Mini-review

The use of autologous blood-derived growth factors in bone regeneration

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Summary

Platelet-rich plasma (PRP) is defined as a portion of the plasma fraction of autologous blood having platelet concentrations above baseline. When activated the platelets release growth factors that play an essential role in bone healing such as Platelet-derived Growth Factor, Transforming Growth Factor-β, Vascular Endothelial Growth Factor and others.
Multiple basic science and in vivo animal studies agree that PRP has a role in the stimulation of the healing cascade in ligament, tendon, muscle cartilage and in bone regeneration in the last years PRP had a widespread diffusion in the treatment of soft tissue and bone healing.
The purpose of this review is to describe the biological properties of platelets and its factors, the methods used for producing PRP, to provide a background on the underlying basic science and an overview of evidence based medicine on clinical application of PRP in bone healing.

KEY WORDS: growth factors; bone regeneration; Platelet-Rich Plasma.

Introduction

Bone regeneration involves the use of cells, biological or artificial biometric scaffolds, and biofactors that promote cell growth and differentiation along complex pathways to repair the tissue.
Growth factors have a crucial role in this process since they influence chemotaxis, differentiation, proliferation and synthetic activity of bone cells, thereby regulating physiological remodeling and bone healing.
That makes the use of the autologous and recombinant growth factors (GF) a rapidly growing field of orthopedics focusing on manipulating GF and secretory proteins to maximize the healing of bone and soft tissues.
A variety of growth factors has been found to play a role in bone healing, but the two most important families can be classified as: Bone-Derived Growth Factors, namely the BMPs family, and the Autologous Blood-Derived Growth Factors.
Most of the growth factors derived from autologous blood is released upon platelet activation, and their clinical use has been popularized with Platelet-rich plasma (PRP); Platelet-rich plasma is obtained from patient’s blood using commercially available devices and, after activation, it could release growth factors for the enhancement of bone and soft tissue healing as demonstrated by basic science and clinical studies.
The autologous nature of PRP, its ease of application and relative low cost are some of the advantages of PRP that have led to research interest and to a wide clinical application.
The purpose of this review, therefore, is to provide back-ground on the underlying basic science, the methods used for producing PRP and an overview of evidence based medicine on clinical application of PRP in bone healing.

Definition of PRP and terminology: Platelet-rich plasma (PRP) is defined as a portion of the plasma of autologous blood having platelet concentrations above baseline. Normal platelet counts in blood range between 150,000/ µl and 350,000/µl and average about 200,000/µl.
Since studies have shown that clinical efficacy can be expected with a minimum increase of 4x of this baseline, a concentration above of 1 million platelets/µl has been suggested to be the working definition of PRP. Greater platelet concentrations have not been shown to further improve healing, although a number of variables affect the biologic activity of various PRP preparations (1).
Others use the term “platelet concentrate”, but platelet concentrate is only a solid composition of platelets which would not clot; “platelet gel” should also be considered incorrect because PRP is nothing more than a human blood clot with increased platelet numbers.
To reverse the order of the words as in “plasma rich in platelets” or “plasma very rich in platelets” and the use of other terms like “Preparation rich in growth factors” (PRGF) and “platelet releasate” does not change anything.
Platelet-rich plasma differs from “fibrin glue” because the clot in PRP contains only the same concentration of fibrin as a normal blood clot.

The biological properties of PRP: The process of healing is composed of three major phases: 1) the acute inflammatory phase, which includes platelet clot formation and degranulation of the growth factors, activation of the coagulation cascade and the migration of granulocytes and macrophages; 2) the mesenchymal cell proliferation and differentiation phase; and 3) the phase of regeneration of the missing tissue by tissue specific cells (2).
The platelets are the main regulators of the inflammatory phase and play an essential role in the proliferation and differentiation phase.
Platelets derive from megakaryocytes and are small discoid blood cells (approximately 1-3 µm) that contain organelles and structures such as mitochondria, microtubules and granules (alfa, delta, lamba). The “alfa” granules, bound by a membrane, are formed during maturation of the megakaryocytes. They are about 200 nm to 500 nm in diameter, and number approximately 50 to 80 performed platelet (3). They contain more than 30 bioactive pro-

