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Effects of Platelet-Rich Plasma and Platelet-Poor Plasma on Human Dermal Fibroblasts

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ABSTRACT

Platelet Rich Plasma is an autologous and safe blood product containing a high concentration of platelets and leucocytes, in a small volume of plasma. Platelets, growth factors, leucocytes and plasma are fundamental fibroblast proliferation agents. The leucocytes' plasticity, reparative qualities, their cross-talk between cells and capacity to orchestrate such diverse outcomes is receiving a considerable commendation. This succession enables fibroblast cells to migrate and proliferate into the wound's surrounding tissue and subsequently deposit granulation tissue to facilitate minimal scarring and also for anti-ageing benefits. The focus on leucocytes in tissue repair has enthused a new approach to tissue regeneration to form a new therapeutic modality, Immuno-Regenerative Medicine.

Keywords:
Leucocyte-Rich-Platelet-Rich Plasma
Platelet-Rich Plasma
PRP
Platelet-Poor-Plasma
Platelet Gel
Platelet Rich Fibrin
Fibroblasts
Regenerative medicine

1. Introduction

The regenerative medicine armamentarium is classified as platelet-rich plasma (PRP), platelet gel (PG), fibrin glue, bone marrow concentrate, stromal vascular fraction, adipose tissue, growth factor (GF) serum and autologous conditioned serum [1]. PRP is a fundamental cellular therapy commonly used by medical specialists due to its' ease of access, minimal manipulation and its' markedly tissue regenerating properties. PRP therapy has progressed substantially in clinical practice, following the rising incidence of connective tissue inflammation and injury, which is vastly becoming a growing cause of chronic pain and disability [2]. A new approach to assist tissue regeneration is to restore locally compromised homeostasis progressively and to modify the biological microenvironment. This can be achieved through administering PRP by percutaneous injection or topical application to the impaired tissue, such as skin, ligaments, tendons, osseous tissue or cartilage. If an impaired tissue is unattended to, faulty healing can cause excessive, abnormal or inadequate scar formation, chronic infection and nerve damage that may predispose to secondary ailments associated with chronic disability [3].

PRP is an autologous and safe blood product containing a high concentration of platelets and leucocytes, in a small volume of plasma. PRP is obtained by density gradient centrifugation of whole blood which separates the plasma, leucocytes and platelets from the red blood cells to form a buffy coat and plasma layer. The buffy coat is suspended in a small amount of plasma to form the final PRP product. Platelet-poor Plasma (PPP) is the residual plasma once the PRP is extracted, which contains beneficial proteins, insulin-GF (IGF) and a low number of platelets [4]. PRP, GFs and leucocytes are fundamental fibroblast proliferation agents; however, their combined impact on challenged fibroblasts has been mostly overlooked, and current publishings are too few to merit a comprehensive review [5]. Therefore, this review will chronologically evaluate the early development and outcomes of PRP research within a variety of medical specialities, and progress to the current research, addressing the methodologies, biological and clinical outcomes of a variety of connective tissue cells, and concluding with recommendations for the future research.

2. Methodology

Searchers were conducted using PUBMED, The Cochrane Library, and MEDLINE using the following search terms: platelet-rich-plasma, leucocyte-rich-platelet rich plasma, leucocyte-poor-platelet-rich-plasma, platelets, plasma, fibrin, growth factors, leucocytes, mononuclear cells, monocytes, macrophages, neutrophils, lymphocytes, fibroblasts, myofibroblasts, regenerative medicine. In addition, the following terms were searched in the context of regenerative medicine: tissue regeneration, stem cell, collagen biostimulation, wound healing, burns, burns treatment, scar, scar revision, wound healing, tissue repair, keloid, hypertrophic, incision wound. English and American English spellings of leucocytes and its derivatives were included. Studies from 1970 to current time were included. Reference lists of reviewed articles were also assessed for other relevant articles. Inclusion criteria were peer-reviewed papers, therapeutic use of PRP, clinical trials and case studies. Non-English articles which were not able to be translated were excluded. Muscular-skeletal studies on tendon were excluded. Title and abstract analysis were performed to identify appropriate studies, and full texts of included studies were assessed. In total, 38 studies were found on leucocyte-

rich-platelet-rich-plasma covering a range of topics including: muscular skeletal medicine, inflammatory markers, healing potential, tissue inhibition, tissue regeneration. Studies relating to leucocyte-rich-platelet-rich-plasma or platelet-rich -plasma and their effects on fibroblasts were included however due to the paucity of data studies from related fields were included.

3. Platelet-Rich Plasma

3.1. PRP Nomenclature

Initially, researchers had an equivocal knowledge of PRP and its' influence on tissue regeneration. For instance, platelets were initially identified as a clotting and haemostatic agent. However, they are now known for a myriad of diverse functions, cytokine signalling, chemokine and GF release, human mesenchymal stem cells (hMSC) mitogenesis and thus contributing significantly to tissue renewal. The PRP studies over the past decade originated as case studies that focused on clinical outcomes and gradually progressed to randomised control trials that also distinguished between the biochemical and biological mechanisms underlying these outcomes [6].

Many different terms have been applied to characterise PRP over the last 10 years, including platelet (plt) gel (PG), PLT concentrate, PLT therapy and PLT releasate, PLT rich fibrin (PRF), Leucocyte – PRP (L-PRP), Leucocyte-Rich (LR)-PRP and Leucocyte-Poor (LP)-PRP and platelet-rich growth factors (PRGF) [7]. Terminology standardisation is yet to be formalised for PRP, and as a consequence, the literature lacks uniformity in dilutions and volume ratios, registers indistinguishable PRP concentrations, which has all substantially affected the interpretations of outcomes. Moreover, Systematic Reviews too have injudiciously presumed the broad view approach in categorising all PRPs as analogous by excluding their apparent differences. These obscurities have confused the literature because such a lack of standardisation permits the term "PRP" to be promoted equivocally, irrespective of the concentrations or the quality of the product [8, 9].

