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REVIEW ARTICLE

Blood Platelets in Tissue Regeneration: Narrative Review

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Abstract

Background: The growing multidisciplinary tissue engineering field aims to regenerate, enhance, or replace damaged tissue in an efficient manner in a plethora of conditions linked to trauma, disease, and senescence. To ensure that tissue engineering methods are widely available in clinical settings, they need to be modified to make them promptly available and relatively easy to use in daily clinical routines. Therefore, all steps from preparation to application must be shortened and optimized to be practical, and the implementation must be realistic. The overall aim is to develop platelet concentrates of natural origin *close* to patients and to accelerate the application process in a way that is financially sustainable for both patients and the National Health System.

Discussion: This semi-systematic review describes and compares the methods used to obtain these materials and exploit their properties and possible uses in personalized treatments. Platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and their derivatives have been employed in a wide array of medical fields for soft-tissue regeneration.

Conclusions: The results of this systematic review highlight the positive effects of platelets contained in PRF compared to PRP in wound healing and after platelet-based regenerative therapy for the management of soft tissue defects found in *wound care* fields.

Keywords: Platelet-Rich Fibrin, Liquid Fibrinogen, Platelet Concentrate, Platelets, Leukocytes, Prp, Low-Speed Centrifuge,Tissue Regeneration.

1. Introduction

Platelets are notorious for their thrombotic functions. However, apart from their function in blood loss control, platelets also supply numerous mechanisms and steps in lesion healing and tissue restoration, such as tenderness, angiogenesis, cell increase, and delineation. Their therapeutic potential to aid in wound repair has led researchers worldwide to examine platelet-containing crops and their ability to support tissue restoration, both *in vitro* and *in vivo*. In this study, we discuss the main growth factors present in platelet granules that can influence tissue regeneration. Furthermore, we consider how platelet-derived products, such as platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), can be employed to enhance tissue regeneration. We will highlight and examine the possible exploitation of this knowledge in experimental studies and *in vivo models*, as well as the ability of platelet harvest to replace the classical mechanism of *in vitro* cell culture media.

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2. Platelet Biology and Expansion Factors

Approximately one trillion platelets are present in an adult human bloodstream at any time (3×10^8 platelets/ml in 4 lt of blood). Platelets have a existence of about 10 days. They are synthesized by megakaryocytic cells in the bone substance of extended frame, and about 10% of them are replenished daily. "Aged" and injured platelets are eliminated by phagocytes present in the liver and spleen. In the bloodstream, platelets scan blood vessels, looking for any verification of injure. If injure is sensed, they take part in haemostatic actions. After platelets are activated by vascular damage, they modify their disc-like shape into a more globular morphology, complete with pseudopodia, and subsequently release their grainy inside [1] (Figure 1).

These granules are not simply linked to coagulation processes and haemostasis, but also take roles in tissue restore functions. Granules are distinguished in 3 different types: α-granules, dense granules and lysozymes.

α−granules are the majority copious type of granules present in human being platelets (around 50-80 per single platelet) and include a very wide protein pool, counting a assortment of molecules exhibiting active organic roles (Table 1) in tissue regeneration.

Table 1. *Major bioactive molecules in platelet α-granules [4].*

CATHEGORY	MOLECULES
EXPANSION FACTORS	TGF-β (Transforming Growth Factor-β)
	PDGF (Platelet-Derived Growth Factor)
	FGF (Fibroblast Growth Factor)
	EGF (Epidermal Growth Factor)
	VEGF (Vascular Endothelial Growth Factor)
ADHESIVE PROTEINS	Fibrinogen
	Fibronectin
	Vitronectin
	Thrombospondin-1

Among all the biologically active molecules found in platelets, the largest quota active in tissue healing and repair processes is attributed to the so-called growth factors, mainly stored in α-granules. These molecules are unconfined subsequent platelet opening and play significant practical roles at sites of vascular injury. Numerous bioactive proteins resultant from platelets play a critical role in irritation, angiogenesis and wound therapeutic. For instance, TGF-β1, which is the majority rich isoform of TGF-β found in platelets, has an significant role in all phases of lesion therapeutic. It coordinates numerous pathophysiological actions, counting initial employment of inflammatory cells to wound sites, angiogenesis, re-epithelialization subsequent injury and the generation of extracellular matrix creation by fibroblasts. PDGF constitutes a chemo attractant fragment for fibroblasts and horizontal muscle cells, and it also induces mesenchymal cell proliferation.

Of all the growth factors stored in platelets, those essential for wound repair are PDGF, with its isoforms -AB and -C (predominant isoforms in platelets); also, vascular endothelial growth factor (VEGF), represented mainly by the VEGF-A form, transforming growth factor β1 (TGF-β1), basal fibroblast growth factor (bFGF), mainly its FGF-2 form; epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1). They all exhibit a short half-life, and are known to cause, in most cases, local effects. Members of the TGF-β family are important in wound repair and scar formation. TGF-β1 is activated starting from a secreted latent form, and can negatively affect angiogenesis, even though it promotes matrix proteins production [2].

FGF-2, the major FGF isoform current in platelets, promotes angiogenesis by sustaining endothelial cells increase. It is also a strong fibroblasts mitogen and is able to provoke hyaluronic acid production, easing up scarless injury curing. EGF enables mesenchymal cell increase, chemotaxis and cytoprotection. VEGF is a proangiogenic biomolecule, stimulating blood vessel development and the appearance of linkage proteins, able to augment leukocyte adhesion. In reality, added than 300 bioactive agents have been recognized that are unrestricted from activated platelets. These agents be different in their derivation, as some mechanism are synthesized in the parent megakaryocyte though others are healthier from the plasma and obtain concentrated in platelet globules (Table 1).