teins, many of which have a fundamental role in haemostasis or tissue healing (4,5).
The properties of PRP are based on the production and release of multiple growth and differentiation factors when the platelets are activated (6,7). It also contains proteins such as fibrin, fibronectin, vitronectin and thrombospondin, which are known to act as cell adhesion molecules, important for the migration of osteoblasts, fibroblasts and epithelial cells (8).
Bioactive Growth Factors in PRP: The growth factors are peptides that promote cell proliferation, differentiation, and chemotaxis inducing the migration of various cells. They play an important role in healing processes, as demonstrated in several studies. The levels of growth factors released from the platelets upon activation are commonly quantified by enzyme-linked immunosorbant assay (ELISA) (9,10,11).
Platelet-rich plasma can potentially enhance healing by the delivery of various growth factors and cytokines from the alfa-granules contained in platelets (Table 1). The basic cytokines identified in platelets regarding bone regeneration include:
PDGF (Platelet-derived Growth Factor) appears to be the first growth factor present in a wound and initiates connective tissue healing through the promotion of collagen and protein synthesis (12).
PDGF is a glycoprotein with a molecular weight of approximately 30 kD. There are three isoforms, PDGF-AA, -BB and -AB (13). The primary effect of PDGF in bone regeneration seems to be its mitogenic activity to mesoderm-derived cells such as fibroblasts, vascular muscle cells, glial cells and condrocytes. It also attracts and activates neutrophils, monocytes and fibroblasts and stimulates the synthesis of additional growth factors.
The most important specific activities of PDGF include angiogenesis and macrophage activation, proliferative activity on fibroblasts, chemotaxis for fibroblasts and collagen synthesis (14).
It has strong chemotatic and mitogenic properties for osteoblasts, and has been shown to exhibit differential spatial and temporal expression in fracture healing (15, 16,17).
TGF-β (Transforming Growth Factor β) and its family members such as Bone Morphogenetic Proteins (BMPs) play an important role in cell division, differentiation, migration, adhesion, organization and apoptosis (18). In humans, three subtypes of TGF-β are present (19), but TGF-β1 and TGF-β2 appear to be the most important with regard to general connective tissue repair and bone regeneration. TGF-beta can inhibit the formation of osteoclasts and therefore favour bone formation over resorption. TGF-β is a potent stimulator of bone formation, promoting osteoblastic differentiation and the synthesis of the osteoid matrix, and inhibiting the synthesis of the proteases (20,21,22,23).
IGF-I (Insuline-like Growth Factor) stimulates osteoblasts. It was shown that IGF-I increases bone turnover in patients with low bone mineral density (24,25,26).
VEGF (Vascular Endothelial Growth Factor) is the major regulator of vasculogenesis and angiogenesis and as such plays an important role in bone tissue regeneration (27).
CTGF (Connective tissue growth factor). This new Growth Factor was described very recently by Kubota and others; Platelets adhere to CTGF at injured tissue wound sites, where it is overexpressed along with the platelet coagulation process. In their experiments they showed that non-activated platelets contain considerable amounts of CTGF and that is released by activated PRP and that CTGF endorses angiogenetic activity, cartilage regeneration and fibrosis (28). Cicha et al. showed that CTGF is expressed in bone marrow cells, but not by platelet producing megakaryocytes, suggesting that the total amount of CTGF in platelets is the result of endocytosis from the extracellular environment in bone marrow (29).
The development of PRP focused on enhancing this rich environment and for this reason it has been proposed to delivery a concentrate of platelets at the site of the injury as a successful strategy for fostering the regeneration pathway during bone wound healing.

Table 1 - The effects of the Growth Factors produced by platelets.

