The Contribution of Leucocytes to the Antimicrobial Activity of Platelet Rich Plasma Preparations: a systematic review

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INTRODUCTION

The topical use of autologous Platelet Rich Plasma (PRP) as a biological accelerator of the healing process has been safely used as a form of treatment for wounds since the 1990s [1,2]. Platelets promote wound healing through their release of a vast array of granular components and biological mediators, including growth factors (which promote the chemotaxis of leucocytes, fibroblasts and myofibroblasts, as well the synthesis of extracellular matrix (ECM) and promotion of angiogenesis [2-4]). Additionally, platelets are well recognized for their role in the host defense system [5], which results from the release of antimicrobial peptides (AMP) contained in the granules, and through inducing expression of antimicrobial proteins from other cells [5-7].

Many different procedures have been used to obtain PRP from whole blood, leading to heterogeneity in preparations [8]. During this process it is also possible to include leucocytes in the final preparation (which would then be referred to as Leucocyte-platelet rich plasma (L-PRP) [9]) at different concentrations, or totally exclude leucocytes from the preparation.

Although a large body of literature exists detailing the wound healing and antibacterial properties of platelets, the inclusion of leucocytes in the PRP preparation has been neglected for many years [9]. The potential role(s) of leucocytes included in PRP has yet to be fully explored despite the well-recognized role for leucocytes in tissue repair and host defense [9-11], and there are contrasting reports regarding the benefits of including leucocytes within PRP preparations [12]. Several small but promising studies have demonstrated benefits in wound care of L-PRP, and antimicrobial properties have been reported [7,13,14]. However other authors discourage their inclusion in PRP, reporting

that this stimulates a pro-inflammatory environment that may negatively influence tissue regeneration [15]. Furthermore, an *in vitro* study recently suggested that the inclusion of leucocytes in PRP preparations does not enhance the bactericidal activity of PRP [16] .

The potential natural antimicrobial properties of a PRP preparation is an alluring addition to a preparation already recognized to the beneficial for wound healing. It is however unclear which components of the preparation are important for the bactericidal effect. Laboratory studies exploring the antimicrobial effect(s) of L-PRP are still ongoing; therefore it is timely to evaluate the current literature on this subject. It is beyond the remit of the work to detail how leucocytes exert their antimicrobial effects, but the reader is directed to the elegant review published in 2012 by Bielecki *et al*.[9].

In this review we aim to summarise and evaluate the literature on the contribution of leucocytes included in L-PRP to the antimicrobial properties of the preparation as a whole. Acknowledging the wide and poorly defined method variations in preparation of L-PRP, and the reported challenges in comparing the clinical and antimicrobial effects of PRP preparations [9, 18], the studies included in this review have been analysed in terms of the methodology used i) for the preparation of L-PRP and the other blood-derived products , ii) to study the antimicrobial activity. This should help to inform clinical practice and additional research in this promising field.

METHODS

Data Sources

A literature review of publications was performed by two independent reviewers in May 2016 in the Ovid MEDLINE, PubMed (1946-2016) and EMBASE (1974-2016) database.

In addition, the reference lists of all identified articles were examined to identify relevant papers that were not captured by electronic searches. MeSH Terms, Headings with Boolean operator for PubMed search were: ["platelet-rich plasma"[MeSH Terms] OR "platelet-rich"[All Fields] AND "plasma"[All Fields]] OR "platelet-rich plasma"[All Fields] OR ["platelet"[All Fields] AND "rich"[All Fields] AND "plasma"[All Fields]] OR "platelet rich plasma"[All Fields]] AND ["anti-infective agents"[Pharmacological Action] OR "anti-infective agents"[MeSH Terms] OR ["anti-infective"[All Fields] AND "agents"[All Fields]] OR "anti-infective agents"[All Fields] OR "antimicrobial"[All Fields]].

In addition, the terms “exp Platelet-Rich Plasma/ OR “exp Platelet-Derived-Growth Factor/” OR the following keywords: “L-PRF” OR “L-PRP” OR “Platelet Rich Fibrin” OR [“PLG” AND “Platelet-Rich Plasma"] OR [“PRF” AND “Platelet-Rich Plasma"] and “exp antimicrobial/” were used in MEDLINE and EMBASE.

Study selection criteria

Study eligibility was defined using the population, intervention, comparator, outcome, and study design approach (PICOS) [17]. The inclusion and exclusion criteria are summarized in Table 1. Study selection was performed through two levels of screening. In the first level, abstracts were reviewed for the inclusion and exclusion criteria. In the second level screening, all articles filtered through the first level were fully read and the selected inclusion and exclusion criteria applied. The eligibility of the studies was assessed independently by two authors, and discrepancies were resolved by discussion. The ultimate list of included articles was designated with the agreement of all the authors. To be included, studies needed to clearly address the leucocyte inclusion in the composition of PRP and report the haematology values. Comments, case reports and review papers were excluded. Studies with duplicate publications were only included

once, using the most recent publication. Only *in vitro* and human studies were included. Animal studies were excluded, since i) none of the animal studies extracted from the literature search compared PRP preparations including leucocytes to those without, and ii) the environment in an animal model is different to a human model and does not translate to the full potential of PRP-like products in terms of bactericidal capabilities for wound treatment.

