

The Usefulness of Second Generation Platelet Concentrates in Regenerative Therapy of Limbs' Complex Wounds

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Abstract

Regeneration is the ultimate aim in the field of multidisciplinary tissue engineering, together with the amelioration or substitution, in a predictable manner, of damaged or missing tissues, an occurrence that presents in a multitude of conditions, including trauma, diseases and aging. To guarantee an ample availability of different tissue engineering techniques, in clinical fields, these need to be changed and adapted in order to render them accessible and relatively easy to apply in everyday clinical routines. Choukroun's Platelet Rich Fibrin (PRF) and its derivatives have been implemented in a vast array of medical fields, as a supranatural concentrate of autologous growth factors, able to simulate tissue regeneration. Platelets have been found inside blood clots, in its entirety, in all its different groups, even if inside the A-PRF group, the platelet counts is higher in the distal portion, distal to the Buffy Coat (BC), compared to L-PRF. T and B lymphocytes, stem cells, and monocytes have been found close to the BC. Lowering the number of spins and increasing the duration of centrifugation in the A-PRF group leads to a higher neutrophil count in the distal portion of the clots. In conclusion, the results of this systematic study have highlighted the positive effects of PRF and its derivatives (A-PRF, i-PRF) in wounds healing, after regenerative therapy of complicated cutaneous foot lesions.

Keywords: Advanced Platelet Rich Fibrin; Growth Factors; Injectible Platelet Rich Fibrin; Platelet and Leukocyte Rich Fibrin; Stem Cells

Introduction

If wounds do not heal in an ordered and timely manner, or if the healing process does not have structural integrity, wounds can be considered as complex. Complex wounds are a significant problem, not only in specialized structures, but also in everyday clinical practice. Complex wound healing takes place through the same mechanisms of an acute wound healing, but in this case, an abundant granulation tissue is usually formed, with an excessive fibrosis that leads to scar contraction and function loss [1-5]. Complex wound healing is involving a complex cascade of events, taking into play a multitude of different cell types, which are able to enter the circulation thanks to the release of soluble mediators and signals able to influence them, and lead them to damaged tissues.

Platelets are responsible for activation and release of important biomolecules, including specific platelet proteins and growth factors, among which: Platelet Derived Growth Factor (PDGF), coagulation factors, adhesion molecules, cytokines/chemokines and angiogenic factors, able to stimulate proliferation and activation of cells involved in wound healing processes, like fibroblasts, neutrophils, macrophages and stem cells. Despite the diffused use of Human Platelet Concentrates (HPC) (Figure1), like Platelet Rich Plasma (PRP), one of the reported disadvantages is the concomitant use of anti-coagulation factors that are responsible for delayed wound healing [4,5]. Due to these limits, further researches had the aim of developing platelet concentrates of second generation, which would not need anti-coagulation factors. As such, a platelet concentrate devoid of coagulation factors, subsequently defined as Platelet Rich Fibrin (PRF), has been developed by Choukroun (2001), with particular reference to its ability to speed up wound healing and tissue regeneration. The biological and clinical prop-

erties of these concentrates make them extremely attractive in regenerative medicine fields. This fibrin scaffold, devoid of any cytotoxic property, is obtained from 9ml of patient's blood, after centrifugation through a PRF-DUO Quattro centrifuge, and the use of gel-free glass vials, and not vacuum, silica-containing PET vials [6]; it holds a variety of blood cells - including macrophages, platelets, B and T lymphocytes, monocytes, stem cells, and neutrophils - and different growth factors. L-PRF (Leukocyte-PRF) and its derivatives (A-PRF, i-PRF and so on), hence, contain white blood cells, necessary during wound healing processes. Furthermore, since white blood cells, including neutrophils and macrophages, are among the first types of cells found in wound sites, their role includes also phagocytosis of cellular debris, microbes and necrotic tissues, preventing infections. Macrophages are also cells of the myeloid cell line, and are considered one of the main protective factors for infection. In 2008, Lundquist [7] was among the first to give an account of PRF effects on fibroblasts coming from human dermis. It was shown that the proliferative effect of PRF on dermal fibroblasts was significantly superior compared to that of fibrin glue and recombinant PDGF-BB. Furthermore, PRF induced a rapid and prolonged release of collagen type I, as well as providing a protection against proteolytic degradation of endogenous fibrogenic factors, important for wound healing.