PDGF	Macrophage activation Angiogenesis Fibroblast chemotaxis and proliferative activity Collagen synthesis Proliferation of bone cells
TGF-beta	Enhances the proliferative activity of fibroblasts Stimulates biosynthesis of type I collagen and fibronectin Induces deposition of bone matrix Inhibits osteoclast formation and bone resorption
IGF-I	Chemotactic for fibroblasts and stimulates protein synthesis Enhances bone formation by proliferation and differentiation of osteoblasts
PDAF	Induces vascularisation by stimulating vascular endothelial cells
PDEGF	Promotes wound healing by stimulating the proliferation of keratinocytes and dermal fibroblasts
PF-4	Stimulates the initial influx of neutrophils into wounds Migration and mitosis of endothelial cells
EGF	Cellular proliferation Differentiation of epithelial cells
VEGF	Migration and mitosis of endothelial cells Angiogenesis Creates blood vessel lumen and fenestrations Chemotactic for macrophages and granulocytes Vasodilation (indirectly by release of nitrous oxide)

PRP Preparation

Regarding the production and application of clinically effective PRP there are a few main principles:
The processing technique: The platelet collection should start before surgery and before the use of an inhalation anesthetic as this may start the activation of platelets. Once the PRP is prepared it is stable for 8 hours. PRP is perioperatively prepared from a unit of autologous whole blood which in the clinical standard setting is drawn from the median cubital vein. The use of a needle diameter larger than 17 gauge avoids trauma to the platelets during blood drawing.
There are 3 techniques for PRP preparation:
a) **Gravitational platelet sequestration systems**: The GPS are a table-top centrifuge system that rely on centrifugation to separate platelets from other blood components; they are the most common technique used and a great number of devices for preparing autologous PRP has become commercially available.
The autologous pre-donated blood is collected in sufficient amounts of anticoagulation citrate dextrose-A solution (ACD-A). The aspirated blood is gently agitated to mix the anticoagulant with the blood.

To truly concentrate platelets from autologous blood, the device must use a double centrifugation. The first centrifugation step separates the red and white blood cells from plasma and platelets. Red blood cells (7 μm in diameter) and white blood cells (7-15 μm in diameter) are much larger than platelets (2 μm in diameter); these cells separate from the plasma and platelets.
The second slower spin is used to further concentrate the platelets and separate the platelets and white blood cells together with a few red blood cells from the plasma. This spin produces the PRP and separates it from the platelet poor plasma (PPP). Platelet-poor-plasma (PPP) and erythrocytes are then discarded (30).
b) **Cell savers/separators:** With cell savers/separators, larger predonation blood volumes (250 mL to more than 500 mL of whole blood) can be obtained, resulting in a PRP volume ranging from 20 mL to more than 50 mL (31). When a cell saver is used, autologous whole blood is collected into standard donor bags. In general, they use a continuous-flow centrifuge bowl or a continuous-flow disk separation technique and both a hard (fast) and a soft (slow) spin, yielding platelet concentrations from two to four times baseline (32). After this procedure the erythrocytes are also separately collected in a blood bag and then returned to the patient (33).
c) **Selective filtration technology:** With this system, also called plateletpheresis, the platelets are captured on a single-use disposable filter and so separated from whole blood without the use of centrifugation (34).
Activation and application of PRP: To initiate the release of the growth factors contained in the alpha granules, platelets must be activated. This is generally accomplished by adding either 1000 units of thrombin or 10% calcium chloride (35). To antagonize the anti-coagulative effect of the citrate present in the pre-donation blood bag the syringe is then mixed for about 10 seconds to initiate clotting.
Thereafter, the obtained PRP can be applied. PRP may be mixed into a bone graft, layered in as the graft is placed, sprayed on a soft tissue surface, applied on top of a graft, or used as a biologic membrane. It has to be considered that approximately 70% of the stored growth factors are released within 10 minutes, and nearly 100% of the growth factors are released within 1 hour (36).
Later it became evident that the use of bovine thrombin to activate the clotting mechanism and to induce platelet activation can lead to complications associated with formation of antibodies against the bovine thrombin. This is a rare but potentially serious complication that can result in an immunomediated coagulopathy.
An alternative system to delay the release of growth factors and not to use Bovine thrombin is possible through the creation of a **"Platelet-Rich Fibrin Matrix" (PRFM)**. With an initial low speed centrifugation we separate the blood cells from the platelets and plasma proteins. A second centrifugation converts fibrinogen to fibrin in the presence of $CaCl^2$ and the fibrin cross-links to form a dense fibrin matrix; intact platelets are subsequently trapped in the fibrin matrix. The design of the PRFM allows a slow release of platelet-derived growth factors into the surgical area for approximately 5 to 7 days (37). With the use of a commercially available devices we can produce matrices that are malleable and can be molded in the desired fashion or even sutured to the lesions.

