BLOOD COMPONENTS AND REGENERATIVE MEDICINE

Review

In vitro evidence supporting applications of platelet derivatives in regenerative medicine

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The role of platelets in haemostasis has long been known, but understanding of these cells' involvement in wound healing/tissue repair is more recent and has given rise to a multitude of translational studies. Tissue repair processes consist of complex, regulated interactions between cells modulated by biologically active molecules, most of which are growth factors released by activated platelets: this aspect represents the rationale on which the use of platelet derivatives for clinical purposes is based.

In the last years, many *in vitro* studies have focused on the mechanisms of action by which these growth factors affect the biological activities of cells, thus supporting tissue healing. Although limited by some drawbacks (two-dimensional *in vitro* monocultures cannot replicate the tissue architecture and organisation of organs or the continuous interplay between different cell types), *in vitro* studies do have the advantages of giving rapid results and allowing precise control of platelet concentrations and other parameters.

This review offers an updated overview of the data obtained from the most recent bench-top studies focused on the effects of platelet derivatives on a wide variety of human cells, highlighting their possible impact for *in vivo* applications. The heterogeneity of the data obtained so far is very evident. This can be explained by the different experimental settings used in each study, which may be the cause of the variability in clinical outcomes. In fact, *in vitro* studies suggest that the composition of platelet derivatives and the method used for their production and activation (or not) and the platelet concentration used can have profound effects on the final results.

Keywords: *regenerative medicine, blood platelets, wound healing, platelet-rich plasma (PRP).*

TISSUE REPAIR PROCESSES

"Tissue repair" is a term that refers to those dynamic processes that normally occur in the body as a physiological response to tissue damage, aiming to restore the normal function and architecture of the damaged area. These processes consist of a complex set of cellular/molecular events that, regardless of the type of damage (acute or chronic) and the extent of tissue loss, is split into three overlapping stages: inflammatory, proliferative and remodelling[1,2].

The first stage occurs soon after the tissue damage as a reaction to blood vessel injury; it begins with vasoconstriction, which lasts a few seconds, followed by platelet clotting. As

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the platelets form a cap to close the vessels temporarily, the coagulation system is activated and an insoluble fibrin matrix is formed to fill the lesion and to become the temporary scaffold for infiltrating cells. Very soon after, the influx of neutrophils begins: these white blood cells are attracted to the area of the wound by inflammatory cytokines released from activated platelets, such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ)[2]. Inflammatory cells play a crucial role in preventing infection and facilitating the clean-up of cellular debris and damaged tissue.

As inflammation moves toward resolution, the proliferative stage begins. This stage consists of new tissue formation and involves the proliferation and migration of several cell types, with endothelial cells and fibroblasts being among the most important. Endothelial cells are needed for angiogenesis, the coordinated process that consists in the formation of new vessels from pre-existing ones. Angiogenesis begins from the sprouting of intact blood vessels present at the edge of the lesion and is sustained by the proliferation of endothelial cells. Along with angiogenesis, vasculogenesis, the formation of new vessels from endothelial progenitor cells, can sustain this neovascularisation too. These processes are regulated mainly by vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), extensively released by activated platelets[3,4]. Meanwhile, fibroblasts can migrate into the clot from the surrounding tissue using the fibrin network as a temporary matrix. Fibroblasts proliferate in the site of the wound in response to PDGF, transforming growth factor (TGF)-β and TNF, originating from leucocytes and platelets, and secrete cytokines and growth factors (GF) that stimulate healing. They also produce a "granulation tissue" secreting precursors of collagen (mainly type III), elastin, proteoglycans and other glycoproteins which then mature outside the cells restoring a three-dimensional extracellular matrix (ECM)[2].

Tissue repair ends with a remodelling stage that aims to restore the normal tissue structure; this process needs reorganisation, degradation, and re-synthesis of the ECM and leads to a tissue that, at last, will be impoverished of cells and vessels but enriched in collagen fibres[2]: blood vessels are removed by apoptosis, the type III collagen is degraded by means of matrix metalloproteinases (MMP) and replaced by type I collagen, and most of the immune cells and fibroblasts disappear. Some of the fibroblasts transform into myofibroblasts, which are rich in smooth muscle actin and are responsible for the contraction of the wound's edges toward the centre[5,6]. PDGF, FGF, and TGF-β are the main coordinators of these final events[1,2].

PLATELET INVOLVEMENT IN TISSUE REPAIR

Although the role of platelets in haemostasis has long been known, the involvement in these cells in wound healing and tissue repair is more recent knowledge. The sequence of all the events leading to wound healing is finely regulated by an extensive communication that is established between the different cellular constituents involved and, as explained above, is mainly mediated by GF and cytokines. Some of these are released by activated platelets as well as by many other types of cells (**Table I**), such as macrophages, mast cells, T lymphocytes, endothelial cells, and keratinocytes; once stimulated, these cells synthesise GF and release them, some hours or days after receiving the stimulus. In contrast, platelets, which store GF inside their alpha-granules, quickly release these factors once activated, modulating many processes involved in wound healing, including:

- angiogenesis: GF stimulate endothelial cell proliferation, migration, and association in tubular structures, as well as the recruitment of perivascular cells providing the damaged tissue with new blood vessels;
- renovation of connective tissue: GF orchestrate all those fibroblast activities needed to restore damaged tissue, from proliferation to migration to collagen synthesis;
- restoration of tissue-specific cell types: GF modulate the proliferation and differentiation of tissue mesenchymal stem cells into tissue-specific cell types[5].

The contents of alpha-granules not only contribute to tissue healing but also take part indirectly in antimicrobial activities since platelets release chemokines and cytokines that recruit and activate immune cells; upon activation they also release molecules with direct microbicidal properties such as reactive oxygen species, kinocidins (i.e. chemokines that exert direct antimicrobial activity such as platelet factor 4 [PF4]), defensins (e.g. β-defensin 2), thrombocidines (e.g. neutrophil-activating peptide-2 [NAP-2] and connective tissue-activating peptide-III [CTAP-III]) and proteases, playing important roles in the defence against pathogens[3,14].