In a second *in vitro* study conducted by Lundquist et al. in 2013 [8], PRF induced a mitogenic and migratory effect on human dermal fibroblasts in culture and also demonstrated that fibrocytes (important cells in acute wound healing) might be cultivated on PRF disks, further favoring wound healing and soft tissue regeneration. Subsequently, Clipet et al. [9] discovered that PRF induces survival and proliferation of fibroblasts and keratinocytes. It was discovered that PRF induces mitogenic effects on endothelial cells through the extracellular activation pathway of signal-mediated kinase. A slow and constant release of growth factor was observed from the PRF matrix, which released VEGF, a notorious growth factor, responsible for endothelial mitogenic response.

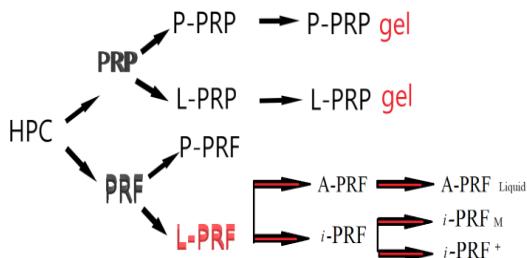


Figure 1: Platelets concentrates (HPC). PRP, platelet rich- plasma; PRF, fibrin rich in platelets.

L-PRF

In the longitudinal section of a L-PRF clot, obtained through the standard centrifugation protocol (2700 rpm for 12 minutes) (325G), with a DUO Centrifuge (PRF DUO PROCESS[®] for PRF, Nice, France) (Kobayashi et al., 2016), a dense fibrin clot has been found, with a minimal inter-fibrous space. Cells have been observed along the entire length of the clot, even if they are reduced in distal portion of the PRF clot [4].

Advanced-PRF

PRF clots formed with the centrifugation protocol Advanced-PRF (A-PRF), in its variants A-PRF+ (1300 rpm, 8 minutes) and A-PRF Liquid (1300 rpm, 5 minutes) [10], following Choukroun's indications, have demonstrated a freer structure, with a bigger inter-fibrous space and a higher number of cells inside the clot. Furthermore, cells are more uniformly distributed inside it, compared to L-PRF, and some cells might be found also in the distal portion. A representative picture of cell distribution inside A-PRF clots has been presented in (Figures 2,3).

Injectable PRF formulation (i-PRF)

The development of an injectable PRF solution (named i-PRF) [11,12] (centrifuged at 700 rpm [60g] for 3 minutes) and of its derivatives i-PRF M (700 rpm for 4 minutes) and i-PRF+ (700 rpm for 5 minutes), has been pursued with the aim of providing physicians with a platelet concentrate that would be easy to use in a liquid formulation, alone or combined with various biomaterials. Taking advantage of a slower and briefer centrifugation speed, it is possible to observe a higher number of regenerative cells, with a higher growth factors' concentration, compared to other PRF formulations obtained through higher centrifugation speeds. Ghanaati et al. [10] referred that velocity and time do not influence the monocyte and stem cells concentrations, but are able to influence platelet and neutrophils concentrations. Consequently, A-PRF contains a higher number of platelet, which are mainly located in the distal membrane portion, and includes more neutrophils compared to L-PRF. This type of concentrate can potentially ameliorate angiogenesis, through the expression of metalloproteinase-9 of the enzymatic matrix. As such, the neutrophilic inclusion inside the PRF membrane, with the use of A-PRF, might be taken into account if angiogenesis is one of the aims. The analysis of Ghanaati et al. [10] studies have also shown that platelets were the only cells present in all areas of the clot, up to 87±13% inside L-PRF group, and up to 84±16% inside the A-PRF group. Also, results have shown that T lymphocytes (L-PRF: 12±5%, A-PRF:17±9%), B lymphocytes (L-PRF: 14±7%, A-PRF:12±9%), CD34+ stem cells (Figure 2) (L-PRF:17±6%, A-PRF:21±11%) and monocytes (L-PRF:19±9%, A-PRF:22±8%) were not found past a certain point, at maximum 30% of the total clot's length, since they were distributed close to the BC, created by the centrifugation process.

