Review article

Title
Platelet Biology in Regenerative Medicine of Skeletal Muscle

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Abstract

Platelet-based applications such as platelet-rich plasma and platelet releasate have gained unprecedented attention in regenerative medicine across a variety of tissues as of late. The rationale behind utilising platelet-rich plasma originates in the delivery of key cytokines and growth factors from α–granules to the targeted area, which in turn act as cell cycle regulators and promote the healing process across a variety of tissues. The aim of the present review is to assimilate current experimental evidence on the role of platelets as biomaterials in tissue regeneration, particularly in skeletal muscle, by integrating findings from human, animal and cell studies. This review is composed of three parts: firstly, we review key aspects of platelet biology that precede the preparation and use of platelet-related applications for tissue regeneration. Secondly, we critically discuss relevant evidence on platelet-mediated regeneration in skeletal muscle focusing on findings from a) clinical trials, b) experimental animal studies and c) cell culture studies; and thirdly, we discuss the application of platelets in the regeneration of several other tissues including tendon, bone, liver, vessels and nerve. Finally, we review key technical variations in platelet preparation that may account for the large discrepancy in outcomes from different studies. This review provides an up-to-date reference tool for biomedical and clinical scientists involved in platelet-mediated tissue regenerative applications.

Key words: growth factors, platelet releasate, platelet-rich plasma, regeneration, skeletal muscle
Introduction

Platelets, also called thrombocytes, are produced from megakaryocyte projections into micro-vessels in mammalian bone marrow. Freely circulating platelets are the first cellular response following damage to vascular or tissue integrity and play a crucial role in haemostasis, innate immunity, angiogenesis and wound healing. The latter aspect is receiving increased attention since the wound healing effects suggest a regenerative ability for maintaining whole body integrity and homeostasis. Until recently, platelet-rich plasma (PRP; defined as a biologically active, autologous concentration of platelets re-suspended in plasma), was extensively used in the medical fields of connective tissue regeneration and thrombosis research, while the study of the regenerative potential of PRP in a clinical context attracted less attention. Although our understanding on the mechanisms linking platelet biology to tissue regeneration is still evolving, at this stage many aspects remain to be established due to inconsistent and conflicting scientific evidence.

Data from clinical studies on the effectiveness of platelet-rich plasma applications appear to be conflicting or limited to outcomes such as improvement of quality of life, reduction of post-operative pain, improved healing or absence of any beneficial effect. This has been attributed to possible methodological differences of preparation, PRP composition, medical condition of the patients, anatomical location of the lesions as well as type of injured tissue and has been discussed in relevant reviews of clinical interest. Growth factors and cytokines have a crucial role in the healing process with regards to early inflammation in tissue regeneration. Therefore, the rationale for utilising autologous PRP originates in the easy availability of numerous cytokines and growth factors to the targeted area, acting as biomaterials to promote regeneration. These factors in turn upregulate proliferation, differentiation and migration of necessary cells in the area of regenerating tissue. Over the past decade, there have been amounting articles contributing to the knowledge surrounding the mechanisms of growth factors in the regeneration of wounded or dysfunctional tissue. Due to increasing understanding in cell signalling and growth factor biology, research and clinical attention has been drawn to the use of autologous PRP as a novel means of delivering growth factors to injured tissue such as liver, bone and skeletal muscle. Skeletal muscle is a highly plastic tissue with remarkable capacity to regenerate in response to injury and trauma. The early acceleration of muscle regeneration, specifically within the first week, is a crucial time point to implement a clinical intervention due to the early inflammatory response as well as the regeneration phase taking place. Therefore, understanding the molecular and physiological mechanisms that link platelet biology to tissue regeneration has the potential to identify novel opportunities in regenerative medicine in the near future.
Readers interested in the clinical aspects of platelet-based applications for orthopaedic regeneration, in muscle, ligaments and tendons are directed to recent relevant reviews \(^6,8,19,20\). In the present review we assimilate current experimental evidence on the role of platelets as biomaterials in tissue regeneration, particularly in skeletal muscle. Firstly, we review key aspects of platelet biology that precede the preparation and use of platelet-related applications for tissue regeneration. Secondly, we provide a critique of the evidence for platelet-mediated regeneration in skeletal muscle focusing on findings from a) clinical trials, b) experimental animal studies as well as c) cell culture studies. Thirdly, we discuss the application of platelets in the regeneration of several other tissues including tendon, bone, liver, vessels and nerve.

1. Overview of Platelet Biology

The use of autologous PRP in clinical research has grown exponentially over recent years due to the gradually increasing understanding in the role of PRP’s growth factors in tissue regeneration \(^5\) (Figure 2). The first publication on PRP was issued in 1954 \(^21\). Ten years later, the first study of PRP being utilised in a therapeutic scenario was published \(^22\). This increasingly attractive therapeutic tool has made considerable advancements in many areas of regenerative medicine, particularly in the wound healing and skin regeneration, dentistry, plastic and cosmetic surgery, minor wounds, fat grafting, bone regeneration, tendinopathies, ophthalmology, hepatocyte recovery, aesthetic surgery, orthopaedics, veterinary, spinal fusion, treatment of soft-tissue ulcers, heart bypass surgery and at last but not least in skeletal muscle injuries \(^2,17,23-34\). Before we embark into the discussion of current evidence on the role of platelets in tissue regeneration, we briefly review key aspects of platelet biology such as platelet formation, activation and aggregation that precede the release of growth factors and the preparation of PRP.

A. Platelet formation and activation: Hematopoietic stem cells in the red bone marrow give rise to common myeloid progenitor cells which further differentiate to megakaryocytes \(^35\). Platelets are anucleated products formed from long extensions into vascular sinusoids after migration of the megakaryocytes to the vascular niche \(^35,36\). Vascular injury leads to exposure of prothrombotic extracellular matrix proteins, which facilitate platelet adhesion and activation. In addition to minimising blood loss, a major function of platelets is to promote healing of the damaged tissue. This is achieved through the release of cytokines, chemokines and growth factors from platelet granules. There are three major types of secretory granules in platelets including: i) \(\alpha\)-granules, containing many growth factors and cytokines ii) dense \(\gamma\)-granules, which release calcium, serotonin, polyphosphates, pyrophosphates, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) and iii) lysosomes, which contain a number of hydrolytic enzymes \(^13\).
In particular, there are approximately 50-80 α-granules per platelet with a typical diameter of 200–500 nanometres that can be released intracellularly or extracellularly 1, 37. Alpha-granule contents secreted by activated platelets release growth factors such as: platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), insulin-like growth factor (IGF), epithelial growth factor (EGF), endothelial cell growth factor (ECGF), and fibroblast growth factor (FGF) 38. Upon platelet activation, degranulation follows and release of trophic factors occurs 39 (Figure 1). It has been suggested that interaction of these factors with the hampered tissue structures causes an ameliorated and accelerated healing response 9.

B. Platelet aggregation: It is important to understand the mechanisms that govern platelet aggregation, as variations in aggregation strength will cause deviations in growth factor release 40. Differing aggregation procedures in clinical trials may be one of the reasons for conflicting results. Damaged cells or injured tissue release soluble platelet agonists such as ADP and thrombin, which act as platelet-activating factors. These signalling events then allow processes such as platelet spreading, consistent adhesion, granule secretion and clot retraction to occur 34. In order to exocytose contents, α-granule’s vesicle-associated membrane protein 8 (VAMP-8), synaptosomal-associated protein 23 (SNAP-23) and syntaxin 2 (a Q-SNARE proteins participating in exocytosis) are involved (Figure 1). After an agonist binds to its receptor, platelet shape-change occurs, followed by aggregation and granule content release.

