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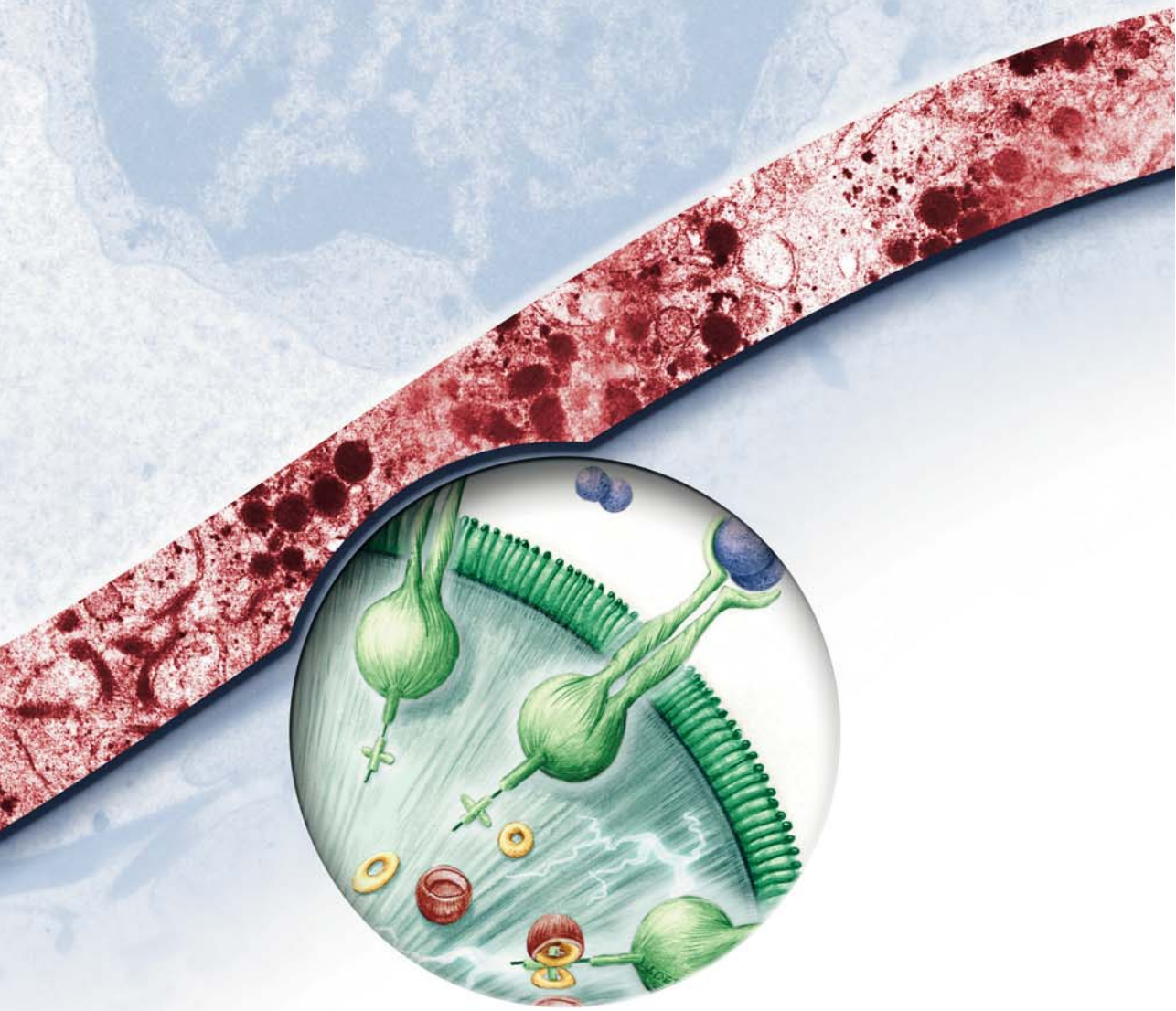

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Chapter 2

Platelet rich plasma and platelet gel. A Review

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Introduction

Few hospitals in Europe routinely use autologous platelet gel application techniques as part of a peri-operative blood management program. In the United States, an increasing number of clinicians tend to employ platelet gel applications in various surgical settings, for both in, and out of hospital surgery. The question why this novel and promising technique for the delivery of autologous growth factors has not yet been adopted on a broader scale need to be addressed. The main reason may be the lack of convincing scientific data that provides information whether or not the use of platelet rich plasma (PRP) and platelet gels (PG) are appropriate in the clinical setting.