Table I - *Main growth factors and cytokines involved in wound healing*

Growth factors and cytokines	Cell source	Effects
Ang-1	Platelets, neutrophils	Induces angiogenesis stimulating migration and proliferation of endothelial cells. Supports and stabilises blood vessel development via the recruitment of pericytes[3,5]
CTGF	Platelets, fibroblasts	Stimulates leucocyte migration; promotes angiogenesis; activates myofibroblasts stimulating ECM deposition and remodelling[6-8]
EGF	Platelets, macrophages	Regulates epithelial migration, fibroblast/epithelial/endothelial proliferation; promotes M2 differentiation[1,4,5,9]
FGF	Platelets, macrophages, mast cells, T lymphocytes, endothelial cells, fibroblasts	Calls macrophages, fibroblasts, endothelial cells; regulates fibroblast/monocyte/epithelial/endothelial migration, fibroblast/epithelial/endothelial proliferation, collagenase synthesis; induces angiogenesis; contributes in wound contraction[1,3,5,9]
HGF	Platelets, mesenchymal cells	Regulates cell growth and motility in epithelial/endothelial cells, supporting epithelial repair and neovascularisation during wound healing[5,10,11]
IGF-I	Platelets and all tissues	Calls fibroblasts[3,5,12]
KGF	Fibroblasts, mesenchymal cells	Regulates epithelial migration and proliferation[1,9,13]
PDGF	Platelets, endothelial cells, macrophages, smooth muscle cells	Calls neutrophils, macrophages, fibroblasts, endothelial cells, mesenchymal stem cells; regulates fibroblast proliferation, collagen and collagenase synthesis; supports angiogenesis; aids in wound contraction; promotes M2 differentiation[1,3,5,6,9]
PF-4	Platelets	Calls leucocytes and regulates their activation. Microbiocidal activities[3,5]
SDF-1α	Platelets, endothelial cells, fibroblasts	Calls $CD34^+$ cells, induces their homing, proliferation and differentiation into endothelial progenitor cells stimulating angiogenesis. Calls mesenchymal stem cells and leucocytes[3,5]
TGF-α	Macrophages, T lymphocytes, keratinocytes	Regulates fibroblast/epithelial proliferation, epithelial migration; involved in angiogenesis[1]
TGF-β	Platelets, endothelial cells, T lymphocytes, keratinocytes, macrophages	Involved in fibroblast proliferation, fibroblast/monocyte migration, collagen and collagenase synthesis; modulates angiogenesis[1,3,5,9]
TNF	Macrophages, mast cells, T lymphocytes	Regulates monocyte migration, fibroblast proliferation, macrophage activation, angiogenesis[1,3,9]
VEGF	Platelets, macrophages, keratinocytes, endothelial cells	Induces angiogenesis stimulating migration and proliferation of endothelial cells; regulates collagenase synthesis and collagen secretion; calls macrophages and granulocytes[1,3,5]

Ang-1: angiopoietin-1; CTGF: connective tissue growth factor; ECM: extracellular matrix; EGF: epidermal growth factor; FGF: fibroblast growth factor; HGF: hepatocyte growth factor; IGF-I: insulin-like growth factor; KGF: keratinocyte growth factor; PDGF: platelet-derived growth factor; PF-4: platelet factor 4; SDF-1α: stromal cell-derived factor-1α; TGF: transforming growth factor; TNF: tumour necrosis factor; VEGF: vascular endothelial growth factor.

PLATELET DERIVATIVES

For many years now, the clinical use of platelet derivatives as an adjuvant to hard and soft tissue healing, in virtue of their GF content, has been widely adopted in various medical and surgical procedures, ranging from ophthalmology, skin ulcers, gynaecological and urogenital disorders to almost all fields of surgery - orthopaedic, oral and maxillofacial, cosmetic, cardiothoracic, vascular, otorhinolaryngological, and neurosurgery[15,16].

Platelet derivatives include platelet-rich plasma (PRP), fibrin glue (FG), platelet gel (PG), plasma rich in growth factors (PRGF), platelet-rich fibrin (PRF), hyperacute serum (HAS), serum eye-drops (E-S), PRP eye-drops (E-PRP) and platelet lysates (PL)[17].

Platelet derivatives can be autologous or allogeneic. The use of autologous platelet derivatives avoids any type of virus or prion contamination and immune reactions associated with allogeneic proteins. Although the volume of autologous platelets may be sufficient for clinical use, limitations of these types of products include wide variability in quality due to changes in platelet counts and GF content that are influenced by the patient's age and biological conditions. In contrast, allogeneic platelet derivatives are prepared from healthy donor blood using standard working procedures that guarantee products enriched in platelets and GF, with minimal contamination from red blood cells and leucocytes than single-donor batches[18,19].

There are no standardised protocols for the preparation of platelet derivatives in clinical practice: the parameters

considered during the preparation include the number and concentration of platelets over baseline, centrifugation conditions and activation of platelets. All these parameters contribute to the composition of platelet derivatives and, ultimately, to their therapeutic effect[20-22].

The general method to prepare platelet derivatives involves sequential steps: whole blood is collected with or without an anticoagulant (e.g. in acid-citrate- dextrose tubes), centrifuged to concentrate the platelets, then activated to allow the alpha-granules to release their biological molecules[23].

The platelets are concentrated according to protocols that include centrifugation steps with different speeds (100-300 *g*), times (4-20 minutes) and temperatures (12-26 °C). The number of platelets in the final product is four to five times greater than the baseline value; all suspensions of platelets in plasma with a platelet count greater than the baseline count can be identified as PRP or platelet concentrates[17,20-23].