Solid globules enclose factors associated to platelet activation, such as Ca^{2+} and ADP, serotonin, histamine, dopamine, and catecholamines. Restricted discharge of these components, in reaction to platelet activation or thrombotic procedures, results in distorted employment of inflammatory cell types and distorted vascular permeability. In conclusion, lysosomes enclose hydrolytic enzymes and catalase. Platelet activation, with associated liberate of granular filling, occurs in equivalent with coagulation or thrombosis. The physiological contribution of platelets in hemostasis and tissue restore has led to the expansion of products that might support in these processes.

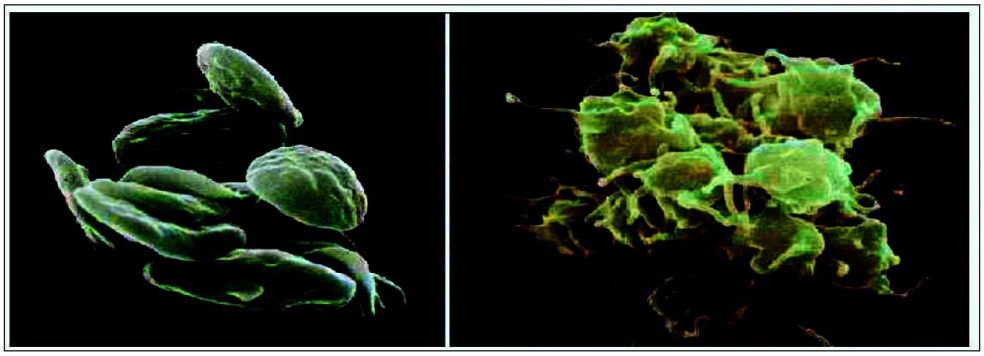

Figure 1. *Platelet activation Legend: Resting platelets are smooth and disc shake (left). Activated platelets have an irregular shape with many protruding pseudopodia (right). The arrow indicates the presence of pseudopodia (activation).*

Given its potential applications and the extensive range of studies conducted, PRP represents the inaugural platelet-based innovation considered for tissue restoration purposes (Figure 2). It is a platelet concentrate within a minimal plasma volume, obtained through the centrifugation of whole blood to remove red and white blood cells. The typical platelet concentration in peripheral blood is estimated to be 150-350 × 106/ml [3,4].

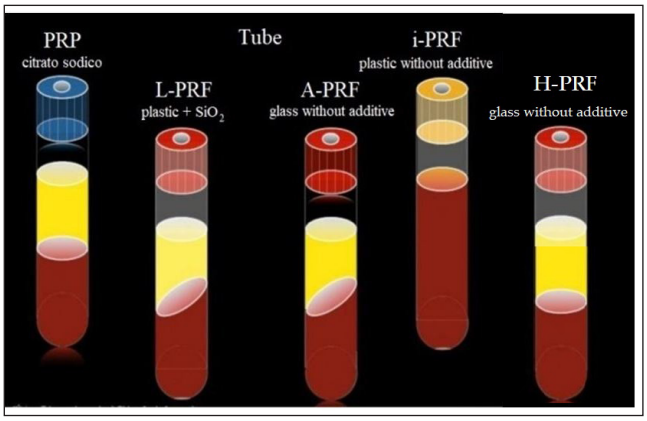

Figure 2. *Various Platelet concentrate types*

In the context of tissue engineering and injury repair, the term PRP (Platelet-Rich Plasma) refers to a plasma platelet concentration exceeding the normal range, which can be injected into a lesion site to influence or expedite repair. The medical application of PRP, primarily in bone and soft tissue regeneration, involves a platelet concentration of at least 10^9 per ml, which is approximately five times higher than physiological levels. At lower concentrations, the effect is suboptimal, while at elevated concentrations, it may have inhibitory effects. The therapeutic action of platelet concentrate arises from the release of growth factors, which are implicated in tissue repair following platelet activation. The clot formed during activation may also serve as a temporary extracellular matrix, facilitating cell proliferation and differentiation. In this context, a high platelet count is likely to lead to a high localized concentration of free bioactive factors. However, the correlation between platelet count and the concentration of released bioactive agents may not be precise, due to variations among blood donors or platelet preparation methods [PRP or PRF (L-PRF, A-PRF+, H-PRF)]. Furthermore, some growth factors involved in tissue repair are also present in plasma, such as HGF and IGF-1. Consequently, the concentrations of these factors at wound sites can be only slightly altered based on platelet count. Generally, platelet concentrations five times higher than peripheral blood concentration may lead to an increased localized concentration of growth factors, ranging from three to five times higher than normal physiological levels. Therefore, serving as a reservoir

of concentrated growth factors involved in cell proliferation and differentiation, platelet concentrates may contribute to tissue growth and repair. Similarly, platelet-derived bioactive products can be utilized in cell culture protocols.

3. Platelet Foodstuffs (PRP vs. PRF, A-PRF, L-PRF, APG, etc.).

Since the advent of cell culture techniques, numerous advancements have been pursued to optimize the process of in vitro cell development, examining their differentiation potential and response to chemical agents and promising pharmaceutical compounds. Recently, significant progress has been made in the development of plastics, glassware, bioreactors, and engineering technologies, alongside advancements in biological sciences. Methods for cultivating cells in vitro aim to accurately replicate the physiological conditions observed in vivo, striving to mimic these conditions in vitro. The culture medium serves as the foundation of soluble factors that facilitate cell growth and survival. Traditional cell culture media consist of a basal balanced salt solution, such as MEM (Eagle's Minimum Essential Medium), DMEM (Dulbecco's Modified Eagle's Medium), IMDM (Iscove's Modified Dulbecco's Medium), RPMI (Roswell Park Memorial Institute Medium), Medium-199, Ham's F12, and McCoy's Medium. Although these media contain essential salts, amino acids, vitamins, and glucose, a protein-rich supplement, such as fetal bovine serum (FBS), is required to provide growth factors. FBS contains carrier proteins that transport hormones (e.g., transcortin), minerals, trace elements (e.g., transferrin), and lipids (e.g., lipoproteins). Additionally, FBS includes accessory and spreading factors, acting as nucleation sites for cell attachment, as well as stabilizing and detoxifying factors necessary to maintain pH or directly inhibit proteases, such as α-antitrypsin or α2-macroglobulin, or indirectly inhibit them by serving as a non-specific barrier for proteases and other potentially toxic molecules.