Data extraction and analysis

Data were recorded using a data extraction table (Table 2). The following data from the full text of included papers were extracted and analysed: study design, methodology used for L-PRP preparation, haematological values of leucocytes and platelets, preparation comparators (different preparations tested), platelets activators, bacterial strains tested, other parameters tested, microbiological assay, overall antimicrobial effect. A formal statistical analysis was not performed because of the methodological heterogeneity, and small numbers of eligible studies.

RESULTS

Articles included

The literature search yielded 686 articles. From a first screening of the titles and abstracts, 643 citations were removed since they were not relevant to the topic or were duplicates. Therefore 43 articles progressed to the second level of screening. After retrieval of full text, review of each article, and application of inclusion and exclusion criteria (and addition of one article discovered by review of references), 11 papers were included in the final analysis [14,16,18-26] (Figure 1 and Table 2).

Study design

All the 11 papers included in the review were *in vitro* studies where various amounts of blood were drawn according to the requirements of the individual L-PRP preparation method (varying from 53 ml to 300 ml of blood from each donor). Blood was drawn from healthy human volunteers in eight studies [14,16,18-21,23,24,27], from patients with diabetic ulcers in one study [22], from horses in one study [25], and from rabbits in one study [26]. Only the *in vitro* data has been analysed for this latter study [26].

Parameters tested

The antimicrobial activity of L-PRP was tested against different bacterial strains including *Staphylococcus aureus* both methicillin-sensitive (MSSA), and resistant (MRSA), *Escherichia coli*, *Enterococcus faecalis, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus epidermidis*, *Bacillus cereus, Bacillus subtilis, Streptococcus pyogenes, Neisseria gonorrhoeae*, and *Propionibacterium acnes*. In addition to the antimicrobial properties of the biomaterial, other leucocyte and platelet parameters were also included in some studies. Moojen *et al*. [19] for example aimed to evaluate the contribution of leucocytes with their oxidative killing action, and therefore included in their study measurements of myeloperoxidase (MPO) activity and MPO release. MPO was gradually released by thrombin-activated, and non-activated PRP preparations in the first hours, withno significant differences detected in the MPO activity, and nocorrelation between MPO concentration, activity and bacterial killing. To test the antimicrobial contribution of leucocytes in the preparation, Chen *et al*., [22] used apocynin, an inhibitor of NADPH oxidase activity, to block the oxidative burst action of leucocytes. The authors did not find difference in the antimicrobial activity of the preparation where leucocytes function was inhibited compared to an activated L-PRP preparation. Release of different growth factors (F-4, TGF-β1, and PDGF-BB) at

various incubation times were also measured in one study [25], in order to test the possible antimicrobial contribution of different concentration of growth factors within PRP preparations, and the ability of bacteria to denature or reduce growth factors levels. Measurement of complement and antibody levels were also included in the study conducted by Wu *et al*. [23]. Using immunoassay kits, Mariani *et al.* [16] measured proteins released by PRP preparations, such as 'Macrophage Inflammatory Protein' (MIP)-1α (CCL3), 'Regulated on Activation Normal T Expressed and Secreted protein'(RANTES), GRO-α, Interleukin (IL)-8, Interleukin (IL)-6, neutrophil-activating protein (NAP)-2, and stromal cell-derived factor (SDF)-1α. When the concentration of the proteins and the bacteria growth inhibition was tested, the proteins showed strong antimicrobial potential. Other parameters tested were the contribution to the antimicrobial effect of activated (with different activators) and non-activated PRP [21-23,25]. In Li *et al*. [26] various concentrations of bovine thrombin were used to activate L-PRP and used to assess the role of thrombin in the antimicrobial properties of PRP.

