The L-PRF membrane and its derivatives useful in wound care surgery

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ABSTRACT

Growing multidisciplinary field of tissue engineering aims to regenerate, improve or replace predictably damaged or missing tissues for a variety of conditions caused by trauma, disease and old age. To ensure that tissue engineering methods are widely applicable in the clinical setting, it is necessary to modify them in such a way that they are readily available and relatively easy to use in daily clinical routine. Therefore, the steps between preparation and application must be minimized and optimized to make them realistic implementation. General objective of developing platelet concentrates of natural origin can be produced *close* to the patient and accelerate the implantation process, being financially realistic for the patient and the health system. Fibrin rich in platelets and leukocytes (PRF) and its derivatives have been used in a wide variety of medical fields for soft tissue regeneration. In conclusion, the results of this systematic review highlight the positive effects of PRF on wound healing after regenerative therapy for the management of various soft tissue defects found in wound care.

INTRODUCTION

The multidisciplinary field of tissue engineering aims to repair, regenerate or restorably repair damaged and supportive tissues, including cells, tissues and organs, due to an assortment of biological conditions, including congenital anomalies, lesions, diseases and/or aging[1,2] During their regeneration, a key aspect concerns the growth of a vascular source that is able to support cell function and the future development of tissues by maintaining a vital nutrient exchange through vessels blood. Although most tissue engineering scaffolds are avascular in nature, it remains essential that all regenerative strategies focus on developing a vascular network to achieve positive clinical outcomes and regeneration in both soft and hard tissues.[3] Wound healing involves a cascade of complex, orderly and elaborate events involving many cell types driven by the release of soluble mediators and signals that are able to influence the return of circulating cells to damaged tissues. Platelets have proven to be important cells that regulate the hemostasis phase through vascular obliteration and facilitating the formation of fibrin clots. It is known that they are responsible for the activation and release of important biomolecules, including specific platelet proteins, growth factors including platelet-derived growth factor (PDGF), coagulation factors, adhesion molecules, cytokines/chemokines and angiogenic factors that are able to stimulate proliferation and activation of cells involved in wound healing, including fibroblasts, neutrophils, macrophages and mesenchymal stem cells. Despite the widespread use of platelet concentrates (HPC) (Figure 1) such as platelet-rich plasma, one of the drawbacks reported is the use of anticoagulation factors that delay normal wound events.[4,5] Because of these limitations, further research has been focused on the development of a second generation platelet concentrate without using anticoagulation factors. As such, a platelet concentrate free of coagulation factors, subsequently termed platelet-rich fibrin (PRF), was developed because of its properties of anticipating tissue regeneration and wound healing. This fibrin scaffold, which has no cytotoxic potential, is obtained from 9 ml of the patient's blood after 1 phase of centrifugation and contains a variety of blood cells – including platelets,

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B and T lymphocytes, monocytes, stem cells, and neutrophil granulocytes – in addition to growth factors. Furthermore, L-PRF (also called leukocyte-PRF) contains white blood cells, necessary cells that are important during the wound healing process.[6] Moreover, since white blood cells, including neutrophils and macrophages, are among the first types of cells present in wound sites, their role also includes phagocytic fragments, microbes, and necrotic tissue, thus preventing infection. Macrophages are also key cells derived from the myeloid lineage and are considered one of the key cells involved in growth factor secretion during wound healing, including the transforming growth factor beta (TGF-β), PDGF and growth factor vascular endothelium (VEGF) (Figure 2). These cells, together with neutrophils and platelets, are key players in wound healing and in combination with their growth factors/secreted cytokines are able to facilitate tissue regeneration, the formation of new blood vessels (angiogenesis) and the infection prevention.