Our work group found them in the proximal 2/3 of a compressed equine A-PRF membrane (Figure 2).

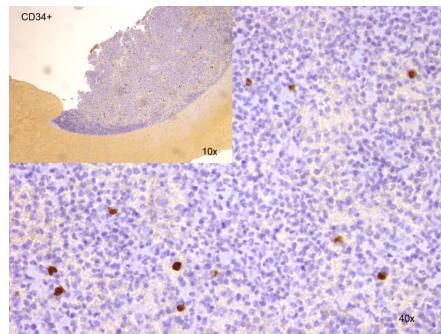


Figure 2: CD34+ stem cells found in the intermediate portion (body) of a horse's auto-compressed A-PRF membrane.

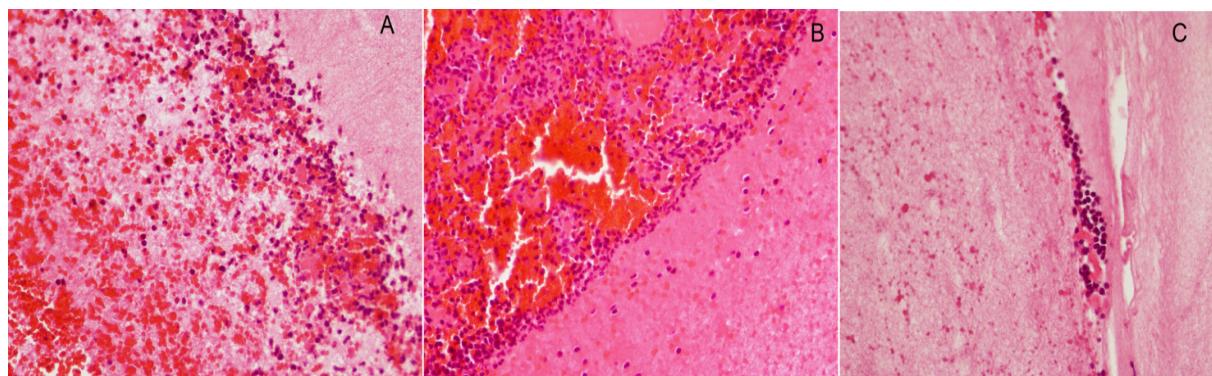


Figure 3: Head (A), body (B) e tail (C) of a horse's auto-compressed A-PRF membrane (40x magnification; H&E). Abundant Leukocytes (possibly neutrophils) are highlighted, together with Erythrocytes, distributed in the fibrin meshwork in A and B. In C, presence of abundant meshwork and scant cells.

PRF: Various Types of Effects On Growth Factor Release

It has been long observed that PRF release a series of growth factors for the micro-environment. TGF- β (transforming growth factor) has an ample efficacy compared to more than 30 known factors, known as fibro genetic agents, and TGF- β 1 is the most described in literature. It is a known stimulator of proliferation for various types, including osteoblasts, and it constitute the most powerful fibrogenic agent among all cytokines. It plays a prominent role in synthesizing matrix molecules, like collagen-1 and fibronectin, both from osteoblasts and fibroblasts. Even if its regulatory mechanisms are particularly complex, TGF- β 1 plays an active role in cutaneous wound healing, in all different districts.

VEGF (Vascular Endothelial Growth Factor) is the most powerful growth factor in tissue angiogenesis. It has powerful effects on tissue remodeling, and the implementation of VEGF in different osseous biomaterials demonstrated an increase in new osseous formation, highlighting the rapid and potent effects of VEGF. Insulin-Like Growth Factors (IGF) are positive proliferation and differentiation regulators, for the vast majority of mesenchymal types of cells, acting as cell-protecting agents. These

cytokines, even if mediators of cell proliferation, also constitute a main player in planned cell death (apoptosis), [13] inducing survival signals able to protect cells from many apoptotic stimuli. Bayer et al. [14] explored for the first time the PRF properties, that might contribute to its anti-inflammatory/antimicrobial activities. It was found that in human keratinocytes, PRF was able to induce hBD-2 (β -defensin 2).

