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Leukocyte-rich PRP versus leukocyte-poor PRP - The role of monocyte/macrophage function in the healing cascade

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ABSTRACT

The mechanism of action of Platelet Rich Plasma (PRP) is thought to be related to the biomolecules present in α-granules. However, for the healing process to occur, an inflammatory phase is also deemed necessary. Leukocytes present in the inflammatory phase release both pro- and anti-inflammatory molecules. The latter may play an important role in the process of "inflammatory regeneration". Thus, we propose that in the context of healing, both platelets and leukocytes play an important role, specifically due to the macrophage's plasticity to switch from the M1 to M2 fraction. Therefore, we propose that PRP products derived from the buffy coat may be more beneficial than detrimental from a standpoint of the regenerative potential of PRP.

1. Introduction

Platelet Rich Plasma (PRP) has been the focus of many published studies in the medical as well as veterinary[1] and dental[2] literature both as a standalone therapy as well as in conjunction with Stem Cells and scaffold materials. Specific to medical clinical trials, there is an increasing interest in PRP as evidenced by the large number of registered clinical trials. Currently there are 302 registered clinical trials for a variety of medical conditions (www.clinicaltrials.com).

PRP contains an autologous mixture of a variety of cells with a primary focus on platelets concentrated above baseline.[3] Platelets contain granules with a wide range of active biomolecules. When the platelets are activated, they release these biomolecules, which stimulate the natural healing cascade.[4] The primary focus of published studies as well as the hypothesis behind the therapeutic efficacy of PRP relies on this biomolecule release from the α-granules.

The cell type and concentration of cells within a PRP preparation other than platelets may also include White Blood Cells, Red Blood Cells and a small fraction of stem cells.[5] The impact of the various PRP cell components other than platelets remains a subject of some controversy in the literature. This specifically applies to the recovery of leukocytes such as neutrophils due to their established release of inflammatory cytokines and metalloproteinases which can exacerbate the early inflammatory response to tissue injury.[6] This way, leukocyte-rich platelet-rich plasma (LR-PRP) and leukocyte-poor platelet-rich plasma (LP-PRP) have been the focus of debate over the past few years without a consensus. However, these and other variables should be considered in the questions for the ideal biologic activity of a PRP product. These variables include platelet number, the presence of white blood cells, the level of growth factors and the use of image guidance for its administration, among others. Recently, Lana et al. (2017) have published an article incorporating a broad variety of variables in a classification system termed MARSPILL. In summary, this new classification focuses on the method of PRP preparation (M), the use or lack of exogenous

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activation (A), the presence or absence of red blood cells (R), the number of spins of the centrifugation (S), the concentration fold of platelets reached in relation to basal number (P), the use of image guidance methods for PRP application (I), the presence or absence of leukocytes (L) and the use of light activation (L).[7]

In this review, the authors would like to focus on the Leukocyte content of PRP as a therapy beyond growth factors.

1.1. Platelets – more than a hemostasis role

Platelets are non-nucleated cell elements present in large numbers in human blood containing hemostatic, immunological and inflammatory properties.[8] They contain a number of different granules, including alpha, dense and lysosomal types that together offer more than a thousand different biomolecules relevant to hemostasis, inflammation, regeneration, and immune function.[9] Alpha granules are not only the most abundant, but they also store high levels of a wide variety of biomolecules while still maintaining proper osmolarity.[10] The biomolecules important for hemostasis and thrombosis include the von Willebrand factor (vWf), fibrinogen, factor V, factor IX and factor XIII. However, platelets also contain anticoagulant proteins, such as antithrombin, protein S and plasminogen. In addition, platelets contain a large number of growth factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor (TGF), Fibroblastic Growth Factor (FGF), among others.[11,12] Chemokines are the most abundant content of the alpha granules, comprising CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA−78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP1α), and CCL5 (RANTES).[13,14] Table 1 presents a summary of the major content of platelet alpha-granules.