At the Catharina Hospital in Eindhoven The Netherlands, we started to utilize PG techniques in 2001 with a small group of patients undergoing complicated cardiac surgical procedures and in patients undergoing a spinal fusion operation. This was carried out as an adjunct to the already existing peri-operative blood management programs with apparently impressive clinical results.

The Department of Peri-operative Blood Management of the Catharina Hospital performs close to 1600 blood management procedures annually, of which 60% are related to obtain whole blood platelets to produce PRP for the utilization of PG procedures. While it's extended use is based upon positive clinical impressions and on clinical judgment, it still lacks a firm scientific basis. Therefore, clinical trials are required to answer questions on the efficacy, efficiency, and on the safety of PG applications under various surgical and medical conditions.

It is clear that a good understanding of the proper preparation and utilization of this specific blood management technique is mandatory for clinicians to adequately evaluate results of its use and to avoid inconsistent results. Conflicting data have been reported in clinical and experimental research on the efficacy of PG treatment[1-5]. To understand how this arises it is essential to be in possession of the details of the preparation of PRP and PG. Knowledge of the following factors are of particular importance: the method of drawing blood, the quality of the PRP used, platelet and growth factor counts, the PRP activation, whether autologous or donor PRP was used, and the overall methodology. With respect to these issues, the clinician should be aware that data may sometimes appear to be conflicting in the eventual outcome.

This review addresses a variety of aspects pertaining to the use of PG; these include background on platelet activity, the pivotal role of platelets in hemostasis, soft tissue healing and bone growth, the whole blood PRP production procedure,

platelet activation with thrombin, and a description of the various actions of platelet derived growth factors. In addition, a discussion of the most recent clinical and experimental articles is presented with respect to these issues. Some safety issues including possible PG mitogenic effects are also addressed.

Platelet anatomy and function

Platelets are small discoid blood cells (approximately 1-3 µm). The average platelet count ranges from 1.5-3.0 x 10^{-5} per mL of circulating blood and the *in-vivo* half-life of platelets is about seven days. Platelets are formed from megakaryocytes and are synthesized in bone marrow by pinching off pieces of cytoplasm. Thereafter, platelets are extruded into the circulation. Platelets have a ring of contractile microtubules (cytoskeleton) around their periphery, containing actin and myosin. Inside the platelet, a number of intracellular structures are present containing glycogen, lysosomes and two types of granules. These are known as dense granules, which contain ADP, ATP, serotonin, and calcium. The α-granules contain clotting factors, growth factors, and other proteins. Platelets are equipped with an extensively invaginated membrane with an intricate canalicular system, which is in contact with the

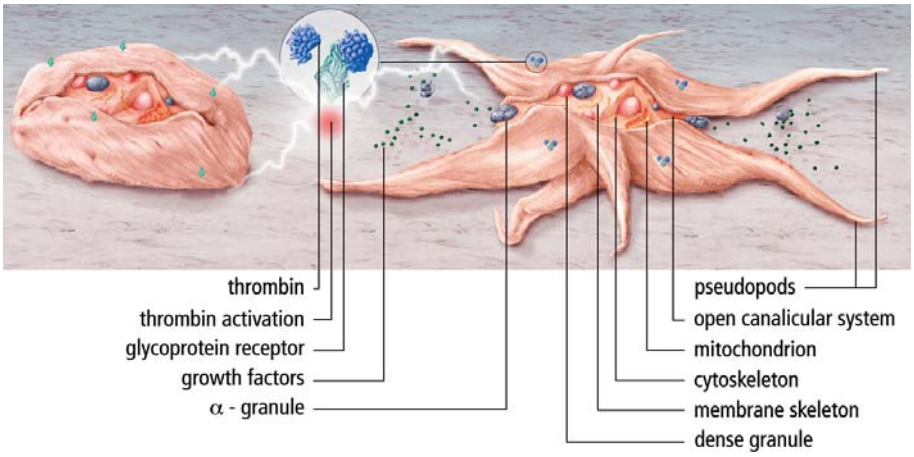

Figure 1. *Schematic overview of a resting and activated platelet.*
Normally platelets are in a resting, non-activated state. Upon activation (e.g. by thrombin) platelets change their shape with the development of pseudopods to promote platelet aggregation and subsequent release of granule content via the open canalicular system. (α-granule: alpha granule).

extracellular fluid[6]. Normally, in the resting state, platelets are non-thrombogenic and require a trigger before they become a potent and an active player in hemostasis and wound healing. Upon activation (e.g. by thrombin) they change shape and develop pseudopodia, which promotes platelet aggregation and the subsequent release of the granule content via the open canalicular system (Figure 1).