Method of preparation of L-PRP and its influence of the haematological values of leucocytes and platelets

The methods of L-PRP preparation used in the selected studies were considerably different, as shown in Table 2: single or double centrifugation, different platforms, different spin and centrifugation values ('g' standing for multiples of earth gravitational field and 'rpm' standing for revolutions per minute) and different centrifugation duration times. Not surprisingly the variation within the methods of preparation caused variations in the quantities of the haematological components of the blood products. In all the included studies the processing methods resulted in enrichment of platelet concentration of the PRP, ranging from as lowest as two-fold [20,25,28] up to ten and eleven-fold [23, 26]. There was also a wide variation in the concentration of leucocytes in the L-PRP

after processing, varying from a low concentration of leucocytes used in an *in vitro* study (3.9-fold decrease from whole blood) [23] to a high concentration (4-fold enrichment) [26]. None of the studies specified the differential white blood counts, so the relative percentage of each type of white blood cell in the L-PRP preparations is unknown. The viability and function of leucocytes after the PRP processing was assessed in the study conducted by Moojen *et al.* [19]. Here the authors measured MPO concentrations after a single step centrifugation method used to prepare L-PRP, and found that neutrophils and monocytes were not only viable but biologically active as shown by the rapid increase in MPO concentration detected shortly after the addition of L-PRP to the bacterial culture.

Activation of L-PRP

Of the 11 studies, 9 prepared L-PRP as a gel form by activating platelets to release their granular components. L-PRP was activated by different materials including calcium chloride, bovine thrombin, calcium gluconate, or a combination of calcium chloride with bovine thrombin (Table 2). Only one study evaluated the antimicrobial effect of L-PRP in its pure, inactivated form [24].

Microbiology assay

The 11 studies included are very similar in terms of the methodologies used to test the biomaterials. Nine of the 11 (Table 2) used a bacterial killing assay, whereby bacterial cultures and the biomaterials were mixed together, incubated under agitation, and aliquots removed at certain time points (up to 24 hours) for serial dilution, plating and subsequent assessment of bacterial counts (in terms of CFU/ml). The evaluation of the zone of inhibition (Kirby-Bauer disk-diffusion method) was used in 3 studies [18,21,23]. As well as the methodologies being similar, there was good concordance in terms of the bacterial isolates tested, with 9 of the 11 papers testing *S. aureus.*

Antimicrobial outcomes

To our knowledge, the first paper addressing the antimicrobial activity of a PRP preparation with a clear recognition of the inclusion of leucocytes, was the study published in 2007 by Bielecki *et al.* [18]. The authors conducted an *in vitro* study drawing blood from healthy human volunteers to evaluate the antimicrobial activity of PRP activated with bovine thrombin in a 10% calcium chloride solution (PRG, Platelet Rich Gel) against the most frequent bacteria responsible of wound and bone infections: MSSA, MRSA, *E. coli* (Extended Spectrum Beta Lactamase, ESBL) and non-ESBL, *K. pneumoniae* (ESBL), *E. faecalis* and *P. aeruginosa*. High concentrations of both platelets and leucocytes were obtained (increased by 760% and 790% respectively) using a single centrifugation method. The antimicrobial activity of the L-PRP preparation was determined using a Kirby-Bauer disc-diffusion method, and showed different effects for different strains, with strong antimicrobial activity detected against MSSA, MRSA and *E. coli*, whilst no bactericidal activity was found against *E. faecalis*, *K. pneumoniae* and *P. aeruginosa*. Alarmingly the addition of L-PRP to *P. aeruginosa* led to an actual increase in growth. Even though the objective of the study was not specifically to evaluate the antibacterial activity of different concentrations of platelets or leucocytes, the authors stated that within the 20 blood samples studied, no correlation between antibacterial activity and the value of platelets and leucocytes in the blood and the platelet-rich plasma was detected. The study conducted by Moojen *et al*. [19] was the first attempt in recognising the specific contribution of leucocytes in L-PRP preparations. The authors used a semi-automated table top centrifuge to obtain PRP, reaching high concentrations of both platelets and leucocytes (platelets and leucocytes more than 7-fold and 3-fold enrichment, respectively). L-PRP was activated with either autologous or bovine thrombin, resulting in a preparation referred by the authors as PLG-AT or PLG-

BT (Platelet-leucocyte gel autologous (AT) or bovine thrombin (BT). Other comparators were PPP (Platelet Poor Plasma), PRP (Platelet Rich Plasma) and PBS (phosphate buffered saline), the latter which acted as a control. The different preparations were used to test antimicrobial activity against MSSA using a bacterial killing assay. Even though all the four blood preparations showed antimicrobial activity, the PRP containing high concentrations of both platelets and leucocytes, and activated with autologous thrombin (PLG-AT), proved to be the most antimicrobial (and to give the longest duration of effect) compared to PRP, PPP and PRP-BT. In this study non-activated PRP and PPP also exhibited some antimicrobial activity, but with a more delayed effect. Autologous activated PRP showed the largest effect for the entire 24 hours. There were no preparations resulting in 100% efficiency of bacterial killing. In order to explore the contribution of leucocytes to the antimicrobial properties shown by the L-PRP preparation, myeloperoxidase (MPO) activity and MPO release at different time points of incubation were measured. MPO was gradually released in PLG-AT, PLG-BT and PRP preparations in the first few hours. The authors speculated that the stronger antimicrobial effect found with the PLG-AT preparation is likely due to the effect of thrombin acting on the antimicrobial peptides released by platelets rather than effect of leucocyte activation. Different preparations including low and high concentrations of platelets (and inclusion or exclusion of leucocytes), were used by Anitua *et al*. [20] to evaluate the bactericidal effect of the different blood products against four bacterial strains. Here a bacterial kill assay was performed to test the antimicrobial activity of PRP products against MSSA, MRSA, methicillin-sensitive *S. epidermidis* (MSSE) and resistant *S. epidermidis* (MRSE). The different fractions obtained from plasma after a single centrifugation step were: F1 (plasma), which contained a 1-fold enrichment of platelets and no leucocytes compared to whole blood, F3 (PRP) containing 2.5-fold enriched