Cell culture usage is robustly suggested as an substitute to animal testing. In addition to moral issues, controlled questions are moreover raised about the use of FBS. First, presence of batch-to-batch variations construct it necessary to test samples before purchasing, as FBS constitution of parties molecular can vary. FBS may interfere with the genotype and phenotype of cells, influencing the experimental outcome. For example, it can promote cell proliferation in fibroblasts, while inhibiting it in epithelial cells. Furthermore, it can be contaminated with viruses, bacteria, mycoplasmas, yeasts, fungi, immunoglobulins, endotoxins and possibly prions, which contraindicates its use for cells needed for human transplants. FBS is not fully defined chemically, as numerous substances in attendance in it include not yet been characterized, the functions of some others has not yet been discovered fully and examined, and some more might smooth be lethal. Serum, obtained from clotted complete blood, is identified to be additional appropriate for cell culture than plasma obtained from the similar creature, although the complexity of collecting it in big quantities. This is probably due to the liberate of proteins and increase factors from activated platelets throughout the coagulation procedure. Consequently, PRP, platelet lysates, PRF and other platelet-derived foodstuffs might be able to replace FBS in cell culture. Because platelets are present in a elevated concentration, expansion factors significant for cell culture are also more intense, as previously discussed in this work [5].

PRF is a second generation platelet concentrate that does not contain anticoagulant factors. Choukroun et al. [6] were the first to define Platelet-rich Fibrin. It can facilitate wound closure, accelerate bone healing [7] and improve graft survival without causing significant complications.

PRF has repeatedly shown several advantages over PRP and include:

- Faster and less expensive preparation time;
- Better handling and usage properties;
- It is non-expensive;
- It has no additional additives such as anticoagulants, thrombin or calcium chloride, which are required for PRP preparation;
- Simple processing and transport to the operative site;

PRF cannot be stored in tissue banks since it contains circulating immune cells, and all plasma antigen molecules are strictly donor specific; therefore it cannot be used as an allogeneic graft material. PRF can be used as a wound closure adjuvant since it meets all the clinical criteria to induce successful wound healing. However, several parameters of PRF still need to be clinically tested before it can be definitively included in current clinical practice.

An evolutionary form of PRF, A-PRF (advanced-PRF), has also been the subject of investigation in recent years. Decreasing the number of centrifuge rotations and the duration of processing resulted in an

increase in the number of neutrophil granulocytes in the distal region of the A-PRF clot (Fig.3) [8].

So far, neither PRF nor PRP are part of routine clinical wound treatment.

4. Applications in Regeneration (Wounds, Bones, Tendons)

In 1987, PRP emerged as an autologous transfusion product before open-heart operation, to obviate the require for a homologous creation. In 1998, Marx et al. described the use of platelets as an accelerator of tissue restore/restoration; in this case, bone creation in bone grafts for maxillofacial surgery. PRF was first mentioned by Choukroun in 2001 [6] as an advanced platelet concentrate. Because next, the majority studies include shown an improvement in bone, musculoskeletal (muscle, tendon and cartilage) and other "elastic" tissue repair after using platelet concentrates.

Human PRP, demonstrated its angiogenic properties in an animal representation with a significant-sized cranial defect. Furthermore, when used in synergy with BMP-2, it showed an increased result on bone medicinal, as observed on histological samples, bone mineral density and bone mineral substance after 8 weeks of grafting. PRP-treated mesenchymal cells are also intelligent to better encourage bone restore and maturation processes in mandibular bone defect models, being indicated as an substitute to autogenous grafts. This has been highlighted when canine mandibular bone defects were filled with autologous PRP gel, autologous PRP gel with bone marrow MSCs (Mesenchymal Stem Cells) or cancellous bone, and autogenous particulate bone marrow (PCBM).

In muscular injuries, various factors found in platelet concentrates such as IGF-1 and bFGF have been shown to speed up tissue restore. In distinction, TGF-β can direct to fibrotic renovate, escalating recurrence rate of novel injuries. Animal studies and human trials for tendon lesions demonstrate positive results when PRP has been used. Although clinical trials with correct methodologies include not yet demonstrated PRP efficacy in this category of wound, contained administration of platelets can provoke circulating cells mobilization at sites of tendon injuries in rat models, among associated augment in collagen production. *In vitro*, platelets can induce proliferation, collagen synthesis, and release of angiogenic factors in human tenocytes. A systematic review stated that there is strong evidence against PRP injection for chronic lateral epicondylar tendinopathy. In a total of 6 studies, 5 showed no significant benefit at final follow-up, meanwhile highlighting possible benefits in favor of PRP injections over corticosteroid injection [3].

L-PRF is preferred over PRP and PRGF (Platelet Rich Plasma and Platelet Derived Growth Factor) since it exhibits a solid structure of fibrin and does not require any biochemical alteration through addition of bovine thrombin or anticoagulants. As a result of fibrin natural properties, growth elements can sustain their action for a generally longer period of time and allow tissue repair. Platelets are able to take on additional roles in tissue fixation and vascular remodeling, as well as covering a dynamic role in the inflammatory and immune responses. Platelet granules are able to store a significant amount of important substances, as effectively highlighted by scanning electron microscopy (SEM) and immunofluorescence staining. Thin fibers contained in HPC (human platelet concentrate) could be identified with the initial high centralization of platelets.

Fibrin glue proteins are quite abundant on the fibrin network mesh: Fibrinogen (Fg), Fibronectin (Fn), Vitronectin (Vn), Thrombospondin-1 (TSP-1). Fibronectin (Fn) initiates and facilitates wound healing and promotes the mitogenic migration of platelet-derived growth factor (PDGF). Members of the TGF family are known to be fundamental in wound healing and scar arrangement. Holding a dominant role in healing, platelets are a rich source of cytokines and chemokines. Platelets are the source of tissue inhibitors of metalloproteinases (TIMP 1-4) which are also found in granules and vesicles of the cytoplasmic layer. Metalloproteinases (MMPs) are a group of enzymes whose fundamental ability is the breakdown of extracellular matrix proteins, including collagen, fibronectin, elastin and proteoglycans. PRF clots can be considered as a bioactive depot.