In 2008, Lundquist[7] was one of the first to evaluate the effects of PRF on human dermal fibroblasts. It was found that the proliferative effect of PRF on dermal fibroblasts was significantly greater than fibrin glue and recombinant PDGF-BB. Furthermore, PRF induced rapid release of collagen 1 and prolonged release and protection against pro-

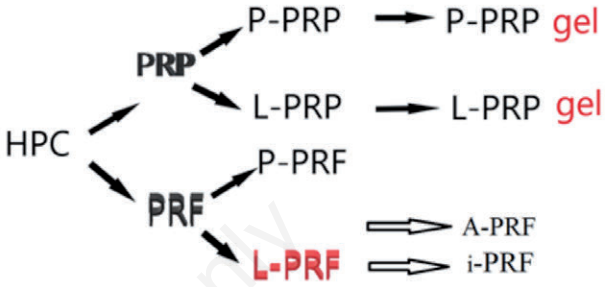

Figure 1. Platelets concentrates (HPC). PRP, platelet rich-plasma; PRF, fibrin rich in platelets.

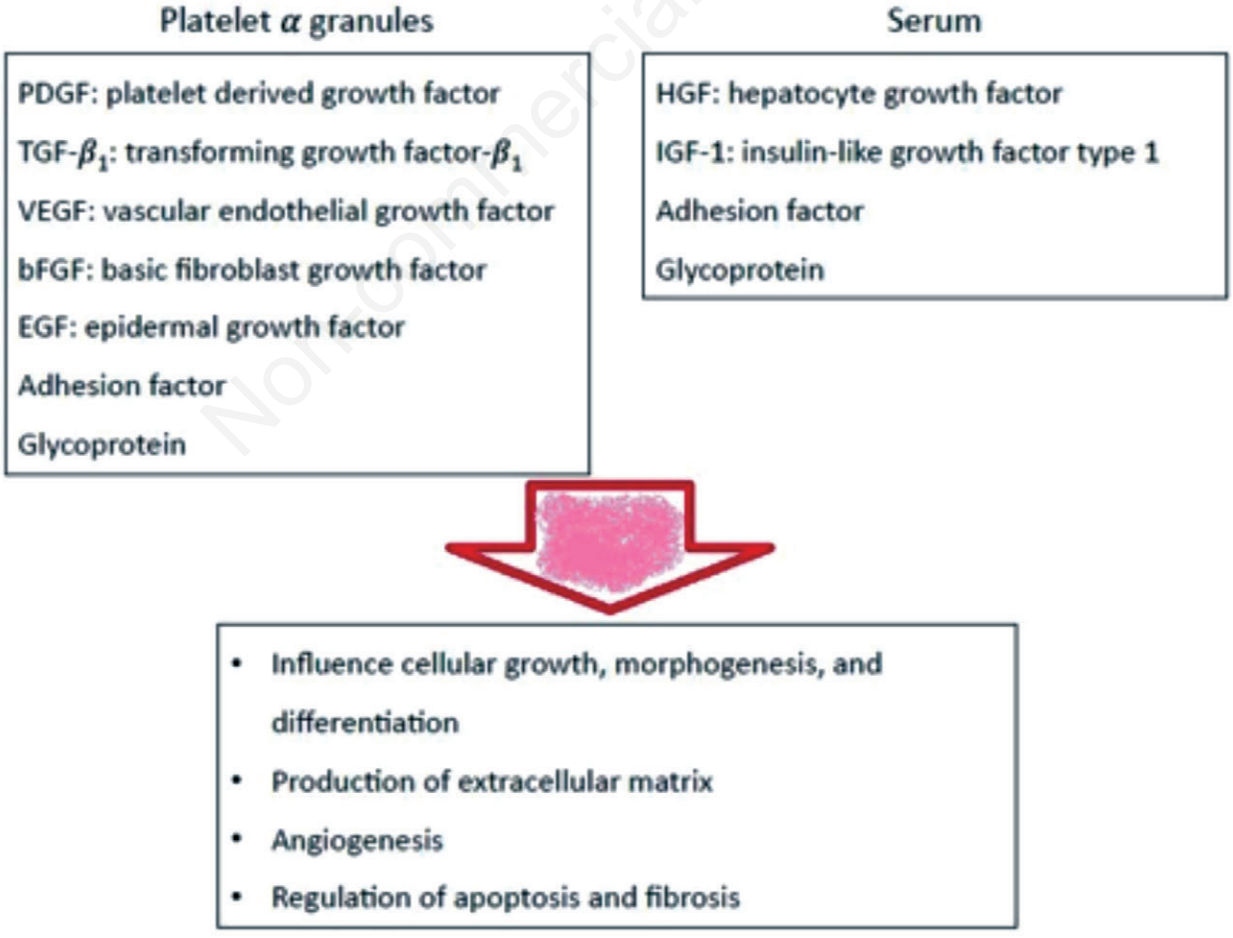

Figure 2. Function of the platelets in wound healing.

teolytic degradation of endogenous fibrogenic factors that are important for wound healing. In a second *in vitro* study conducted by Lundquist *et al*. in 2013,[8] PRF induced the mitogenic and migratory effect on cultured human dermal fibroblasts and also showed that fibrocytes (a type of cell important for healing acute wounds) could be cultured within disks PRF, further promoting wound healing and soft tissue regeneration. Subsequently, Clipet *et al*.[9] found that PRF induces the survival and proliferation of fibroblasts and keratinocytes. The PRF has been found to induce endothelial cell mitogenesis via the extracellular pathway of signal-regulated kinase activation. A slow and steady release of growth factors from the PRF matrix was observed that releases VEGF, a known growth factor responsible for the endothelial mitogenetic response.

L-PRF AND ITS DERIVATIVES IN THE HEALING OF CHRONIC WOUND ULCERS

L-PRF

In the longitudinal section of the L-PRF coagulum, produced according to the standard centrifugation protocol (30" of acceleration, 2' at 2700 rpm, 4' at 2400 rpm, 3' at 3000 rpm, and 36" of deceleration and stopping),[4] a thick fibrin clot is present with minimal inter-fibre space. Cells are observed throughout the blood clot, although decreasing towards the most distal parts of the PRF clot (Figure 3).

Advanced-PRF