platelets and no leucocytes, and F3+leucocytes:1.8-fold enrichment of platelets and 3.9-fold enrichment of leucocytes. The authors found that after four hours all the fractions revealed bacteriostatic properties against MSSA and MRSA and MRSE. The preparation including leucocytes was revealed to be superior against MSSE, where it was the only fraction able to reduce the growth in the experiment. In order to explore the nature of the antimicrobial activity of PRP and specifically to investigate which components exert this effect, Burnouf *et al.* [21] tested the effect of different blood preparations against eight strains of wound bacteria (four Gram positive and four Gram negative bacteria). The authors compared PRP (containing leucocytes), calcium chloride activated PRP (PG: Platelet Gel), PPP (platelet poor plasma), solvent/detergent-treated PLT lysate (S/D P-L) and complement inactivated preparations . All the preparations were immediately frozen and kept frozen at ≤-20°C until use. PRP was processed by apheresis for the blood samples from the two donors obtaining a 6-fold enrichment for platelets and 1-fold for leucocytes. The authors performed a bacterial plate assay followed by colony counting, and assessed log reductions at 3 hours when the test sample was compared to the PBS controls. *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* all showed an initial log reduction in bacterial numbers 3 hours after spiking into the different preparations, however the reductions were lowest with the PG, and bacterial regrowth was seen at 48 hours with all preparations. The preparations were most effective against *E. coli*, where there was a large inactivation of bacteria (7.51 to >9.01 log), and no viable colonies for 48 hours after spiking. Furthermore, similar antimicrobial activity was seen in different preparations regardless of the concentrations of the platelets and leucocytes. As the PG preparation appeared to have the lowest antimicrobial effect on these strains, it seems that the use of calcium chloride for activation of coagulation could have decreased the bactericidal property of the PRP. Although there were some antimicrobial effects, none of

preparations were able to inhibit, *B. cereus, B. subtilis,* and *S. epidermidis*. Reassuringly, complement-inactivated control preparations did not show any antimicrobial activities. Having taken all into account, the authors concluded that plasma complement, rather than specifically platelets or leucocytes, are the elements responsible for the antimicrobial activity seen against *E. coli, K. pneumoniae,* and *S. aureus.* Chen *et al.* [22] investigated the *in vitro* antimicrobial activity of APG (autologous platelet-rich gel) extracted from the blood of patients with diabetic dermal ulcers, against the most common bacteria found in diabetic chronic wounds: *S. aureus, E. coli* and *P. aeruginosa*. To mitigate any confounding effects of previous IV antibiotics that the patients may have received as part of routine care, the blood for the PRP preparation was drawn from patients 8-12 hours after antibiotic administration. PRP was obtained through a double step centrifugation procedure and activated with thrombin and calcium gluconate. Comparator preparations were: APG, PRP (inactivated), PPP, and APG-APO (autologous platelet gel with apocyanin). Apocyanin is an inhibitor of NADPH oxidase activity, and is thus a way to study L-PRP antimicrobial activity, whilst excluding the possible antimicrobial contribution of leucocytes producing superoxide. PBS was used as a control. Both PLG and APG-APO were effective in reducing the bacterial counts of *S. aureus* compared to the PBS control in the first four hours, and were more effective during 24 hours compared to PRP and PPP. Since the APG, APG-APO and PRP preparations showed antibacterial effects against *E. coli* and *P. aeruginosa* compared to PBS (but there was no effect with PPP), the authors attribute the antimicrobial effects seen to the prior IV antibiotics administered to the patients, rather than the biomaterial preparations. The authors concluded that the antimicrobial activity seen against *S. aureus* is likely not due to the inclusion of leucocytes in the PRP preparation, as APG and APG-APO showed a

similar antibacterial effect. It is thought that the activity may be attributable to the thrombin and calcium gluconate used to activate the platelets.