3.2. Platelet concentration

The preliminary literature brought PRP therapy to the forefront of regenerative medicine in endeavouring to establish customised protocols for the medical specialities. These studies initiated discussions on the ambiguity of platelet characteristics, which lead to the hypothesis that various PRP concentrations influence the proportion of tissue regeneration [10, 11]. This hypothesis was further evaluated via testing increased PRP concentrations and their corresponding dose-response curve in a variety of studies. An early study [12] assessed the effects of PRP (338% greater than the whole blood baseline count), plus platelet-derived-GF (PDGF) and transforming-GF (TGF) on soft tissue healing and the regeneration of mandibular bone defects of 88 patients. The results demonstrated that the maturation rate was 2.16 times higher after the PRP treatment compared to the control; and achieved significantly faster radiographic maturation and denser bone regeneration. A further study evaluated the effects of various PRP concentrations on hMSCs mitogenesis and their corresponding dose-response curve [13]. The study reported that PRP concentrations of up to 5-10 fold higher than whole blood generated 848% and 720% more hMSCs on day seven than the growth medium, whereas the hMSCs exposed to 1.25 - 2.5 fold PRP responded with lower increases of 325% and 356%. Similar

observations occurred for human stromal stem cells as the PRP dose increased, plus a marked increase in the proliferation and differentiation of the osteoblastic lineage occurred for up to nine days [14]. These findings are consistent with current researchers that found PRP concentrations from 1.5 - 3 x 10^6 plt/µl exhibited optimal cell proliferation with no inhibitory effects observed [15-19]

3.3. Platelet degranulation

The platelet dose-responses and their effects on cell proliferation had developed further enquiries into the underlying biological mechanisms, thus, the effects of platelet-released GFs [20]. For instance, a study investigating the signalling pathways of PRP demonstrated that PDGF-AB, FGF, and TGFβ activated AKT which subsequently stimulates the AKT/Smad2 pathway and thenceforth induces adipose stem cell proliferation via increasing the cyclin D1 [21]. These findings generated a particular focus on the TGF family (TGF-β1, TGF-β2 and TGF-β3) and SMAD for their contribution to reduced dermal scarring. Other aspects that induce scarless healing include the downstream mediation of a multitude of GFs, such as the angiogenic vascular-endothelial GF (VEGF). VEGF restores angiogenesis and subsequently activates endothelial cell receptors and the release of matrix metalloproteinases (MMPs). This succession enables fibroblast and endothelial cells to migrate and proliferate into the wound's surrounding tissue and subsequently deposit granulation tissue to facilitate optimal wound healing and minimal scarring [22].

PRP processing employs a multiple of *ex vivo* platelet activation methods (incubation, thrombin or calcium) and centrifugation specifications. These processes may impact the efficacy of platelet activation and incidentally damage the platelets, initiating their premature degranulation and limiting their capacity to release GFs. Consequently, this may compromise sufficient GF dosing and effects the physiochemical events in the treated tissue and hence, minimises wound healing [23, 24]. Platelet activation, *in vivo*, occurs at the time of percutaneous injection which instigates an interaction with the glycoprotein, von Willebrand factor (vWF). Sequentially, vWF structures a connection from the exposed endothelium collagen to the platelet glycoprotein Ib-IX-V receptor complex, and thenceforth interacts with adenosine diphosphate (ADP) and thrombin, which subsequently activates the degranulation of the alpha and dense granules. The degranulation of alpha granules results in downstream GF signalling [3]. The GFs interconnect with the tyrosine kinase receptor to induce signal transduction, adenosine triphosphate (ATP) synthesis, chemotaxis for hMSC, effectors and macrophages, an increase in further GFs and fibroblast proliferation. Upon activation, the dense granules release catecholamines, such as, ADP, ATP, calcium, dopamine, noradrenaline, histamine and serotonin, which regulate haemostasis, thrombosis and pain relief. The second stage during activation is the clotting process in which fibrinogen forms into fibrin to stabilise the tissue and inhibit excessive bleeding. Hence, the wound healing cascade, the immune response, angiogenesis, stem cell activation, nerve repair and subsequently tissue regeneration transpire [25].

Some researchers have presumed the *ex vivo* pre-activated PRP (using calcium or thrombin) is more effective than *in vivo* PRP activation (contact with endothelial collagen) for the optimal release of GFs. To investigate these activation processes in detail, the GF releases of three platelet activation methods (thrombin, no activation (control) and endothelial collagen) were assessed over seven days. The degranulation process expressed a direct proportionality between platelet numbers and GFs; plus,

the endothelial collagen activation method gradually released GFs in a time-dependent manner for up to five days; and this method proved superior to the alternative methods, showing an 80% greater increase for TGF-β. [26]. Further still, a quantitative study compared the GF release of activated PRP (1.6×10^6 plt/μl and 31.1×10^3 leucocytes/μl) to the GF release of whole blood from the same sample. At first, the study confirmed platelet activation had not occurred during processing via a P-selectin test; therefore the GFs remained within the platelet granule, thus producing the maximum possible GF dose, *in situ.* The outcome determined that the GF levels were significantly higher in the PRP supernatant compared to the whole blood; demonstrating VEGF increased from 155 to 995 pg/ml; PDGF-BB from 3.3 to 17 ng/ml; epidermal GF from 129 to 470 pg/ml; TGF-β1 from 35 to 120 pg/ml but no increase for IGF-1 [27].