High hematocrit or low platelet count may be a limiting component, and further analysis is needed to establish an ideal mean platelet concentration of the material to be used in the methodology [9]. PRF clotting is achieved by a characteristic polymerization process, during centrifugation, with the common fibrin design being effectively responsible for moderate releases of GF and network glycoproteins (≥7 to 28 days). PRF clots are used directly to fill plastic wells and in general medical procedure medications. Although platelet GFs play a fundamental role in PRF functions, fibrin design, presence of leukocytes and proximity of stem cells are three key parameters [8].

PRP and PRF have also been exposed to stimulate mesenchymal stem cell (MSC) production.

Investigating stimulation of osteogenesis on MSCs, mouse MSCs were treated through human being PRP activated by thrombin/calcium mixture or washed platelets (WPLT), in which platelets were poised in phosphate and salt solution instead of plasma, through identical platelet attentiveness, 4 times higher than baseline. Fascinatingly, equally agents stirred cell production at previous moment in time, although WPLT induced greater increase than PRP at afterward moment in time. Otherwise, ALP (Alkaline Phosphatase) activity and form I collagen appearance, indicative of osteogenic delineation, were found to be amplified in cells treated with PRP relatively than WPLT. In other research, PRP clot might stimulate osteocalcin and type 1 collagen appearance in rat MSCs, just like activated PRP, among platelet concentration 4-fold superior than baseline, induced increased mineralization in human MSCs. The confinement of platelets within the PRF gel was inspected by immunostaining and scanning electron microscope (SEM) guidance. Furthermore, platelet-derived factors are shown to initiate and control the expansion and migration of several cell types, associated with tissue fixation, similar to smooth muscle cells (SMCs) and immature mesenchymal stem cells (MSCs). Activated platelets release a full spectrum of chemokines and advance the assimilation, uptake and expansion of basal adult microcells, including CD-34+ stem cells, MSCs, SMC generators and endothelial stem cells [10].

Histomorphometric examination was performed using an optical magnification with 100 × all out amplification. A 10 mm eyepiece, with a 100-division grid, was used to quantify the level of inclusion of the absolute length within each event of cell layers, in each area. With Masson's trichrome staining (adjusted by Godman), platelet accumulations are found in dark blue, but RBCs are effectively recognized, as they are recolored in red. With this association, it is difficult to recognize the contained cellular elements. The greater amount of platelets and leukocytes were found in the initial millimeter of the yellow clot, at the limit with the red clot. This result has a gigantic clinical effect since the number of leukocytes present inside the membrane is considerable, and small lymphocytes are particularly involved in inflammatory responses. Based on these findings, the most useful segment, under careful perspective, is the initial whitish part of the PRF membrane (Figure 3A). Therefore, it is important to include a small layer of RBC at the edge of the clot, which contains the platelet and leukocyte load [11].

5. Low Speed Centrifugation Concept (LSCC)

A systematic study by Choukroun and Ghanaati (2017) highlighted the influence of the reduction of relative centrifugation force (RCF), on the number of leukocytes and platelets in PRF-based fluid matrices (i-PRF)(Fig.4), as well as their role in the release of growth factors, following the low speed centrifugation concept (LSCC); it shows that reduction of RCF value increases the number of cells and the release of growth factors within PRF-based matrices [12, 13]. The distribution of platelets in PRF matrices, identified via immunohistochemical staining by CD-61 antibodies, was performed by El Bagdadi et al. (2017) [14] (Figure 5) to determine the platelet distribution in three different PRF-based matrices cross-sections [15]. Platelet distribution was assessed in relation to the position in the clot. Platelets were found to be accumulated within all three segments of the clot. L-PRF, which was prepared with a high RCF (2400 rpm, 708 g, for 12 min) showed a different distribution pattern based on location. The upper (C) and central (B) portions of the clot showed only a few platelets, while most of the platelets were distributed in L-PRF lower part (A) (Figure 3).

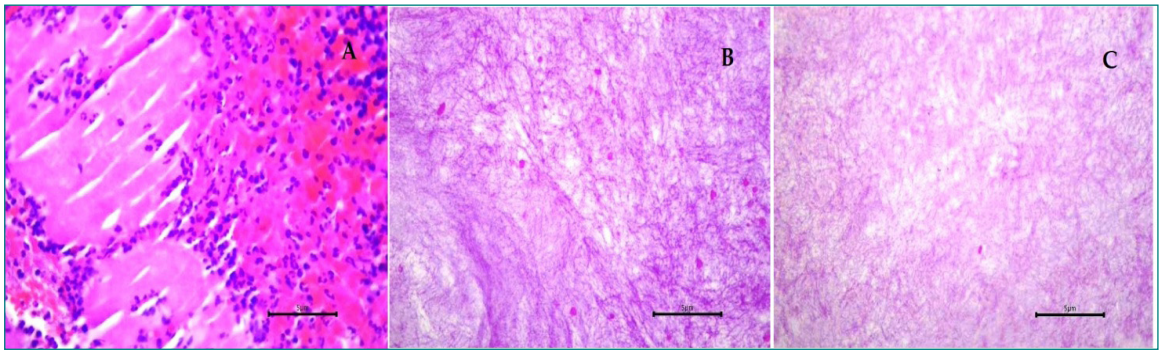

Figure 3. *Membrane L-PRF 0 min after compression (hematoxylin-eosin staining). (A) III proximal 40× fibrin on the right, the center lymphocytes, erythrocytes, and granulocytes neutrophils on the left; (B) average III 40× pattern of fibrin; (C) III distal 40× pattern fibrin [13].*

By contrast, A-PRF$^+$, prepared via reduced RCF (1300 rpm, 208 g, for 8 min), exhibits a totally different scheme of distribution. Platelet are spaced within the whole clot. A-PRF$^+$ with reduced RCF and reduced time of centrifugation, shows an equal distribution pattern within the clot (Figure 4, 5, 6, 7).