The PRF clots formed with the A-PRF centrifugation protocol (Advanced-PRF) (1500 rpm, 14 minutes)[10] showed a freer structure with more inter-fibre space and more cells can be counted in the fibrin-rich clot. Furthermore, the cells are more evenly distributed in the clot than L-PRF, and some cells can also be found in the most distal parts of the clot. A representative image for cellular distribution within A-PRF is shown in Figure 4.

PRF injectable formulation

The development of an injectable formulation of PRF (referred to as i-PRF)[11,12] (centrifuged at 700 rpm [60 g] for 3 minutes) was pursued with the goal of delivering a platelet concentrate easy to use to doctors in liquid formulation that can be used alone or easily combined with various biomaterials. Taking advantage of slower and shorter centrifugation speeds, a greater presence of regenerative cells with higher concentrations of growth factors can be observed compared to other PRF formulations using higher centrifugation rates.

Ghanaati *et al.*[10] reported that velocity and time do not affect monocyte and stem cell concentrations, but influence platelet and neutrophil concentrations. As a result, A-PRF contains more platelets, most were found in the distal part of the PRF and L-PRF membrane include more neutrophils. This type of concentrate has the potential to improve angiogenesis by expressing the enzymatic matrix metalloproteinase-9. Therefore, the inclusion of neutrophils in the PRF could be considered if angiogenesis is of interest.

Analysis of the study by Ghanaati *et al*.[10] also revealed that the platelets were the only ones present in each coagulum area up to 87±13% in the L-PRF group and up to 84±16% in the A-PRF group (Figure 4). Furthermore, the results showed that T lymphocytes (L-PRF: 12±5%, A-PRF: 17±9%), B lymphocytes (L-PRF: 14±7%, A-PRF: 12±9%), CD34 positive stem cells (L-PRF: 17±6%, A-PRF: 21±11%), and Monocytes (L-PRF: 19±9%, A-PRF: 22±8%) not more than 30% of the total length of the clot have been found beyond a certain point, since they are distributed near the BC generated by the centrifugation process (Figure 4).