3.4 Correlation of plasma volume in PRP

The research in platelet concentration efficacy has overlooked the value of plasma and neglected to appreciate that a sufficient plasma volume will maintain the nutrients for both the platelets and GFs [28]. Plasma is predominantly water containing serum albumins, globulins, fibrinogen, hormones, clotting factors, electrolytes, glucose, gases and IGF-1. IGF-1 is principally transported throughout the blood, and are therefore in higher amounts in the plasma compared to the platelet granules. It is hypothesised that the plasma's contribution to tissue regeneration is likely due to the IGF-1, an endocrine hormone and the mediator of the effects of growth hormone, plus stimulates proliferation of the TGF-β1-activated myofibroblasts and thus influences scarless wound healing [29]. In a study undertaking the comparison of plasma, whole blood and PRP, it was found the plasma (3.1×10^5 plt/μl) stimulated procollagen type 1 carboxy-terminal peptide, protein production and higher MMP -1, -3 expressions, although the PRP (1.7×10^6 plt/μl) was significantly higher [15]. Plasma's regenerative effects are also noted in further research [13, 16], although less regenerative than PRP. To thoroughly represent the mechanisms of regenerative medicine all components of PRP need equal representation; and, for the platelet dose to be directly linked to the plasma volume and that neither should be interpreted alone [30].

4. Leucocytes: LR-PRP and LP-PRP

Leucocytes are granulocytes and mononuclear cells (monocytes, T and B lymphocytes), which are heterogeneous effectors and versatile progenitor cells that emigrate from the blood into the tissue via diapedesis. The leucocytes' plasticity, reparative qualities, their cross-talk between cells and capacity to orchestrate such diverse outcomes has enthused a new approach to tissue regeneration to form a new therapeutic modality, Immuno-Regenerative Medicine [31].

Neutrophils are the first immune cell recruited to injured sites for the phagocytosis of invading pathogens. After the pathogens are destroyed the neutrophil will undergo efferocytosis. Successively, the monocytes are recruited to mediate inflammation, and as they migrate into the tissue they differentiate into dendritic cells; including the pro-inflammatory M1 macrophage which facilitates phagocytosis and stimulates angiogenesis; and, the anti-inflammatory, reparative, macrophage 2 which enhances fibroblast proliferation, extracellular matrix assembly, peripheral nerve and connective

tissue repair [32]. The innate and adaptive immune systems' cross-talk is synchronised via monocytes, thus engaging the natural killer cells, and the B- and T-lymphocytes. Natural killer cells secrete cytokines and predominantly direct, control and kill viruses and cancer cells. The B-lymphocytes facilitates the production of antibodies which binds to and destroys antigens. Macrophages and dendritic cells present these antigens to T-lymphocytes to be terminated and thus generate pro- and anti-inflammatory cytokines. These leucocyte interactions constitute the inflammatory response and are pivotal to minimising tissue damage and establishing homeostasis [31].

Researchers have questioned the use of highly concentrated leucocytes within PRP and profess they may contribute to a higher incidence of adverse events and post-treatment pain side effects. It is hypothesised that these side effects are caused by the catabolic potential induced by the interleukin-1β (IL-1β) and the tumour necrosis factor-α, and their subsequent stimulatory effects on the nuclear factor κB signalling pathway. Furthermore, it is theorised that high concentrations of leucocytes in PRP may intensify MMP expressions and therefore cause excessive degradation of the extracellular matrix tissue and thus inhibit healing [5, 33, 34]. Conversely, though, tissue regeneration is equally reliant on the interactions and cross-talk between surrounding cells and leucocytes. For example, peripheral blood monocytes propagate pluripotent stem cells which display the markers of CD14, CD34, and CD45. Subsequently, the proliferation of these pluripotent stem cells is induced by the macrophage colony-stimulating factor [35]. Furthermore, the peripheral blood CD14 + monocyte differentiates into a fibrocyte, the non-active fibroblast, which successively produces cytokines, chemokines, and GFs for the promotion of tissue healing [36]; plus, the CD14 monocytes transdifferentiate into anti-fibrotic keratinocyte-like cells and subsequently regulates the inflammatory MMP-1 expression in dermal fibroblasts [37].

Hence, leucocytes and especially mononuclear cells embody the mechanisms that stimulate fibroblasts and thus have a direct relationship with tissue remodelling, and this, therefore, challenges the viewpoint that leucocytes are principally catabolic. To date, there are few publications focused on leucocytes and PRP studies and for those that exist have generally omitted the data distinctions between neutrophils, monocytes and lymphocytes. Following is a brief review of LR-PRP studies which are summarised in Table 1.

4.1. Leucocytes impact on tissue

Leucocytes within PRP were evaluated for their destructive tissue effects, and fibrosis induction and accordingly would, therefore, render the tissue's mechanical properties weaker and less resilient to force. LR-PRP and leucocyte-poor (LP-PRP) were employed to test for tissue resilience in a specially designed plexiglass dog-bone-shape mould, which was fabricated to make the fibrin specimens, identical in size, volume and figure. As a result, the LR-PRP for all incidences produced a higher significant difference in tensile strength, toughness and stiffness with stronger mechanical properties compared to the LP-PRP [38]. A study that focused on the synergy between platelets and leucocytes revealed that the platelet has a direct relationship with balancing inflammation, for they express the chemokine receptors that regulate inflammation, thus, CCR1, CCR3, CCR4 and CXCR4 and TGF-β [39].

4.2. Mononuclear cells and Fibroblast

Thus far, platelets, GFs and plasma combined with leucocytes has been the focus of PRP research. However, in the following study, the platelets and plasma have been eliminated to determine the synergistic healing potential of mononuclear cells and cultured fibroblasts combined. This experiment applied single and multi-layered mixed sheets containing autologous fibroblasts and peripheral blood mononuclear cells on the cutaneous ulcers of mice. On day 14, it was reported that the sheets released significantly higher VEGF, hepatocyte GF, TGF and C-X-C motif chemokine ligand -1 and -2, in comparison to the single layer sheet or the fibroblast sheet alone. These results were re-evaluated in an *in vitro* experiment, which too demonstrated angiogenic potency and fibroblast migration proved higher with the mononuclear cells and fibroblasts multi-layered mixed sheets [40].