A-PRF+	A-PRF+	1300 rpm, 8min
i-PRF	I-PRF	700 rpm, 3min
i-PRF M	I-PRF *M*	700 rpm, 4min
i-PRF+	I-PRF+	700 rpm, 5min
A-PRF Liquid	A-PRF Liquid	1300 rpm, 5min
Custom	Custom	1300 rpm, 3min
	L-PRF	2700 rpm, 12min
	H-PRF	1300 rpm, 8min orizontal centrifuge

Figure 4. *Various types of Second Generation Platelet Concentrate*

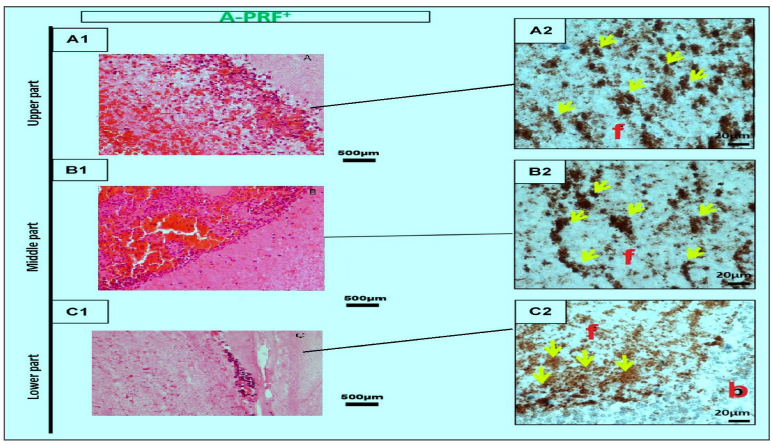

Figure 5. *CD-61 immunohistochemical analysis of A-PRF$^+$ according to the different regions. A1, A2 upper portion; B1, B2 middle portion; C1, C2 lower portion (A1, B1, C1 total scan sections; ×100 magnification, scale bar 500 µm). A2, B2, C2 Show the distribution pattern of platelets (yellow arrows) in higher magnification (f fibrin; b buffy coat; ×400 magnification; scale bar 20 µm) [14].*

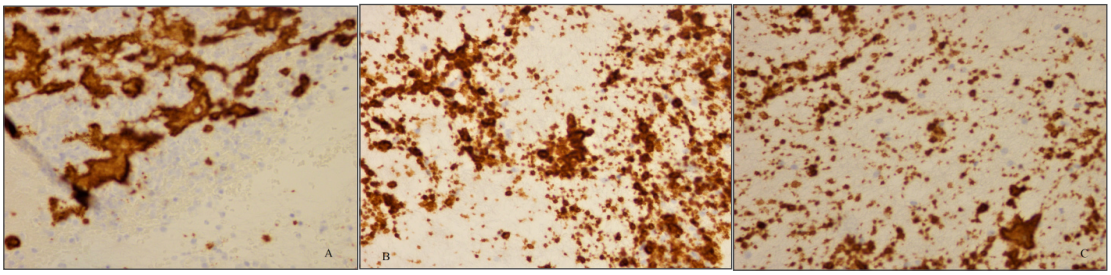

Figure 6. *CD 61+ 40× A=head B=body C=tail*

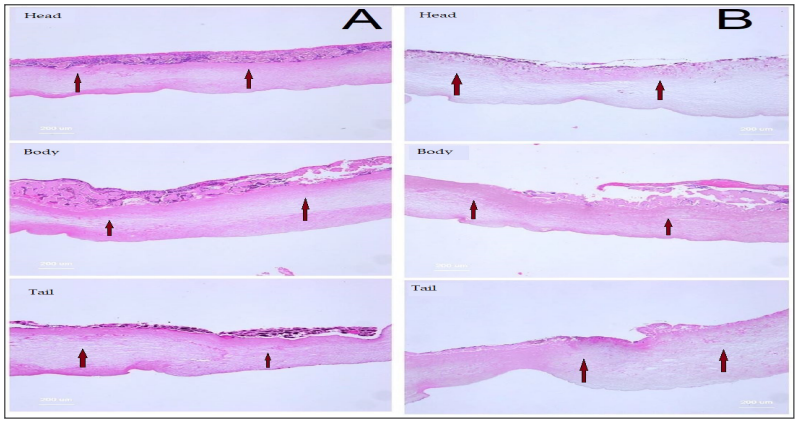

Figure 7. *Platelet distribution in PRF matrix prepared using A-PRF$^+$ glass tubes via low- (A: 700 rpm for 14 min) and high-speed centrifugation (B: 30 s, acceleration; 2 min, 692 × g; 4 min, 547 × g; 4 min, 592 × g; 3 min, 855 × g; 36 s, deceleration). The arrows indicate the direction of the centrifugal force.*

6. Ideal Platelet Concentration in Platelet Concentrates

An unresolved issue is represented by the optimal platelet concentration in platelet concentrates, able to influence the healing processes and repair of damaged tissue. Some authors suggest that a density fluctuating 3-4 times the baseline is optimal for tissue healing, which has been demonstrated via the example of bone tissue. Other authors, however, consider as an optimal therapeutic level a concentration of platelets in PRP at least five times the basal value of whole blood. Although currently available devices are able to obtain densities more than 8 times higher, there are studies demonstrating an inhibitory effect on regeneration processes with high concentrations of platelets in PRP compared to whole blood. This is confirmed by tests using PRP (at a density 9-11 times higher than whole blood) in the treatment of bone tissue caries. To explain the inhibitory effect of the application of platelet-rich plasma for regenerative processes, the authors hypothesized a cytotoxic effect given by large concentrations of growth factors contained in PRP. However, Lee et al. [15] where able, in their research based on platelet-rich plasma with platelet density more than 8 times the baseline values, to obtain satisfactory results in experimentally regenerating damaged knee joint cartilage in rabbits. The species of platelet concentrates called platelet-rich fibrin (PRF), obtained by immediately centrifuging the patient's blood to maximum speed, is colleted in vials not containing any anticoagulant, differently from PRP. The coagulation process activation in collected blood is caused by both centrifugation and the formation of a fibrin clot in the middle of the tube, that is between the mass of red blood cells at the bottom and the plasma depleted of platelet, which are instead involved clot formation. The product thus obtained has the same gelatinous consistency and also contains cytokines and growth factors found in platelet-rich plasma, without the need to activate it.