C. The biological role of platelet releasate: Several studies have focused on releasate, which is a refined, centrifuged and purified sample of growth factors released from aggregated platelets when the supernatant is collected, and cellular debris removed (Jiang 14, 41, 42. Soluble bioactive molecules in the releasate secreted by the α-granules of platelets are known to enhance matrix synthesis (e.g. TGF- β), upregulate chemo-attraction and proliferation in several cell types (e.g. PDGF, VEGF, IGF-I and II, EGF and ECGF) (Figure 3) and angiogenesis (e.g. VEGF, FGF and ECGF) 4, 43. It is interesting to note that proteins released by the α-granules, such as platelet factor 4, are inhibitors of angiogenesis, additionally; endostatins are inhibitors of endothelial cell migration. These bioactive molecules may be involved in negative feedback mechanisms to fine tune the main growth factors such as VEGF which upregulates angiogenesis, or adhesive proteins such as fibronectin that promotes cell migration and differentiation 40. This opposing effect is essential to create the ability for homeostasis within the injured area capable to react to the surrounding environment.

Growth factors found in releasate are involved in promoting tissue regeneration, such as EGF which causes cell growth, recruitment and differentiation as well as cytokine exocytosis and secretion. Similarly, the growth factor PDGF-BB (homodimers PDGF -AA, -BB, -CC, and –DD and the heterodimer PDGF-AB) has a physiological effect such that causes significant cell
growth, cell migration, blood vessel growth, granulation, growth factor secretion and matrix formation with bone morphogenetic proteins (BMPs) \(^{43}\). The role of PDGF-BB following muscle damage is still yet to be determined \(^{44}\). It has been shown, however, that PDGF-BB upregulates the proliferation of satellite cells (i.e. skeletal muscle stem cells) and may affect differentiation negatively \(^{45, 46}\). Conversely, PRP application on satellite cells was shown to improve differentiation, indicating that the role of PRP on muscle progenitor cell differentiation has yet to be elucidated \(^{47, 48}\). Additionally, the VEGF and PDGF pathways both interact through the Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway to induce proliferation of satellite cells (Figure 3)\(^{45, 49-51}\). Another growth factor associated with releasate is TGF –\(\beta_1\), this factor is known to stimulate collagen synthesis, growth inhibition, apoptosis, differentiation and activation \(^{27}\). TGF- \(\beta_1\) acts in both an autocrine and paracrine fashion, inhibiting macrophage and lymphocyte proliferation, stimulating mesenchymal stem cell proliferation, while also regulating endothelial fibroblastic and osteoblastic cell mutagenesis, collagenase secretion and collagen synthesis. Additionally, releasate contains IGF-I and II, which is commonly known to cause cell growth, differentiation, recruitment and collagen synthesis when recruited with PDGF. VEGF and ECGF both target endothelial cells to cause cell growth, migration, new blood vessel growth as well as anti-necrotic properties. Finally, FGF in releasate has been shown to cause cell growth of blood vessels, smooth muscle, fibroblasts and endothelial cells as well as cell migration and blood vessel growth \(^{3,4,40}\). The combination of these growth factors has been shown to be beneficial for many types of tissue regeneration (Table 2). However, there is lacking evidence surrounding the role of these growth factors from platelet-based applications in muscle regeneration \(^8\).

It has been previously argued that discrepancies in releasate content may be accounted for by the varying methods used for activating platelets. In this context, four main types of platelet activation (i.e. 10% of either collagen type I, \(\text{CaCl}_2\), autologous thrombin, or a mixture of \(\text{CaCl}_2\) + thrombin), may have an impact in the amount of growth factors and cytokines (e.g. TGF\(\beta_1\), TNF-\(\alpha\), IL-1\(\beta\), PDGF-AB, and VEGF) released by activated platelets, when collecting releasate specifically for regenerative applications \(^{40}\). Other methods such as freeze-thaw for activation of platelets have provided insights regarding the shelf-life of platelet products. It has been reported that human platelet releasate can be stored for 21 days and retain its proliferative properties in fibroblasts as much as 2-day-old platelets \(^{42}\). Standardisation of platelet activation is a crucial step in order to optimise the various growth factors and cytokines released and implemented in experimental procedures. Different protocols for different tissue or cell types have been outlined (Table 3). Other experimental variables such as platelet count and centrifugation speed have also been recognised and are schematically represented in Figure 4. \(^{52}\). In terms of platelet count, the variation is donor-specific, which is why re-
suspending platelets to a standardised concentration is important. Moreover, the release of PDGF and TGF-β from platelet concentrates is pH-dependent, and therefore standardisation of platelet preparation is critical, given that TGF-β1 is one of the growth factors involved in deterring stimulation of differentiation of myoblasts.

Current preparation methods of platelet-based applications are outlined in Table 3. There seems to be significant variations between preparation methods from different laboratories, even when producing the same product such as PRP. Typically, either the whole blood is inhibited with an anticoagulant such as citrate dextrose solution (ACD) or sodium citrate and then either centrifuged or processed using a kit. The first centrifugation step can be processed at a speed between 160 to 1800 x g and between 4 to 30 minutes. The resultant platelet-rich plasma is then either isolated and used as it is, or inhibited with further anticoagulants such as prostaglandin or prostacyclin and centrifuged between 5 to 20 minutes at 250 to 3000 x g in order to pellet the platelets. Pelleted platelets are then resuspended in either a buffer or platelet-poor plasma (PPP) to a standardised concentration, varying between physiological levels (2 x 10^5 platelets μL^-1) to 3 x 10^6 platelets μL^-1. This resuspension is then aggregated using many methods including thrombin and calcium chloride, thrombin protease-activated receptor (PAR)-1, PAR-4, freeze-thaw cycles, zeolite, calcium, calcium gluconate/batroxobine, ADP, thromboxane A2 or exposure to cells. Thrombin is very frequently used in PRP preparations; however, one characteristic of thrombin is that it is a protease and may cleave or damage proteins in the releasate sample. For this reason, using a thrombin protease-activated receptor -1, or -4 (PAR-1) or (PAR-4) peptide may be an appropriate method for releasate preparation, activating the thrombin receptors without the potential of damaging the sample’s contents enzymatically. Finally, the releasate can be spun down to remove cellular debris. Expectedly, there is a large variation between publications for this final centrifugation step. In fact, the final centrifugation, excluding platelet exosome isolation, varies from 1400 to 100,000 x g between 10 and 30 minutes. Such discrepancies in the preparation methods are illustrated in Figure 4. At present, it cannot be ruled out that technical inconsistencies such as the ones mentioned above may possibly account for the variable outcomes reported in several studies.

D. PRP Alternatives: It is essential to characterise the current forms of platelet-based applications that are derived from PRP methods in order to cope with the current methodological limitations (Table 3). One alternative to PRP that is noteworthy to mention is platelet mediator concentrate (PMC). PMC contains similar factors as PRP such as PDGF-BB, TGF-β1, VEGF, BMP-2, BMP-4, TNF-α BMP-7, and IL-6. However, it has been reported that low levels of TNF-α and IL-6 in PMC are suitable for tendon healing and reduced scar formation. Platelet-rich fibrin (PRF; a fibrin clot in which contains the platelet cellular debris with their cytokines) also contains many of the above-mentioned growth factors such as
PDGF, TGF-β, VEGF, EGF and IGF-1. These alternatives may be particularly useful in patient-dependent cases where local injection may not be applicable. Several alternatives to PRP could also be implemented such as advanced platelet-rich fibrin (A-PRF) as well as a concentrated growth factor (CGF) which have higher levels of TGF-β1, PDGF-BB and VEGF as well as higher platelet counts. Leukocyte-rich PRP and pure PRP (leukocyte-poor PRP) is an emerging new delineation in platelet-based applications in which describes the hampering effects of having white blood cell contamination in PRP preparations. Other novel platelet therapy methods could be implemented if PRP continues to show promising results in terms of skeletal muscle regeneration. One may speculate that delivering higher concentrations of these factors to the localised injury site may cause further recovery, however an undesirable ratio of growth factors and cytokines may cause an imbalance in homeostasis which may be detrimental. Attempting to get this ratio correct results in many variations and alternatives in the platelet preparation methods. Novel platelet-based applications such as CGF or A-PRF would have to be a localised implantation. These physical manipulations, as well as leaving the injury site open for minor surgery, may cause dissimilar factor releasate from the platelets, resulting in non-comparable results to PRP. It is important to outline the role of platelet aggregation when we are denoting the ratio of the release of growth factors that are crucial in relation to tissue regeneration, and choosing the correct method of platelet-based application.