4.3. LP-PRP and whole blood impact on fibroblast

PRP processing specifications vary amongst manufacturers, and thus the PRP can be formulated to produce low or high concentrations of leukocytes, platelets and plasma. The lack of PRP standardisation allows manufacturers to promote their PRP formulation as superior in its' regenerative effects and in particular due to the exclusion of leucocytes without providing sufficient evidence. This notion was negated in a study that compared LP-PRP (427 x 10^3 plt/μl; 2.61 x 10^3 leucocyte/μl) to LR-whole blood (257 x 10^3 plt/μl; 6.96 x 10^3 leucocytes/μl). Fibroblast proliferation transpired for both samples, however, with no significant difference from day four to eight. Additionally, post the activation of each sample, the PDGF-AB/BB, VEGF, IGF-1 and TGF-β1 levels increased in correlation to the fibroblast proliferation increase; and furthermore, the GF release was within a time-dependent manner from eight to ten days, indicating an ongoing GF release *in vivo* [41].

4.4. LR-PRP, LP-PRP, MMP, fibroblast proliferation and GFs

Leukocytes, cytokines, and chemokines influence MMPs, which are partially responsible for the regeneration of the extracellular matrix, however, in excess MMPs are potentially catabolic to tissue [42, 43]. The following two studies investigated the effects of MMPs within their PRP experiments. The first study examined fibroblast proliferation, MMP expressions and GF release after the treatment of LR- or LP-PRP within the dose ranges of 0.3 - 3 x 10^6 plt/μl [44]. The outcomes demonstrated fibroblast proliferation increased 2.5-fold for both samples compared to the untreated cells; however, no significant differences occurred between the PRP groups. In the wound healing assay, fibroblast migration was most significant from PRP of 0.3 - 1.5 x 10^6 plt/μl; the most significant effect on motility was achieved by PRP of 1.5 x 10^6 plt/μl compared to the LP-PRP; however, fibroblast motility inhibition was observed at 2.5 x 10^6 plt/μl, compared to the untreated cells. The MMP-2 and -9 were expressed from both the LR-PRP and LP-PRP, with no significant difference. GF release displayed no significant differences between the samples, except for VEGF which exhibited a significant decrease from the LP-PRP. IFN-γ and PDGF-B expression in LP-PRP showed a substantial increase vs the LR-PRP, whereas TNF-α and fibroblast–GF (FGF)7 levels were not detected. A second study undertaking the expressions of MMP -2, -3, and -9 content in LP-PRP vs LR-PRP demonstrated

that each sample mutually released all active MMPs for up to six days post PRP treatment. In the second stage of this study and to further investigate the potentially damaging effects of leucocytes, the fibroblasts were challenged with IL-1β and successively treated with LP-PRP and LR-PRP. The outcome reported that the MMP-2 expressions increased while MMP-3 expressions decreased. However, the study could not establish whether the leucocytes were the catalysts and therefore the result was inconclusive [42].

4.5. Defining LR- PRP

LR-PRP**,** thus far, has an ambiguous definition in the literature and the leucocyte components are not characterised as distinct cells with unique and interconnecting functions. However, in a recent systematic review, to assign preparations unambiguously, LR-PRP was defined as PRP with a white blood cell (WBC) concentration of more than 100% that of the whole blood. The LP-PRP was defined as PRP with a WBC concentration less than 100% than that of whole blood. Within the 34 muscular skeletal studies included in this review the majority did not record their cell characteristics, and subsequently, the researchers obtained this data from the manufacturers. The investigation established that the patient functional outcome scores are only partially affected by leucocytes; and that the local inflammatory reactions to PRP injections are unrelated to the presence of leucocytes, but rather due to the patients already existence of osteoarthritis pain [5].

Collectively, these studies demonstrate that the exclusion of leucocytes does not achieve greater efficacy for tissue repair and that their inclusion does not cause adverse events or tissue destruction. From the evidence in these studies, it is highly plausible to propose that leucocytes in PRP have a cumulative on-going anabolic and ergogenic effect; and corollary generates numerous interactions, chemotaxis and migration, enhances cell proliferation, stimulates the differentiation and expansion of fibroblasts and thus contribute equivalently to new tissue growth.

5. Fibroblasts in tissue healing

The fibroblast's multifaceted capabilities, adaptabilities and proliferative effects, establish fibroblasts as pivotal in skin tissue repair. Fibroblasts are resident hMSCs and the principle progenitor cells in mammals that facilitates the maintenance and regeneration of the body's architectural framework. In the event of injury, fibroblasts will proliferate and subsequently differentiate into myofibroblasts and successively migrate into wounds to secrete type I and III collagen to facilitate healing [45]. In wound repair, the skin tissue's structure and morphology rely on fibroblasts to interact with collagen fibrils and to continue accumulating collagen until the extracellular matrix and surrounding tissue reach equilibrium. To avoid abnormal scarring optimal fibroblast proliferation is necessary for the stimulation and production of gene expressions, glycosaminoglycan and collagen synthesis into the wound [15]. However, in impaired tissue these fibrils fragment, thus inhibiting the fibroblasts' size, dispersal and interactions; plus TGF-β signalling and wound contraction are hindered, all of which increase the probability of abnormal scarring. AKT phosphorylation activates fibroblast cell migration, proliferation and matrix synthesis and this action is induced when fibroblasts are exposed to platelets [46]. In the healing cascade, platelets influence fibroblast proliferation via the

degranulation of PDGF–BB, VEGF and TGF-β1, and -β2. Fibroblasts too secrete FGFs for extracellular matrix regulation, hepatocyte-GFs for wound repair, VEGFs for angiogenic stimulus and proteases MMP-9, -13; and can differentiate into an endothelial cell-like phenotype. Fibroblasts will recruit, modulate and regulate the behaviour of immune cells and via the expression of chemokines, their duration in the injured tissue will determine the outcome of an acute or chronic wound [47]. Collectively, these interactions mediate standard tissue repair and demonstrate the emphasis for optimal fibroblast function to avoid impaired healing and abnormal scarring. The crosstalk between platelets, leucocytes and fibroblasts is a complex cascade that orchestrates immune responses, inflammation, anti-inflammation, proapoptotic, antiapoptotic, debridement and regeneration which is outlined in Figure 1.