To obtain a product with a higher concentration of GF, some protocols produce platelet concentrations up to ten times higher than the baseline value by combining low temperatures, high speeds, and various centrifugation cycles[6,23,24]. These conditions can, however, induce premature activation of the platelets, thereby altering the properties of the final product.

In order to produce pure platelet-rich plasma (P-PRP), also known as leucocyte-poor platelet-rich plasma (LP-PRP), the whole blood is collected and centrifuged at low speed to separate the red blood cells - which settle at the bottom of the tube - from white blood cells/platelets and a upper plasma layer, which sediment as an intermediate layer (called the buffy coat) and higher layer, respectively. The upper layer is composed of plasma and a gradient of platelets: poor on the surface, intermediate in the middle and rich near the buffy coat[23]. The upper layer and just the superficial layer of buffy coat are transferred into a sterile tube and then centrifuged at high speed to obtain the P-PRP, which consists of the small volume at the bottom of the tube (about the lower one-third) and is mainly composed of platelets; the resulting supernatant (about the upper two-thirds) constitutes platelet-poor plasma (PPP)[25] (**Figure 1A**).

PPP has a very low cellular content; after induction of the coagulation cascade, fibrinogen polymerises into fibrin monomers which finally form a three-dimensional network called FG that has a high content of fibrin along with a paucity of platelet-derived factors, except for insulin growth factor-1 (IGF-1) and hepatocyte growth factor (HGF)[20,26,]. In spite of this, in some animal models, FG was shown to be more effective than PG for the preservation of sockets with buccal dehiscence[27]. This may be because fibrin can act as a natural biomaterial scaffold, having a structure very similar to the native ECM and thus a good capacity to bind cells. It has also been proven that it is biocompatible and biodegradable, which are essential features for its use as a scaffold in regenerative medicine applications[28].

In order to produce leucocyte- and platelet-rich plasma (L-PRP), after the low speed centrifugation of whole blood, the entire buffy coat (avoiding red blood cell contamination) along with the upper layer is transferred into a tube and then centrifuged to obtain the L-PRP (enriched in platelets and white blood cells) at the bottom of the tube and PPP above (**Figure 1B**).

The process of activation of the PRP, which is due to the action of thrombin and the generation of fibrin, is generally achieved by the addition of calcium chloride or calcium gluconate and leads to the production of the corresponding PG accompanied by release of the biologically active molecules contained in the alpha-granules[29,30]. The activation protocols define a time from 20 min to 1 hour at 37 °C or room temperature[30.]

PG is a platelet derivative that is mostly used to treat ulcers and wounds. Forty minutes after its activation, a liquid exudate forms. This exudate, also called PRGF (**Figure 1**), contains plasma proteins and the molecules released by activated platelets[17].

PRF products are second-generation platelet derivatives resulting from coagulation induced during centrifugation. The advantages of these products compared to the PRP are due to the autologous process of platelet degranulation that occurs physiologically without biochemical modifications: this allows the creation of a fibrin network that can better support the release of cytokines and cell migration[31,32].

A further activation method, leading to the "photo-activated PRP", consists of exposing platelets to ultraviolet (UV) light irradiation. Although the mechanism is not well understood, there is evidence of positive effects of intra-articular injections of UV-activated PRP in the orthopaedic field[33].

To obtain pure PRF (P-PRF), Fibrinet PRFM tubes are used; whole blood is collected in the presence of anticoagulant (tri-sodium citrate) and a separator gel and centrifuged. After transferring the buffy coat and the upper layer into a second tube, calcium chloride is added to induce clotting and the tube is centrifuged immediately, leading to the formation of a fibrin-rich and very stable clot (**Figure 2**). The separator gel removes mostly white blood cells enabling P-PRF to be obtained[31].

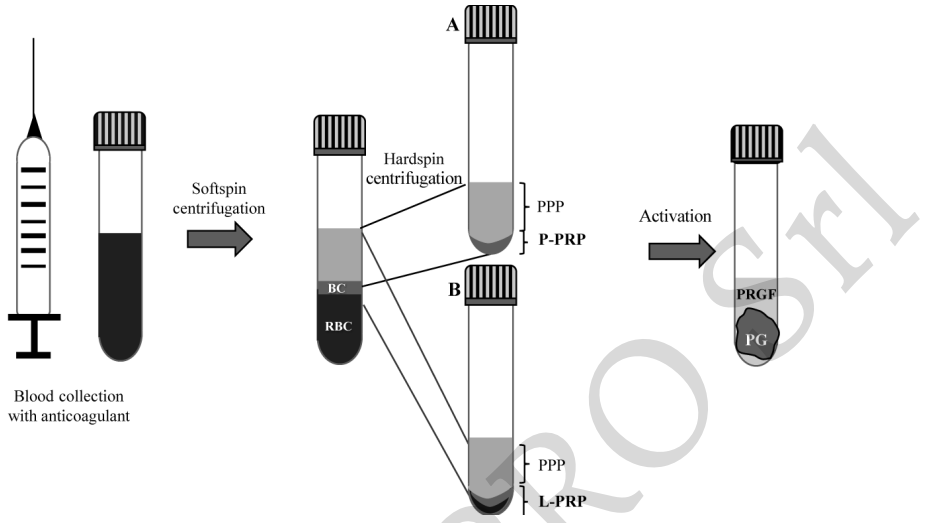

Figure 1 - Two-step centrifugation protocol to obtain pure platelet-rich plasma (P-PRP), leucocyte- and platelet-rich plasma (L-PRP), platelet gel (PG) and plasma rich in growth factors (PRGF)
The first step consists of "softspin" centrifugation of the whole blood that leads to three layers: an upper layer containing mostly platelets and white blood cells, an intermediate "buffy coat" (BC) layer rich in white blood cells and a bottom layer consisting mostly of red blood cells (RBC). To produce the P-PRP the upper layer and superficial BC are transferred to a new tube and "hardspin"-centrifuged to obtain P-PRP at the bottom of the tube and platelet-poor plasma (PPP) on the top (A). To obtain L-PRP, instead, the upper layer and the whole BC along with some RBC are transferred to a new tube and "hard spin"-centrifuged to obtain L-PRP at the bottom of the tube and PPP at the top (B). The PPP is removed and P-PRP or L-PRP eventually activated to induce clotting and produce the PG and PRGF. Fibrin glue (FG) is obtained from coagulation of PPP[17,23,25].