2. Platelet-mediated skeletal muscle regeneration

Current evidence on the role of platelet-based applications in skeletal muscle regeneration derives mainly from: a) human clinical trials, b) experimental animal studies, as well as c) in vitro cell studies.

A. Clinical trials with platelet-based applications targeting skeletal muscle: Given the recent development of platelet-based applications, the number of studies in this area has increased exponentially, with more than 1000 articles being published in the last two years (Figure 2). The studies reviewed in this article were identified through a PubMed search using combinations of the following key words: platelet-rich plasma, regeneration, skeletal muscle or any other tissue as discussed. Relevant references were reviewed to identify further original research on platelet-based applications and regeneration, focusing on cellular, animal and human studies. There have been several clinical trials examining the use of PRP for muscle regeneration (see Table 1). Human athletes with muscle lesions (partially torn) were injected locally with autologous PRP every 7 days for 21 days. Despite not having a control group, this study determined that the autologous PRP injections remained a safe and effective treatment for varying muscle lesions. Ultrasound-guided injections of PRP in professional athletes had an augmented pain relief score as well as increased pain on resisted flexion, strength and
range of motion after only 7 days versus conventional treatment. Similarly, pre- and post-treatment of proximal hamstring injuries with PRP was carried out using the Nirschl Phase Rating Scale and Visual Analogue Scale for pain rating. The results of this clinical study showed that pain reduction was augmented in the PRP group over the conventional treatments. A recent study analysed hamstring injuries in professional football players over a 31-month period. This study conversely reported that lesions showed a non-significant healing rate over patients treated with Actovegin; however it reported the safety in use of PRP in human patients.

It has been previously argued that the conclusion of several trials against the use of PRP in sport injuries may be attributed to technical inconsistencies and methodological limitations of the studies. Such limitations are poor sample sizes, non-blinded studies, lack of control studies, inconsistency in PRP preparation methods, platelet concentration and growth factor levels are inconsistent, selection bias in clinical studies as well as a specific demographic used, such as healthy and fit male athletes as opposed to the general population or patients. One of the major flaws in PRP studies for muscle healing in human subjects is the lack of physiological data and mechanistic insights as an outcome measure, rather, pain scores and return to respective sporting fields have been used.

There have been many speculations made in recent scientific reviews upon the beneficial use of platelet-based applications in musculoskeletal injuries. These previous reviews of clinical trials using PRP in orthopaedic injuries seem to consistently indicate insufficient results while they recognise that platelet-based applications may hold promise in future applications. A systematic review covering the effects of PRP on muscle lesions in both humans and horses showed that PRP has positive results in 46.7% of the clinical studies. Further to this, Sanchez et al. delineate a common protocol used for PRP in muscle injuries in clinical settings as well as the post-infiltration protocol for follow up potential complications. The protocol here for human patients seems thought out, thorough and effective in follow up treatment. In fact, this may be a promising approach to go forward for optimisation in clinical trials. With the gathered evidence over recent years, one may surmise that there is an effect between platelet-based applications and in the early stages of inflammation with and increased skeletal muscle healing rate.

Experimental evidence from animal and cell studies remains to be applied to clinical practice. Optimisation of platelet preparation is essential to be standardised or tailored for individual patients, such as depletion of deleterious cytokines. Additionally, the timing of PRP application is pertinent. For example, addition of PRP on days 1 and 4 post-injury hinder skeletal muscle regeneration, but on day 7 has been shown to be beneficial in a rat model. Of note, inconsistencies between clinical and experimental data need to be narrowed, given
the current diverse methodologies in platelet preparation among different laboratories as listed in Table 3.

**B. Animal Studies with platelet-based applications targeting Skeletal Muscle:**

Despite the limitations in human trials, with respect to PRP, there have been numerous progressions in the field of skeletal muscle regeneration in rodent models 2, 9, 16, 70. Accumulating data from animal studies on the role of PRP on skeletal muscle recovery after varying types of injury has emerged (Table 1). Dimauro et al. showed promising data on the delivery of PRP on cell proliferation and differentiation as well as satellite cell recruitment that resulted in improved skeletal muscle regeneration 2. They propose that PRP downregulated myo-miR-133 and increased Pax7 and other MRFs involved in both myoblast proliferation and differentiation in vivo. Myo-miR-133 upregulates myoblast proliferation through the suppression of serum response factor (SRF) and impede myotube differentiation 71, 72. Conversely, myotube differentiation was upregulated with the application of 20 nM of double-stranded miRNA for myo-miR-1, 133 and 206 73. The conflicting data suggest that the role of miRNAs on muscle regeneration remains to be fully elucidated. It has been reported that the concentrations of the specific growth factors TGF-β1, PDGF-AA, PDGF-AB, and PDGF-BB in human PRP are highly elevated over goat and rat growth factors per platelet 74. Interestingly, TGF-β1 was found to be the highest growth factor concentration across all 3 species’ PRP. Both b-FGF and IGF-1 have been found in the α-granules of platelets; these factors are known to independently promote regeneration in vivo in a murine model. For example, gastrocnemius muscles of mice injected with (100ng ml−1) IGF-1 or with (100ng) b-FGF on days 1, 3 and 5 showed faster muscle healing and tetanic strength recovery 75. An influential study addressing the effect of TGF-β1 neutralisation in PRP on a rat muscle injury, reported that modified PRP with depleted TGF-β1 boosted myofibre regeneration and decreased fibrosis 17. The study also reported an increase in angiogenesis and greater M2 macrophage localisation in the injury site, which are known to have an anti-inflammatory function and regulate wound healing. Additionally, satellite cell number was increased in response to TGF-β1-depleted PRP 2 weeks post-injury. This finding indicates that PRP composition may be modified in order to optimise benefits towards skeletal muscle regeneration. In a previous mouse study from the same group, it was shown that human muscle-derived progenitor cells cultured in either PRP or foetal bovine serum (FBS) had the same capacity to regenerate myofibres in vivo upon transplantation into injured gastrocnemius muscle 34.

Interestingly, local delivery of PRP can shorten recovery time after a muscle strain or multiple muscle strain injuries in rat models leading to faster functional recovery of the tibialis anterior muscle 70. Additional evidence from a mouse injury model suggests that the optimal time point for a platelet-pure PRP (i.e. a leukocyte-poor PRP) injection was 7 days post-injury, leading to reduced fibrosis and better exercise tolerance. However addition of PRP on 1 or 4 days
post-injury that coincide with the period of myoblast fusion and commitment to differentiation
causes fibrosis and shortens exercise tolerance. The mechanistic insights of this finding
remain to be determined. One possibility is that platelet releasate has a more potent effect on
myoblast proliferation, while its use during the early phases of regeneration or cell
differentiation may be compromised by inflammatory pathways. This notion is supported
by in vitro data, where PRP releasate upregulated myoblast proliferation but inhibited myoblast
fusion.

Two recent articles relate the role of reactive oxygen species (ROS) and muscle regenerative
capacity. The role of ROS in myogenic differentiation is multifaceted as cellular responses
alter acutely to minute changes in ROS stress levels. Martins et al. showed that PRP is
capable of modulating the oxidative impairment determined by muscle contusion, defined as
a section of damaged tissue where capillaries have been ruptured. The prevalence of
contusions is very common both in the general population and in sporting athletes affecting
the function of the musculoskeletal system. Contusion, by dropping a 200g mass directly
onto the gastrocnemius muscle, was shown to increase the levels of oxidative stress markers
(i.e. thiobarbituric acid and oxidized dichlorofluorescein) in both muscle tissue and in
erthrocyte preparations. Application of PRP was able to attenuate oxidative stress and
increase enzymatic antioxidant defence in injured skeletal muscle. These data suggest that
the beneficial effects of PRP on muscle regeneration may, at least in part, be brought about
by lower levels of oxidative stress.