5.1. Impact of PRP on Fibroblasts

The diverse experimental designs in the following fibroblast studies have made it difficult for interpretation for the reader. For instance, the final PRP platelet concentrations range from 0.3-5 x 10^6 plt/μl with inconsistent leucocyte data and plasma volumes, which are then diluted with media, altering the original strength and volume. The new PRP formula is reported in percentages rendering the final dose as unknown and therefore non-translatable for the clinical setting, as displayed in Table 2. These studies indicated that the higher PRP percentages resulted in a decrease in fibroblast proliferation and collagen production, and proposed that this be due to the high concentrations of TGF-β1 (81-fold) [15, 48, 49]. However, the TGF-β1 was solely measured and omitted the TGF-β2 and -β3 that are the principal mediators for scarless tissue healing. Fibroblast inhibition is likely due to the experiments' limited evaluation time of 24 to 72-h, and therefore not allowing sufficient time for cell proliferation. Longer PRP evaluation periods may be required as fibroblast cell inhibition occurred between 0 to 24-h, and on day seven fibroblast proliferation achieved statistical significance [16]. Another study realised no significance occurred in fibroblast proliferation from 24 to 72-h after the co-culture of PRP, and at this time the higher concentrations exhibited cell inhibition [50]. To the contrary, another study displayed that high concentrations (0.3-5 x 10^6 plt/μl) did not cause cell inhibition for up to 72-h and achieved the greatest proliferation up to 2 x 10^6 plt/μl. Furthermore, the greater fibroblast motility and migration, and MMP-2, -9 expressions exhibited at 1.5 x 10^6 plt/μl; plus, collagen production increased 15 times more than the lower concentrations; and, the GF release exhibited 107 times greater than the normal serum [17]. The controversy that high platelet concentrations cause adverse events has no evidence to support this claim and is more likely to be representative of the manufacturer's commercial bias than a reflection of clinical results.

5.2. Unchallenged vs Challenged Fibroblasts

In dermatology practice, PRP therapy is administered by percutaneous injection or topical application to treat burns, photoaged skin, acne, acne scars, keloid and hypertrophic scarring, incision wound, alopecia and ulcers [51, 52]. To represent the clinical setting, the measurements of survival and proliferation rates of senescent fibroblasts cells are essential; however, few studies evaluate PRP's

effects on impaired fibroblasts [53]. The following studies have induced cell senescence to provide the much-needed tissue-engineered solutions for the damaged skin.

5.2.1. UVA Challenged Fibroblasts

Chronic UVA irradiation exposure on skin cells shortens telomeres, inhibits the TGF-β1 receptor gene expression, increases MMP-1 gene expression, plus suppresses collagen synthesis and fibroblast proliferation. Photo-ageing skin is presumably irreversible, however, from the influence of leucocytes and GFs partial correction for this skin condition may be possible. The effects of various PRP doses on UVA-irradiated senescent fibroblasts determined 25% and 50% PRP both significantly increase fibroblast proliferation, collagen deposition and migration rates. Although the challenged fibroblasts proliferation rates are less than the unchallenged fibroblasts, survival and proliferation are nonetheless partially restored. The authors proposed that the fibroblasts restoration was due to the MMP-1 tissue inhibitors released from the PRP and TGF-β1 signalling ameliorated suppressed proliferation [54].

5.2.2. PRP and fibroblasts in chronic ulcers

Chronic ulcers present with depressed collagen deposition, suppressed fibroblast proliferation and migration and may necessitate the amputation of the affected appendage. In the following study, Fibroblasts were serum-starved for 24-h to mimic the challenged fibroblasts similar to that found in chronic ulcers. Thereafter, the PRP was diluted with media to concentrations of 100%, 50%, and 25% of PRP and subsequently applied to the challenged fibroblasts in which all samples ameliorated suppressed proliferation. The highest migration capacity was restored via the 50% PRP, and the 100% PRP (no additional medium) restored collagen deposition in challenged fibroblasts [55]. These results propose PRP as an adjunct therapy for chronic ulcers and that higher platelet concentrations proved the most efficacious. Previous studies have reported that higher PRP concentrations inhibit cell proliferation; however, this study' results oppose this notion. For the fair interpretation of results, it is essential to assign particular attention to the methods details which can potentially influence the experiment’s outcome.

6. PRP and clinical applications: dermal regeneration

The skin is the largest organ of the body and visibly reveals the effects of extrinsic and intrinsic ageing. Medical specialists underestimate the dermal tissue's significance because skin regeneration is often considered cosmetic and not critical for the on-going health of the patient. The dermis is the connecting link between the epidermis and hypodermis and contains nerve endings, sweat and oil glands, immune and regenerative cells, and protects the underlying structures. The dermis is unable to regenerate in the same way as the bone, the epidermis or liver and if impaired so too is the body’s temperature control, immune response, and the protective barrier which can lead to dermatitis, eczema, psoriasis, allergies and possible skin cancers. In recent times, PRP is injected into the dermis to order to restore the skin’s function and health; however, results vary due to the inestimable variables in tissue surfaces and fluctuating PRP formulas [9, 56].

6.1. Burns - PRP Treatments

Fresh skin burns and their subsequent scars comprise of senescent fibroblasts and on-going tissue impairment. Reducing the burns' impact at the time of the incident minimises long-term medical care, lessens keloid scar risk, tissue pain and photosensitivity. The theoretical benefits of PRP for chronic ulcers suggests this healing benefit could be transferred to burn wound therapy; however, PRP treatments for burns' patients have not been as successful and still inconclusive. A PRP review as a treatment for burns found that fibrin sealant, a by-product of PRP, was mostly applied to treat split skin grafts, however, due to the lack of sufficient studies no conclusive evidence for its' use was found [57]. Furthermore, in an *in-vivo* randomised, double-blind study enlisting 52 burns patients already undertaking skin grafts to trial LR-PRP topical therapy, combined with the graft or without, resulted inconclusively. From seven days to twelve months, a DermoSpectoMeter acquired the measurements of the epithelialisation and graft uptake, which displayed no significant statistical difference for all time intervals and no improved or superior scar formation between the test areas. Several variables presented by the participants, such as vastness in age, the total surface burn areas and wound sepsis, all impact the wound healing capacity.