Despite a large number of α-granule molecules and the diversity of functions, there are few proteins specific to platelets, like platelet factor 4 (PF4). The α-granule cargo proteins are also constituents of plasma and secreted by other cell types.[16]

Dense granules can be observed using an electron microscope even in the absence of staining, due to the high electron-dense core.[17] They contain elevated levels of calcium contributing to its electron density, and magnesium and potassium in their free form or complexed with anions. Their role in coagulation is well established.[18] Also, there are anions in dense granules such as adenine nucleotides, transporter proteins, bioactive amines and membrane proteins.[19] Some of the components of dense granules also play roles in immune cell-modifying effects besides platelet activation and thrombus formation. For example, after exposure to pathogens in skin, platelets do not only assist with hemostasis but also with immune responses against potential infectious agents, through their interaction with white blood cells and endothelial cells. This can promote an overstimulation of the inflammatory phase.[8] The list of dense granules content is described in Table 2.

Platelets can induce a multitude of inflammatory effects both locally and systemically.

Recently, the literature points to platelet exosomes and microparticles as messengers able to mediate a great variety of processes. Exosomes are molecules of 50–120 nm of diameter secreted by platelet endosomes with little participation of α-granules.[20] The cargoes inside exosomes include cytokines, chemokines, growth factors, lipoproteins and other lipids, and RNA types. Also, exosomes can protect its internal contents from degradative enzymes or chemicals, and have low immunogenicity and great stability, representing optimal carriers for nanodelivery treatments.[20] Their membranes express glycoproteins (GPIb, GPVI, αIIbβ3), CD40 ligand and P-selectin, common for platelets.[21] Their release is increased modestly after stimulation with thrombin or calcium. Studies suggest that exosomes are the major mediators of platelet intercellular interactions (cell-to-cell or platelet to cell).[22] For instance, platelet exosomes increase the adherence between platelets and monocytes, converting these cells to an inflammatory phenotype. Furthermore, through contact with endothelial cells, platelet exosomes enhanced expression of adherence proteins in the endothelial cells, increasing adherence.[22]

It has also been previously suggested that exosomes consistently contribute to the activity of platelet lysates, in addition to the latter's advantageous nano delivery role for cell-free regeneration therapies.[19] Evidence reveals that varying exosome concentrations

Table 2
Biomolecules present in platelet dense granules (from Flaumenhaft and Koseoglu, 2017).[15]

Type	Examples
Ionic species	Ca2+, Mg2+, K+, polyphosphate, Pyrophosphate
Membrane proteins	CD63 (granulophysin), LAMP-2, GPIb, αIIbβ3
Nucleotides	ATP, GTP, ADP, GDP
Bioactive amines	Serotonin, histamine
Transporter proteins	MRP4, VNUT, VMAT2

Abbreviations: Lysosome-associated membrane protein 2 (LAMP-2), Adenosine Triphosphate (ATP), Guanosine Triphosphate (GTP), Adenosine Diphosphate (ADP), Guanosine Diphosphate (GDP), Multidrug Resistence-associated protein 4 (MRP4), Vesicular Nucleotide Transporter (VNUT), Vesicular Monoamine Transporter 2 (VMAT2).

Table 1
Biomolecules present in platelet alpha-granules (adapted from Flaumenhaft and Koseoglu, 2017; Morrell, 2014).[15,8]

Type	Examples
Growth factors	TGF-β, PDGF-AA, PDGF-BB, PDGF-AB, VEGF, EGF, HGF, FGF, insulin-like growth factor (IGF), platelet-derived angiogenesis growth factor (PDAF), platelet-derived epithelial growth factor (PDEGF)
Chemokines	Stromal-derived factor (SDF-1), pro-platelet basic protein (Ppbp β-thromboglobulin nap-2, PF4, CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA−78), CXCL7 (β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP-1α), and CCL5 (RANTES)
Hemostasis	Factor V, factor IX, factor XIII, protein S, plasminogen, plasminogen activator inhibitor I, antithrombin
Membrane proteins	P-selectin, αIIβ3, GP1βα-IX-V, fybrocistin
Adhesion proteins	Fibrinogen, von Willebrand factor, thrombospondin
Immune mediators	Complement C3 precursor, complement C4 precursor, β1H Globulin, factor D, factor H, C1 inhibitor, IgG, CD40L. MP1-α
Proteases	MMP-2, MMP-9
Microbicidal molecules	Thymosin-β4, thrombocidins 1 and 2 (from NAP-2)

Abbreviations: Transforming Growth Factor Beta (TGF-β), Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), Fibroblast Growth Factor (FGF), Platelet Factor 4 (PF4), Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein (MIP-1α), Metaloproteinases (MMP), Neutrophil Activating Peptide (NAP-2).