It is evident that both animal and human studies have revealed largely dissimilar results when
analysing the impact of PRP in various tissues and treatments. The supportive evidence on
platelet-based applications from experimental animal studies remains to be validated and
extrapolated into human studies with a more thorough experimental design and biological or
functional end-point measures. Identification and in depth assimilation of the mechanisms
behind the effect of platelet releasate on these tissues is crucial in order to design and conduct
better human trials.

C. Cell Studies with platelet-based applications: Studies now emerging are finding
beneficial results with platelet releasate in muscle regeneration similar to PRP treatments.
One crucial and recent study added platelet releasate to a primary culture of rat
gastrocnemius muscle cells with the aim of investigating the impact of releasate on cell
proliferation. It was revealed that releasate increases the proliferative potential of the cells
in a dose-dependent manner. This finding was attributed to a continuation of the cell cycle
from the G1 phase to the S phase, driving progression through expressions of cyclin and
cyclin-dependent kinase (cdk) protein.

One recent study examined the role of releasate on both myogenesis and adipogenesis in
rats as well as in a C2C12 in vitro cell line. C2C12 cells are a mouse myoblast cell line
capable of differentiation. The localised sub-acromial injection of PRP proved significantly effective in reducing the instance of adipogenic gene expression as well as supressing adipogenic differentiation. In the C2C12 cells, there was substantial proliferation when PRP was administered as well as inhibiting muscle and adipocyte differentiation. This finding mirrors the effect of PRP on myoblasts, namely an upregulation in proliferation but presumably an inhibitory role in differentiation. Thrombin-activated PRP has been reported to be detrimental in both Saos-2 cells (Sarcoma osteogenic cell line) and marrow stromal cells when activated with thrombin in terms of cell viability through a 48-hour MTT assay. However, a recent study has shown that co-cultures of adipose-derived stem cells (ASCs) or PRP with myogenic progenitor cells had an augmented effect on myogenic proliferation. Notably, this study reported that the ASCs promoted both myogenic progenitor and C2C12 cell proliferation with PRP. An interesting tissue engineering study looked at C2C12 cells in a PRP treatment embedded in fibres of polydioxanone and polycaprolactone which were electro-spun. This study showed proliferative benefits using the electro-spun scaffold in myoblasts. Co-culturing myoblasts with a micro-environmental niche such as with ASCs or with a fibrous scaffold may be a more accurate representation of myogenesis in vivo than single cell culture. Conclusively, myoblast cell lines proliferate in response to platelet releasate; however the role of releasate in cell differentiation is still being discussed.

In order to determine if there is any merit to using platelet-based applications as an effective form of regulating cell proliferation and differentiation with an ultimate goal to support regeneration, key cell culture studies were analysed as outlined in Table 3. With the exception of endothelial progenitor cells in one study, platelet preparations seemed to produce a positive proliferative effect on various cell types across species. Some of the cell types in Table 3 include myogenic progenitor cells, bone-derived periosteal cells, osteosarcoma, endothelial, trabecular bone cells, human adipose-derived mesenchymal stem cells, fibroblasts, tenocytes, myo-endothelial cells, pericytes, C2C12 cells, adipose-derived stem cells and muscle satellite cells. Variable levels of differentiation have been reported for different cell types; e.g. increased differentiation for rat bone marrow cells, human skeletal muscle myoblasts, rat muscle satellite cells, rabbit bone marrow mesenchymal stem cells, and C2C12 myoblasts or maintained effect with hMDPCs, myo-endothelial cells and pericytes compared to control conditions. Conversely, some studies reported that platelets inhibited differentiation of C2C12 myoblasts. This discrepancy in the current literature may provide the basis for a thorough consideration of the technical aspects in platelet applications that may affect the final outcome in a study. For example, despite the majority of studies applying 10% PRP, a recent study used higher concentrations of platelets in smaller volumes (i.e. 1-2%) to induce differentiation, assuming that the concentration of growth factors was not altered. Finally, an interesting study carried out by Miroshnychenko et al. has led
to a new insight in PRP and PPP effects \textit{in vitro} on human skeletal muscle myoblast cells \cite{48}. This study looked at PRP, PRP with depleted TGF-β1 and myostatin and PPP in culture with the myoblasts. TGF-β1 and myostatin were depleted due to their detrimental effects on muscle regeneration \cite{2}. The study has reported that PPP and leukocyte-poor PRP with a second centrifugation to remove whole platelets induced myoblast differentiation, however unmodified leukocyte-poor PRP increased myoblast proliferation \cite{48}. An interesting aspect of this study is that PRP did not seem to induce muscle differentiation; rather it was more inclined to induce a proliferative property. This study is pertinent due to the method of removing unwanted growth factors from the PRP in which resulted in altered biological properties on the myoblast cell line used. Further studies to eliminate additional releasate factors could be implemented to optimise skeletal muscle regeneration and possibly expand in a clinical application in the near future.

3. Current evidence on platelet-mediated regeneration in other tissues

There has been an intense interest in determining the effect of platelets and platelet-related application on the regeneration of several other tissues such as tendon, adipose tissue, bone, liver, nerve, vascular tissue, wound healing etc.

**Tendon regeneration:** One recent study analysed PMC, a centrifugation-free method of preparing human platelet releasate, co-cultured with Achilles tenocytes \textit{in vitro} from both human and murine tendons \cite{29}. This study reported that PMC concentrations caused an elevation for important growth factors and markers for cell viability in tenocytes, suggesting that autologous PMC may be a future useful therapy in tendon recovery. However, a cross-comparison with PRP or releasate would be useful in determining which treatment at what dose would be optimal for tendon recovery \textit{in vivo}. An \textit{in vivo} study looked at platelet gel; a resuspended pellet of platelets activated with thrombin and calcium, in relation to the effect on the transected Achilles tendon of female rats after 14 days \cite{28}. The results, when compared to a saline injection, showed a 42% increase in the force at tendon failure when subject to being pulled at a consistent speed of 1mm second\(^{-1}\). There was also a 61% increase in ultimate stress (MPa) observed in the tendons when compared to the saline injections, suggesting an increased tendon recovery time. Notably, the platelet gel seemed to have a significantly lower force at failure score than the platelet gel, 24% and 42% respectively. This suggests that the activated PRP with thrombin and calcium is required for optimised tendon recovery. Currently, leukocyte-poor PRP progresses tendon healing and is considered a more viable option for the clinical treatment of tendinopathy after a comparative use in a rabbit model \cite{85}. In general, the current consensus on platelet-based applications on tendon regeneration is a positive one with tenocyte proliferation reported \textit{in vivo}, as well as structural optimisation of the tissue being upregulated.
Adipose and Endothelial tissue regeneration: A clinical and in vitro study analysing the application of PRP in terms of tissue engineering, specifically fat grafting, for the purpose of plastic surgery, reports an accelerated chronic skin ulcer re-epithelisation. Co-culture of adipose tissue-derived stem cells (ADSCs) with PRP show a proliferative effect. The clinical application of fat grafting with PRP showed consistently higher re-epithelisation from 3 weeks until 18 months over a control group. Fat grafts were maintained with PRP up to 69% restoration rate when compared to a 31% control. In an earlier study, activated PRP co-cultured with human umbilical vein endothelial cells or with transformed human osteoblasts showed increased endothelial proliferation. Interestingly, the non-activated PRP group in the osteoblast cells were more proliferative than in the activated group, suggesting that PRP secretions gave no supplementary benefit on osteoblast proliferation over the 3 groups. However, when compared to the minimal medium group there was a substantial increase in all PRP conditions showing that platelet extracellular growth factors were expressed in adequate quantities to induce substantial proliferation. These results indicate towards an increased proliferative rate of ADSCs with platelet-based applications, with a possible benefit for cosmetic utilisation.