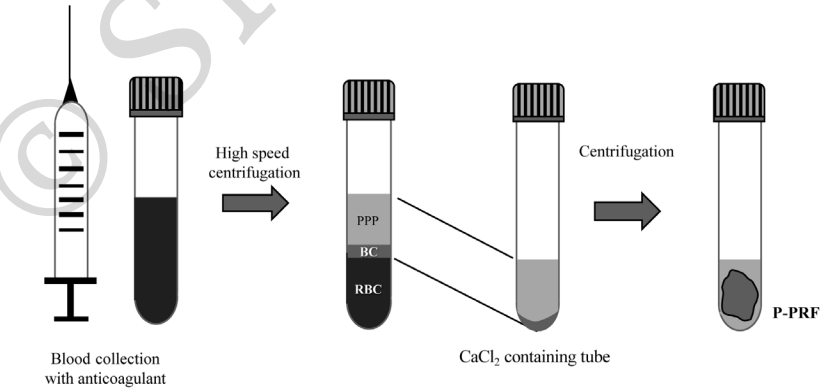

Figure 2 - Schematic description of the procedure to obtain pure platelet-rich fibrin (P-PRF)
Whole blood is collected into specific Fibrinet PRFM tubes (Cascade Medical Enterprises Inc, Wajne, NJ, USA) and centrifuged at high speed. After transferring the buffy coat (BC) and upper layer of plasma into a second tube, $CaCl_2$ is added and centrifuged immediately, allowing the formation of a fibrin-rich and very stable clot[31]. PPP: platelet-poor plasma; RBC: red blood cells.

Leucocyte- and platelet-rich fibrin (L-PRF), on the other hand, is obtained from whole blood collected without anticoagulant and centrifuged: platelet activation and fibrin formation take place immediately. After centrifugation three phases are formed: the red blood cells at the bottom, the plasma on the surface and the L-PRF clot in the middle[17,31]. L-PRF is mainly composed of a dense fibrin matrix that enables enmeshment of pla telets and leucocytes and is used as a three-dimensional scaffold for tissue regeneration. Unlike the other platelet derivatives PRF incorporates all the cells that remain entrapped in fibrin clots and also contains several molecules and GF of therapeutic interest (FGF-2, VEGF, PDGF, TGF-β1) other than fibrin, making it very useful as a scaffold to support wound healing[34].

If at the end of the PRF clotting the serum is squeezed out from the PRF clot, hyperacute serum (HAS) is obtained, which, despite its method of preparation method being very similar to that of PRP, has a peculiar ionic and protein composition that could be beneficial for some cell functions (**Figure 3**)[35].

E-S and E-PRP are two further formulations used in ophthalmology because GF and proteins promote proliferation, migration, and adhesion of corneal epithelial cells[36].

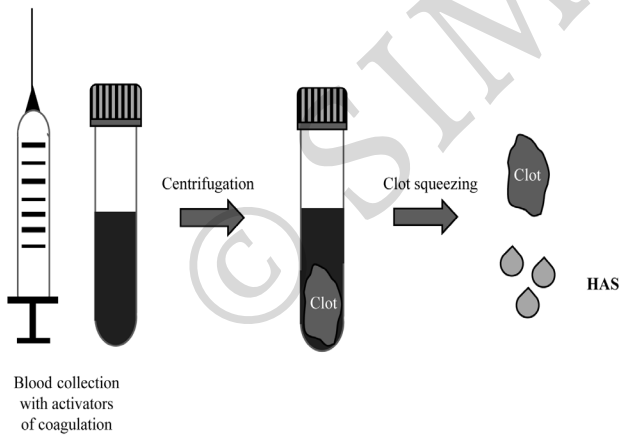

Figure 3 - Schematic procedure to obtain hyperacute serum (HAS)
In order to prepare HAS, blood is collected into specific tubes containing coagulation activators (VACUETTE® Z Serum C/A tubes, Greiner Bio-One International GmbH, Kremsmünster, Austria) and centrifuged. Once the fibrin clot has been formed, it is placed in a new container and squeezed to obtain the serum portion, resulting in HAS[35].

E-S derives from the spontaneous coagulation of whole blood; after centrifugation the clot separates from the serum, which is diluted with saline solution or antibiotics to constitute E-S. E-PRP derives from PRP: after the first centrifugation of whole blood the upper portion is aspirated with a syringe taking care to avoid contamination by red blood cells. The syringes thus prepared are stored at 4 °C until used as normal eye drops[36].

PRP can be used to prepare PL: alpha-granules are disrupted by freezing-thawing cycles or by ultrasound and thus release their content of biologically active molecules. The temperatures used in the treatment range from –80 °C to 37 °C, then centrifugation follows to separate the cellular debris; the supernatant containing the GF constitutes the PL[17,37] (**Figure 4**). The GF contained in PL include VEGF, TGF-β1, and FGF-2 at levels comparable to those in other platelet derivatives; the level of PDGF in PL is, however, significantly lower than that in PRGF[38]. PL has the advantage that it can preserved without the levels of GF being changed; it has been demonstrated that the stability of the GF in PL is maintained during storage for 5 months at –20 °C[39] or for up to 9 months at 4 °C, if previously lyophilised, with the GF maintaining their biological activities[40].