PLATELET ACTIONS

Platelets and PG in hemostasis

Hemostasis is a balanced interaction of platelets, vasculature, plasma clotting proteins and low molecular weight substances. Following an injury (e.g. by surgical trauma), the most important initial reactions leading to immediate blood coagulation are mainly mediated by platelets and blood vessel wall changes. In surgery, damaged blood vessel walls expose sub-endothelial collagen, binding von Willebrand factor in the plasma, subsequently changing the structure so that the platelets can adhere to the blood vessel wall. This process, known as platelet adhesion, acts via the glycoprotein Ib and IIb/IIIa receptors, which are present in the platelet membrane. After this event platelets become activated and aggregate. Upon activation, the platelet cytoskeleton changes from discoid to a spherical shape with protruding pseudopods which then spread over injured tissues at the site of injury, a phenomenon called *platelet aggregation*.

After aggregation, the granular contents are released via the canalicular system. Secreted serotonin probably assists in tissue vasoconstriction. Adenosine diphosphate (ADP) promotes release of granule contents from other platelets and makes the platelets sticky, thus forming a hemostatic plug. Many other agents are able to cause platelet aggregation and also to activate phospholipase A_2 present in the platelet membrane.

Subsequently, as a result of the latter, membrane phospholipids release arachidonic acid, which is converted into thromboxane A_2 leading to platelet aggregation and platelet growth factor (PGF) release. Independent of thromboxane and ADP, another mechanism that causes platelet aggregation and platelet granule release, is induced by the presence of thrombin. Thus, by these three mechanisms of platelet activation, the platelet plug is extended in an attempt to stop blood loss from damaged vessels. Furthermore, the coagulation system is activated by secreted and budded particles[7,8]. The most well understood platelet function, at the onset of primary hemostasis, is the formation

of a platelet plug. Thereafter, secondary hemostasis is initiated with the activation of coagulation factors and the formation of a fibrin network that stabilizes the platelet plug[9]. The final step is the activation of leukocytes invading the affected area with the release of cytokines which then activate the fibrinolytic system leading ultimately to clot lysis (Figure 2). Since platelet α-granules secrete platelet derived growth factors at the wound site almost at the instant of injury, repair of injured vasculature and tissue is directly initiated with the formation of new connective tissue and re-vascularization. Furthermore, the temporary formation of platelet and fibrin plugs at the wound site prevents the entry of micro-organisms.

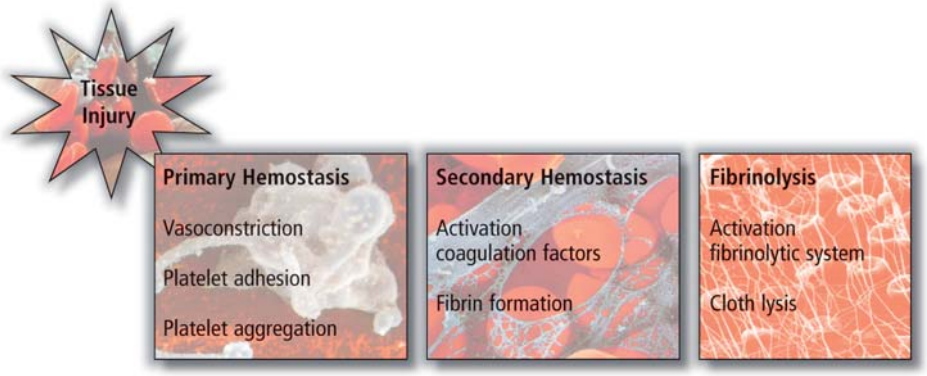

Figure 2. *The different cascade stages in hemostasis after tissue injury.*

Based on the fundamental role of platelets in hemostasis, as discussed above, it may be hypothesized that exogenously applied PG would contribute to a more effective hemostatic condition of (surgical) wound surfaces, where it attaches to tissues as a solid platelet plug. Stover *et al.*, prospectively evaluated the use of PG as a dural sealant, in patients undergoing craniotomy or thoracolumbar procedures, and noted successful closure in 39 of 40 treated patients[10]. Another therapeutic application is to use PG as a wound sealant when it is sprayed by an aerosol technique over larger wound surfaces and suture lines in patients who are at risk of postoperative wound leakage or fistula formation. Furthermore, in patients who are at risk of impaired wound healing, such as diabetics, and thus at risk for postoperative wound complications, a sprayed PG may deliver a high concentration of PGF to the wound, thus boosting and supporting the natural healing process.