Osteoblast and Chondrocytic tissue regeneration: Many studies now see the potential mechanisms of PRP acting on osteoblast proliferation and migration. Using the SaOS-2 osteoblast line, both TGF-β and PDGF were analysed for both cell proliferation and cell migration. Notably, TGF-β appeared to have an inhibitory effect on proliferation, while PDGF was reported to upregulate migration. Similarly, Kanno et al., have shown the link between a PRP treatment and osteogenesis in vitro in a dose-dependent method. This study suggests that growth factors in PRP, such as TGF-β, prompt pre-osteoblasts to undergo division increasing their quantities through chemotaxis, stimulating differentiation into mature osteoblasts. In connection to osteogenesis, a study makes the connection to bone and cartilage regeneration. In this study, the mechanisms connecting PRP to chondrocyte differentiation and regeneration were assessed by the means of regulating local inflammation in cartilage through decreasing chemotaxis of anti-inflammatory agents such as HGF. Releasate from the PRP was found to be accountable for the inhibition of NF-kB-transactivating activity due to the upregulation of HGF. It is still unclear whether the stimulatory effects of PRP, in osteoblast proliferation for example, are connected to the growth factors present or to other factors present in the cytoplasm or cell membranous structures from activated platelets. This study also stated that cell-to-cell contact was not reportedly required for upregulated osteoblastic proliferative effects of platelets. In summary, recent studies indicate that there is a positive proliferative and differentiative effect of platelet-based applications in osteogenesis. PRP also promoted growth and proliferation in chondrogenesis and may be beneficially applicable in cartilage repair.
**Hepatocyte tissue regeneration:** A study evaluated the in vivo effect of platelet-rich plasma on carbon tetrachloride-induced hepatotoxicity in male rats. Animals received PRP treatment twice a week for 8 weeks. After the 8 weeks, the rats were bled and their livers were analysed histopathologically, showing a hepatocytic protection of the PRP as well as showing that PRP itself is not toxic for at least a 3-week period. Further to this, a recent review highlighted the factors released by PRP such as VEGF, HGF and IGF-1 to promote hepatocyte proliferation. It has been hypothesised that an unidentified receptor on the liver sinusoidal endothelial cells interacts directly with the platelets in PRP in which stimulate proliferation in the hepatocytes. Similarly, a recent study followed up from this, analysing patients who underwent hepatic resection. It was shown that a rapid accumulation of platelets to the resection was correlated to regeneration of the liver. Interestingly, an unfavourable ratio of growth factors such as an increased TSP-1 level as well as a lower VEGF level displayed hampered regenerative properties. These studies reveal a proliferative effect in hepatocytes when using platelet-based applications.

**Nerve tissue regeneration:** One of the first experimental uses of platelet-based applications was the use of PPP in a rabbit model in 1973. This study analysed a plasma clot welding of nerves with regained myoneural function and no sign of substance rejection. A more recent study performed a bilateral sciatic neurotomy in rats, followed by being promptly re-anastomosed with a cyanoacrylate glue used in order to study the regenerative properties of PRP in relation to nerve regeneration. The biopsies were harvested 12-weeks post-operation with the aim to see if the PRP treatment promoted peripheral nerve healing. The article suggests that through distal axon counts, neurotisation indexing and density analysis, a PRP-treated group has potential in enhancing peripheral nerve regeneration. In terms of facial nerve regeneration, a study analysing the effect of PRP and fibrin sealant in a rat model was conducted. Male rats were subject to transection in survival and non-survival surgery groups of the left facial nerve and treated with either PPP, PRP or fibrin sealant using the right facial side as the control. Axon counts and facial nerve motor action potentials were analysed resulting in a faster recovery in the PRP group, the study reported overall that PRP was notably the better option when sutured compared to the other two interventions. A more recent study explored the benefit of PRP lysate on an ischemic stroke in rats. The outcomes were measured by means of analysing neurological deficit score and infarct volume. One of the more interesting points that this article tackles is the use of human PRP lysate in a rat model and how it shows a significant benefit in recovery after an induced stroke. Overall, platelet-based applications show a beneficial effect on nerve regeneration in animal models.

**Angiogenesis:** In a recent study to analyse angiogenesis on a PRP-seeded poly(ε-caprolactone) scaffold, it was reported that this PRP application method may be beneficial for tissue engineering due to the consistent delivery of growth factors without loss of activity.
Not only was there an increase in angiogenesis, the chicken chorioallantoic membrane (CAM) model also increased the hydrophilicity, attachment of mesenchymal stem cells and cell proliferation on the scaffold. PRP-seeding is looking like a promising tissue engineering method for integration into a host. The therapeutic value of PRP in angiogenesis can be seen in a study aiming to evaluate the application of platelet-enriched plasma in oral mucosal healing in terms of capillary count and density in a randomised split-mouth design in patients. The results showed that for the initial two weeks, capillary density and capillary count was higher in the PRP treatment over the placebo treatment administered to the contralateral side. A gelatin hydrogel was used with releasate in a recent study to analyse the aspect of angiogenesis in wound healing. This study used male mice to demonstrate that capillary formation was enhanced after 2 weeks in the gelatin hydrogel with PRP group, supporting angiogenesis when compared to a control saline group and a single PRP injection group. The article also reported that augmented wound healing through wound area analysis and angiogenesis using anti-VWF immunohistochemical staining was significantly higher in the treatment group. This study suggests a more specified application of PRP through a hydrogel with releasate can steadily release the growth factors over a period of time being more beneficial than a single PRP injection. It would be interesting to see if the beneficial effects of platelet-based applications on angiogenesis were directly due to the concentration of VEGF released from the α-granules in platelets, or due to the ratio of growth factors released. Taking these studies into consideration, platelet-based applications are seen to increase angiogenesis.

**Cutaneous wound healing:** PRP has been shown to be increasingly used in wounds that are difficult to heal such as tissue injuries. In order to address if PRP was beneficial for acute cutaneous trauma wounds such as open and closed fractures as well as epithelial necrosis and friction injuries, a study looked at patients receiving a local injection of PRP. With conventional treatments given to patients as a control group, the PRP group showed a faster rate of recovery in comparison; measured by the time taken for the wound to heal to such a degree that plastic surgery is applicable. This trend of a faster regenerative rate can also be seen in chronic cutaneous ulcers. A study has shown that a localised 100-200 μl injection of autologous platelet-enriched plasma in patients proved that the percentage area of healed cutaneous ulcer over a standard-care group between 4 and 8 weeks was highly significant. This study suggests that topical application of platelet-enriched plasma is cheap and effective treatment at tackling chronic ulcers in modern healthcare.

**Conclusions**

In conclusion, there is mounting evidence on the use of platelet-based applications in tissue regeneration. Inevitably, there is currently a large discrepancy in the effectiveness of platelet-
based applications in the scientific literature, especially between human and experimental animal studies. This may be attributed to methodological differences in platelet preparation and platelet releasate composition among different research groups. At present, there is an intense interest in the field worldwide with tremendous possibilities for exploitation in regenerative medicine. The current consensus with the use of PRP and especially modified PRP (where individual factors are depleted) in skeletal muscle regeneration remains promising, despite an incomplete understanding of mechanistic insights in both knowledge of platelet-satellite cell interactions, as well as PRP preparation optimisation. Most importantly, the molecular mechanisms linking platelet biology to skeletal muscle, or other tissue regeneration, have just begun to unravel and are expected to transform our understanding in using platelets as a biomaterial for tissue healing.

