3	Inactivated PRP

2.3 | Bacterial kill assay

This experiment was designed to assess the effect on planktonic bacteria at 1 and 4 hours after contact. This assay is considered the best practice to quantitatively analyse antimicrobial activity over time and has been used in multiple previous studies.

Five experimental samples were prepared in 15 mL screw cap tubes for each bacteria as follows—1500 μL BHI, 250 μL bacterial inoculum, and 250 μL of test variable These samples were prepared to achieve an inoculum concentration of 1×10^5 CFU/mL, which is the laboratory marker for diagnosis of infection. Tubes were then placed in the shaking incubator at 200 rpm at 37°C. At 1 and 4 hours, the tubes were removed, vortexed for 10 seconds, and aliquots of 200 μL were removed and placed into one row of a 96-well plate. Serial dilutions were performed to obtain eight concentrations. A 20 μL aliquot of each dilution was applied to half of a BHI agar plate and spread evenly using L-shaped spreaders. Plates were incubated for 24 hours and bacterial colonies were then counted and concentration was expressed as 10 Log CFU/mL. Bacteriostatic activity was defined as no increase in growth of bacteria from the original inoculum concentration. Bactericidal was defined as a reduction of at least 99.9% of the total count of CFU/mL in the original inoculum. Data generated from the experiment were mean concentration of bacteria at 1 and 4 hours (10 Log CFU/mL). Concentrations for each preparation at each time point were compared using statistical analysis.

2.4 | Plate colony assay

This experiment was designed to examine the effect of PRP against established bacterial colonies. The protocol for this assay was adapted from the Kirby-Bauer disk diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[41] Overnight bacterial cultures were prepared as in step 2.2 and were then diluted to a concentration of 1×10^5 CFU/mL after measuring with the spectrophotometer. A sample of each inoculum was then applied evenly to agar plates using a sterile swab and left for 15 minutes. A 50 μL volume of each test variable was applied to 6-mm-diameter sterile filter paper discs (180 μm thick, 11 μm pore size), and the discs were then applied to each bacterial lawn. After 15 minutes, the plates were placed in an aerobic incubator at 37°C for

24 hours. Standardised images of each plate were taken and the number of colonies present within 18 mm of the embedded discs were counted using ImageJ (National Institute of Health) to ensure more consistent results when compared to manual counting.[42] The mean numbers of colonies counted for each preparation were compared using statistical analysis.

2.5 | Colony biofilm assay

This experiment was designed to examine the effect of PRP against biofilm colonies. The protocol for this assay was modified from a validated biofilm growing protocol,[43] which ensures that bacterial colonies once formed are less likely to detach. This ensures that variations in colony counts are more likely due to cell death rather than detachment.

Overnight bacterial cultures were prepared and were then diluted to a concentration of 1×10^5 CFU/mL after measuring with the spectrophotometer. Sterile 25-mm nitrocellulose membrane filter paper discs were placed in agar plates using sterile forceps. The discs were inoculated with 5 μL of each inoculum, and these were then incubated upside down to prevent condensation at 37°C for 24 hours. After 24 hours, 100 μL of each test variable was applied to the discs and the plates were transferred back to the incubator for a further 24 hours. Each disc was then added to a sterile 50 mL screw cap tubes with 5 mL of sterile PBS and six 3-mm glass beads. The tubes were vortexed twice for 60 seconds to detach the bacteria. Aliquots of 200 μL were removed and placed into one row of a 96-well plate. Serial dilutions were performed to obtain eight concentrations. A 20 μL aliquot of each dilution was applied to half of a BHI agar plate and spread evenly using L-shaped spreaders. Plates were incubated for 24 hours and bacterial colonies were then counted and concentration was expressed as 10 Log CFU/mL. The use of solid agar plates to grow biofilm colonies in this method allows growth of a fractal colony with numerous morphologies via the diffusion limited aggregation process.[44] Data generated from the experiment were mean concentration of bacteria (10 Log CFU/ml). Concentrations for each preparation were compared using statistical analysis.

2.6 | Statistical analysis

All experiments were performed in triplicate. Data were presented as mean ± SD. Statistical analysis was performed using IBM Statistical Package for the Social Sciences (SPSS) Version 24 (IBM Corp., Armonk, NY). Comparison of means (bacterial concentration and colony count) was undertaken using an unpaired *t*-test or a Mann-Whitney *U* test for non-parametric data. A *P* value of <.05 was considered significant.