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(0.6 μg, 5 μg, and 50 μg) increase the proliferation of mesenchymal stem cells (MSC) in a culture of bone marrow stromal cells in a dose related-manner. In the osteogenic differentiation assays, it was demonstrated that variations of exosome concentrations affected the ability of MSCs to deposit mineralized matrix differently, inducing a significant increase in MSC proliferation, reaching the highest rates at 50 μg of exosomes.

The role of PRP-derived exosomes has been studied in chronic wounds of diabetic animals both in *in vitro* and *in vivo* experiments.[23] While i*n vitro,* exosomes can effectively induce proliferation and migration of endothelial cells and fibroblasts. I*n vivo*, they promote healing of chronic wounds in diabetic animals through activation of extracellular signal-regulated kinase (Erk) and Akt signaling pathways, suggesting that PRP-induced re-epithelization may be due to the activation of yes-associated protein (YAP).[23]

Platelet microparticles are 150−1000-nm fragments derived from the plasma membrane of platelets that are undergoing physicochemical stress, or apoptosis.[19] While circulating blood contains microparticles derived from erythrocytes and leukocytes, platelet derived microparticles are the most abundant.[24] Elevated levels of microparticles are found in some platelet-related disease states such as thrombosis and sickle cell disease.[25,26] Upon activation, microparticles express phosphatidylserine and tissue factor on their surface, which can mediate thrombus formation.[27] The release of these compounds is related to platelet storage, processing and manipulation. Platelet apheresis increases the microparticle count, and platelet activation, as described by Nouslry et al. (2017).[28] The microparticles can contribute to intercellular communication and may have an effect on target cells by direct stimulation or transfer of surface receptors from one cell to another.

1.2. Platelet Rich Plasma therapy (PRP)

Due to the therapeutic potential of the large biomolecular release from activated platelets, PRP has been the focus of a large body of basic and clinical science research. Over the years, many questions have been raised in the literature regarding the heterogeneity of results, culminating in attempts to standardize PRP products. One important variable that needs further attention is the PRP type. The use of LR-PRP (or leukocyte-rich platelet-rich plasma) or LP-PRP (or leukocyte-poor platelet-rich plasma) is not well described in the articles and there is no consensus about what type is the best for clinical use. For example, in an *in vitro* study, both types of PRP cultured with tendon stem/progenitor cells (TSCs) isolated from the healthy patellar tendons of rabbits induced similar TSC differentiation into active tenocytes. However, while LR-PRP induced predominantly catabolic and inflammatory changes in differentiated tenocytes such as increased matrix metalloproteinase (MMP) 1, MMP-13, and the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, LP-PRP induced predominantly anabolic effects such as increased gene expression of anabolic genes, alpha-smooth muscle actin and collagen types I and III. The authors suggested that LR-PRP may exert detrimental effects due to its catabolic activity, while the use of LP-PRP in acute injuries may result in excessive scar formation due to the strong potential of inducing inordinate anabolic effects.[29] In another *in vitro* study, either LP-PRP or LR-PRP were cultured with synovial fibroblasts isolated from patients with osteoarthritis undergoing joint surgery.[30] LR-PRP induced a greater increase in the pro-inflammatory factors IL-1β, IL-8 and fibroblast growth factor-2, and also a greater decrease in anticatabolic mediators in cartilage such as hepatocyte growth factor and tissue inhibitor of metalloproteinase − 4. Since these studies were *in vitro*, whether the greater inflammatory response induced by LR-PRP affects the healing process could not be determined.