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Conflict of interest
The authors declare no conflict of interest.
| Reference | Species     | Intervention                                      | Findings                                                                                                                                                                                                 |
|-----------|-------------|---------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 9         | Rat         | PRP on a flexor sublimis lesion                   | ↑ leucocyte infiltration; ↑ early inflammatory response post muscle injury                                                                                                                           |
| 2         | Rat         | PRP on flexor sublimis incision                   | ↑ mRNA of pro-inflammatory cytokines, MRFs & IGF-1Eβ; ↓ myo-miR-133a                                                                                                                                   |
| 70        | Rat         | PRP on tibialis anterior under muscle strains     | ↑ myogenesis ↓ time-to-recovery after a muscle strain                                                                                                                                                    |
| 16        | Rat         | PRP on gastrocnemius muscle injury                | ↓ pain/claudication score                                                                                                                                                                |
| 15        | Rat         | PRP in gastrocnemius contusion                    | ↓ oxidative stress and ↑ enzymatic antioxidants in injured skeletal muscle                                                                                                                              |
| 82        | Rat         | PRP-derived growth factors on rat muscle satellite cells | ↑ proliferation & osteogenic differentiation ability of satellite cells from rat masticatory muscle.                                                                                                    |
| 54        | Rat         | Rat releasate on rat gastrocnemius muscle cells in vitro | ↑ proliferation; ↑ Cyclin A2, B1, cdk1, cdk2 and PCNA of protein expression (dose dependently)                                                                                                          |
| 17        | Rat         | TGF-β1 neutralisation in PRP on a cardiotoxin-induced muscle injury model | ↑ muscle regeneration; ↓ fibrosis; ↑ angiogenesis; prolonged satellite cell activation; ↑ M2 macrophages to the injury site                                                                                               |
| 78        | C2C12 myoblasts and Rat | Human releasate on C2C12 murine myoblasts. B. Rat PRP on rat rotator cuff tear | ↑ Proliferation; Inhibited myogenic differentiation; ↓ expression of adipogenic genes & lipid droplet formation in vivo                                                                                     |
| 69        | Mouse       | Muscle contusion injury & PRP at different time points | PRP injection 7 days after injury ↑ exercise time; ↓ fibrotic tissue; PRP at 1 and 4 days after injury ↓ exercise time; ↑ fibrotic tissue                                                                 |
| 94        | Mouse       | Gelatin hydrogel with platelet releasate in wound healing | ↑ levels of angiogenesis ↑ wound healing rate                                                                                                                                                          |
| 34        | Mouse       | Human releasate on muscle-derived progenitor cells | ↑ Proliferation of hMDPCs; PDGF further increases the proliferative effects of PRP                                                                                                                     |
| 80        | Rabbit      | Rabbit PRP with ASC extracts on rabbit myogenic progenitors and Human fibroblast culture | ASCs extracts had a stronger effect on proliferation of MPCs than PRP                                                                                                                                  |
| 61        | Human athletes | PRP in grade II muscle lesions                    | ↓ pain in all patients and improved muscle function in 85% of patients after first injection. ↓ VAS two weeks post-treatment. 100% return to sport activities after 35 days (Non-controlled study) |
| 62        | Human athletes | PRP in acute muscle injury                        | 93% ↓ pain after 28 days versus 80% in control; ↑ range of motion and strength                                                                                                                         |
| 63        | Human Patients | PRP in proximal hamstring injuries               | ↓ VAS and NPRS scores                                                                                                                                                                                  |
| 96        | C2C12 myoblasts | Human PRP lysate on C2C12 murine myoblasts       | ↑ C2C12 proliferation up to 20% PL but mildly cytotoxic at 100%; ↑ C2C12 scratch wound closure                                                                                                           |
| 48        | Human (Ex Vivo) | 1) PRP 2) Releasate with depleted TGF-β1 and myostatin 3) PPP; in human skeletal muscle myoblasts | PPP and Releasate with depleted TGF-β1 and myostatin induced myoblast differentiation; ↑ myoblast proliferation with PRP                                                                                   |
| 47        | C2C12 myoblasts and murine satellite cells | PRP + BM-MSC                                       | ↑ proliferation & differentiation                                                                                                                                                                        |
| Reference | Intervention | Tissue Type | Findings |
|-----------|--------------|-------------|----------|
| 29        | PMC          | Tendon      & Human & murine tenocytes | ↑ proliferation |
| 28        | Thrombin and platelet gel | Tendon | Rat | ↑ tendon repair (↑ in force at failure & ultimate stress) |
| 18        | PRP          | Tendon equine flexor digitorum superficialis tenocytes | ↑ TGF- β1 and PDGF-BB; ↑ expression of matrix molecules in 100% PRP; no effect on catabolic molecules (MMP-3 and MMP-13) |
| 25        | PRP suspended on collagen | Adipose (Fat grafting) | Chronic lower-extremity ulcers: 100% re-epithelisation, versus 40-60% of controls |
| 86        | Human PMC    | Osteoblast (CAL-72) & fibroblast (NIH-3T3) | ↑ proliferation both cell line; osteoblast secretion of IL-6; ↑ differentiation of fibroblasts |
| 26        | Calcium & thrombintreated PRP | Osteoblast and endothelial cells | ↑ proliferation |
| 27        | PRP          | Osteoblast cell line SaOS-2 | ↑ chemotaxis & proliferation dose-dependently; PDGF from PRP involved in stimulating cell migration; TGF- β from PRP inhibited cell proliferation. |
| 31        | PRP          | Osteoblast and SaOS-2 cell lines | ↑ mRNA: Procollagen type I, osteoprotegerin, osteopontin, and cbfa1; ↑ bone regeneration |
| 87        | Platelet concentrates | Human Trabecular Bone Cells | ↑ proliferation of bone cells independent of cell-to-cell contact |
| 74        | Goat, Rat and Human PRP-coated wells | Rat Bone Marrow Cells | ↑ initial cell growth; Human PRP had the most growth factors per platelet; TGF- β1 was the highest growth factor in all PRPs |
| 97        | PRP & BMSCs  | Bone marrow stromal cells in Rat femoral defect | ↑ BMSC proliferation; a concentration of platelets at 100 x 10^4 μl^-1 with BMSCs in a collagen mixture: ↑ newly formed bone. |
| 98        | Platelet Lysates and Platelet exosomes | Bone marrow stromal cells | ↑ proliferation & migration dose-dependently |
| 99        | PRP in a polylactic-glycolic acid | Osteochondral articular cartilage defects in rabbits | ↑ osteochondral formation |
| 38        | PRP          | Chondrocyte lbpva55 cells | ↓ activity of NF-kB, regulating the inflammatory process; ↓ COX-2 and CXCR4 target genes; ↑ HGF, IL-4 & TNF- α |
| 55        | Leukocyte- and (L-PRP) and pure (P-PRP) PRP | Chondrogenesis of rabbit bone marrow mesenchymal stem cells | P-PRP ↑ both proliferation and differentiation over L-PRP group |
| 88        | (Review)     | Hepatocyte | Proliferation | PRP stimulates hepatocyte proliferation by activating the Akt and ERK1/2 signalling pathways in hepatocytes. |
| 30        | PRP          | Hepatocyte (rat hepatic injury) | Hepatoprotective effects of PRP counteract the effects of CCl_4 on liver fibrosis |
| 89        | Platelet releasate | Hepatocyte (post-operative patients) | Patients with high TSP-1 and a low VEGF release profile have ↓ liver regenerative capacity |
| 90        | PPP & fibrinogen, thrombin or CaCl_2- | Sciatric Nerve Rabbit Model | Presence of functional nerve regenerates when fibrinogen, in high concentrations, plus factor XIII were used |
| 32        | PRP          | Peripheral Nerve (rat) | ↑ number of regenerating nerve fibres |
|   | PRP and Fibrin Sealant | Facial rat Nerve Regeneration | PRP with a suture established an increase in axon counts and neurotrophic effects |
|---|------------------------|-------------------------------|----------------------------------------------------------------------------------|
| 92 | Human PRP | Brain Reperfusion | Decreases brain injury after focal ischemia; Significantly reduces infarct volume |
| 100 | PRP | Nerve-grafted defects (rat) | ↑ nerve gap reconstruction with a 1-cm long nerve graft |
| 57 | PRP-PCL Scaffold | Angiogenesis in a CAM model | ↑ mesenchymal stem cell attachment and proliferation on scaffold; ↓ differentiation |
| 93 | PRP (human patients) | Oral mucosal wound healing | ↑ capillary regeneration in mucosal wound healing |
| 23 | PRP (human) | Cutaneous wound healing | ↑ rate of wound healing |
| 94 | Gelatin hydrogel & releasate | cutaneous wound healing murine | ↑ wound area epithelialisation rate |
| 95 | Preparation PRGF | Cutaneous ulcers | ↑ healed surface area in PRGF group |
| 53 | PRP-Exos (Rat) | Cutaneous wound healing, Endothelial & Fibroblast cell | ↑ proliferation and migration of endothelial cells and fibroblasts |
| 42 | 1-21 day-old Human platelets | Fibroblast | ↑ proliferation; retention of proliferative activity with old platelets |
| 56 | PAR1-PR & PAR4-PR | Endothelial progenitor cells | No effect on EPC proliferation; both ↑ cell migration; PAR1-PR ↑ vasculogenesis |
Table 3. Platelet-based applications on cell culture studies