EFFECT OF PRF ON THE RELEASE OF GROWTH FACTORS

It has long been observed that the PRF releases a number of growth factors for the microenvironment.

The TGF-β has a broad efficacy of over 30 factors known as fibrosis agents, with TGF-β1 which is the most described in the literature. It is a known stimulator of the proliferation of various types of mesenchymal cells, including osteoblasts, and is the most powerful fibrotic agent among all cytokines. It plays a pre-eminent role in the synthesis of the matrix molecule such as collagen1 and fibronectin, both from osteoblasts and fibroblasts. Although its regulatory mechanisms are particularly complex, TGF-β1 plays an active role in wound healing.

VEGF is the most powerful growth factor responsible for tissue angiogenesis. It has powerful effects on tissue remodeling and the incorporation of VEGF alone into various bone biomaterials has shown increases in new bone formation, thus indicating the rapid and powerful effects of VEGF.

Insulin-like growth factor is a positive regulator of proliferation and differentiation for most types of mesenchymal cells, which also act as cell protection agents. Although these cytokines are cell proliferative mediators, they also constitute the main axis of programmed regulation of cell death (apoptosis),[13] inducing survival signals that protect cells from many apoptotic stimuli. Bayer *et al*.[14] explored for the first time the properties contained in the PRF that can contribute to its anti-inflammatory/antimicrobial activities. It was discovered that in human keratinocytes, PRF induced the expression of hBD-2 (β-defensin 2).

EFFECTS OF PRF ON WOUND HEALING AND *IN VIVO,* ANGIOGENESIS

The effects of PRF have in particular been studied on the healing of soft tissue wounds and on angiogenesis in various animal models. In other medical procedures, the use of PRF has mainly been combined for success in the management of leg ulcers that are difficult to heal, including diabetic foot ulcers, venous ulcers, and leg ulcers. Furthermore, the PRF has been studied for the management of hand ulcers and soft tissue defects.[15,16]