PRF Effects On Cutaneous Foot Wound Healing and *in Vivo* Angiogenesis

Growth factors effects on tissue growth, and in particular PRF and its derivatives, have been profusely studied in reference to healing and angiogenesis of soft tissue wounds in various animal models and in humans. In many medical procedures, PRF usages have been combined, mainly to obtain an efficacious management of leg ulcers, that previously displayed a difficult healing pattern, including ulcers of diabetic foot, venous ulcers and the arterio-pathic ulcers. Furthermore, PRF was studied by the Authors in the management of diabetic hand ulcers and in scarring defects of foot tissues (Figures 4-8) [15,16].



Figure 4: Post-surgical dehiscence following Achilles' tendon reconstruction (A) treated with L-PRF (B) after seven days from therapy (C) and after complete healing (D).

Our work group has proposed the use of platelet and leukocytes rich fibrin (L-PRF) also in ulcerated diabetic foot osteomyelitis, hypothesizing a recovery from this severe pathology. In this study, the objective was to standardize the use of L-PRF in patients with osteomyelitis, to use this second generation platelet concentrate, easing out healing processes [15-20]. Authors produced and utilized L-PRF membranes made from peripheral blood, in patients with osteomyelitis, with lower limb cutaneous lesions for at least 6

months. Membranes, in conjunction to the liquid formed from the compression with Wound L-PRF Box, were inserted inside the cutaneous lesion, reaching the bone, after surgical debridement. The evolution of lesions was subsequently analyzed in its time progression (Figures 5-7).



Figure 5: Use of Leukocyte Platelet (L-PRF) Rich Fibrin in diabetic foot ulcer with osteomyelitis. (A, C, D, E) Different moments of the wound healing, stable after two years; (B) NMR of the patient with the bone lesion (from Crisci et al.2018) [16].

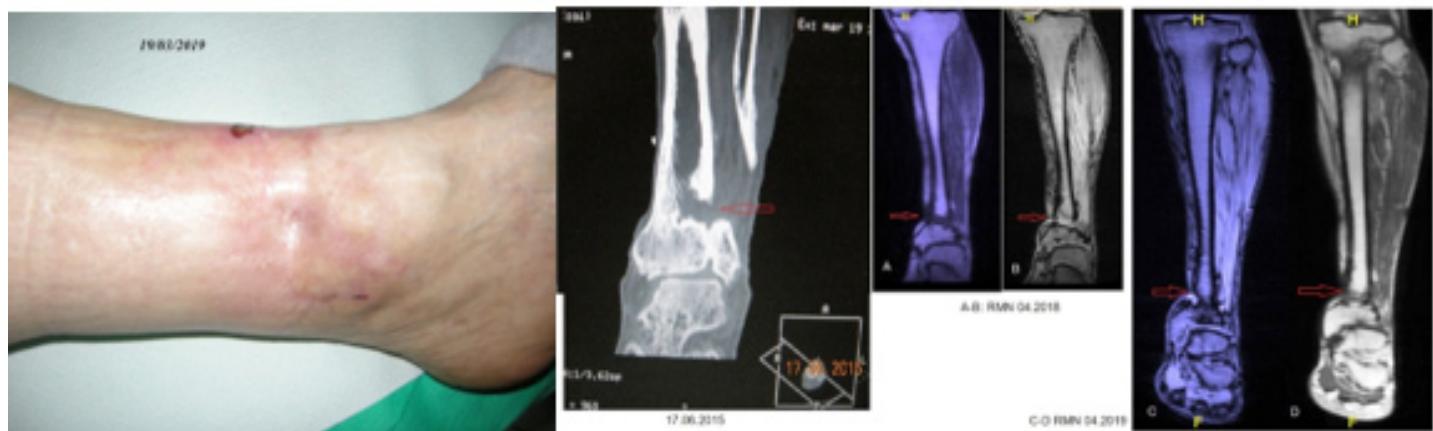


Figure 6: The same patient 4 years after L-PRF therapy. NMR demonstrates osseous regeneration of the lesion (from Crisci et al.2018) [16].