In order to test the antimicrobial activity against Gram negative bacteria that are commonly present in enterocutaneous fistula tracts (including *E. coli*, *P. aeruginosa* and *K. pneumoniae*), Wu *et al*. [23] compared three different biomaterials, and a commercial fibrin glue called Bioseal®. Blood drawn from 14 healthy volunteers was centrifuged and processed to obtain: PLF (platelet-leucocyte fibrin) which is a preparation containing a high concentration of platelets (10-fold enrichment from whole blood) and low leucocytes (3.9 fold decrease from the baseline) activated with thrombin; PRP (similar concentrations of platelets to whole blood and 3.9 fold decrease in leucocytes from the baseline) and PPP (poor platelet concentration and no leucocytes). The Kirby-Bauer disc diffusion test and bacterial killing assays were used to compare the antibacterial activity of the different preparations. Also the levels of complement and antibodies (IgA, IgG, IgM, C3, C4) were measured in PLF, PRP and PPP and no significant difference was found between the different preparations. For the microbiological assays a greater antibacterial effect was found in with PRP and PLF compared to PPP. However, it was noted over time that PLF seemed to lose its antimicrobial effect more than PRP (although this was not statistically significant). The commercial fibrin glue (Bioseal®) did not show any antimicrobial effect. For the bacterial killing assay, the effect seemed only bacteriostatic, with the maximum killing of bacteria observed in the first four hours, followed by regrowth up to peak numbers at 24 hours. The antimicrobial activity against *P. aeruginosa* was dose-dependent, requiring higher concentrations of the PLF preparation than those that were effective for the other two bacterial species. In the *in vitro* study conducted by Intravia *et al*. [24], different PRP preparations with low and high concentrations of platelets and leucocytes were tested to verify the antimicrobial

properties against bacteria commonly found in arthroplastic surgery (MSSA, MRSA, *S. epidermidis* (MSSA) and *P. acnes*). Two different preparations were obtained using different centrifugation conditions (Table 2). The preparations contained low (PRPLP), or high (PRPHP) concentrations of platelets and leucocytes, and both showed a significant decrease in bacterial growth at 8 hours for all of the bacterial samples when compared to the controls. PRP rich in leucocytes and platelets (PRPHP) gave a superior decrease of *S. epidermidis* and *P. acnes* at 8 hours compared to the preparation with negligible inclusion of leucocytes and lower platelets (PHPLP). Also, at 1 and 4 hours after MRSA incubation, the preparation enriched with leucocytes (PRPHP) showed a stronger growth inhibition than the leucodepleted biomaterial (PRPLP). After 24 hours, significant inhibition was still seen for MSSE, MRSA, and *P. acnes* with both preparations. Similarly Lopez *et al*. [25], compared different preparations of PRP (with low and high levels of leucocytes, activated or in their pure form), in terms of their antimicrobial properties against MSSA and MRSA. Blood was drawn from 18 healthy horses, and using double centrifugation, the authors obtained a range of preparations containing different concentrations of platelets and leucocytes. These were enriched respectively by 1.64 and 2-fold from the blood baseline in the PRP preparation whereas in the LPP (leucocyte poor plasma) preparation, the leucocytes count was negligible. The pure form of these two products was also compared to the activated form (activated via calcium gluconate), and to plasma and to heat-inactivated plasma (IP). With the exception of IP, at six hours all blood components significantly inhibited bacterial growth. Furthermore, the non-activated form of PRP showed a better bacteriostatic activity against MRSA when compared to the activated form (LPG: leucocytes-poor plasma gel) and plasma. MSSA showed higher sensitivity to the treatments than MRSA, and PRP against MSSA showed the highest bactericidal effect followed by plasma, then LPP, LPG, and PRG.

Against MRSA, at 6 hours PRP was again the preparation showing stronger antimicrobial effect, whilst at 24 hours LPG and plasma had stronger bacteriostatic effect against MRSA. The release of growth factors (PF-4, TGF-β1, PDGF-BB) by the different preparations at different incubation times were also measured, but no correlation between bacterial counts and leucocytes, platelets, PF-4, TGB-β1, PDGF-BB was recorded suggesting that bacteria were not able to denature the growth factors, and that plasma components could be responsible for the antimicrobial effects observed. A recent publication by Mariani *et al.* [16] specifically investigated the possible contribution of leucocytes in a PRP preparation against different bacteria of relevance to bone, soft tissue and wound infections (including MSSA, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *E. faecalis*). Two different PRP preparations (including or excluding leucocytes) were obtained from 150ml of whole blood, drawn from 10 healthy donors. The preparation involved different centrifugation steps as shown in table 2, and resulted in L-PRP (leucocyte and platelet rich plasma), and P-PRP (pure platelet rich plasma). Moreover, to investigate whether or not the cryopreserved blood product influenced the antimicrobial effect, a third preparation (L-PRP cryo) was obtained by freezing the L-PRP preparation at −30°C for 2 hours. All the products were then used in their activated form for the experiment. As the three different preparations showed similar antimicrobial results (with bacterial growth inhibition observed for to the first 4 hours for all the preparations), the authors concluded that the inclusion of leucocytes does not contribute significantly to the antimicrobial activity of PRP. The inhibition varied between 1-4 log, according to the bacterial strain and experimental conditions tested. After treatment with the three plasma fractions, a time-dependent inhibition of bacterial growth (for up to 4 hours) and at low bacterial count for *E. coli*, and *P. aeruginosa*, and at higher numbers for *S. aureus*, and *Enterococcus faecalis* was detected. In contrast, there was not a time-