IN VITRO EVIDENCE

Platelet derivatives are now used widely in most clinical fields. Over time, many *in vitro* studies have been conducted to elucidate the biological processes triggered by platelets in cell types involved in *in vivo* wound healing, providing, mostly but not always, indications of their supportive effect for clinical applications. Numerous cell lines, sometimes of animal origin, have been used as the

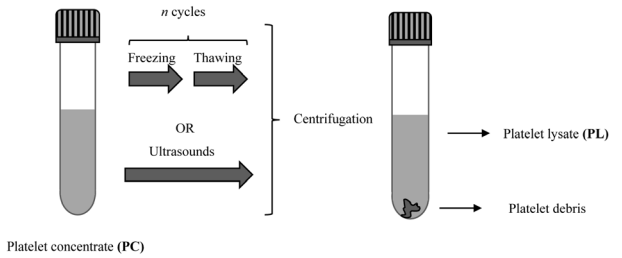

Figure 4 - Schematic procedure to obtain platelet lysate (PL) from platelet concentrate or platelet-rich plasma
Platelets and alpha granules are broken using freezing-thawing cycles or by ultrasound; centrifugation is performed to separate the cellular debris from the supernatant rich in growth factors (i.e. the PL)[37].

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target of platelet derivatives. The derivatives have been tested in activated or not-activated forms, prepared by different techniques and starting from different platelet concentrations. For these reasons, opposing outcomes have often been obtained, making the interpretation of results very difficult. The information gained from rigid, controlled, reproducible *in vitro* studies is useful for a clearer understanding of the biological mechanisms triggered, to give indications on the most effective doses and conditions and, ultimately, to support or discourage the clinical use of platelet derivatives.

Fibroblasts are among the cells most extensively studied *in vitro* because they are involved in the healing of almost all damaged tissues in the body. They are dispersed in connective tissue contributing to tissue architecture and function by secreting ECM, especially type I/III collagen; they play pivotal roles in all three stages of wound healing, being involved in wound contraction, in the deposition of ECM and its subsequent remodelling[41]. When a tissue is injured, the surrounding fibroblasts proliferate, migrate into the wound and produce ECM. As such, they are involved in many clinical conditions related to wound healing; for example, fibroblasts from chronic wounds exhibit decreased proliferation, altered patterns of cytokine release, as well as decreased release of MMP-1 and active MMP-2 associated with increased levels of tissue inhibitor of metalloproteinases-1 and -2 (TIMP-1 and TIMP-2)[42]. In contrast, fibroblasts from keloid display increased proliferation and resistance to apoptosis associated with alterations in collagen production and degradation[43]. Some studies have highlighted how platelet derivatives could be of support in regenerative medicine, promoting cell growth and collagen biosynthesis[44], and a potential therapeutic tool for those diseases in which fibrosis plays a major aetiological role, activating negative feedback signalling for TGF-β1 which, in turn, downregulates connective tissue growth factor (CTGF) expression[45,46]. In fact, excessive collagen synthesis and deposition by fibroblasts are regulated by cytokines, especially TGF-β and its downstream mediator CTGF[47].

Fibroblasts also represent one of the precursor cells for myofibroblast differentiation. Myofibroblasts, being able to produce ECM and exert contractile forces, are among the most important cells in the creation of provisional scar tissue, a crucial step in the healing process. They usually disappear once the tissue has been repaired; the temporary scar is degraded and replaced by regenerated tissue. In contrast, in fibrotic diseases, myofibroblasts persist in an activated state, producing excessive ECM that can damage tissue architecture and function[46]. PRP has been considered as one of the possible therapeutic options to oppose fibrosis, but studies have produced contrasting results. Some of them indicated that PRP induces cell differentiation of fibroblast-like cells to myofibroblast-like cells[48], while others suggested that PRP can prevent and inhibit TGF-β1-induced differentiation[49]. The contradiction in these findings probably stems from the different concentrations of platelets in the PRP used or the heterogeneity in the procedures employed to prepare the PRP, which yield formulations containing different doses of GF that can have pro-fibrotic (TGF-β) or anti-fibrotic (FGF-2) effects. For these reasons, further *in vitro* studies are needed to generate clear evidence on the role of PRP in fibrosis.

Fibroblasts also play an important role in photo-aging, a term used to indicate the changes to skin induced by exposure to solar radiation: indeed, UV irradiation induces a senescence-like phenotype. In photo-aged skin, collagen bundles undergo structural changes partly due to decreased collagen synthesis accompanied by increased degradation by MMP, and partly due to the reduced proliferation of fibroblasts[50]. For these reasons, many *in vivo* and *in vitro* studies have tried to determine the possible benefits deriving from the use of PRP, highlighting that this treatment counteracts the negative effects of UV irradiation[51].

PRP is also extensively used in dentistry, as a boost to grafting materials to increase regeneration of bone and periodontal tissues; nevertheless, many researchers have found no benefits from its use. These observations generated controversy about the effectiveness of PRP in clinical procedures, but also encouraged research to better understand, using gingival fibroblasts *in vitro*, the biological basis for the use of PRP[52,53].

Musculoskeletal cells have also been extensively studied. Platelet derivatives are widely used in orthopaedic procedures to facilitate wound haemostasis and to treat several musculoskeletal injuries and disorders, including tendinopathies and rotator cuff disease[54,55]. Tendons, in fact, have poor regenerative capacity because

of their limited vascularisation and low cell density, making tendon injuries a difficult clinical problem and encouraging studies to evaluate the effect of PRP on their healing.