All patients showed positivity to Probe-to-Bone test, and Nuclear Magnetic Resonance highlighted a cortico-periosteum thickening and/or foci of osteolysis in the cortical-spongious portion, adjacent to the ulcer. Gram-positive bacteria were found in 52% of our patients. Among the different found, infective agents, there were Gram-positive bacteria like *S. Aureus* (15.6%), β -hemolytic Streptococci (12.1%), *S. Viridans* (7.1%) and Gram-negative bacilli, like *Pseudomonas* (10.6%), *Proteus* (7.8%), *Enterobacter* (5.7%). *Candida* was found in 2.8% of cases. To follow-up, cutaneous osteomyelitic lesions were found to be healed in all treated patients, with no signs of infections or relapses. In one of the treated patients, during the latest clinical controls, an initial stage of osseous regeneration was found at NMR (Figure 6).



Figure 7: Patient with a 2 years old Diabetic ulcer and osteomyelitis. Results after a single application of A-PRF as therapy.

The use of L-PRF in the management of cutaneous foot lesions by the AA showed the reported results, with a minimal effort in terms of surgical techniques and economical costs for the health structure where patients were treated. Moreover, also the surgical risk to which each patient was exposed is low (our patients were all treated under loco-regional anesthesia).

Discussion

Regenerative properties of L-PRF and its derivatives (A-PRF, i-PRF) (Figure 1), as surgical co-adjuvant material, received notorious attention, since the introduction of the material, in the first years of this millennium. However, there is no clear evidence to explain the antimicrobial potential of this biomaterial, that is structurally and biologically different compared to other HPC forms. Ghanaati et al. [10] described A-PRF as an extracellular matrix, seeded on fibrin, containing various types of blood cells, including: platelet, lymphocytes (B and T), monocytes, stem cells, and neutrophils, which are able to release a series of growth factors. Theoretically, biological components and physiological

mechanisms able to exert the antimicrobial activity are similar among all types of HPC and are similar to clotted blood.

However, all these autologous biomaterials differ among them for: 1) the variable mix of cell types; 2) vitality of contained cells; 3) their activation pathway, either natural or chemical; 4) density of the fibrin meshwork; 5) interactions between cellular and extracellular components; 6) the release of a variety of proteins. All these differences might have a significative result on the respective anti-inflammatory and antimicrobial activities. Moreover, the mechanisms and dynamics of the individual antimicrobial components present in these biomaterials cannot be easily understood.

A-PRF shows antimicrobial activity against all single organisms tested in this study, over a 24 hours interval of time. These results are coherent with those obtained in previous studies, evaluating the antimicrobial properties of other HPC preparations. As A-PRF shows antimicrobial properties, it is necessary to invest-

tigate and establish if this activity is significantly higher than an entire blood clot. Future researches are needed to investigate the A-PRF antimicrobial spectrum, and that of all L-PRF derivatives, as well as to ascertain the possibility that it might act as substratum to facilitate growth of specific organisms. In particular, for surgeons, it is necessary to remember that *Staphylococcus Aureus* is one of the main causes of nosocomial acquired infections, internal medical devices associated infections and surgical wounds infections. A significative research is nowadays focused on alternative therapies for *S. Aureus* infections, to reduce the risk of selecting antibiotic resistant strains [21-25]. This is the reason why *S. Aureus* is still the most frequently tested organism in literature, taking in exam the antimicrobial activity of HPC [19]. Different HPC preparations have demonstrated antimicrobial activity, for both methicillin-resistant and methicillin-sensitive *S. Aureus* strains. *Candida Albicans* is the fungal species most commonly isolated in microbiomes. Compromised immune response might allow these opportunistic fungi to give rise to infection [26,27]. A-PRF has a higher capability to constantly inhibit *C. Albicans* growth, compared to whole blood clots. Furthermore, *C. Albicans* is less sensible to antimicrobial components of platelets and it confirms discoveries made in 2002 by Tan et al. [28], who noticed how human platelets' antimicrobial peptides were more powerful against bacteria compared to fungi. A-PRF shows a greater potential of *Streptococcus* mutant inhibition, compared to natural blood clots.