The idea that LR-PRP induces a greater inflammatory response than LP- PRP is also supported by *in vivo* studies. For example, it has been reported that LR- PRP induces a greater acute inflammatory response than LP- PRP at 5 but not 14 days after injection in healthy rabbit tendons.[6] However, whether this greater acute inflammatory response induced by LR-PRP affects the healing process could not be determined because the study used a healthy tendon. In another study using a chronic tendinopathy model induced by a local injection of collagenase in the Achilles tendon of rabbits, the effect of both types of PRPs on healing outcomes was evaluated 4 weeks after the intra-tendon injection of either LP-PRP or LR-PRP.[31] It was demonstrated that LP-PRP induced better histological scores with large collagen fibril diameters than LR-PRP. However, the effectiveness of the type of PRP treatment may depends on the healing stage and the type of injury. LR-PRP may be helpful for the treatment of acute tendon injuries as previously suggested and LP-PRP for the treatment of chronic tendon injuries.[32] The effect of LR and LP-PRP on the healing of other types of injuries and pathologies remains to be investigated in other pre-clinical studies. Furthermore, there are no clinical studies comparing the use of LR and LP-PRP. In fact, many clinical studies that are available in the literature do not cite the type of PRP used.

It is important to point out that the inflammatory phase is crucial to the healing process to promote the other healing phases such as remodeling and tissue contraction. In the next section, we will discuss another cell population that plays important roles in the inflammatory phase and may comprise the major workers in PRP orchestration: mononuclear cells, especially monocytes.

1.3. Mononuclear cells − the real workers in PRP therapy

Peripheral blood mononuclear cells (PBMCs) comprise lymphocytes (T and B), natural killer and monocytes.[33] All these cells have important roles, however, due to the monocyte's differentiation into macrophages as well as their plasticity, a unique cell population is generated. The ability to switch phenotypic expression and the display of different functions because of environmental stimuli are critical monocyte properties.[34] Tissue or peripheral macrophages play an important role in the phagocytosis of cells undergoing apoptosis and protect the host through innate immunity. Upon tissue migration, peripheral blood monocytes differentiate into tissue macrophages. The adaptive immunity development initiates with reciprocal interactions between macrophages and activated lymphocytes T and B, promoting regulation and enhanced antimicrobial resistance.[35]

Macrophage (M) may express two major phenotypes depending on type of activation. The M1 phenotype is induced by microbial agents and acts like a proinflammatory cell while the M2 phenotype is produced by a type 2 response and acts like an anti-inflammatory cell. The type 2 immunity is characterized by an increase in IL-4, IL-5, IL-9 and IL-13. Depending on the specific setting, the type 2 response determines the M2 macrophage role in host protection versus pathogenicity.[36] In addition, this response is directly involved in regeneration after injury and tissue repair. The M2 type 2 response is also found in diverse cells such as eosinophils, mast cells, basophils and Th2 cells. M2 macrophages present three subtypes based on their functionality: M2a − phenotype verified after stimulation with IL-4 or IL-13; M2b − dependent on the immune complex made of IL-1 and LPS; M2c − dependent on IL-10, TGF-β or glucocorticoid action.[35] Macrophages have a protective immunological function and promote angiogenesis through the production of angiogenic factors and cytokines.[36] These pro-angiogenic properties are not shared between all subsets of macrophages. For example, one *in vitro* study showed that the M2 phenotype expresses higher levels of growth factors and cytokines

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that drive the angiogenic process.[35] Basically, there is an important difference in metabolism induced by the different macrophage phenotypes. For example, M2 produces ornitine and polyamines, while M1 synthetizes mainly nitric oxide (NO), inducible nitric oxide synthase and citrulline.[37] Also, M1 and M2 present differences in the pattern of the cytokine profile and glucose and iron metabolism. The M2 type promotes cell proliferation and repair through polyamine and collagen synthesis, besides other tissue remodeling functions, releasing IL-10 and IL-4. On the other hand, the M1 type, through NO mechanisms, shows microbicidal activity and inhibition of cell proliferation, releasing IL-6 and TNF-α.[38] It appears that M2 is the type adopted by most of the resident macrophages.