The healing of injured muscle, on the other hand, involves many cells, including muscle satellite cells, quiescent stem cells with very important features for muscle healing. These cells contribute to muscle regeneration because, once activated by an injury, they proliferate, undergo self-renewal, migrate to the damaged site and differentiate to generate new myofibres[56]. Over the last years many *in vivo* and *in vitro* studies have been conducted to test the effect of PRP on stimulating musculoskeletal tissue healing, supported by the hypothesis that the GF contained in the products could enhance regeneration, for example by modulating cell migration, proliferation, differentiation and acting on satellite cells. It must, however, be highlighted that evidence of the efficacy of PRP has been highly variable and the products studied have led to very heterogeneous outcomes[54,57-60].

In the past few years, great interest has also arisen regarding the effects of platelet derivatives on stem cells. Due to their differentiation potential and high capacity for self-renewal and *in vitro* expansion, mesenchymal stem cells have been considered for possible use in wound healing processes. The main function of mesenchymal stem cells in wound healing relies on their ability to release cytokines and GF that act in a paracrine way and modulate several processes: they activate dermal fibroblasts, their proliferation, and migration; promote collagen production; activate keratinocytes; and increase angiogenesis and neovascularisation. Mesenchymal stem cells can also contribute to wound healing by differentiating into other cells such as fibroblasts, keratinocytes, and epithelial cells[61]. Many studies, to date, have explored the effects of PRP on various biological activities of mesenchymal stem cells, highlighting the ability of the products to stimulate proliferation and preserve multipotency[62-64]. Numerous studies have also been conducted to investigate how to use platelet derivatives in order to improve the performance of *in vitro* expansion and differentiation potential of stem cells and thereby obtain a number of cells sufficient for subsequent *in vivo* transplantation[65,66]. Stem cells isolated from synovium, for example, can be harvested for clinical applications rather easily without causing donor site morbidity and have been confirmed to have chondrogenic, osteogenic, and adipogenic potential; however, it is not possible to harvest enough cells for immediate use and prior *in vitro* expansion of the cells is always necessary.

In the case of cartilage, platelet derivatives have not only been considered to induce the healing/regeneration of injured tissue directly[67], but also to provide a scaffold to support cartilage regeneration[68] and to be carriers of biochemical stimuli able to overcome the state of de-differentiation of chondrocytes cultured for autologous chondrocyte implantation. Articular cartilage, in fact, is not able to regenerate itself sufficiently to repair injuries or defects that occur after a trauma. One clinical treatment available to overcome this problem is autologous chondrocyte implantation: a small piece of the patient's cartilage is removed and the chondrocytes are isolated, grown in the laboratory and re-implanted, with the hope that they will be able to repair the damaged area, restoring a new layer of articular cartilage[69].

Table II reports a summary of some of the most recent *in vitro* studies, performed on a wide variety of human cell types involved in the repair/regeneration of different tissues and organs after treatment with platelet derivatives. It can be noted that many preparation methods, activation strategies, and platelet concentrations have been explored[37,44-46,51-53,63,70-81].

LEUCOCYTE CONTENT AND ANTIMICROBIAL ACTIVITIES

Although the use of platelet derivatives is justified by the release of platelet-derived GF leading to an acceleration of wound healing processes, many researchers have started to highlight the importance of leucocyte content in relation to antimicrobial activity. The use of platelet derivatives enriched in leucocytes leads to a decreasing number of infections in many clinical applications, which is an important aspect considering that, despite all the advances in surgical techniques and antibiotic use, the treatment of infected wounds is still associated with a high rate of complications[82]. Platelets are, in fact, an important source of antibacterial peptides (such as fibrinopeptide A and B, thymosin beta 4, platelet basic protein, connective tissue-activating protein 3, RANTES [regulated upon activation, normal T-cell expressed, and secreted] and PF4), but their antimicrobial role is not yet

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Table II - *Summary of some of the most recent in vitro studies performed using different platelet derivatives to treat a wide variety of human cell types involved in tissue repair/regeneration processes of different tissues*