Furthermore, systemic changes transpire within the patients' as a consequence of the burn. Such as, the platelets are in a state of heightened activity and hyper-coagulated for up to one-week post burns, thus compromised [58]. Patients' too may require immediate general anaesthetic, which pre-activates the platelets *in vivo*. It is therefore advised to delay PRP treatment for burns patients until their platelets have returned to their normal state. More so, it would be expected a deep wound, such as a burn, would require multiple PRP treatments to address each wound healing phase; the phagocytosis and debridement of infected and necrotic tissue, angiogenesis, granulation and re-epithelisation. Also, the burn wounds' exudate will inhibit PRP absorption, and in the event of an imbalanced exudate wound breakdown occurs; all of which inhibits debridement and epithelial migration. For this reason, the PRP percutaneous injection into the burn's underlying healthy tissue, which contains less damaged and functioning fibroblasts would have a higher proliferation potential and migrate more proficiently to the burn wound. A comparison study of PRP injection vs topical application for the effective treatment of burn wounds would evaluate this hypothesis [59].

6.2. PRP and Collagen Regeneration

In aesthetic medicine, PRP is applied as an adjunct therapy in replace of or in conjunction with dermal fillers to stimulate collagen regeneration and volume augmentation. In a prospective cohort pilot study, combined PRP and cultured fibroblasts were injected into nasolabial rhytids to evaluate the collagen rejuvenation effects. Subsequently, at nine months post-treatment, there was still notable softening of the nasolabial fold and the surrounding skin. Dermal fillers augment collagen defects and remain in the tissue from three to twelve months, however due to their foreign body status and gel substance, they can present with adverse events, such as granulomas, artery occlusion causing necrosis, and uneven volume affecting a distorted appearance. Considering that PRP is autologous, anti-microbial and a bio-stimulant these complications are unlikely to occur and hence effecting PRP as an alternative and safer treatment [60].

6.3. PRP and Post-Surgical Tissue Defects

In a dental study of 102-patients undergoing surgery to remove an odontogenic mandibular cyst, thus an aggressive and destructive wound locally, the patients received one LR-PRP treatment (1.7×10^6 plt/μl; 39×10^9 leucocytes/L) to augment the excision tissue defect. Results demonstrated notably decreased bleeding throughout the surgery, advanced healing of the oral mucosa, less visible erythema within the suture margins and no inflammation at the surgical site 14 days post-surgery. In stage two of this study, 18 patients undergoing surgery for a double mandibular fracture were divided into two groups: half the group received no fluids while the other half received LR-PRP. There was minimal difference between the groups; however, the LR-PRP group healed more efficiently [61].

6.4. Combination Therapy: PRP and Cells

PRP research is progressing beyond platelets and GFs to supplement with tissue cell combinations. A combination of human keratinocytes and fibroblasts and PRP was applied to full thickness wounds in mice. The results displayed greater re-epithelialization at day 7 and 14; some wounds achieved complete closure, whereas the non-treatment group (saline) expressed no epidermal growth in the wound centre. TGF-β1 expression was higher in the PRP + fibroblast + keratinocyte group, and PDGF-BB and VEGF expressed higher in the non-treatment group compared to the treatment groups [62].

7. Research Limitations

Throughout the studies, the PRP point-of-care devices vary from conventional laboratory tubes to highly advanced automated cell separating apparatuses, and changeable centrifugation parameters. Step-by-step protocols for PRP processing are often not included or mostly non-reproducible for clinical practice. The preparation methods activated PRP to produce GF concentrate gel as a supernatant to the medium, without considering that each preparation has a different initial platelet concentration. Although plasma volumes within PRP formulae carry equal importance to the platelet concentration, it is often data omitted. In clinical practice, such variances prevent precise dosing and may well affect the PRP quality, and thus, reducing the regenerative benefits.

Collectively, these studies validated that PRP induces fibroblast migration and proliferation and this process is dose-dependent. The ambiguous dilutions, methods and conflicting results throughout the studies obscure interpretation for the clinician. Throughout the studies, concentrations vary from 250%, 20%, 10% to 5% and hence claim to be the most proficient, but these data are not of equal value. The original platelet count and plasma volumes vary between experiments; and, the ratio percentage of platelet secretome and plasma volumes or dilutions are not recorded. Therefore, it cannot be determined if disproportions affected outcomes, affecting it impossible to make a valid comparison between studies.

Furthermore, the PRP studies are mostly compared through their platelet concentration and thus, overlooking the platelet yield concentration in the plasma volume; thus, the mean platelet volume. Whole blood volumes and cell count differ for each study and subsequently so will the buffy coat cell count, and plasma volumes; plus, the platelet mass is only contained in the lower third of the plasma, and therefore the formulas will differ [28]. Studies employed short evaluation periods which proved insufficient for significant fibroblast proliferation compared to the experiments that evaluated for up to 14 days and did observe substantial proliferation [63]. These obscurities make it challenging to perform comparisons or to draw conclusions and potentially allows for misinterpretation and bias in studies. Due to these inconsistencies, the evidence is not yet established to support that the higher concentrations of platelets or leucocytes have an inhibitory effect on fibroblasts.