The process of inflammation that occurs in an injured area is carried out by activated macrophages, with the objective to eliminate microbes [during the] inflammatory phase of wound healing. In this phase there is also an initial role of pathogen removal and clearance of cellular debris. Following this phase, macrophages become deactivated and unresponsive to inflammatory stimuli, in order to promote angiogenesis, cell proliferation and matrix deposition for the remodeling phase.[39]

1.4. Macrophage polarization

There is a lack of consensus regarding whether or not the M1 and M2 factions are distinctly different and, because of that, different hypotheses have been proposed. The first hypothesis is that M1 and M2 macrophages are two distinct cell populations, acting on different phases of the inflammatory reaction and healing process.[40] The second hypothesis is that M1 and M2 macrophages are the same cells, capable of altering the functional phenotype in response to microenvironmental signals.[41] According to the first hypothesis, the Ly6C + monocytes become the M1 macrophage in tissue with inflammatory functions, and the Ly6C- monocytes or tissue-resident macrophages become M2 macrophages with reparative roles. However, this hypothesis is not supported by some studies.[42] The second hypothesis proposes that the macrophages can polarize in different subtypes according to the microenvironment, that is, M1 subtype in the early phase of healing, and M2 subtype in the late phase. Italiani et al. (2014) observed that monocytes that polarized to M1 can mature into M2 in a culture system that had induced sequential changes in the microenvironment. In summary, the release of cytokines and signals in the microenvironment appears to be responsible for the shift in macrophage subtypes.[43]

1.5. Stimuli for M1 role

Currently, there are three main stimuli for M1 polarization recognized in the literature. In cytokine activation, the main protein involved is IFN-γ, also a Th1 cell product. Type 1 response is defined by the activity of T helper 1 cells such as lymphoid cells, neutrophils and macrophages, that release proinflammatory cytokines (IFN-γ) to elicit an inflammatory reaction. This response is used to protect the organism against intracellular pathogens, viruses, bacteria and other microorganisms.[44] This cytokine is produced by cells like natural killer and macrophages.[35] IFN-γ controls specific gene expression programs using cytokine receptors, markers of cell activation, adhesion molecules.[45] The innate immunity initiates with the recognition of pathogens by receptors in macrophages. The pattern of recognition is similar to that in toll-like receptors (TLR) isolated, with the same genes programs. LPS is the most studied M1 macrophage signal recognized by TLR4. This activation induces a strong pro-inflammatory cytokine and chemokine release and antigen presentation molecules. The latest stimulus is the granulocyte macrophage colony stimulating factor (GM-CSF). This factor is produced by a variety of cells ranging from macrophages to parenchyma cells. The activated GM-CSF recruits a significant number of signals and regulators that are part of IFN-γ and TLR signaling, promoting antigen presentation, phagocytosis mediated by complement-enhanced microbicidal capacity and leukocyte migration. Also, GM-CSF induces the production and release of pro-inflammatory cytokines in monocytes and macrophages.[46]

1.6. Stimuli for M2 function

The main interleukin related to the M2 phenotype is the IL-4, which is produced by Th2 cells, eosinophils, basophils and macrophages and is recognized by three different receptors, IL-4Rα, IL-4Rγc, IL-13Rα1. The role of IL-4 in macrophages is the decrease in its phagocytic activity. Another important stimulus is the crosstalk between macrophages and B cells, turning off IL-12 and inducing IL-10 release, promoting Th2 responses.[47] IL-10 following receptor binding mediates inhibition of pro-inflammatory cytokine expression. IL-10 is a potent inhibitor of Th1 cells.[48] Macrophage colony-stimulating factor (M-CSF) is an M2 stimulus and, after binding to its receptor, significant changes occur resulting in over-representation of cell cycle genes and downregulation of human leukocyte antigen (HLA). In summary, the M2 group is activated by diverse stimuli at different levels. Some stimuli lead to a switch in macrophage phenotypes, while others affect the interaction between macrophages and immune cells, and yet, others lead to the resolution level of macrophages against a pathogen.[35] All these M2 properties relate to wound healing and regeneration.

In this way, due to the plasticity of macrophages and importance of leukocytes for the healing process, we encourage the authors to use leukocyte-rich PRP.