Platelets and PG in wound healing

Wound healing is a well orchestrated and complex series of events involving cell-cell and cell-matrix interactions, with growth factors serving as messengers to regulate the various processes involved. The "wound healing process" as a whole has to be considered from the point of view of the type of lesion, which will then in turn dictate the degree of healing that can be obtained. A partial-thickness skin abrasion heals almost entirely by epithelization, whereas deep pressure chronic ulcers rely mainly on matrix synthesis, angiogenesis, and fibroplasia and wound contraction. The significant action of platelet derived growth factors in wound healing has been widely reviewed. With wounds, and also after surgical incisions, repair begins with platelet clot formation, activation of the coagulation cascade, and platelet degranulation with release of the growth factors. During the first two days of wound healing an inflammatory process is initiated by migration of neutrophils and subsequently macrophages to the wound site. In turn, activated macrophages release multiple growth factors, including transforming growth factors-alpha and -beta (TGF-α, TGF-β), platelet derived growth factor (PDGF), interleukin-1 (IL-1), and fibroblast growth factor (FGF)[11]. Angiogenesis and fibroplasia starts shortly after day three, followed by the beginning of collagen synthesis on day's three to five. This process leads to an early increase in wound breaking strength, which is the most important wound healing parameter of surgical wounds, followed by epithelization and the ultimate remodeling process. During the various stages of wound healing PGF play a key role, as demonstrated in several studies[12,13]. In Figure 3, an illustration of the role of platelet derived growth factors during the different stages in the wound healing process is represented.

Platelet degranulation: After tissue damage, PDGF and FGF are already being produced by the injured cells[14]. Once the platelet plug is in place, platelets will start to degranulate with the release of growth factors, PDGF and TGF-β, being the most important growth factors at the wound site in the start of the wound healing process. A characteristic of PGF molecules is that they are also chemotactic and mitogenic with regard to inflammatory cells, i.e. neutrophils, monocytes and macrophages[15].

Inflammatory action: Pierce *et al.*, demonstrated that a single application of PDGF used in incisional wounds amplifies the inflammatory response with an increased wound influx of neutrophils and macrophages[16].

Matrix deposition: During the phase of matrix synthesis and matrix deposition, PGF again plays a predominant role. Mustoe and co-workers showed, in an experimental model, that a single dose of PDGF increased the volume of tissue granulation by 200% after 7 days[17].

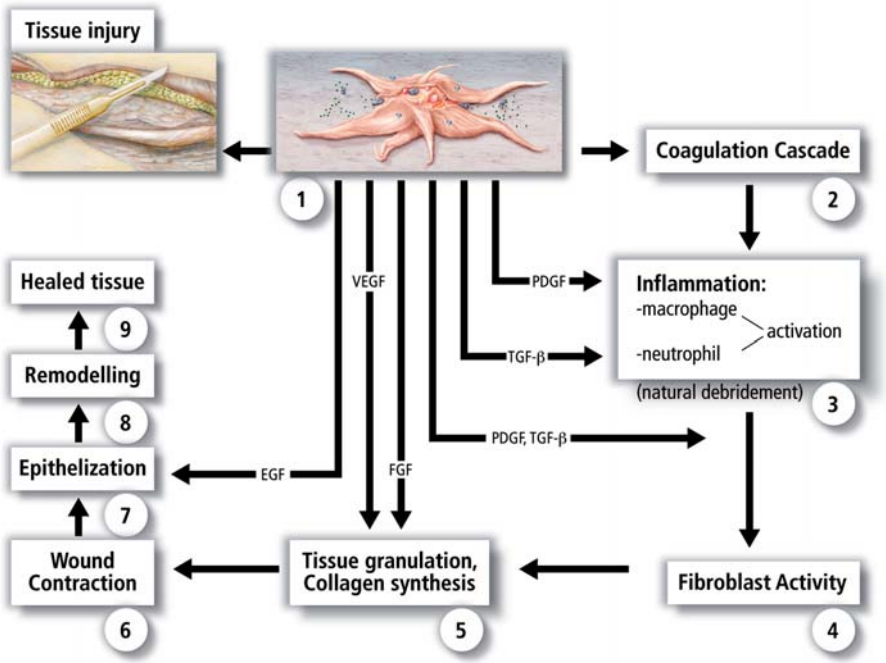

Figure 3. *Schematic illustration of the role of platelet derived growth factors (the numbers indicate the sequence of the wound healing actions) during the different stages of the wound healing process.*
(EGF: epidermal growth factor; FGF: fibroblast growth factor; PDGF: platelet derived growth factor; TGF-β: transforming growth factor bèta, VEGF: vascular endothelial growth factor).

With the application of TGF-β alone on wounds, it was revealed that the matrix mainly consisted of new collagen[15]. Furthermore, in steroid treated or irradiated wounds, it was demonstrated that the application of TGF-β reversed the healing deficit with restoration of wound breaking strength[18].