Cell type	Experimental setting	Main results	Possible impact from *in vivo* application
Foreskin fibroblasts	10% activated PRP	No promotion of proliferation, slight stimulation of motility	No effects[70]
Hypertrophic scar dermal fibroblasts	5% activated PRP	Activation of negative feedback signalling for TGF-β1 which, in turn, downregulates connective tissue growth factor expression	Improvement of hypertrophic scars[45]
Skin fibroblasts	1% and 5% PRP, not activated or Ca^{2+} -activated PRP supernatant	Increase of collagen synthesis and stimulation of prolidase activity; increase of β1-integrin receptor, focal adhesion kinase and phosphorylated mitogen-activated protein kinases.	Promotion of cell growth and collagen biosynthesis, which could be of support in regenerative medicine; PRP was the most effective platelet derivative among those analysed[44]
Dermal fibroblasts	Activated PRP	Negative regulation of fibroblast-to-myofibroblast transition inhibiting TGF-β1/Smad3 signalling	PRP could be a potential therapy in those diseases in which fibrosis plays a major aetiological role[46]
Dermal fibroblasts	Chronic UVA irradiation followed by 25% and 50% platelet-rich fibrin lysate treatment	UVA irradiation decreased the biological activities of fibroblasts (collagen deposition and migration rate). Treatment with platelet-rich fibrin lysate lessened this negative effect.	Platelet-rich fibrin lysate could be a good candidate for treating UVA-induced photo-aging of skin[51]
Keratinocytes	PRGF (1:10-1:20-1:50)	Decrease of keratins-1 and -10 (early markers) and increase of involucrin and transglutaminase-1 (late markers). Induction of antimicrobial peptides human β-defensins-2 and -3 and psoriasin	PRGF induces keratinocyte differentiation, which is an important step, being the endpoint in wound re-epithelisation. It offers broad antimicrobial effects leading to a greater ability to promote wound closure[71,72]
Gingival fibroblasts	10%, 25%, 50%, 75% Ca-activated and non-activated PRP	Increase in proliferation rate, with the strongest stimulation reached with the 10% activated PRP	Activated PRP has greater efficacy compared to non-activated PRP[52]
Gingival fibroblasts	1%, 2%, 5% Ca-activated PRP	Increase in proliferation and migration	Stimulation by PRP could be very important in gingival tissue repair and wound healing[53]
Fibroblast-like tenocytes	Activated PRP combined or not with IL-1β (which simulates tendon inflammation)	In the absence of IL-1β, PRP induced expression of pro-inflammatory cytokines and MMP (stimulating an inflammatory state) whilst in IL-1β-induced inflammation it improved inflammation, downregulating pro-inflammatory cytokines and MMP and upregulating some anti-inflammatory cytokines and inhibitors.	PRP has pleiotropic effects on tenocyte biology, depending on the presence or absence of an inflammatory state induced by IL-1β: it has an anti-inflammatory effect on tenocytes only in inflammatory states typical of tendinopathic conditions while, otherwise, it stimulates a pro-inflammatory state[73]
Fibroblasts from rotator cuff tendons	LP-PRP	Induction of proliferation.	LP-PRP has a positive effect on tendon wound healing and could be useful to treat tissue degeneration[74]
Tenocytes	AlloPL, PRP, PC, PL	PC and alloPL, characterised by a higher content of growth factors, were not the products stimulating greatest tenocyte viability or expression of ECM proteins but did have the strongest effects on HGF expression and downregulation of COX-1 expression.	Both HGF and COX-1 are pain-associated molecules: HGF is a pain antagonist and COX-1 is a pain marker, thus, their modulation could result in pain reduction when using PRP in clinical applications[37]
Tenocytes	Co-culture of tenocytes with mesenchymal stromal cells adding 10% fresh/frozen $CaCl_2$-activated PRP	MSC alone could increase tenocyte migration and ECM production (fibronectin, collagen I and aggrecan); PRP acts as an adjuvant inducing greater effects, with the fresh PRP being more effective than the frozen one	PRP combined with MSC could lead to better tendon wound healing[75]
Synoviocytes	0%, 5%, or 10% PRGF with or without TNF-α (to create an *in vitro* model of rheumatoid arthritis).	Increase of cell viability by addition of PRGF; reduction of TNF-α, IL-6 and IL-1β release and increase of VEGF and IL-10 secretion when cells were pre-stimulated with TNF-α	Since TNF-α and IL-6 are usually increased in inflammatory states of synovia whereas IL-10 and VEGF are reduced, the data suggest an anti-inflammatory, and thus anti-arthritic, effect of PRGF[76]
Fibroblast-like synoviocytes	20% PPP or activated PRP in a cell model of rheumatoid arthritis induced by LPS.	PRP, more than PPP, counteracted the LPS-induced expression of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6). LPS also stimulated cell viability and reduced the percentage of apoptotic cells but PRP reversed this trend	Synovial tissue hyperplasia is typical of rheumatoid arthritis and synoviocyte growth plays a key role in this pathology, thus their inhibition by PRP is reassuring for *in vivo* applications[77]
Articular chondrocytes	10% PRP or HAS	Proliferation increased compared with HAS but not with PRP supplementation. PRP was capable of re-differentiating the de-differentiated chondrocytes while FCS or HAS led to further de-differentiation	Although HAS has positive effects on chondrocyte proliferation, PRP could be a better choice than standard FCS for chondrocyte expansion for clinical use in autologous chondrocyte implantation, being able to stimulate both proliferation and re-differentiation[78]
Endometrial stromal fibroblasts and MSC	5% PRP or PPP, in their activated and not activated forms	Increase in cell viability and migration after treatment with activated PRP; not activated PRP and PPP stimulated these processes but to a lesser extent compared to controls. No evidence of mesenchymal-to-epithelial transition. Activated PRP treatment upregulated several MMP (MMP-1, -3, -7, -26) and inflammatory markers (IL1A, IL1B, IL1R2, CCL5, CCL7, CXCL13), modulating inflammation and chemotaxis	PRP could be used to promote endometrial regeneration in clinical situations with compromised endometrial growth (such as Asherman's syndrome and atrophic/thin endometrial lining in infertile patients)[79]
Adipose-derived stem cells	10% PRP	No promotion of proliferation or migration	No effects[70]
Adipose-derived stem cells	5% PRP	Increased chondrogenic/osteogenic differentiation	Support for translational applications for the management of osteochondral defects in the field of regenerative medicine[80]
BM-MSC	1:50 and 1:100 PRP	BM-MSC viability and proliferation were positively regulated. The PRP/BM-MSC combination was more effective than PRP alone in sustaining the proliferation and myogenic differentiation of myoblasts	PRP alone or, to a greater extent, in combination with BM-MSC, may favour repair mechanisms in damaged skeletal muscle tissue, promoting myogenic differentiation[63]
MSC isolated from amniotic fluid	10% PRP	Migration and proliferation rate increased	PRP could act as a regulator of processes involved in *in vivo* wound healing[81]

AlloPL: autologous platelet lysate from pooled donors; BM-MSC: bone marrow-derived mesenchymal stromal/stem cells; MSC: mesenchymal stem cells; CCL5: chemokine (C-C motif) ligand 5; CCL7: chemokine (C-C motif) ligand 7; CXCL13: chemokine (C-X-C motif) ligand 13; COX-1: cyclo-oxygenase 1; ECM: extracellular matrix; FCS: foetal calf serum; HAS: hyperacute serum; HGF: hepatocyte growth factor; IL: interleukin; LPS: lipolysaccharide; LP-PRP: leucocyte-poor platelet-rich plasma; MMP: matrix metalloprotease; PC: platelet concentrate; PL: platelet lysate; PPP: platelet-poor plasma; PRGF: plasma rich in growth factors; PRP: platelet-rich plasma; TGF-β1: transforming growth factor-β1; TNF-α: tumour necrosis factor-α; VEGF: vascular endothelial growth factor; UVA: ultraviolet A.