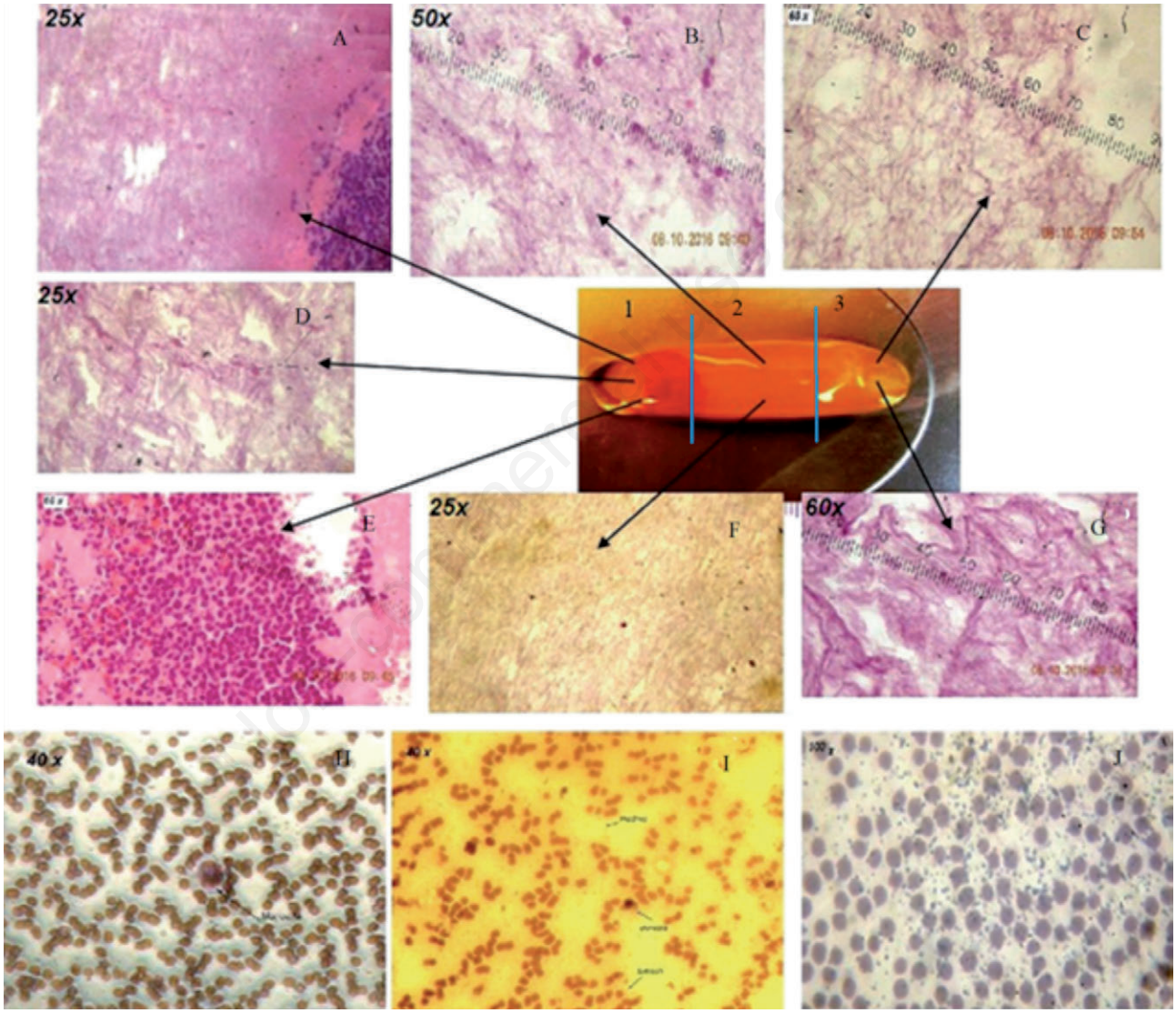

Figure 3. Horse L-PRF membrane at 0 minutes from compression (eosin-hematoxylin color). The L-PRF layers were fixed in 10% formalin buffered neutral solution at pH 7.2 for 48 hours and incorporated in paraffin according to the standard procedure. Twenty serial sections (7 µm thickness) of each sample were cut using a microtome. A) III proximal ingr. 25× white blood cells - fibrin reticulum; B) III average ingr. 60× erythrocytes-fibrin pattern; C) III distal ingr. 60× fibrin reticulum; D) III proximal ingr. 25× erythrocytes-fibrin; E) III proximal ingr. 60× fibrin on the right, lymphocytes in the center, erythrocytes and neutrophil granulocytes on the left; F) III medium ingr. 25× fibrin lattice; G) III distal ingr. 60× fibrin reticulum; H) Red clot smear ingr. 40× presence of monocita in a carpet of erythrocytes; I) smear red clot ingr. 40× presence of erythrocytes, monocytes and platelets; J) smear red clot ingr. 100× platelets in a carpet of erythrocytes (May-Grunwald-Giemsa stain). *Reproduced from Crisci* et al.,[4] *licensed under the terms of Creative Commons Attribution 4.0 International License.*

FURTHER RANDOMIZED CLINICAL TRIALS

One of the advantages reported by the PRF is the ability of the fibrin network to contain leukocytes, to resist and fight infections. Chronic unhealed wounds represent a significant medical challenge and the pathogenesis of unhealed wounds, therefore, requires new therapeutic options to improve clinical outcomes. Macrophages have proven to be key actors during tissue regeneration, wound healing and infection prevention. Furthermore, they contain antimicrobial effects that are able to reduce bacterial contamination after surgery.