3 | RESULTS

3.1 | Bacterial kill assay (planktonic bacteria)

Using both *S aureus* and *S epidermidis*, both activated and inactivated PRP showed a significant reduction ($P < .05$) in bacterial concentrations compared with control at both 1 and 4 hours. Activated PRP also showed a significant reduction at both 1 and 4 hours compared with inactivated PRP. The results are summarised in Figures 2 and 3. The results for *S aureus* indicate that both PRP samples are bacteriostatic—with activated showing the greater effect—and with the maximum effect coming in the first hour, but neither PRP is bactericidal. The results for *S epidermidis* show that both PRP samples are relatively bacteriostatic, again with the maximum effect coming within 1 hour, but less so compared with their effect on *S aureus*.

3.2 | Plate colony assay (bacterial colony)

The mean numbers of *S aureus* colonies identified in both activated (103.44 ± 17.47) and inactivated (110.11 ± 30.74) were significantly lower when compared with control (144.11 ± 31.02). However, there was no significant difference between types of PRP Figure 4.

The mean numbers of *S epidermidis* colonies identified in both activated (91.22 ± 26.82) and inactivated (91.88 ± 26.81) were significantly lower when compared

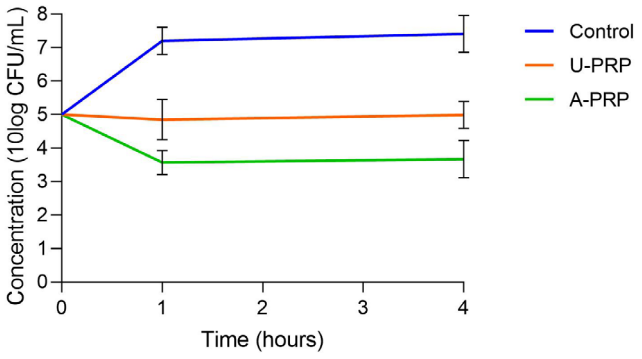

FIGURE 2 Change in planktonic *Staphylococcus aureus* concentrations from baseline to 4 hours

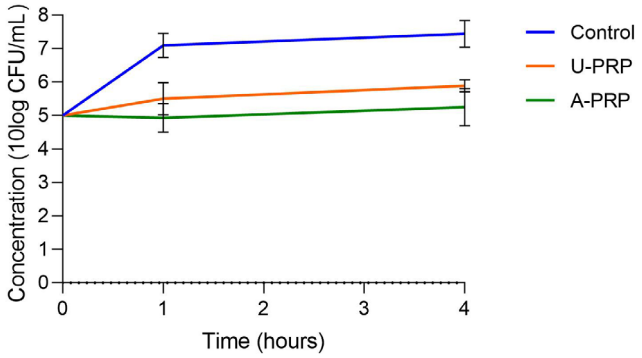

FIGURE 3 Change in planktonic *Staphylococcus epidermidis* concentrations from baseline to 4 hours

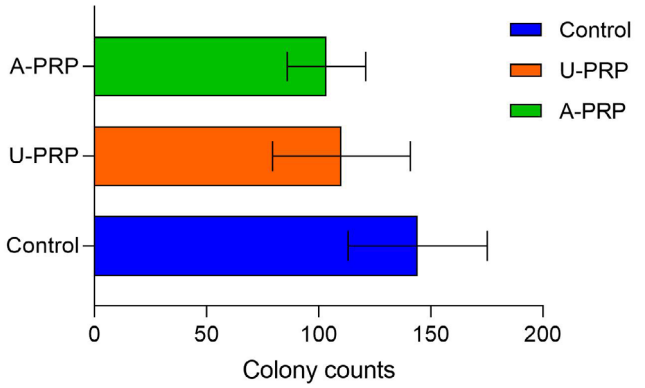

FIGURE 4 Mean number of *Staphylococcus aureus* colonies on plate colony assay

with control (143.66 ± 47.20). However, there was no significant difference between types of PRP Figure 5.

When PRP supernatant from both activated and inactivated PRP was tested against control using a plate colony assay, there was no difference between the number of colonies observed between any of the groups.