fully understood and requires further studies. Just as one example, Cieślik-Bielecka *et al.* found that L-PRP had an *in vitro* microbiocidal effect on some of the tested strains[83]. As far as concerns healing processes, it is still a subject of debate whether leucocytes contained in platelet derivatives have a positive or negative effect. On the one hand, leucocytes should hypothetically have beneficial effects, sustaining the immune response against infections and increasing GF release (thus contributing to angiogenesis, matrix production, and hypercellularity)[84]. On the other hand, they may release inflammatory cytokines (TNF-α and reactive oxygen species) exacerbating the inflammatory response, thus delaying tissue healing[85], and inducing an increase in MMP levels, which could cause excessive matrix degradation and, consequently, inferior repair of wounded tissues and scar formation[86]. In fact, while several studies, mainly conducted in the orthopaedic field, have suggested that LP-PRP could induce more effective tissue healing when compared to leucocyte-rich, platelet-rich plasma (LR-PRP)[86,87], other studies found no significant differences between them[88,89]. Given that the issue is not yet completely settled, clinicians should consider using LP-PRP or LR-PRP according to the specific pathology in order to achieve better clinical results from PRP therapy.

CONCLUSIONS

The heterogeneity of the results obtained from *in vitro* experiments on cells treated with platelet derivatives is mirrored by the variability of clinical outcomes and can be explained by the different experimental settings used each time. In fact, the type of platelet derivative and the method used for its production can have profound effects on the final results.

For example, an important parameter to consider is whether or not the platelets are activated. Some authors reported that the maximum effect on cells was achieved after the activation of PRP[52,90]. Furthermore, the very method chosen to activate platelets could affect the availability of GF, indeed, it has been shown that PRP activation performed with different protocols influences the amount and kinetics of GF release[91]. This suggests that the PRP activation strategy should be chosen according to the pursued biological effects in the tissue to be healed.

Another, often underestimated but very important, parameter is the platelet concentration, which is partly dependent on marked differences in baseline platelet counts between individual patients, leading to variability in PRP composition and, therefore, concentrations of GF. Several studies have demonstrated, *in vitro*, that cells respond in a dose-dependent manner, but that very high concentrations of GF are not necessarily a prerequisite for optimal stimulation of cell processes, and may in fact be counterproductive. Numerous studies have shown that high GF concentrations can have a detrimental effect and can be more an obstacle than an advantage[53,58,92-94]. It is possible that the quantity of receptors on the cell surface is limited and thus, once the levels of GF are too high for available receptors, they became excessive and affect cell function negatively[53].

For example, in human primary tenocytes an excessively high concentration of platelets was shown to have an inhibitory effect on proliferation, migration, and the production of collagen type I. In contrast, MMP production increased with increasing platelet concentration, which could be detrimental because excessive proteolysis may impair the mechanical stability of tendons[58]. Similarly, we showed that PG supernatant was able, *in vitro*, to stimulate all the necessary mechanisms for fibroblasts to restore normal tissue during wound healing *in vivo*, such as proliferation, migration, and invasion, but that in this case, too, excessively high concentrations had an inhibitory effect on the processes[92]. Many other studies have indicated similar repercussions. Choi *et al.* reported a similar effect on the viability and proliferation of alveolar bone cells[95]. Graziani *et al.* demonstrated that the maximum effect on cell proliferation was achieved with a 2.5× concentration of of activated PRP, while higher concentrations resulted in a reduction of cell proliferation[96]. Kakudo *et al.* observed that 5% activated PRP maximally promoted cell proliferation of human dermal fibroblasts and adipose-derived stem cells, but that activated PRP at 10% or 20% had a lesser effect[90]. Creeper *et al.* demonstrated that PRP could exert a positive effect on osteoblast and periodontal ligament cell migration, proliferation, and differentiation, but that the effects were concentration-specific with the maximal concentration of 100% being less effective than the 50% concentration[97]. Tavassoli-Hojjati *et al.* observed that 0.1% or 5% PRP supplementation was significantly more effective than 50% PRP supplementation in

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inducing fibroblast proliferation[98]. Klatte-Schulz *et al.* demonstrated that the higher concentrations of GF in two different platelet derivatives did not result in greater cell viability when compared with that induced by platelet derivatives containing lower levels of GF[37].

These data strongly underscore that a high platelet or GF concentration is not necessarily related to a strong stimulatory effect but, instead, can have stagnating or inhibitory effects. Thus, *in vitro* studies have been and continue to be very useful for revealing how not all concentrations are equally functional to wound healing: maximal concentrations do not necessarily result in optimal clinical outcomes. Moreover, the inhibitory effects observed *in vitro* suggest that high concentrations could be counterproductive for wound healing *in vivo* too, prompting careful consideration of the clinical settings in which the products are to be used. The clinical effectiveness of different concentrations of platelet derivatives on different cell types still warrants further investigation to reach full standardisation.

It should not be forgotten that *in vitro* studies, although having many advantages (such as precise control of parameters and rapid results), also have some drawbacks. In fact, due to their architecture and organisation of all organs, there is a continuous interplay between different types of cells within an organ which it would be difficult to replicate in a two-dimensional monoculture *in vitro*: for example, cell density in the *in vitro* setting is usually less than 1% of the tissue situation (which affects cell signalling) and cell contacts with ECM are lacking. The accumulation of waste products, paralleled by a continues consumption of nutrients, is typical of culture conditions but is not a homeostatic condition, nor is the oxygen supply or the sudden exchange of media typical of *in vitro* cultures. Nonetheless, as highlighted by the large number of published papers in this field, *in vitro* studies remain important to provide indications on the biological processes sustained or hampered *in vivo* by using platelet derivatives[99] and to support their clinical use.

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