6.1 Fibrinogen Role in Platelets

Fibrinogen, synthesized primarily by hepatocytes, is the most abundant procoagulant factor in plasma. Fibrinogen constitutes 10% of the α-granule protein and is the predominant adhesive protein secreted by platelets (Table 1). Recent data indicate that platelet fibrinogen is not synthesized by megakaryocytes, that is platelet precursor cells in the bone marrow, as previously thought, but is acquired exclusively from plasma by endocytosis. Since fibrinogen is present in α-granules at a higher concentration than other plasma acquired proteins (for example, albumin), endocytosis of fibrinogen appears to be receptor-mediated. Because the primary receptor for fibrinogen present on cells of platelet-megakaryocyte lineage is $\alpha_{IIb}\beta_3$, and since Glanzmann's thrombosthenics, lacking this receptor, also lack platelet α-granular fibrinogen, it was hypothesized that fibrinogen was endocytosed through this receptor. However, it is also possible that a small amount of fibrinogen may enter α-granules through alternative receptors (that is, $\alpha_v\beta_3$) and/or by fluid-phase endocytosis.

Studies suggest that fibrinogen can bind to $\alpha_{IIb}\beta_3$ megakaryocytes (and possibly platelets) in the absence of cellular activation. This is an intriguing possibility, as it was previously thought that platelets can bind soluble fibrinogen only after being "activated." Stable fibrinogen, however, binds to $\alpha_{IIb}\beta_3$ on unactivated platelets. Recent data indicate that fibrinogen bound to an integrin (that is, $\alpha_v\beta_3$) can bind to $\alpha_{IIb}\beta_3$ on the same or another cell without prior activation. Similar mechanisms may operate *in vivo* [16].

Fibrinogen is involved in atherosclerosis and thrombosis, and reflects inflammatory changes and endothelial dysfunction in vascular injury, contributing to a hypercoagulable state of the blood, which is a condition found in thrombosis and subclinical atherosclerosis. It is an important determinant of blood viscosity and platelet aggregation and may play a role in endothelial damage, formation of low-osmolar fibrin clots, thrombosis, blood flow abnormalities, and platelet hyperactivity. Different studies suggest that fibrinogen is significantly associated with vascular injury and thrombosis. Fib is also closely related to Diabetic Foot (DF). The relationship between α angle, *k*-value at thromboelastography, fibrinogen and diabetic foot is an index of the risk of diabetic foot onset and progression, with the aim of investigating the optimal cut-off point value of the above three tests (Li et al. 2016) [17]. Platelet hyperreactivity, coagulation status and abnormal fibrinolytic function are prevalent in diabetic foot patients and worsen with disease progression. Metabolic abnormalities increase fibrinogen, causing a state of blood hypercoagulability in diabetic foot patients, which predisposes to thrombi formation and causes microvascular and lower limb macroangiopathy [18].

6.2 Antimicrobial Properties

Bacteria are heterogeneously distributed in wound tissue, and thus the identification and abundance of bacteria are influenced by collection techniques such as inoculation or tissue biopsy [19, 20]. The

individual bacteria identification also depends on the employed methodology. Bacteria can be identified by culture, phenotypic characterization, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), DNA and 16S rRNA polymerase chain reaction (PCR), and sequencing. There is a lack of knowledge about the interactions present between different microorganisms and whether coexistence, symbiosis, or hostile coexistence are present. Many *in vitro*, *in vivo*, and animal models exist to assess wound microbiology, but bacteria are found to behave differently in different models [21], limiting inference between them.

When considering specific bacterial species, results obtained using platelet concentrates (PC) have sometimes been contradictory. These discrepancies could be due to several reasons, such as intrinsic characteristics of bacterial strains considered, which can present a different susceptibility to platelet concentrates per se, as well as different test sensitivities, among those used to evaluate the antibacterial activity. Other important reasons to explain the high variability observed in results, could be attributed to the types of platelet concentrate used that can differ in form (gel or liquid), as well as in platelet concentration, in leukocyte content, in fibrin network density, in mode of activation, which might occur naturally by contact with tissues or may be induced by thrombin or calcium chloride [22]. These data seem to suggest that some plasma components (such as complement) are main responsible for antimicrobial action and activity of platelet concentrates, and that cooperation between platelets and plastic elements is necessary. Examining the obtained study results from various working groups [23-26], leukocyte-rich preparations certainly exhibit antibacterial activity, but only few studies compared leukocyte-poor and leukocyte-rich platelet concentrates: results obtained seem to suggest that there are no substantial differences in antimicrobial activity between the two formulations. Thus, it could be inferred that white blood cells possess phagocytic activity and constitute a rich source of antimicrobial molecules (e.g.: defensins, cathelicidins, lysozyme, myeloperoxidase) [27].

Bacterial strains, in fact, as previously described [10], have been isolated from infected wounds; specifically, strains listed in Figure 8 have been found.