8. Conclusion

There is a paucity of data of leucocytes in PRP and for those that exist have mostly presented these immune cells as catabolic, or neglected to classify them, and have not yet fully appreciated their critical influence in tissue regeneration. In summary, the studies have presented with varying and undefined methodologies, complex processing, uncertain cellular parameters, insufficient data, obscure valid endpoints, lacked a translatable relationship between platelet concentrations and platelet gel supernatants coupled with inconsistent results. Consequently, this allowed for unfair comparisons, the misinterpretation of results and provided for bias. Researchers will better serve the medical community scientifically by adhering to reproducible methodologies and the reporting of accurate clinical data. Nonetheless, the studies demonstrated clear evidence that PRP does affect fibroblast proliferation and migration and that this varies according to the dose. The effects of leucocytes combined with PRP are yet to be established; however, it is evident that the leucocyte is not tissue destructive.

9. Future Directions

The recent research generation has predominantly focused on platelet concentration, activation methods, GFs and the controversy of leucocytes. However, PRP classification systems are poorly characterised in data reporting. To assess clinical efficacy and to distinguish between formulation variances, it is essential to employ accurately calculated PRP characteristics and to report more comprehensive data. Nonetheless, as the studies progressed to understand the pathways of these biological factors, so too reflected a more extensive knowledge of molecular signalling and inter/intracellular communications, hence providing the next generation with a stronger scientific foundation. Moreover, an increased focus on the combination of leucocytes, PRP and plasma combined tested on challenged fibroblasts would better evaluate their synergistic regenerative potential. Collectively, these data will provide the physiological changes that occur in the challenged cell and how these tissue healing mechanisms on a molecular level differ to the unchallenged cell. These combined methods will set a new standard for data reporting and a precedent for the next generation of regenerative medicine researchers. Hence supporting self-healing mechanisms with Immuno-Regenerative Medicine.

Conflict of Interests: JD educates clinicians in a variety of cell separating systems.

Contributors: JD wrote the article under the guidance of VA, KN, DK, SK. All authors edited and reviewed the article.

Funding: No funding was received for this review article.

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Table 1. The effects of Leucocytes and Platelets on Tissue Regeneration				
Cell type	**LR- PRP**	**Time**	**Outcome**	**Ref**
a. Expression of MMP-2, -3, -9 b. Fibroblast challenged by IL-1β	LP-PRP vs. LR-PRP Data omitted ACP (Arthrex) GPS (Biomet)	24 – 144 hours (h)	LR-PRP exhibited higher total MMP-2, -3, -9 concentrations for up to 144 h. LR-PRP - significantly higher total and endogenous MMP-2 activity. Once normalisedd to platelet count, differences in MMP activity were not significant between L Pv. LR-PRP. Cells stimulated with IL-1β and treated with LP-PRP showed significantly higher MMP-2, -3 concentrations at 24 hs. LR-PRP. 48 h - IL-1β stimulated cells treated with LR-PRP exhibited higher MMP-2 concentration, but no difference in MMP-3. 48 h - significantly higher concentration of MMP-9 in the LP-PRPvs. LR-PRP treated cells.	[42]
A specially designed plexiglas smould was fabricated to make the fibrin specimens identical in size, volume, and figure, into a dog-bone- shape.	LR-PRP vs. LP-PRP Data omitted (Endoret)	NA	LR-PRP - greater tensile strength, toughness and stronger mechanical properties. LR-PRP - Tensile strength of early group was significantly higherthan thee PRP group. Early LR- PRP group was stiffer than LP-PRP group but not statistically significant. Thetoughnesss ofthe early LR-PRP group was significantly higher than LP-PRP group.	[38]
Split Skin Graft (SSG)	LR-PRP Topical application 2139.3 x 10^9 plt/L; 51.5 x 10^9 leucocyte/L	1 year	No significant statistical difference for all time intervals. No improved or superior scar formation. Several variables - vastness in age, the total surface burn areas and wound sepsis	[58]

Nasolabial rhytid injection	PRP and fibroblasts	9 months (m)	16 patients - 80 % increase in skin thickness at 9-month. 15 patients - increase in dermal density. 7 patients - increase in skin hydration. 16 patients, improved sebum quality adjacent to the nasolabial fold. Nasolabial fold wrinkle depth reduced in 15 patients. 85%of patients were satisfied.	[60]
a.Thedefectt resulting from an odontogenic mandibular cyst excision b. Double mandibular fracture	LR-PRP 1.7 x 10^6 plt/µl; 39 x 10^9 leucocyte/L	14 days (d)	Decreased bleeding in surgery. Advanced healing. No inflammation,less visibles erythema in suture margins. The LR-PRP group healed more efficiently.	[61]
Angiogenic potency and fibroblast migratio	mMultilayeredd sheets fibroblasts (1.25 x 10^5) and peripheral blood mononuclear cells (2.0 x 10^5)vs. single layer sheet	2 d	Multilayeredd sheet - greater angiogenic potency and fibroblast migration.	[40]
a. Fibroblast proliferation	LP-PRP 427.61 x 10^3 plt/µl; 2.26 x 10^3 leucocyte/µl LR-whole blood 257 x 10^3plt/µl; 6.96 x 10^3 leucocyte/µl	10 d	Fibroblasts showed a significant increase in proliferation compared to untreated cells - up to 8 days. No significant difference between the groups,exceptpfort a significant increase for both groups compared to untreated cells - from day 4-8.	[41]
b. GF release	LP-PRP 427.61 x 10^3 plt/µl; 2.61 x 10^3 leucocyte/µl LR-whole blood; 257 x 10^3plt/µl	10 d	PDGF-AB/BB increased immediately before fibroblast proliferation and decreased over 10 days. IGF-1 and TGF-β1 increased as fibroblasts proliferation increased,peaked atn 4 and 6 d up to 8 d.	[41]