However, since no other HPC was tested against this organism, the inhibition mechanism and its clinical potential require further studies. Even if results of various studies suggest that A-PRF shows antimicrobial activity, there are several limitations. As first instance, the in vitro exam dose not imitate a clinical situation where A-PRF might be employed, in an environment, surrounded by tissues that react to a surgical intervention. In this scenario, A-PRF is able to interact with different cells and cytokines that are involved in wound healing processes, and can modify the initial immune response and the healing phases. Growth factors, released by activated platelets inside the fibrin meshwork, might modify the expression of antimicrobial peptides by surrounding tissues. It is possible that numerous factors, patient related, might influence A-PRF quality, Yajamanya et al. [29], demonstrated that the fibrin matrix formed in PRF from older patients was more generically organized compared to fibrin matrix formed in younger patients. The entity of this discovery is yet to be determined. Types of cells, number of cells, and plasma component concentration differ inside each clot, and among single clots, each sampled disk cannot be identical to another. One of the problems to be considered and further evaluated is that there is still, up to this date, no way to determine if the tested material is bactericidal or bacteriostatic. Our work group is currently working on this topic. Without considering the disadvantages, the disk diffusion method proved sufficient to demonstrate that A-PRF, like all other L-PRF derivatives, shows antimicrobial activity [30] (Figure 8).



Figure 8: Diabetic lesions of the hand, treated with PRF (from Crisci A., 2018) [31].

Conclusion

There are still many things that are not known about PRF and its derivatives' (A-PRF, i-PRF) antimicrobial properties, and only a scant number of studies highlighted, up to this date, this kind of phenomenon. Under a tissue engineering point of view, it is interesting underlying how no research project focused on the strength, the rigidity and resilience of PRF, notwithstanding its clinical usage during the last 15 years. Hence, an interesting future prospect would be a better characterization of this biomaterials' properties, and future research should focus on those factors that might further improve its characteristics, for its various biomedical employments. It is of fundamental importance that future research, focusing on PRF usage as a co-adjuvant in soft tissue regenerative therapies, should design appropriate studies, with the required controls, to further estimate the regenerative potential for PRF in soft tissue wound healing, in particular for foot wound healing. A-PRF usage in clinical practice showed great potential in improving healing and surgical outcomes, since it works as an autologous scaffold, able to host cells and bioactive compounds. However, the antimicrobial potential of this material has been demonstrated, and it could constitute an important property, further contributing to accelerated, non-complicated healing processes, clinically ascertained. Results of this study show that A-PRF is displaying, nonetheless, antimicrobial activity against *S. Aureus*, *S. Mutans*, *Enterococcus faecalis* and *C. Albicans*. Moreover, spectrum and potency as antimicrobial agent are largely inferior to those of a surgical antimicrobial compound (specific antibiotic). Our work group is also conducting researches, investigating A-PRF and its derivatives, to ascertain the entire spectrum of their antimicrobial activity *in vitro*, their participation *in vivo* and the influence of patient's characteristics on their biological activity. Furthermore, we are exploring PRF's clinical potential as a topical drug administration route in infected sites. Future studies should increase patient's variability and sample's dimensions for all studies based on HPC.

Further clinical, histological and statistical studies are required to fully comprehend the advantages of this novel technique. However, it is important to highlight how, once obtained from autologous blood samples, L-PRF and its derivatives have a reduced volume, and can be used in limited quantities. This limits PRF's systematic usage in major cutaneous lesion. Even if there are ample possibilities for PRF applications, there is need of deep knowledge of this biomaterial's functioning, as well as knowledge of its biology, efficacy and limits, to better optimize its use in everyday clinical practice.

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