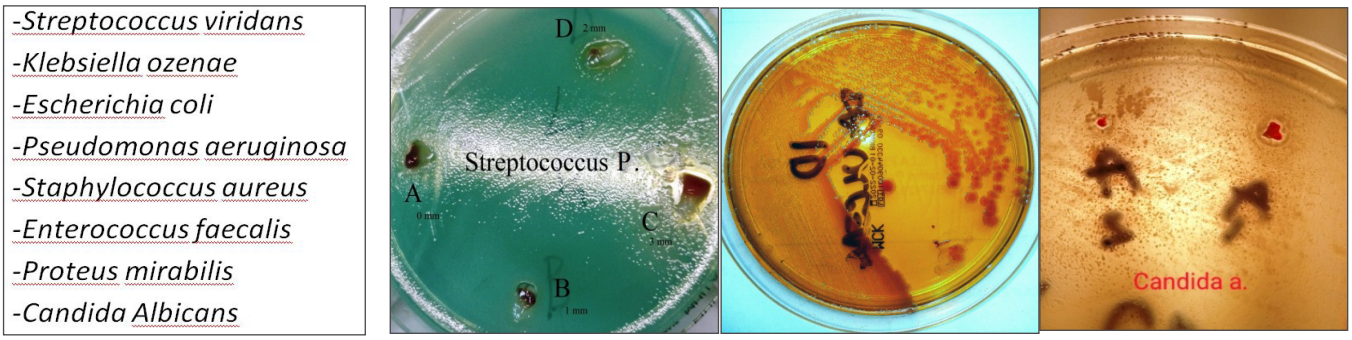

Figure 8. *Bacterial and fungal strains observed and isolated from skin ulcer cultures: in the central picture, Pseudomonas Aeruginosa*

The antibacterial activity was tested using the kill test, that is counting the number of bacterial colonies surviving after incubation at different time points, namely baseline, 4 and 8 h. Results showed that all fractions remained bacteriostatic for up to 4 h, both for methicillin-sensitive and resistant strains of bacteria, but after 8 h a certain bacterial regrowth could be observed.

In fact, a recovery of bacterial growth was always observed after an initial reduction of the inoculum, suggesting that platelet concentrates could exhibit inhibitory (bacteriostatic) rather than microbicidal activity. Regarding individual bacterial species, S. aureus was the most intensively tested microorganism and it always showed sensitivity to platelet concentrates, while Escherichia coli, Proteus Mirabilis, Candida Albicans, Streptococcus Viridans, Pseudomonas aeruginosa and Klebsiella pneumoniae were less frequently tested and produced contradictory results: in some studies antimicrobial activity was observed, but in others no inhibitory effect was highlighted against these species. In this work, our group wanted to investigate the inhibitory effect in relation to these bacterial strains as well as to the type of platelet concentrate employed. Therefore, the antibacterial activity of both PRF and PRP was investigated; however, the inhibited area was significantly larger when PRP was used.

The main limitation of these studies is the artificial *in vitro* system used to produce the platelet concentrates. *In vivo*, a physiological tissue environment would influence the behavior of the platelet concentrate

in terms of structure, cellular crosstalk, exposure to degradation enzymes and release of growth factors. However, the characterization of L-PRF and its derivatives *in vitro* remains an important step towards understanding the *in vivo* effects. For example, knowledge of the antimicrobial effect of L-PRF and derivatives could provide important guidelines for the choice of tissues and/or lesions this product can be effectively employed for in future preclinical and ultimately clinical studies.

Even with slight variations in timing for different strains and for different preparations, all studies seem to agree that 4 hours is the optimal incubation time, as it results in the greatest bacterial colony decrease. All studies agree that the preparations are bacteriostatic, ultimately resulting in regrowth, however it must be noted that PRF preparations are useful tools, more likely to be used in prophylactical clinical settings rather than therapeutically, in stable infections treatment and management. Authors suggest the use of formulations containing leukocytes and platelets, after surgical debridement, to both reduce bacterial load (killing bacteria and inhibiting biofilm formation [2]) as well as to stimulate healing processes.

Further researches and clinical studies are needed to evaluate PRF potential benefits, in terms of antimicrobial activity. It is evident, relying on available data, that PRF exhibits antimicrobial activity against microbial pathogens. Based on study reviews [28], it can be concluded that, among all various PCs, PRF in particular has higher potential antimicrobial activity than all other concentrates. This activity can be enhanced by specific preparation protocols, such as lowering the centrifugal speed and reducing the time of centrifugation, or even by incorporating antibiotics into the fibrin matrix, acting as a slow-release drug delivery system. Considering its antimicrobial effect on pathogens, PRF should be considered as a potential local drug delivery system as well as a natural regenerative material; these findings would make this biological material unique compared to all other biomaterials currently used. Future research should focus on the exact role as antimicrobial agents of each growth factor and molecules present in PRF, when taken in consideration together with patient specific parameters, such as age, gender, hematochemical values and drug interactions.

First point-of-care diagnostic tests generation classify protease activity as normal or elevated. This represents a therapeutical challenge: treatment should block the activity of pathological MMPs but should not affect MMPs required for normal wound healing. Experimental studies in humans suggest that MMP-1 is important for epithelialization [20]. A systematic review showed that MMP-1 levels were found to be higher in chronic wounds which tend to heal compared to chronic wounds with no tendency to heal [2]. The only MMP inhibitor approved for human use is doxycycline (Bassado), which is administered in subantimicrobial doses. This inhibitor was tested in 20 patients with non-healing venous leg ulcers as an adjuvant to compression therapy. However, treatment did not significantly reduce MMP activity, perhaps because of the low doxycycline concentrations in the wound fluid.

7. Examples of Platelet Concentrates Employment on Medicine: Limitations and Challenges

In both human and veterinary medicine, PRP and PRF are most commonly used in the treatment of: periodontal diseases and oral surgery, maxillofacial surgery, orthopedics and in the so-called surgical trauma. PRP has also found applications in difficult to heal wound management, such as diabetic foot ulcers and burns. In addition, they are used in cardiac surgery, neurosurgery and plastic surgery. The use of platelet-rich plasma in orthopedics began with maxillofacial surgery, where PRP was used to accelerate bone regeneration in the implantation of prosthetics. Good results are achieved in this area of expertise, through PRF and PRP growth factor employment, for treatment of: bone fractures, pseudoarthrosis and osteomyelitis [29]. The proliferation of tenocytes is stimulated via the employment of platelet-rich plasma, thanks to the stimulating effect of molecules contained in PRP, in soft tissue disease treatment of the musculoskeletal system, that is tendons and ligaments. PRP is used in the treatment of tendinopathy and has been used both in humans and animals (mainly horses). Platelet-rich plasma has also been used in humans to improve (tissue) implants healing, in particular the anterior cruciate ligament after intra-articular reconstruction surgeries. These studies have shown an improvement in the reconstruction and healing of the implants used, which was evident at computer tomography and magnetic resonance imaging.