Orthopaedic applications of platelet rich plasma

The first reports of clinical use date back to 1998, when PRP was used in mandibular reconstruction (8,38).
Since that time, the application of autologous PRP has been safely used and documented in many fields including: orthopedics, sports medicine, craniofacial surgery, dentistry, ENT, neurosurgery, ophthalmology, urology and wound healing, as well as cosmetic, cardiothoracic and maxillofacial surgery.
The purpose of this paper is to review the applications of platelet rich plasma in bone regeneration, however in the last year PRP had a widespread diffusion in the treatment of ligament, tendon, muscle and cartilage pathology and lesions.
Multiple basic science and in vivo animal studies agree that PRP has a role in the stimulation of the healing cascade in ligament, tendon, muscle and cartilage (39,40,41), and the success of clinical use of PRP is supported by different studies (Table 2).
However the majority of the human studies that have been performed have a small number of participants and controls and do not have a statistical power to document a true statistical difference, for these reasons further studies on the effects of platelet-rich plasma on soft-tissue and cartilage healing are required (42).

Platelet-rich plasma and bone healing

The effects of platelet-rich plasma on bone healing have been mixed in the literature and sometimes controversial.
"In vitro" and animal studies: Platelet-rich plasma has been shown in several "in vitro" and animal studies to play a role in promoting new bone formation.
Nash et al. studied the effect of PDGF on bone healing using a unilateral tibial osteotomy in rabbits. Microscopically, platelet growth factor-treated tibiae displayed a more florid and advanced state of osteogenic differentiation, both endosteally and periosteally, than the control osteotomies. Radiographic, mechanical and histopathological data suggest that exogenous PDGF has a stimulatory effect on osteotomy healing (43).
Kim et al. (2002) in a bone defect in the iliac crest of dogs demonstrated that PRP combined with demineralised bone powder enhanced bone formation around titanium implants (44).
Rai et al. (2008) tested composite scaffolds with PRP into 8 mm rat non-union femoral defects: they demonstrated accelerated early vascular ingrowth and improved significantly higher torsional stiffness for PRP-treated defects compared to empty scaffolds without using PRP (45).
Other publications in the oral and maxillofacial surgery confirm the ability of PRP to promote bone healing (8,46,47,48).
In summary, platelet derived growth factors support bone regeneration primarily via chemotactic and mitogenic effects on preosteoblastic and osteoblastic cells. The osteopromoting effects of the PRP appear in particular to be manifested in the early phase of bone healing (46,49).
A critical analysis of other studies shows, however, that a beneficial effect of PRP on bone healing could not always be demonstrated.
There is some evidence that PDGF was shown to inhibit intramuscular osteoinduction and chondrogenesis by demineralised bone matrix in immunocompromised mice. In a similar model PRP reduced the osteoinductivity of demineralised bone matrix implanted in immunocompromised mice (50,51).
Chaput et al. suggested that PRP is not a major contributing factor to bone ingrowth at the bone-implant interface in the distal femur of rabbits (52).
Furthermore platelet-rich plasma also has been shown to interfere with the complete differentiation of human osteoclast precursors (53) and it has been demonstrated in a rat model that PRP during early healing, whether alone or mixed with autogenous bone, did not lead to greater bone remodelling, as compared to coagulum (54).
A lower beneficial effect on bone healing was particularly noted when PRP was used alone.
Sarkar et al. utilized a model of a critical size defect (2.5 cm) in the tibial diaphysis of 16 sheep that was supplied either with autogenous PRP in a collagen carrier or with collagen alone as controls. At 12 weeks bone volume, mineral density, mechanical rigidity and histology of the newly formed bone in the defect did not differ significantly between the PRP treated and the control group,

Table 2 - Results of clinical use of PRP in tendon, ligament and cartilage healing.