| Reference | Cell type                        | Findings (Proliferation/ Differentiation)                                                                 | Platelet preparation method                                                                 | Sera (e.g. FBS%) in culture conditions                                                                 |
|-----------|----------------------------------|----------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|
| 74        | Rat bone marrow cells            | ↑ proliferation between days 0 and 4; ↑ differentiation between days 8 and 12                           | 3.8% SC @800 rpm for 15 min @25°C activated with thrombin (300 IU) & 10% CaCl₂.              | 10% FCS 10,000 cells/well (24-well) pre-coated with a PRP gel                                       |
| 97        | Rat bone marrow stromal cells (BMSCs) | Higher PRP concentration ↑ cell proliferation; ↑ newly formed bone with 100 x 10⁴ platelets μl⁻¹ & BMSCs in a collagen mixture @ 8 week | Whole blood @ 600xg for 10 mins, then 2,840xg for 15 minutes; activated with 2% CaCl₂ & thrombin. | α-MEM with 15% FBS; Cells were seeded on a 100-mm dish                                              |
| 11        | Human alveolar bone-derived periosteal cells | A-PFR and CGF extracts ↑ proliferation; PRP @ 2.5% showed the most proliferative properties with ↓ @ higher doses | PRP: Whole blood & ACD @ 1800g for 4 min then stored @ −80 °C; PRGF: whole blood & SC @ 580g for 8 min; A-PRF & CGF clots: whole blood without anticoagulants @ 198g and 692g respectively, frozen, minced, homogenised @ 3000 rpm for 10 min | Human periodontal tissues in 10% FBS for single cells; cells were seeded at 1 × 10⁴ in 6-well plates for 24 h in 1% FBS; PRP, PRGF, A-PRF extract or CGF extract and the cells were further incubated for 48 h |
| 86        | NIH-3T3 cells & CAL-72 cells | PMC ↑ proliferation of murine fibroblasts and human osteoblasts; ↑ NIH-3T3 angiogenesis              | Platelet mediator concentrate (PMC) using an ATR system kit: human whole blood & anti-coagulant & sedimentation accelerator and aggregator | NIH-3T3 culture: DMEM with 10% FCS; CAL-72 culture: 10% FCS over-night, then 2% FCS for 24 hours followed by 10% PMC or 10% FCS |
| 98        | Bone mesenchymal stem cells | Bone marrow stromal cells treated with platelet exosome concentrations ↑ proliferation and migration; ↑ bFGF, VEGF, PDGF-BB & TGF-β1 in platelet exosomes than in PL. | Whole blood & heparin @ 1000 rpm for 5 mins, then 1900 rpm for 15 mins; Human PL: PRP was frozen and thawed then @ 2600xg for 30 mins; & heparin for exosome isolation; PL @ 2000xg for 10 mins & heparin; Exosome isolation: PL @ 500xg for a series of spins then @ 30000 rpm for 1h with repeats. | αMEM & 10% FBS; 4 days of culture; adherent mesenchymal stem cells in αMEM & 10 % FBS. |
| 87        | Human trabecular bone cells | ↑ mitogenic activity of BC, independent of cell-to-cell contact.                                         | Whole blood & ACD spun to concentrate platelets (3x10⁹); then leuco-depleted by a pall filter; washed in Tyrode’s buffer, @ 1400g for 10min and resuspended in serum-free medium; | DMEM/F12 with 10% FCS |