3.3 | Colony biofilm assay (biofilm)

For the *S aureus* colony biofilm assay, there were no significant differences between activated PRP (11.89 ± 0.47 log CFU/mL), inactivated (11.99 ± 0.37 log CFU/mL), and control (12.20 ± 0.31 log CFU/mL. These results also indicate an increase in the number of bacterial colonies from the baseline inoculum concentration of 5 log CFU/mL over 24 hours in all three groups. Figure 6.

For the *S epidermidis* assay, there were also no significant differences between activated PRP (11.95 ± 0.35 log CFU/mL), inactivated (11.97 ± 0.48 log CFU/mL) or control (12.37 ± 0.39 log CFU/mL). Again all three groups

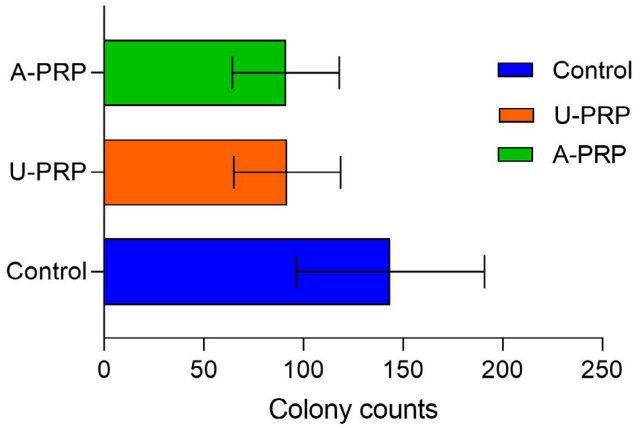

FIGURE 5 Mean number of *Staphylococcus epidermidis* colonies on plate colony assay

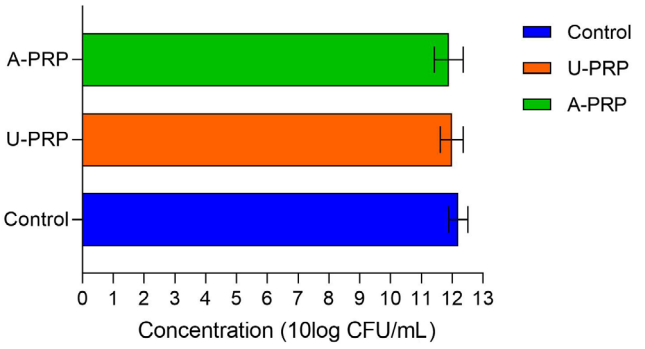

FIGURE 6 Mean *Staphylococcus aureus* biofilm concentration

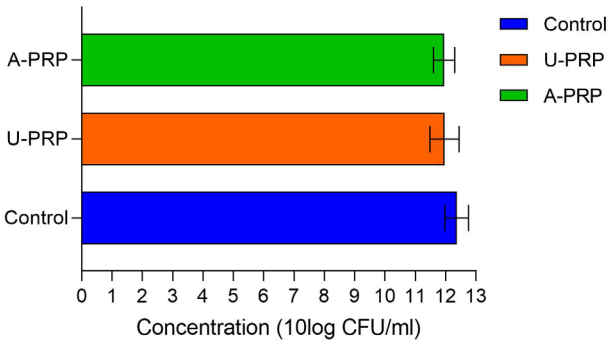

FIGURE 7 Mean *Staphylococcus epidermidis* biofilm concentration

showed an increase in colony concentration from the inoculum baseline Figure 7.

4 | DISCUSSION

Bacterial colonisation and infection of chronic wounds can have a significant impact on the wound healing

process and are likely to contribute to poor healing rates. The use of PRP as a wound healing treatment is becoming more popular, and there is reasonable evidence to show that PRP confers an antimicrobial effect, which in turn may assist in wound healing. Several previous studies have found that PRP can reduce the growth of *S aureus* and *S epidermidis* bacteria in vitro. However, no previous studies have investigated whether activation of a PRP preparation can affect its antimicrobial activity against planktonic bacteria, bacterial colonies, or biofilm in a single study.

Our findings suggest that both inactivated and activated PRP are bacteriostatic against both strains of bacteria when tested against simple planktonic bacteria. The maximum effect is seen within 1 hour, which is supported by the findings of a previous study,[25] but the effect is also maintained up to 4 hours. Neither of the preparations showed a strong enough inhibitory effect to be classed as bactericidal with the log CFU/mL remaining similar to the baseline inoculum concentration. However, no study in the literature has previously demonstrated a bactericidal effect against *S aureus* and *S epidermidis*, with only one study demonstrating bactericidal activity against *Enterococcus faecalis, Streptococcus agalactiae, and Streptococcus oralis*.[34] The bacteriostatic effect of PRP was also demonstrated in this study by the plate colony assay, which illustrated that both preparations of PRP significantly reduced the number of bacterial colonies compared with control.