Authors (year)	Diagnosis	Design	Size	Results
Sanchez et al. 2003	Cartilage lesion/degeneration	Case report	1 patient	Rapid resumption of symptom free athletic activity
Mishra et al. 2006	Chronic elbow tendinitis	Cohort	15 patients	Decreased pain at 2 years
Sanchez et al. 2007	Achilles tear healing	Case control	6 repairs with PRP	Increase ROM and early return to activity by ± 4-7 weeks
Sanchez et al. 2008	Cartilage lesion/degeneration	Retrospective cohort study	60 patients	Better pain control and physical improvement
Randelli et al. 2008	Rotator cuff tear	Prospective study	14 patients	Good and stable clinical results after asc clinical repair
Maniscalco et al. 2008	Rotator cuff tear	Case report	1 patient	Pain relief and Rom recovery after surgical repair; complete integrity
Orrego et al. 2008	ACL tear	Prospective randomized control	108 patients	Enhancing maturation
Silva et al. 2009	ACL tear	Prospective study	40 patients	No difference compared to controls
Kon et al. 2009	Patellar tendinopathy	Prospective study	20 patients	Marked better knee function and quality of life
Kon et al. 2010	Cartilage lesion/degeneration	Prospective study	91 patients	Clinical improvement; better results in early degeneration and younger patients
Radice et al. 2010	ACL tear	Prospective single blind study	50 patients	Reduction in time required for homogeneous graft signal with MRI
Filardo et al. 2010	Partial Achilles lesion/tendinopathy	Prospective study	1 patient	Fast tissue repair and return to competitive sports
De Vos et al. 2010	Achilles lesion/tendinopathy	Double-blind, randomized, placebo-controlled clinical trial	54 patients	Same results in pain and improvement compared with saline injection
Peerboms et al. 2010	Elbow tendinopathy	Randomized controlled trial	100 patients	Reduced pain and increased function

and no effect of PRP upon bone formation was observed (55). However recent research on animal models has demonstrated that PRP, when used in combination with a proper scaffold, is a potent growth factor that promotes bone formation in vivo.
Kasten investigated the efficacy of PRP in improving bone healing of a critical-size diaphyseal radius defect in a rabbit model when utilized with different scaffolds. PRP yielded better bone formation than the empty scaffold as determined by both histology and microcomputer tomography ($p < 0.05$) (56).
Jungbluth et al. investigated the effect of PRP in combination with calcium phosphate granules (CPG) on bone defect healing in a metaphyseal long bone defect; at 6 weeks the radiological and histomorphometrical evaluations showed significantly more bone formation in the PRP group in the central area of the defect zone ($p<0.01$) as well as the cortical defect zone ($p<0.04$) (57).
Bi et al. investigated the mechanical and biological properties of an injectable composite scaffold by combining tricalcium phosphate and chitosan with platelet-rich plasma (58).
Trying to summarize the in vitro and animal study on PRP, one may argue that when used in larger defect, and alone, whereas BMPs have been shown to induce bone healing in similar defect models (59), PRP was not able to enhance osteogenesis. But when PRP was used combined with autografts, or even better, with a proper scaffold, it has shown to promote and enhance bone regeneration.

Clinical studies: The first clinical study using PRP for bone reconstruction therapy was performed by Marx et al. In this randomized study, 88 patients with mandibular defects were treated with autogenous cancellous bone grafts with or without the addition of activated PRP (8).
Both the radiographic and the histomorphometric evaluations show a significantly greater percentage of bone with the addition of PRP Considering only orthopedic surgery a limited number of studies on use of autologous PRP has been published (Table 3).
There are reports on the use of platelet rich plasma for enhancing fracture healing, treatment of existing non-union, enhancing bone repair in spinal and ankle fusion, high tibial osteotomy and distraction osteogenesis.
One of the first clinical study, but with no controls, was perfomed in spinal fusion where PRP was added to autogenous bone grafts to enhance bone healing. At the final X-ray control the results were excellent, obtaining union in all their patients (60).
The first randomized, controlled study with PRP in humans was performed to compare the osteogenic potential of lyophilized bone chips combined with platelet gel, or with platelet gel and bone marrow stromal cells or with of lyophilized bone chips alone, in the hea-

Table 3 - Results of clinical use of PRP in bone healing.