dependent inhibition of growth with *K. pneumoniae*. Generally the quantities and abundance of the microbicidal proteins correlated well with growth inhibition, where higher quantities of proteins correspond with greater inhibition. Thomsen *et al.* [14] have analysed the phagocytic fitness and bactericidal activity of leucocytes included (at a concentration of $55x10^6$/patch) in a multilayer matrix of fibrin and platelets, called a leucopatch. The authors observed that the neutrophils included in this preparation are active and capable of chemotaxis, phagocytic activity and respiratory burst. When *P. aeruginosa* was mixed with the leucopatch, the production of ROS (measured by chemiluminesce) of leucopatch PMNs, was substantial, and the response was concentration-dependent. The production of ROS during phagocytosis of *P. aeruginosa* was also tested in isolated PMNS at a concentration of10^7cells/ml). The resulting chemiluminescence signal was twice as high compared to the signal from leucopatch PMNs. When ROS production was tested for a longer time period of 7 days, ROS production was still observed at day 4. The chemotactic leucocyte migration was also investigated using transwell chambers. The authors found that the leucopatch PMNs were capable of migration towards *P. aeruginosa*. Moreover, bactericidal assay tested Pseudomonas aeruginosa in both planktonic and in an alginate-embedded model, simulating the biofilm mode of bacteria growth. Serum-opsonized P. aeruginosa was exposed to leucopatch, diluted samples were plated and after 24 hours CFU (Colony-forming unit) were counted. Compared to control (Buffer with *P. aeruginosa*), strong reduction of colonies was observed after 20 minutes of leucopatch exposure and reduced further reduced after 90 minutes. Two concentration of bacteria were tested (5×10^7 CFU/ml and 5×10^8 CFU/ml). The loss of bacteria availability was more evident (99% loss of bacteria with respect to the initial inoculum) when the lower concentration of bacteria was exposed to the leucopatch. When bacterial growth was tested in alginate

beads and disc, mimicking a biofilm model, also antimicrobial activity of the patch were detected. The alginate beads and discs with Pseudomonas were exposed to leucopatch for 2 hours, transferred to tubes containing PBS (phosphate-buffered saline), homogenized, serially diluted, plated, and the next day the CFU were counted. After 2 hours, colonies count was reduced by 70% when simulating biofilm in form of disc when exposed to leucopatch and leucopatch inhibited bacterial growth completely compared to non-leucopatch control in the alginate beads model.

In vivo and *in vitro* studies were conducted by Li *et al*. [26] to test the antibacterial effect of PRP gel and the ideal concentration of bovine thrombin for activation of the PRP. For the *in vitro* study, a killing curve assay was used to test the effect of PRP and PPP gels against MSSA, MRSA, *Group A Streptococcus, N. gonorrhoeae*, *Pseudomonas* spp. and *E. coli*. PRP gel was obtained from rabbit blood through a two stage centrifugation process, and activated using a range of different concentrations of thrombin in calcium chloride. Results were compared to PBS controls and to PPP-gel. The concentration of platelets and leucocytes in the PRP gel preparation was extremely high (approximately 10-fold and 4-fold increase from the baseline respectively), and this preparation was shown to inhibit MSSA, MRSA, *Group A streptococcus* and *N. gonorrhoeae* growth in the first 4 hours, after which an increase of bacterial growth was identified. Only high concentrations of thrombin produced sufficient antimicrobial activity of the PRP gel against *N. gonorrhoeae*. There was no antimicrobial activity of the PRP gel against *Pseudomonas* spp. and *E.coli*, and the PPP gel was ineffective for all species.

DISCUSSION

In recent years, one of the hottest areas in regenerative medicine has been research focusing on enhancing and promoting tissue repair and regeneration [29]. Burns and chronic wounds represent a continuing challenge for physicians in terms of treatment,

time, and for healthcare services in terms of elevated costs [30,31]. Wound infection is one of the major contributors to delays in wound healing and tissue regeneration [32,33]. Moreover, as multidrug resistance to antibiotics is becoming a serious threat [34,35], research in this field has focused on finding new agents and strategies to fight infection [36] and additionally to reduce healing times. New approaches to promote rapid wound healing and prevent infection at wound sites are now urgently needed.