DISCUSSION

The regenerative capacities of the PRF and its derivatives (A-PRF, i-PRF) (Figure 1) as a surgical adjuvant, have received considerable attention since its introduction in the early years of the new millennium. In contrast, no clear evidence remains to clarify the antimicrobial potential of this particular biomaterial that differs both structurally and biologically from other forms of HPC. Ghanaati *et al.*[10] described histologically A-PRF™ as a matrix of cells seeded on fibrin-containing a variety of blood cells including: platelets, lymphocytes (B and T), monocytes, stem cells and neutrophil granulocytes able to release a set of growth factors.[17,18] In theory, the biological components and physiological mechanisms for antimicrobial activity are similar within various types of HPC and even coagulated blood. However, these autologous biomaterials differ in terms of i) the variable mix of cell types; ii) the vitality of the contained cells; iii) their mode of activation, natural or chemical; iv) the density of the fibrin network; v) interactions between cellular and extracellular components; vi) and the release of a variety of proteins. These differences may have a significant impact on their respective anti-inflammatory and antimicrobial properties.[19-23]

Furthermore, the mechanisms and dynamics of the individual antimicrobial components contained in these biomaterials are poorly understood.

A-PRF™ shows antimicrobial activity against all single organisms tested within this study over a 24-hour period. These results are consistent with those of previous studies evaluating the antimicrobial properties of other HPC preparations.[19-22,24] Because A-PRF™ shows antimicrobial properties, the need to determine whether this activity is significantly greater than that of a natural blood clot has emerged. Future investigations are needed to explore the antimicrobial spectrum of A-PRF™ and explore the possibility that it may act as a substrate to facilitate the growth of specific organisms.

Of particular relevance to the surgeon is that *Staphylococcus aureus* is a major cause of hospital-acquired infections, infections related to internal medical devices and infection of surgical wounds.[25] Significant research is focused on alternative treatment strategies in *S. aureus*-guided infections to reduce the risk of developing antibiotic-resistant strains.[22,26] For this reason, *S. aureus*

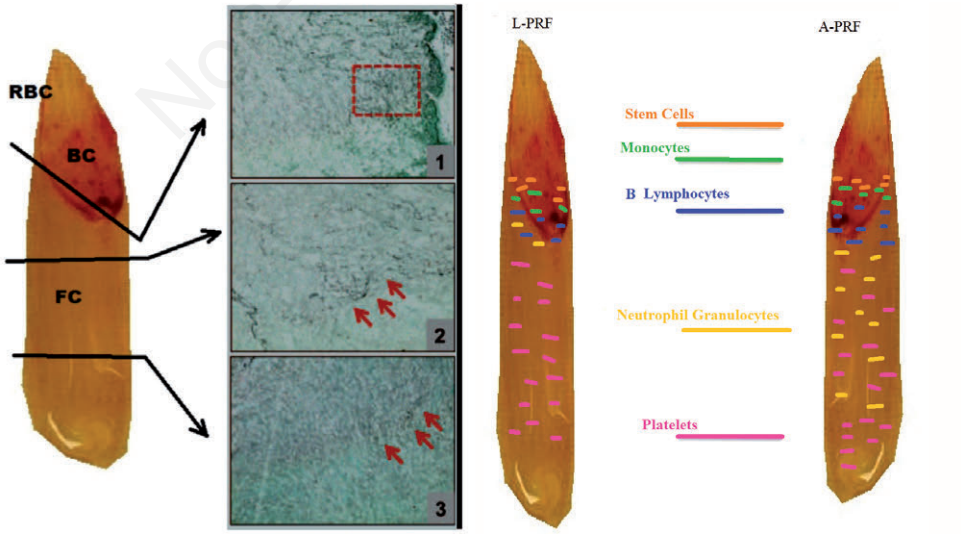

Figure 4. Advanced-PRF (A-PRF) total scan of a fibrin clot along its longitudinal axis (Masson-Goldner staining). RBC represents the fraction of red blood cells. The buffy coat (BC) is the transformation zone between the fraction of RBC and the fibrin clot and FC represents the fibrin clot. The three bars within the scan and the arrows show the first floors of the respective areas. The red arrows mark cells that are trapped inside the fibrin network.