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| Page | Description |
|------|-------------|
| 55   | Rabbit bone marrow mesenchymal stem cells (rBMSC) activated with thrombin; @ 1400xg; supernatant @ 100,000xg |
| 55   | Whole blood & ACD-A; L-PRP: 250xg for 10 mins then @ 1000xg for 10 mins, re-suspended PPP; P-PRP: 160xg for 10 mins then 250xg for 15 mins resuspended in PPP |
| 31   | Human bone marrow mesenchymal stem cells (rBMSC) P-PRP ↓ concentrations of leukocytes and pro-inflammatory cytokines, ↑ proliferation & chondrogenesis of rBMSCs when compared to L-PRP |
| 31   | Human whole blood & SC. Centrifuged at 2,000 rpm for 5 minutes. PRP Centrifuged again at 2,000 rpm for 20 minutes. |
| 26   | HUVECs | Whole blood & ACD-A; L-PRP: 250xg for 10 mins then @ 1000xg for 10 mins, re-suspended PPP; P-PRP: 160xg for 10 mins then 250xg for 15 mins resuspended in PPP |
| 26   | SaOS-2 (Sarcomatous osteogenic) SaOS-2 were cultured in RPMI-1640 medium 10% FCS |
| 27   | SaOS-2 & U937 cells PRP ↑ chemotaxis & cell proliferation; PDGF ↑ cell migration & TGF-β ↓ proliferation |
| 27   | Whole blood & Citrate phosphate dextrose @ 180xg for 20 mins, then 580xg for 15 mins; resuspended in PPP; Jellified with calcium gluconate/batroxobine; then @ 1400 x g for 10 mins |
| 38   | Ibpa55 & U937 cells PRP in chondrocytes ↓ activity of NF-kB & ↓ expression of COX-2 & CXCR4 target genes; ↑ HGF, IL4 & TNF-a; PRP in U937-monocytic cells ↓ chemokine transactivation & CXCR4-receptor expression |
| 38   | Whole-blood prepared using the platelet concentrate collector system GPS II; activated with thrombin and CaCl2·2H2O x 1, x 5 and x 25; @ 4000xg for 10 min |
| 56   | EPCs No benefit on proliferation; ↑ EPC migration; ↑ vasculogenesis |
| 56   | Whole blood @ 190xg for 20 min; then @ 900xg for 10 min & PGE; resuspended in Tyrode’s HEPES buffer @ 2 x 10⁹ mL⁻¹; PAR1-AP or PAR4-AP for 10 min; @ 15000xg for 10 min |
| 161  | MCF-7 breast cancer Platelet releasates activated with 1) ADP: ↑ migration & formation of macrophages |
| 161  | Human platelets @ 2x10⁸ mL⁻¹ activated by ADP, Thromboxane A₂, PAR4, PAR1-AP or PAR4-AP for 10 min; @ 15000xg for 10 min |
| 23   | HUVECs (1 x 10⁴ mL⁻¹) in serum free media on 0.5% gelatin pre-
| cells & HUVECs | capillary structures by HUVECs; 2) TXA2: inhibited migration & formation of capillary structures or exposure to MCF-7 cells @ 3 x 10⁶ mL⁻¹ coated/ transwell plate with 2 x 10⁶ mL⁻¹ platelets in the bottom chamber |
|-----------------|-----------------------------------------------------------------------------------|
| Human adipose-derived MSCs | MSC seeded on the PRP–PCL nanofibers showed an increased adhesion and proliferation compared to pristine PCL fibres. The buffy coat was centrifuged @ 400xg for 15 min; 3x10⁶ platelets μL⁻¹ activated by freeze–thaw cycles; @ 12000xg for 10min |
| Adipose tissue-derived stem cells | PRP accelerates chronic skin ulcer reepithelization; ↑ proliferation SC as an anticoagulant with a 1100xg for 10 min spin; Ca²⁺ for activation DMEM with 10% FBS @ 2500–5000 cell/cm². |
| Rabbit Myogenic progenitors & ASCs & Human fibroblasts | ASCs had an anabolic paracrine effect on proliferation of MPCs; PRP ↑ proliferation of MPCs; ASC-extracts ↑ proliferation more than PRP Rabbit whole blood & SC; @ 400xg for 10 mins; then PRP @ 800xg for 10 mins. Rabbit MPCs & ASCs cultured in EGM-2MV containing 5% FBS; Human fibroblasts in DMEM with 10% FBS |
| Rat gastrocnemius muscle cells | Releasate ↑ proliferation of skeletal muscle cells by transitioning cells from G1 phase to S phase and G2/M phases Whole blood & ACD @ 800xg for 30 mins then @ 3000xg for 20 mins; 10% thrombin with CaCl₂; Then @ 5500xg for 15 mins and filtered DMEM with 10% FBS & 5% chick embryo extract |
| Murine C2C12 myoblasts | PRP ↑ proliferation & inhibited both myogenic and adipogenic differentiation Whole blood & SC @ 2400 rpm for 10 mins; then @ 3600 rpm for 15 mins; activated by freeze–thawing then @ 10000 rpm for 10 mins DMEM with 10% FBS; for myogenesis, DMEM without FBS was used @ 5.0×10⁴ cells/well |
| Murine C2C12 myoblasts | PRP ↑ both myogenic proliferation & differentiation PRGF: human whole blood in SmartPREPW 2 centrifugation system; Then a freeze–thaw–freeze process to lyse platelets and release their granule contents High-glucose DMEM with 10% FBS for Proliferation; High-glucose DMEM with 2% horse serum for Differentiation |
| Murine C2C12 myoblasts | PL ↑ C2C12 proliferation and motility Platelet lysates; centrifuged, washed, repeatedly frozen and thawed & centrifuged to eliminate debris DMEM with 10% FBS |
| Human myoblasts | ↑ in proliferation; both PPP & MSTN and TGF-β1 depletion in PRP ↑ myoblast differentiation Pure PRP kit; 1. PPP 2. PRP 3. Mod-PRP with TGF-β1 and MSTN depletion 4. PRP second spin 550g 5 mins 5. 10,000 cells/cm²; 2% horse serum for differentiation; 10% FBS for proliferation |
| Abbreviations for tables 1, 2 & 3: %; percentage, °C; degree celcius, ↑; an increase, ↓; a decrease, A-PRF; advanced-platelet-rich fibrin, ACD; anticoagulant citrate dextrose solution, Akt; protein kinase B, ASC; adipose derived stem cell, BC; bone cell, bFGF; basic fibroblast growth factor, BMSCs; bone marrow stromal cells, C2C12; mouse myoblast cell line, C57bl6/J; C57 black 6 mouse, CAL-72; human osteoblast cell line, CAM model; chicken chorioallantoic membrane model, cbfa1; core binding factor alpha 1, CCI4; chemokine (C-C motif) ligand 4, cdk1,2; cyclin-dependent kinase 1,2, CGF; concentrated growth factors, COX-2; prostaglandin-endoperoxide synthase 2, CXCR4; C-X-C chemokine receptor type 4, DMEM; dulbecco's modified eagle's medium, DMEM/F-12; dulbecco's modified eagle medium: nutrient mixture F-12, EGF; Epidermal growth factor, EGM-2 SingleQuots complete medium; endothelial cell growth medium, EGM2-MV; endothelial cell growth medium, ELISA; |
enzyme-linked immunosorbent assay, **EPC**; endothelial progenitor cell, **ERK1/2**; extracellular signal–regulated kinases, **FBS**; foetal bovine serum, **FCS**; foetal calf serum, **HEPES**; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, **hFOB**; transformed human osteoblasts, **HGF**; hepatocyte growth factor, **hMDPCs**; human muscle-derived progenitor cells, **HMEC-1**; human microvascular endothelial cell line, **HOS**; human osteosarcoma cells, **HSMM**; human skeletal muscle myoblast, **HSP**; heat shock protein, **HUVECs**; human umbilical vein endothelial cells, **Ibpva55**; normal human articular chondrocytes, **IGF-1Eb**; insulin-like growth factor-1 isoform-Eb, **IL-4**; interleukin 4, **IL-6**; interleukin 6, **IU**; international unit, **L-PRP**; leukocyte- and platelet-rich plasma, **MCF-7**; breast cancer cell line, **mL**; millilitres, **mM**; millimolar, **MMP-13**; matrix metalloproteinase-13, **MMP-3**; matrix metalloproteinase-3, **Mod-PRP**; modified PRP, **MPCs**; muscle progenitor cells, **MRFs**; myogenic regulatory factors, **mRNA**; messenger ribonucleic acid, **MSTN**; myostatin, **Myf5**; myogenic factor 5, **MyoD1**; myogenic differentiation 1, **myomiRNAs**; myo-micro ribonucleic acids, **NF-κB**; nuclear factor kappa-light-chain-enhancer of activated B cells, **NIH-3T3**; mouse fibroblast cell line, **NPRS**; Nirschl Phase Rating Scale, **P-PRP**; pure platelet-rich plasma, **PAR1**; thrombin protease-activated receptor 1, **PAR1-PR**; PAR1-protein releasate, **PAR4**; thrombin protease-activated receptor 4, **PAR4-PR**; PAR4-protein releasate, **Pax7**; paired box protein 7, **PBS**; phosphate-buffered saline, **PCL**; poly(ε-caprolactone), **PCNA**; proliferating cell nuclear antigen, **PDGF**; platelet-derived growth factor, **PL**; platelet Lysate, **PMC**; platelet mediator concentrate, **PPP**; platelet-poor plasma, **PRGF**; plasma rich in growth factors, **PRGF**; Platelet Rich in Growth Factors, **PRP**; platelet-rich plasma, **PRP-Exos**; platelet-rich plasma-exosomes, **PRS**; platelet-released supernatant, **rMSCs**; rat muscle stem cells, **rpm**; rotations per minute, **RPMI-1640**; Roswell Park Memorial Institute 1640 medium, **SaOS-2**; Sarcoma osteogenic cell line, **SC**; Sodium Citrate, **SkBM-2**; skeletal muscle growth basal medium 2, **TGF-β**; transforming growth factor beta, **TGF-β1**; transforming growth factor-beta1, **TNF-α**; tumour necrosis factor-alpha, **TSP-1**; thrombospondin 1, **TXA2**; thromboxane A2, **U937 cells**; myeloid lineage cell line, **uL**; microliters, **VAS**; visual analogue scale, **VEGF**; vascular endothelial growth factor, **αMEM**; α-modification of minimum essential medium, **μg**; micrograms
Figure legends

Figure 1. Schematic of a platelet together with organelles, highlighting key surface receptors, aggregation factors and an overview of known α-granule releasate factors. These contain: adhesive proteins, clotting factors and their inhibitors, fibrinolytic factors and their inhibitors, proteases and antiproteases, growth and mitogenic factors, chemokines, cytokines, membrane glycoproteins and anti-microbial proteins. The platelet releasate may be further used as a biomaterial in numerous applications of regenerative medicine.

Figure 2. PubMed search for publications on a) “platelet-rich plasma”; b) “platelet-rich plasma” AND regeneration; and c) “platelet-rich plasma” AND regeneration AND muscle between 1954 and 2017. The diagram reveals that the publication of articles on platelet-rich plasma (PRP) have increased exponentially in the last two decades (white bars), while concomitantly scientific interest emerged for exploiting PRP in regenerative applications (red bars). Additional interest to use PRP for skeletal muscle regeneration has developed over the last decade (black bars).

Figure 3. A schematic diagram showing a skeletal muscle stem cell’s (i.e. satellite cell) possible response to growth factors, based on current published evidence. Located between the basal lamina and the sarcolemma of the muscle fibre, the satellite cell may come into contact with hundreds of growth factors and cytokines in response to platelet-rich plasma (PRP) administration. For simplicity we are presenting platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which are known to be contained in PRP. PDGF and VEGF interact with tyrosine kinase receptors and induce the Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway to drive cell proliferation through transcription factors such as Cyclin D1. The impact of PRP on muscle progenitor cell differentiation is currently debated and remains to be established.

Figure 4. A schematic diagram showing the different stages in preparation of platelet-based applications, highlighting possible steps for experimental variations based on published evidence (see Table 3). Variations may include; donor-specific variability, variability in centrifugations, use of different platelet-based applications (e.g. PRP or leucocyte-rich PRP or platelet releasate), platelet concentration, buffer of resuspension, platelet agonists used for activation and the storage conditions.
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