L-PRP versus PRP

Only four studies have specifically compared the antibacterial properties of autologous PRP in the presence and absence of leucocytes or L-PRP [16,20,24,25]. The overall conclusion of the authors was that no significant difference between the two preparations was found. Most of the authors suggest that the temporal bacteriostatic properties of L-PRP seem to be caused by either plasma and/or platelet components rather than the leucocytes themselves [21,22,25]. However, looking for results on specific bacterial strains, Anitua *et al.* [20], showed that the fraction containing a very high concentration of leucocytes (almost 4 times the baseline) was the only preparation able to effectively reduce MSSE. Furthermore Intravia *et al.* [24], reported that the L-PRP preparations (with a high concentration of leucocytes) were superior to PRP (with no leucocytes) against MRSA, MSSE and *P.acnes*. Also compared to whole blood, both preparations limited bacterial growth, but for MSSA L-PRP showed a longer inhibition of MSSA (of 24 hours duration). Moreover, Lopez *et al*. [25] showed that the non-activated preparation enriched with leucocytes gave a better bacteriostatic effect against MSSA (at 6 and 24 hours) compared to preparations poor in leucocytes. In contrast, the activated form of the preparation with poor leucocytes and plasma seemed to perform better than the other preparations tested at 24 hours against MRSA. In contrast Mariani *et al.* [16],

concluded that 'leucocyte presence does not increase microbicidal activity of Platelet-rich Plasma *in vitro*' in a study where the quantity of leucocytes included in the L-PRP preparation was low.. Whole blood was used as a positive control by Intravia *et al.* [24], and in their study the authors showed the poor antimicrobial activity of the whole blood compared to the biomaterial enriched with platelets and leucocytes. Moreover, significant antimicrobial effect of PRP was seen when leucocytes were at least 2-fold enriched from baseline [26]. To evaluate the potential antimicrobial contribution of leucocytes, Chen *et al.* [22] used apocynin in a L-PRP preparation to exclude the possible contribution of leucocytes producing superoxide. The authors showed similar antimicrobial activity regardless of apocynin, concluding that the antibacterial effect of the biomaterial was not due to the inclusion of leucocytes.

Activation of PRP preparations

Results are controversial regarding possible relationships between antimicrobial activity and the activation of the PRP preparations. Burnouf *et al.* [21], suggested that the activation of platelets by calcium chloride decreased antimicrobial properties against selected bacterial strains. Similarly Wu *et al*. [23] showed thrombin-activated PRP preparations have less antimicrobial activity against *E.coli, P. aeruginosa* and *K. pneumoniae* compared to the inactivated preparations. These findings are thought to be due to consumption of complement during the coagulation activation. Also Lopez *et al*. [25] indicated that after 24 hours of incubation, calcium gluconate-activated PRP preparations exhibited less antimicrobial activity against MSSA compared to inactivated preparations. When the preparations where tested against MRSA at 6 hours, the strongest antimicrobial effect against MRSA was exerted by the non-activated form of the leucocyte enriched preparation, whilst at 24 hours the activated form of leucocytes poor plasma showed stronger antimicrobial effect compared to the other preparations tested.

Controversially, other studies indicate that strongest antibacterial activity is reached when PRP is activated with thrombin [22], with the highest concentration of thrombin exerting the strongest antimicrobial effect, and with autologous thrombin performing better than bovine thrombin [26].

L-PRP/PRP preparation methods and microbiology assays

Due to the heterogeneity of the preparations and the multiple variables in the study settings, it is difficult to compare results of studies and reach definitive conclusions regarding the relevance of the leucocytes in antimicrobial activity of L-PRP preparations. The proportion of the different components included in the biomaterials that exerted the strongest antimicrobial properties clearly still remain to be identified. It is important to remember that the *in vitro* studies may not reflect the clinical scenario, since this environment may not mimic the dynamic condition of an *in vivo* setting where the antimicrobial properties of each individual components of the biomaterial may be increased, prolonged or even reduced by the complex interaction of cellular signals, and dynamic fluid exchange. An example of different results of platelet gel effects against bacteria obtained *in vitro* and in an *ex vivo* skin model is clearly shown in a recent published study. Edelblute *et al*. [37] demonstrated different antimicrobial performance against three strains of bacteria in different settings. No inhibition was seen for *Pseudomonas* spp. in the *in vitro* experiment, while inactivation of the same strain was detected in the *ex vivo* model. Moreover as suggested by Burnouf *et al*.[21], ATCC (American Type Culture Collection) bacterial strains used in most of the studies, may not reflect the bacterial behaviour of clinical isolates. Also different strains showed different responses to diverse blood products. This might due to the antimicrobial activity of AMPs directly related to the intrinsic AMP susceptibility phenotype of the infecting

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bacterial strain, reflecting the point that different bacterial strains have dissimilar susceptibility to inhibition by platelet AMPs [38].