1.7. Leukocyte-rich PRP

PRP preparations due to their heterogeneous content of cell populations such as leukocytes have been a subject of debate regarding the ideal cell proportions resulting in optimal therapeutic value. There is no consensus on this subject since leukocyte-rich PRP has shown both positive and negative results according to different clinical scenarios.[32,49] Some authors propose the utilization of PRP without neutrophils, or controlled influx of neutrophils, in order to eliminate the generation of ROS (reactive oxygen species) and metalloproteinase production, which could be detrimental for the healing process.[50] For *in house* preparations, it is not possible to separate the neutrophils from the buffy-coat. However, the evolution of commercial PRP preparations presents improved cell separation technology. For example, the Angel System (Arthrex) allows the production of PRP products with adjustable platelet and white blood cells concentrations, potentially avoiding larger neutrophil concentrations. This system utilizes technology to recognize the absorbance of the different cell types and separation into a sterile bag. The Emcyte Pure PRP product offers similar technology for cell separation and custom PRP production. In a comparative evaluation between these two systems, the Emcyte Pure PRP achieves a higher yield in platelet amount, mononuclear concentrations and granulocyte cells in comparison to Angel System. Until now, a gold standard methodology for the production of PRP products is lacking in both commercial and manual processes, however, newer systems with adjustable leukocyte concentrations are encouraging.

Recently, the literature has demonstrated that the interaction between neutrophils and activated platelets can release anti-inflammatory molecules, and the preoccupation with the noxious role of neutrophils is changing. Parrish et al. (2017) published an

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article regarding the interaction of platelets and other cell types in healing, showing the anti-inflammatory potential of the interaction of platelets and neutrophils.[51] Initially, the activated platelets release arachidonic acid, which is picked up by neutrophils and converted into leukotrienes and prostaglandins, which are inflammatory molecules.[52] On the other hand, platelets in association with neutrophils, which produce leukotrienes, can pick up this inflammatory protein and convert it into lipoxins. Lipoxins are a potent anti-inflammatory protein, which can limit neutrophil activation, preventing diapedesis, and drive the resolution phase of the healing cascade [59]. The production of lipoxins through platelets is only possible due to the presence of leukotrienes produced by neutrophils. The ability to change the production of pro-inflammatory to anti-inflammatory molecules prevents neutrophil recruitment and subsequent inflammatory activation, while contributing to the resolution process of the healing cascade.[53]

In conclusion, due to the importance of leukocytes, contributing to both the inflammation and signaling for other cells types, prompt they are crucial for regeneration. Neutrophils secrete cytokines for the chemotaxis of macrophages. Macrophages present plasticity properties which are important in promoting the inflammatory process required to then initiate the healing process. The authors termed this process “regenerative inflammation”, an inflammation required for regeneration. In addition, macrophages are also indispensable for remodeling and repair phases. The release of platelet granules also signalizes to leukocytes. Thus, PRP products may benefit from the inclusion of leukocytes in the buffy coat harvest, as they appear more likely to be a beneficial rather than detrimental component. We hypothesize that macrophages act like instructors of the healing orchestra. The effectiveness of the type of PRP treatment may depends on the healing stage and the type of injury, and furthermore, the authors conclude that the role of leukocytes in PRP products may determine the success of the regenerative intervention provided by PRP.

1.8. Future perspectives

The rise in cellular therapy is gaining evidence in the amount of publications available in the literature. It is important to note that the majority of publications regarding PRP focuses on the role of platelets and the growth factors inside of the alpha-granules. However, we have more than 1000 biomolecules inside the platelet, which can assist in the regenerative process through chemical signals, initiating the recruitment and migration of diverse cell types to promote healing. In addition, the buffy-coat possesses key cell types: the mononuclear cells, which act and release significant proteins that start the “regenerative inflammation”, a necessary step for the healing process to occur. The role of the macrophage is of great importance due to its plasticity (M1 and M2) and contribution for regeneration. Another important factor is the combination of activated platelets and neutrophils. This attachment promotes an anti-inflammatory event, important for healing cascade. All these concepts must be shown so that the researchers report all the cells and molecules that PRP has as well as the amount of variance in quality and quantity of molecules according to their preparation (LP-PRP or LR-PRP), resulting in heterogeneous results in the literature.

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Conflict of interest

The authors declare that they have no conflict of interest.

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