	6.96 x 10^3 leucocyte/µl		VEGF was the lowest but increased along with cell proliferation.	
a. Fibroblast proliferation, migration and motility	LP-PRP 1.5 - 3.0 x 10^6 plt/µl 0 – WBC LR-PRP 1.5 - 3.0 x 10^6 plt/µl 8.1-13.2 x 10^3 leucocyte/µl	72-h	Both supernatants stimulated proliferation (2.5-fold) compared with untreated cells. No significant differences in the proliferative response between LP and LR-PRP treated cells.	[44]
b. Wound healing assay	LR-PRP 0.3 - 2.5 x 10^6 plt/µl	72-h	Greater fibroblasts migration from the 0.3-1.5 x 10^6 plt/µl compared to untreated cells. Fibroblast motility inhibition - observed at 2.5 x 10^6 plt/µl. Greatest motility at 1.5 x 10^6 plt/µlvs. LP-PRP. MMP-2,, -9 expressions - present for both LR- and LP-PRP, no significant differences. No significant difference - GF release. VEGF exhibited a significant decrease from the LP-PRP. IFN-γ and PDGF-B in LP-PRP showed a significant increas ev.Thee LR-PRP. TNF-α and FGF-7 levels could not be detected.	[44]

Table 2. Platelet concentrations and their effects on tissue cells

Cell	PRP	Dilution %	Time	Outcome	Ref
hMSCs	315 - 1600 x 10^3 plt/µl	DMEM + 10% & 20% PRP releasate. DMEM +10% PRP releasate diluted in PPP. Final PRP releasate concentration ranged from 0.625 -2.5 fold	7 days (d)	Significant difference for hMSC mitogenesis - 1 x 10^6 plt/µl. 5 to 10-fold PRP - 848% and 720% more hMSCs by day 7vs. growth medium. Cells exposed to 2.5 or 1.25-fold PRP - increases of 325% and 356%. 1.25-fold PRP was more mitogeni cv the freshh blood clot serum, which stimulated proliferation by 208%.	[13]
Fibroblast	1416 x 10^3 plt/µl	0, 0.2, 2, 10, 20%	96 hours (h)	Lower % proved insufficient. 20% PRP - highest proliferation. No cytotoxicity occurred	[48]
Fibroblast and hMSCs Determination of TGF-β and PDGF levels	132.26 x 10^4 plt/µl PPP- 16.74 x 10^4 plt/µl WB- 5.12 x 10^4 plt/µl	1, 5, 10, 20%	1, 4, 7 d	5% aPRP - Proliferation peaked on day 7 10% and 20% PRP - proliferation decreased from higher concentrations. The mean PDGF-AB level in nonactivated PRP was 0.773 pg/ml and increased to 184-fold to 144.46 pg/ml. The mean TGF-β1 level in nonactivated PRP was 0.982 pg/ml and increased 81-fold to 96.38 pg/ml.	[16]

Fibroblast VEGF, HGH, HA, TGF-β1 in type 1 collagen and HA synthesis Fibroblast Phenotype: Skin, Synovium and Tendon	PPP- 6 x 10^6 plt/ml PRP- 404 x 10^6 plt/ml PRP(2) -767 x 10^6 plt/ml	20% PPP 20% PRP 20% PRP(2)	72 h	Each sample, statistically significantly stimulated each phenotype compared to the non-stimulated cells. PRP and PRP(2) - maximum fibroblast proliferation. VEGF release - the highest in tendon cells. A different pattern for Hepatocyte-GF production. Enhanced HA synthesis, but did not alter collagen type I production. TGF-β may be involved in enhanced HA, but not in type I procollagen synthesis.	[49]
Endothelial Angiogenesis	1.5 to 3 x 10^6 plt/µl		72 h	PRP of 1.5 x 10^6 plt/µl - optimal angiogenesis. No inhibitory effects up to 3 x 10^6 plt/µl.	[18]
Fibroblast; type 1 carboxy-terminal peptide production; type 1 collagen mRNA, MMP-1, -3 expression.	PPP – 3.1 x 10^5 plt/µl vs. PRP – 1.7 x 10^6 plt/µl	1, 5, 10, 20 %	5 d	5% - most effective for all outcomes. 10 to 20% - a proliferation decrease. All samples exhibited growth in procollagen type 1 carboxy-terminal peptide, protein production and higher MMP -1, -3. PRP - significantly higher cell expressions.	[15]
UVAirradiatedd fibroblasts compared to normal fibroblasts	Data omitted	25, 50%	72 h	25% and 50% PRP significantly increased fibroblast proliferation, collagen deposition and migration rates. The challenged fibroblasts proliferationrate wass less than the unchallenged fibroblasts, survival and proliferationwase nonetheless restored.	[54]
24-h Serum starved fibroblasts	Data omitted	25, 50, 100%		All samples ameliorated suppressed proliferation. Highest migration capacity - 50% PRP. 100% PRP restored collagen deposition.	[55]

Fibroblast	980,000 plts in 2 ml	10, 25, 50, 75 %	24, 48, 72 h	No significance in proliferation for all % 10% aPRP - the highest proliferation and optical density at all time points.	[50]
Fibroblasts +keratinocytess + PRP applied to full thickness wounds of mice	Data omitted	10%	7, 14 d	Greater re-epithelialization - 7 & 14 d Some wounds achieved full closure. The nontreatmentt group expressed no epidermal growth. TGF-β1 expression was higher in the PRP + fibroblast + keratinocyte group. PDGF-BB and VEGF expressed higher in the non-treatment group compared to the treatment groups.	[62]

Figure Legend

Figure 1. Cellcrosstalkk, expressions and secretions in tissue healing processes.
Abbreviations: bFGF: basic Fibroblast growth factor; CTGF: Connective tissue growth factor; CXCL8: C-X-C motif chemokine ligand 8; ECM: Extra Cellular Matrix; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; HGF: Hepatocyte growth factor; IFN: Interferon; IGF: Insulin growth factor; IL: Interleukin; KGF: Keratinocyte growth factor; MDGF:Monocytederivedd growth factor; MMP: Matrix metalloproteinase; PDGF: Platelet-derivedgrowth factoro; SDF: Stromal growth factor; TGF: Transforming growth factor; Th: T helper; TNF: Tumor Necrosis Factor; Vascular Endothelial growth factor.