Authors (year)	Diagnosis	Design	Size	Results
Bibbo et al. 2005	High risk foot and ankle patients	Case series	62 patients	Short time to union with PRP + ABG vs PRP
Carreon et al. 2005	Bone healing in instrumented spinal fusion	Retrospective cohort study	76 patients	High rate of non union vs control (not significant)
Calori et al. 2006	Long bone critical size defects	Randomized controlled study	29 patients	n/a
Savarino et al. 2006	Bone healing in varus HTO	Randomized case control	5 patients	No functional or clinical difference
Dallari et al. 2007	Bone healing in varus HTO	Prospective randomized control	23 patients 11 with PRP 12 with bone chips, BMC and PRP	No clinical difference
Kitoh et al. 2007	Bone healing in distraction osteogenesis	Retrospective comparison case control	32 patients	Short average healing time with PRP versus control
Kitoh et al. 2007	Osteotomies for limb length discrepancies	Case series	46 patients	Healing index better with BMC + PRP vs control
Calori et al. 2008	Persistent fracture non-unions	Randomized controlled trial	120 patients 60 PRP 60 PRP + BMP	Lower median clinical and radiographic healing time observed in the rhBMP-7 group
Sanchez et al. 2009	Bone healing in non unions	Retrospective case series	16 patients	84% healing, unclear if PRP made a difference

ling of a high tibial osteotomy. Radiographs revealed a significantly higher rate of osteointegration of the first two groups than in the controls at six weeks (61).
Kitoh et al. used PRP and bone marrow cells (BMCs) during osteogenesis distraction.
Compared to a control group the average healing index of the BMC-PRP group (27.1±6.89 d/cm) was significantly lower, shortening the treatment period by accelerating new bone regeneration (62).
PRP was used in high-risk patients undergoing elective foot and ankle surgery. Overall, a 94% union rate was achieved at a mean of 41 days. The authors suggest that adjuvant PRP may be a useful adjunct to promote osseous healing in high-risk patients undergoing elective foot and ankle surgery (63).
The use of percutaneous injection of PRP in the treatment of delayed union and nonunion was perfomed as a minimally invasive method alternative to open grafting techniques. Union was achieved in all cases of delayed union. In the nonunion group, union was observed in 13 of 20 cases (64).
It has also been demonstrated that the percutaneous application of PRP normalised the early callus in diabetic fractures bringing it to levels comparable to those in non-diabetic controls (65,66,67).
However when Platelet-rich plasma (PRP) was compared to recombinant bone morphogenetic protein 7 (rhBMP-7) in the treatment of persistent fracture non-union the results were not so encouraging (68).
At final follow-up 52/60 (86.7%) non unions treated with rhBMP-7 and 41/60 (68.3%) non-unions treated with PRP progressed to clinical and radiological union. The authors conclude in favour of the use of rhBMP-7 in the treatment of persistent long bone non-unions compared to PRP (69).
In summary the enhancement of bone formation by using augmentation with PRP is still a growing area of research. The success of clinical use of PRP is supported by different studies, but the definite role of PRP and also its production and characterization has still to be defined. None of the clinical studies examined have a strong degree of evidence and in most of the cases PRP was not used alone, but in combination with other osteoinductive scaffolds, cells or growth factors.

Future perspective and conclusions

As a future perspective PDGF is now available as recombinant human PDGF (rhPDGF) and in particular its isoform PDGF-BB is currently used for non orthopedic application (70). Preclinical studies have demonstrated that RhPDGF-BB is a key regulatory molecule in bone homeostasis, repair and regeneration; it is chemotactic and mitogenic for osteoblasts and undifferentiated osteoprogenitor cells, for cytokines that are crucial to bone and soft-tissue healing and regeneration. Preliminary clinical results of recombinant PDGF in fracture healing have been promising and clinical trials are ongoing (71).
In conclusion platelet rich plasma is a safe, autologus, easy to prepare and to use and relative low cost procedure to deliver growth factors for bone and soft tissue healing.
Basic science supports the enhancement of healing with the use of PRP, but the results are still a subject of controversy. Although most of the clinical studies have good outcomes favoring the use of PRP, most of the studies are limited to case reports and there are only a few controlled, clinical studies that provide a high level of medical evidence.
Many clinical questions have to be clarified, particularly with regard to the timing of therapy, the volume and frequency of treatment, and the ideal scaffold for distribution of the platelet-rich plasma. However, because the majority of the clinical trials have shown encouraging outcomes, further controlled clinical trials will help to elucidate the effects of platelet rich plasma.

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