Collagen production: Also important in wound healing is collagen production, which is initiated by the chemotactic and mitogenic actions of fibroblasts by FGF.

Epithelization: Topically applied epidermal growth factor (EGF) leads to accelerated epithelization, as demonstrated in a model by Nanney and associates[19]. In the beginning of the epithelization process PDGF receptor genes were found, indicating that PDGF is also important during epithelization[20]. During the last phase of wound healing both FGF and PDGF increased contraction and remodeling time[21,22].

Based on the actions of the various PGF during the different stages in the wound healing cascade, the use of PG to stimulate wound repair is an interesting proposition (Figure 3). Compared to recombinant single growth factor applications, PG has the supreme advantage that it offers multiple synergistically working growth factors promoting mitogenesis of mesenchymal stem cells at the wound site[12,23-25].

Promising indications for topical PG applications might be for treatment of chronic non-healing wounds and supportive healing after incisional wounds that occur, for example, in diabetic patients who are at risk of impaired wound healing. PG has been used successfully in wound care patients to close chronic non-healing (diabetic) ulcera[26,27]. Margolis and others demonstrated, in a large cohort of patients, that the application of the substances released from platelets was more effective than standard care methods in wound healing. The treatment was even more effective in patients with deeper wounds[28]. Another interesting finding in one study was the effect of PG on the reduction of pain, an effect which is still not understood[29]. In conclusion, there is sound evidence indicating that the use of PG in patients with chronic non-healing wounds can be useful and there is now a need to conduct clinical trials to study its effect on wound rehabilitation and earlier functional recovery in different surgical procedures.

Platelets and PG in bone healing

Bone is defined as a biological tissue composed of dynamically active cells which are integrated into a rigid framework. Bone cells consist of osteoblasts, osteoclasts, osteocytes, osteoprogenitor cells and hematopoetic components[30]. The bone healing process, whether in fracture repair or any given fusion model, is a delicate balance between bone deposition, resorption, and remodeling. This is influenced by numerous biochemical, biomechanical, cellular, and pathological mechanisms. During bone healing, mature bone forming osteoblasts secrete growth factors which are also present in platelets[31]. Osteoclasts, by contrast, are bone-resorbing cells, a process controlled by hormonal and cellular mechanisms. Under normal circumstances the activity of osteoblasts and osteoclasts is in balance.

In fracture repair and bone healing (i.e. callus formation) platelets act as an exogenous source of growth factors stimulating the activity of bone cells, based on their particular relevance to bone growth[32,33]. As in wound healing, bone-fracture healing also incorporates the three stages of inflammation, proliferative repair, and remodeling. At bone fracture sites, platelets release PDGF, TGF-β, and EGF, providing an ideal system for the delivery of growth

factors to the injury site. The richest source of TGF-β is found in platelets, bone, and cartilage. Two isoforms, TGF-β1 and TGF-β2, are present in the platelets. TGF-β1 has the greatest potential for bone repair since both chondrocytes and osteoblasts are enriched with receptors for TGF-β1. In fact, TGF-β may contribute to bone healing at all stages[34,35]. It has been demonstrated that with a combination of platelet growth factors TGF-β, FGF, and EGF, an optimum is created for the stimulation of differentiation and proliferation of osteoblasts to osteogenic cells[36,37]. Similarly, proliferation was increased by the mitogenic action of PDGF in mesenchymal stem cell differentiation when TGF-β and EGF was added[38].

The ability of bone to heal is based on three concepts: osteogenesis, osteoinduction and osteoconduction.

Osteogenesis is described as the ability to produce new bone and is determined by the presence of osteoprogenitor cells and osteogenic precursor cells in the area. PGF are present in three of four stages during the bone healing process[31].

Osteoinduction is defined as the ability to stimulate stem cells to differentiate into mature cells through the stimulation by local growth factors such as PDGF and TGF-β[39,40].

Osteoconduction is determined by the presence of a scaffold that allows for vascular and cellular migration and is usually achieved by the use of autologous harvested bone (autograft), homologous graft materials (allograft) or artificial matrixes like demineralized bone (DMB), hydroxyl apatite, tricalciumphosphate, and collagen[41]. In the regulation and stimulation of these biochemical and cellular processes, PDGF plays a dominant role with regard to mitogenesis, chemotaxis, and stem cell differentiation. Recently, PRP has been successfully subcutaneously applied in a diabetic femur fracture model, were it normalized the early cellular proliferation and chondrogenesis, while improving the late mechanical strength[42].