remains the most frequently tested organism in the literature examining the antimicrobial activity of PC.[19] Many different HPC preparations have shown antimicrobial activity for both methicillin-resistant and methicillin-susceptible *S. aureus* strains.[19,22,24]

Candida albicans is the most frequently isolated of the fungal species in the microbiome. The impairment of an individual's immune response may allow these opportunistic fungi to cause infections.[27,28] A-PRF™ has a greater ability to consistently inhibit *C. albicans* growth than a normal blood clot. Furthermore, *C. albicans* is less susceptible to the antimicrobial components of platelets and confirms the findings of Tang *et al*.[29] who noted that human platelet antimicrobial peptides are more potent against fungi bacteria.

A-PRF™ shows greater potential to inhibit *Streptococcus mutans* than a natural blood clot. However, since no other HPC has been tested against this organism, the mechanism of its inhibition and clinical potential requires further exploration.

Limitations

Although the results of many studies indicate that A-PRF™ shows an antimicrobial activity, several limitations have emerged. Firstly, the *in vitro* investigation does not mimic a clinical situation in which A-PRF™ will be placed in an environment surrounded by tissues that respond to a surgical event. In this scenario, A-PRF™ can interact with a series of cells and cytokines involved in the wound healing process and modify initial immune responses and healing events.[12,20,30] The release of activated platelet growth factors within the fibrin matrix may also modify the expression of antimicrobial peptides from surrounding tissues.[14] It is possible that many patient factors can influence the quality of A-PRF™. Yajamanya *et al*.[31] demonstrated that the fibrin matrix formed by their version of PRF in elderly patients was more generally organized than the fibrin matrix of younger subjects. The impact of this discovery has yet to be determined. The cell type, the number of cells and the concentration of the plasma components differ within each coagulum and between each coagulum,[10,32] each sample disk cannot be identical to the other. One problem to be defined is that it is not yet possible to determine whether the tested material is bactericidal or bacteriostatic. Regardless of these drawbacks, the disc diffusion method was sufficient to demonstrate that A-PRF™ shows antimicrobial activity.

CONCLUSIONS

Very little is known about the antibacterial properties of the PRF and its derivatives (A-PRF, i-PRF) and very few studies have investigated this phenomenon. From a tissue engineering point of view, it is interesting to note that so far no research has focused on the strength, rigidity or resistance of the PRF despite its clinical use for over 15 years. Therefore, interest remains to better characterize its biomaterial properties and future research should focus on which factors could further improve its characteristics for various biomedical applications. It is essential that the next wave of research using PRF as an adjunct to soft tissue regenerative therapies develop appropriate studies with the necessary controls to further evaluate the regenerative potential of PRF for the healing of soft tissue wounds.

The use of A-PRF™ in clinical practice has shown great potential to improve healing and improve surgical outcomes as it serves as an autologous scaffold that hosts cells and bioactive compounds.[12,33-35] However, the antimicrobial potential of the material has been demonstrated and may be an important property contributing to clinically detected accelerated and uncomplicated healing events. The results of this review indicate that A-PRF™ shows, however, an antimicrobial activity against *S. aureus*, *S. mutans*, *Enterococcus faecalis* and *C. albicans*. Furthermore, the spectrum and potency as an antimicrobial agent are far lower than those of an established surgical antimicrobial (specific antibiotic). Future investigations involving A-PRF™ are therefore necessary to determine the full spectrum of it's *in vitro* antimicrobial activity, it's *in vivo* participation and the influence of the patient's characteristics on its biological activity. Furthermore, its clinical potential should be explored as a vehicle for the local administration of drugs within infected sites.[19]

Future studies should increase both patient variation and sample sizes for all future HPC-based studies.

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