In literature, there are reports in which authors highlight liquid PRP/PRF positive effects when administered intra-articularly during the arthrosis treatment, including knee joint treatment in cruciate ligament injury in dogs. Satisfactory results are also shown when analyzing the effects of intra-articular

platelet-rich plasma employment, in experimental studies on rabbits where knee joint inflammation was induced by introducing intra-articular collagenase; these studies have shown a protective effect of platelet-rich plasma on articular cartilage and a beneficial effect on the quality of synovial fluid. The results of the presented research are consistent with the results of *in vitro* tests, confirming the stimulating effect of growth factors contained in PRP on the proliferation and metabolism of articular cartilage. The use of PRP in the treatment of joint and cartilage degenerative changes and defects is also practiced in human medicine. An example is the arthroscopic treatment of articular cartilage defects via microfractures in which, after the operation per-se, platelet-rich plasma is introduced in gel form. This action leads to better results compared to control group, even in individuals of over 40 years of age undergoing surgery, while groups not employing PRP showed no significant clinical improvements. The future direction of PRP and PRF use is their application in tissue engineering, which allows necessary tissue growth for the employment of growth factors connected to isolated or laboratory grown cells and mounted on a special support. Among these studies, it is relevant the evaluation of bone defects treatment efficacy, when such defects are filled with bone marrow mesenchymal stem cells associated with PRP and PRF, offering both scaffolding function and a growth factor source. Obtained results were significantly better when compared to control groups or a group treated with only platelet-rich plasma. Other proven properties of platelets are: their anti-inflammatory and analgesic effect, which has been confirmed in both experimental and clinical studies.

8. Conclusions

Several scientists compared PRF, PRP and the induced-bleeding technique effectiveness. Narang et al. [30] concluded that PRF has much more potential in tissue regeneration processes when compared to PRP and blood clots. Using the patient's own blood for regeneration can be a valiant source of psychological support for both the patient and also the attending surgeon, resulting in an overall benefit to mankind.

It can be concluded that PRF like other platelet concentrates (APG, platelet gel) can be easily prepared during surgery and possesses bactericidal and antibiofilm activity [31, 32]. The APG activation protocol is able to increase the mechanical characteristics of blood derivatives and could be clinically useful for improving regenerative procedures. It may act as an antimicrobial peptide and a potential bioactive agent in order to prevent postoperative infections at surgical sites. Further research is needed to deeply evaluate the broad-spectrum of PRF antimicrobial properties using *in vivo* models. We hope that our conclusion will be supported by the findings of other researchers. Further studies on this topic are needed.

APG membranes demonstrated beneficial physical and tensile properties in vitro, while the activation protocol with autologous thrombin and calcium chloride did not appear to have any impact on their mechanical behavior.

In conclusion, the application of platelet gel or of PRF in membranes could be a promising and useful treatment method for reconstructing bone defects and nonunions, and future clinical studies are encouraged to further investigate the efficacy of this promising treatment method.

It can already be said that the future of medicine is largely based on regenerative medicine. This field's limit is represented, among all other challenges, by the in depth knowledge and use of all growth factors individual properties; largest and most easily available source currently able to be exploited by physicians is represented by platelet-rich fibrin. These growth factors are of great interest to scientists around the world, as numerous reports of scientific studies testify. This interest arises from the share of growth factors and their potential use in the healing and repair processes of many body tissues. Despite the large amount of available scientific material and the vast knowledge derived on the topic of platelet concentrates and their possible applications, there are still many questions and doubts remaining. Predominant example is the previously mentioned degree of compaction, and especially optimal platelet values in the specific injuries treatment or tissues and organs individual regeneration. Therefore, further research and experiments are deemed necessary, to develop basal standards in Platelet-rich fibrin handling for treating specific injuries and regenerating selected tissues.

The authors also hope that this work will be one of the foundations for future studies, aiming to further explore the contribution of leukocytes in PRF preparations, in order to achieve optimal preparation, both to fight infections and to effectively promote wound healing.

Abbreviations

A-PRF+ - Advanced PRF
APG - Platelet Gel
FBS - Fetal Bovine Serum
H-PRF - Horizontal PRF
TIMP - Tissue Inhibitor of Metalloproteinases
EGF - Epidermal Growth Factor
FGF - Fibroblast Growth Factor
BMP-2 - Bone Morphogenetic Protein
P-PRF - Pure Platelet-Rich Plasma
TGF - Pure Platelet-Rich Plasma
HGF - Hepatocyte Growth Factor
HPC - Human Platelet Concentrate
IGF - Insulin-like Growth Factor
L-PRF - Platelet-Rich Fibrin Leukocytes
MMP - Matrix Metalloproteinases
PDGF - Platelet-Derived Growth Factor
PRF - Platelet-Rich Fibrin
i-PRF - Platelet-Rich Fibrin for Injection
PRGF - Growth Factor-Rich Plasma
VEGF - Vascular Endothelial Growth Factor

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Ethical Statement

This study does not contain any studies with human or animal subjects performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

Data Availability

Any inquiries regarding supporting data availability of this study should be directed to the corresponding author.

Author Contributions Conceptualization

C.M., C.F. and C.A.; Methodology: Flag.Fab.; Formal analysis: L.G. and Fel.Fed.; Writing—Preparation of original draft: C.A. and D'A.A.; All authors have read and accepted the published version of the manuscript.

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