Our findings also found that activation of PRP with calcium chloride and ethanol significantly improves the bacteriostatic effect on planktonic bacteria. Two previous studies have shown that activation with thrombin (autologous and bovine) shows improved bacteriostatic effect when compared against inactivated PRP[26] and PPP.[36] However, ours is the first study to demonstrate the antimicrobial effect of activation with calcium chloride and ethanol on a preparation of PRP against different strains of bacterial culture. The literature suggests that activation is likely to confer an enhanced antimicrobial effect due to the increased release of alpha granules, which contain PMPs and kinocidins. PMPs enhance the immune response by recruiting immune cells to the site of infection and exaggerate the inflammatory response and are therefore unlikely to have a significant effect in vitro. However, some PMPs such as NAP-2/CXCL7 and PF-4/CXCL-4 have been shown to have a direct bactericidal effect.[19,20] Kinocidins also have a direct bactericidal effect and may contribute to the effect in vitro. The finding that PRP supernatant known to be rich in growth factors had no effect on colony growth in this study (even when activated) also supports the theory that it is the platelets and the PMPs that provide the antimicrobial effect. However, our study found that the enhanced effect of activation was not seen in more complex bacterial colonies. This may be due to the more complex structure of these bacteria, which are less vulnerable to direct topical application of PRP without an immune response to provide additional antimicrobial support.

This finding is further demonstrated in the lack of effect of both PRP preparations against biofilms. Ours is the first study to evaluate an activated and inactivated PRP preparation against more complex biofilm cultures. The lack of effect is most likely due to the fact that biofilms are known to be up to ×1000 more resilient to antimicrobial agents than planktonic bacteria and therefore require much higher concentrations to show an effect.[45,46] This is because biofilm bacteria are able to protect themselves within the EPS. The antimicrobial agents secreted by alpha granules are less likely to penetrate the biofilm cultures because of the role of diffusion-limited transport of substances through the biofilm EPS, which dilutes them as they pass through the colony. Our study also used relatively low concentrations of PRP against biofilms, which would have limited the amount of PMPs and kinocidins that could take effect. One previous study has demonstrated that there is a clear "window of opportunity" to take effect against biofilms, between the initial adhesion and irreversible binding of the bacteria, when a treatment has a chance of eradication.[2] During this period, the bacteria remain susceptible; however, once developed they are difficult to penetrate. Therefore, topical PRP treatment seems unlikely to be effective against established biofilms. In clinical practice, PRP may be more effective in more established colonies when used along with antibiotic therapy.

4.1 | Limitations

One limitation of the study was the examination of PRP against only two bacterial strains in isolation. *S aureus* and *S epidermidis* are commonly involved in chronic wound colonisation, biofilm formation, and antibiotic resistance.[47,48] However, chronic wounds are often colonised by a myriad of organisms, which unite in a complex biofilm structure. Therefore, the testing against these bacteria alone is not directly representative of the clinical environment. in vitro models for multi-species biofilms do exist; however, there is a lack of consensus over the accuracy and clinical applicability of these models.[49] In order to effectively test PRP against established biofilms, in vivo animal models or clinical studies would be required.

Another limitation is the small number of preparations of PRP tested. There have been several different

PRP preparations evaluated by previous authors for their antimicrobial effect. There are also a plethora of commercial devices and methods of PRP preparation and activation,[50] meaning the numbers of permutations in preparation are infinite. To our knowledge, no previous study has investigated antimicrobial effect of PRP harvested by the Angel device or activated by the ActiVat device. This study could have varied the platelet concentration, the haemocrit, and the activation method of the PRP; however, only a randomised controlled trial of the many different PRP preparations will provide more conclusive answers.

5 | CONCLUSION

This study has demonstrated that PRP has a bacteriostatic effect on *S aureus* and *S epidermidis* when tested against planktonic bacteria and bacterial colonies. Activation of the PRP with calcium chloride and ethanol also gives an enhanced effect against planktonic bacteria. However, there was no effect of either PRP preparation against more complex biofilm colonies.

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CONFLICT OF INTEREST

No author has any conflict of interest

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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