Bone grafts are widely utilized to overcome bone continuity defects and to enhance a variety of fusions. For this reason, they are often used as a supportive tool in fracture repair and for the treatment of fractures. It can be hypothesized that mixing PRP and thrombin (PG), along with sequestered autologous bone graft materials, might create a bio-engineered graft (Figure 4). The result is a bone graft enriched with a high concentration of platelets releasing growth factors. Due to the viscous nature of PG, the bone chips will stick together, thus avoiding migration of bone particles.

This may be a promising technique that could support and promote bone growth and accelerate fracture healing, particularly in patients who are at risk

of the development of *non-unions*. The mixture of PG with bone grafts might also be an attractive alternative in the treatment of fractures, spinal fusion, and in bone tissue engineering strategies.

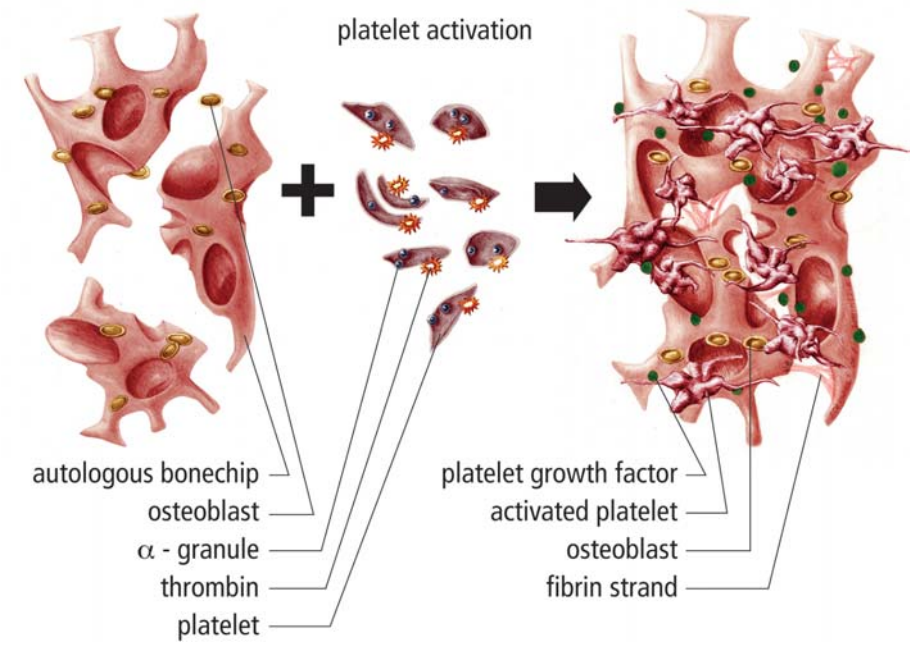

Figure 4. *Graphical representation of a "bio-engineered" bone graft with platelet gel. Sequestered autologous bone chips are mixed with platelet-rich plasma and thrombin. The result is a bone graft that is enriched with a high concentration of platelets, releasing growth factors. Due to the viscous structure of platelet gel, the bone chips will stick together, thus avoiding migration of bone particles.*

The preparation of PRP

PRP is perioperatively prepared from a unit of autologous whole blood by means of extra-corporeal blood processing techniques. PRP can be prepared either through standard blood banking techniques, or through point-of-care devices, including blood cell savers/separators or table-top devices. The preparation of PRP by blood banks, through discontinuous plasmapheresis

methods, should be limited because of higher production costs and delayed availability of PRP, when compared to bedside devices. Furthermore, blood bank prepared PRP is out of reach of the clinician and demands a highly controlled logistic system to avoid product mismatch before application to the patient.

Two different point-of-care blood centrifugation machines were introduced to the market recently that achieves optimal blood separation for the production of PRP. With cell savers/separators, larger pre-donation 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.

Device Name	Manufacturer	Characteristics	Flow	Bowl size (mL)
Brat 2	Cobe Cardiovascular Inc Arvada, CO, USA	Baylor bowl	Discontinuous	55,125,175 225,240
Compact A Electa	Sorin Group Mirandola, Italy	Latham bowl	Discontinuous	55,125,175, 225
Fresenius CATS	Fresenius Kabi AG Bad Homburg Germany	Separation chamber	Continuous	N/A
Haemonetics CS 5 Plus	Haemonetics Corporation Braintree, MS, USA	Latham bowl	Discontinuous	70,125,225
Sequestra 1000	Medtronic Inc. Minneapolis, MN, USA	Latham bowl	Discontinuous	125, 225

Table 1. *Overview of currently available cell saver/separator devices.*

Table-top centrifuges have been used to manufacture smaller volumes of PRP from lesser amounts of whole blood (50mL-150 mL). The choice for either system is mainly dependent on the type of surgical procedure and the anticipated need for the amount of PG. It seems reasonable that cell savers are used when both wound blood cell salvage and PG application are indicated. By contrast, table-top devices are used when only small amounts of PG are required during minimal blood loss surgical procedures. In Table 1, an overview of currently available cell saver/separator devices is shown, and in Table 2 an outline of table-top systems is shown.