Possible use of L-PRP

Even with minor variation in timing for different strains and for different preparations, the studies included in our review seem to agree that 4 hours is the optimal time of incubation, when the maximum decrease in bacterial numbers is achieved. All studies agree that the preparations are bacteriostatic, resulting in regrowth, but recognise that L-PRP preparations are a helpful addition more likely to be used in a clinical setting for prophylaxis rather than therapeutically for the treatment of established infection. Authors [14,23] suggest to use formulations containing leucocytes and platelets in combination after surgical debridement to reduce both the bacterial load (killing bacteria and inhibiting biofilm formation [14]), and stimulate healing.

Are the included leucocytes viable and active?

The methodology described in the studies to centrifuge and prepare blood products should not theoretically damage leucocytes. It is likely that in Burnouf *et al.* [21], leucocytes included in the PRP preparation were no longer viable as the preparations were kept frozen until use, therefore the contribution to the antimicrobial effect showed by the PRP preparation is likely not due to leucocyte inclusion.

In the study conducted by Moojen *et al*. [19] the authors found that neutrophils and monocytes were viable and active as showed by the rise of MPO concentration detected shortly after the addition of L-PRP to the bacterial culture. Thomsen *et al.* [14] investigated the phagocytic fitness of leucocytes included in a multilayer matrix of fibrin and platelets. The authors observed that the neutrophils included in this preparation were active and capable of chemotaxis, phagocytic activity and respiratory burst. Interestingly, the authors contradict the general assumption that neutrophils have a short lifespan of less

than 24 hours. Neutrophils in fact showed reduced but oxidative burst activity even after 7 days, confirming previous published work revealing a neutrophil lifespan of more than 5 days [39].

L-PRP in wound healing: concerns for scarring

Mindful of all the limitations of the studies included in our review, the overall conclusion seems to be that the contribution of leucocytes in a PRP preparation is still poorly understood or at least not fully appreciated in the studies so far conducted. On the other hand, it is possible to state that none of the papers included in our review strongly suggest a remarkable antimicrobial effect specifically due to the leucocytes included in PRP preparations. Leucocyte inclusion in the biomaterial should be carefully further evaluated especially when the biomaterial is used for wound repair and when scar formation is a major concern. Among all the factors that affect scar quality [33,40], the one that seems to have the greatest impact is the time that it takes a wound to heal [40-42]. A large body of literature supports the assertion that achieving wound healing within 21 days will minimize the scarring. Therefore, a major focus in burns and wound healing research is to clarify the pathophysiology of the healing process of a wound, the risk factors related to the scarring process, and the conversion of this knowledge into therapeutic solutions. The use of PRP in wound healing as an accelerator of wound repair seem to justify its use. Several studies are now evaluating the role of leucocytes in wound repair[11,43].Cytokines, such as TGF-β1 and TGF β2, released by leucocytes, have been shown to be involved in in cutaneous scarring [44-46]. Their inclusion for wound healing and scar formation should therefore be carefully considered. In particular, further investigations should be performed into the antimicrobial and immune-metabolic effects of all the subpopulations of leucocytes included in the preparations, in order to create the optimal combination.

CONCLUSION

Although the presence or absence of leucocytes in PRP preparations was previously neglected, in the last decade more attention has been paid to their role and several studies have been conducted to explore both their immuno-metabolic effects and their antimicrobial properties.

In this review, despite a number of studies showing that preparations including leukocytes have antimicrobial properties, there is not enough evidence to attribute this bactericidal effect to the presence of leucocytes in the biomaterial. PRP preparations, with or without leucocytes demonstrated bacteriostatic properties against the majority of the bacterial strains tested. Diverse strains of bacteria respond differently to PRP and L-PRP, some of them requiring the presence of leucocytes (MSSE), and some being dose dependent (*Pseudomonas* spp.). The authors hope that this review will be a groundwork for future studies to further explore the contribution of leucocytes in PRP preparation in order to obtain an optimal preparation to both fight infection and effectively promote wound healing.

LIST OF ABBREVIATIONS

ESBL: Extended Spectrum Beta Lactamase, IGF-1: insulin-like growth factor 1, MRSA: methicillin resistant *Staphylococcus aureus*, MRSE: methicillin-resistant *Staphylococcus epidermidis*, MSSA: methicillin sensitive *Staphylococcus aureu*s, MSSE: methicillin-sensitive *Staphylococcus epidermidis* PBS: phosphate buffered saline, PDGF-BB: platelet-derived growth factor BB, PPP: platelet-poor plasma, TGF-β1: transforming growth factor beta 1, VEGF: vascular endothelial growth factor.

DECLARATION OF INTEREST

The authors report no declarations of interest.

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