Device Name	Manufacturer	Characteristics	Components	PRP Volume	RPM
Angel™	Sorin Group Mirandola, Italy	Variable chamber disk	RBC,PPP, PRP	5-18 mL	Max 4000
Genesis CS™	Emcyte Corporation, Ft. Myers, FL, USA	Concave Aspiration Disc	BMC, PPP, PRP	4-10 mL	2400
GPS II™	Biomet Warsaw, IN, USA	Container + buoy	PPP, PRP	5-6 mL	3200
Magellan™	Medtronic Inc Minneapolis, MN USA	Chamber	RBC, PPP, PRP	1-8 mL	Max 4000
Secquire™	PPAI Medical Fort Myers, FL, USA	Container	RBC, PPP, PRP	7 mL	3500
Symphony II™	dePuy Inc Raynham, MS, USA	Two chambers	PPP, PRP	7 mL	Fixed two step
Vivostat™	Vivolution A/S Birkeroed, Denmark	Preparation chamber	PRF, FS	5 -7 mL	N/A

Table 2. *Overview of currently available table top platelet rich plasma devices (BMC: bone marrow concentrate; FS: fibrin sealant; N/A: not applicable; PPP: platelet poor plasma; PRF: platelet rich fibrin; PRP: platelet rich plasma; RBC: red blood cells).*

PRP preparation methodology

In the clinical standard setting, blood is drawn from the median cubital vein. When a cell saver is used to manufacture PRP, autologous whole blood is collected into standard donor bags filled by gravity, not exceeding the maximum allowable pre-donation volume in relation to the citrate volume in the blood bag (Figure 5a). When table top devices are used the blood is carefully collected by aspiration techniques into syringes, avoiding "negative pulling" in order to fill the syringes quickly. The use of a needle diameter larger than 17 gauche avoids trauma to the platelets during the blood draw. The autologous pre-donated blood is collected in sufficient amounts of anticoagulation citrate dextrose-A solution (ACD-A). In general, a ratio of 1mL of ACD-A to 7-8 mL of whole blood should be maintained. The aspirated blood is gently agitated to thoroughly mix the anticoagulant with the blood.

Currently, most cell savers use a Latham (tapered) bowl, in stead of Baylor (straight) bowl, ranging in volume between 50-225 mL. Furthermore, continuous autotransfusion systems, not using a bowl, can also be used to prepare PRP.

These cell savers/separators sequester the whole blood in a semi-automatic controlled operating mode by centrifugation at 5,600 rpm, separating the platelet poor plasma (PPP) from the buffy coat layer and erythrocytes. The PPP volume is separately collected in a blood bag. Thereafter, centrifugation is slowed down to 2,400 rpm to obtain the buffy coat layer consisting of PRP and leukocytes, which is collected in a separate blood bag or syringe. After this procedure the erythrocytes are also separately collected in a blood bag. The collected PPP and erythrocytes are re-infused during surgery at a time determined by the anesthesiologist. The collected PRP is used to prepare PG for application to tissues.

With table top devices a similar protocol of a high and low speed centrifugation is followed. Depending on the brand of table top device, one may collect all blood components separately, or collect only PRP. In those cases where no re-transfusion of blood components is feasible, the PPP and erythrocytes are discarded.

Regardless of the type of PRP preparation method, the aim of working with whole blood is to prepare PRP with a platelet count in excess of the baseline platelet count values at the patient's bedside (Figure 5b).

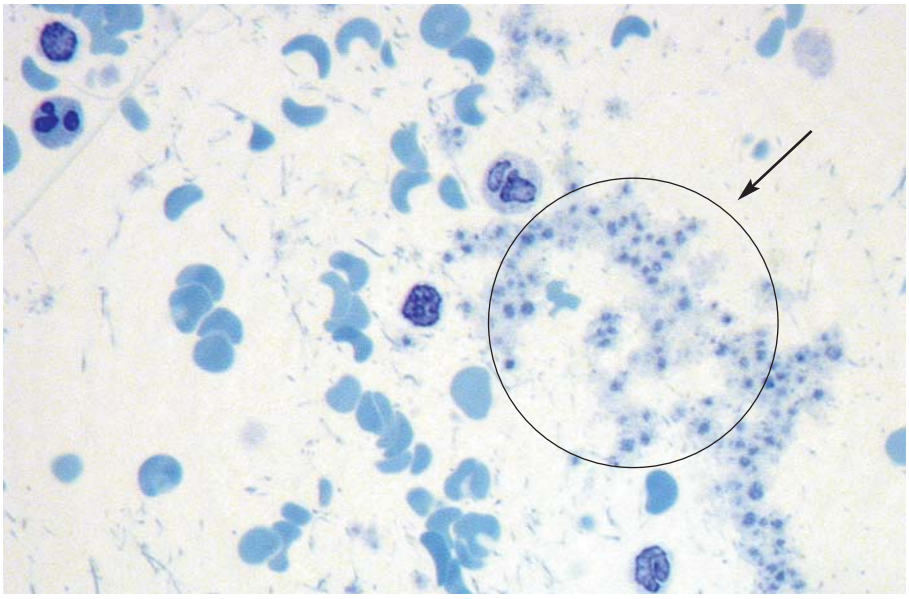

Figure 5a. *Peripheral blood smear of whole blood with a platelet count of 276.000 per µL. Inside the circle platelets are visible.*

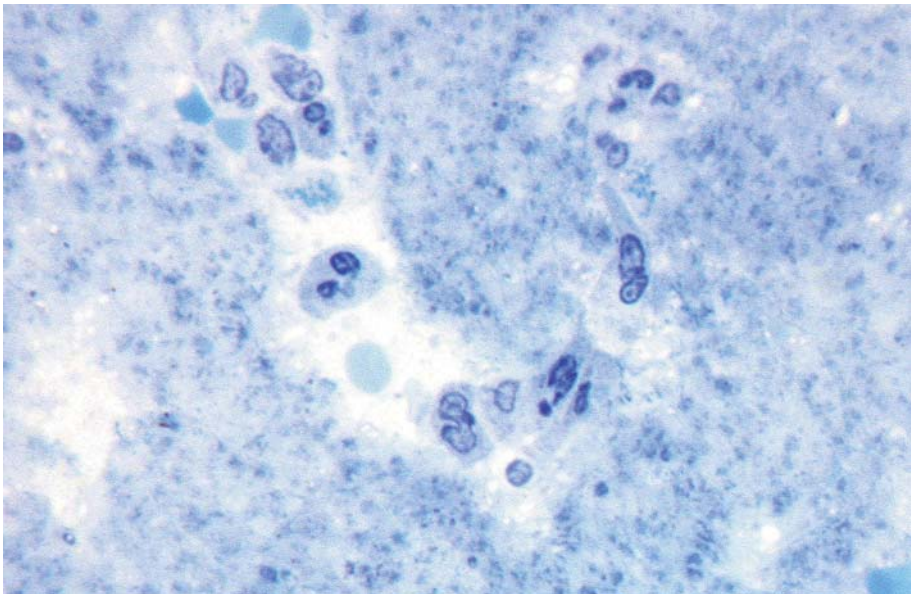

Figure 5b. *Platelet rich plasma smear. Dense platelet concentrations correlating to a platelet count of 2.750.000 per µL, in a 7 mL volume. Prepared with the Angel PRP system.*

PRP ACTIVATION

Alpha granules of the non-activated platelets in the PRP contain PGF, and are thus non-functional, since they are not released or in contact with the tissue. To initiate the release of these growth factors, platelets must be activated. Thrombin, the most potent platelet activator, will induce immediate PGF release from the PRP in a dose-dependent fashion[43,44]. In the USA, commercially available thrombin, derived from bovine plasma is used as a 'gold standard', despite the fact that bovine thrombin has been associated some years ago with the development of antibodies to clotting factors V, XI, and thrombin, which had occasionally lead to life-threatening coagulopathies[45]. Alternatively, PRP can be activated by autologous thrombin, produced with commercially available thrombin production kits, which either use autologous whole blood sequestered PPP or PRP (Table 3). Recently, Tsay *et al*.[46] reported that the use of a synthetic peptide that mimics thrombin known as peptide-6 SFLLRN (TRAP). Activation with TRAP results in a more sustained release of the PGF with less PG retraction and higher PDGF-AB and TFG-β concentrations.

The mechanism of this sustained release phenomenon is unclear, but it may possibly be useful in the development and maturation of platelet enriched bone grafts and also in tissue healing.

Autologous thrombin kit Manufacturer	Required volume. Product	Thrombin volume	Activator Reagent	Thrombin Activity	Ratio AT:PRP
ActivAT™ Sorin Group, Mirandola Italy	12 mL PPP	5 – 6 mL	ethanol 17%, glass beads calcium chloride 10%	40-90 IU	1:10
Magellan™ Medtronic, Minneapolis, MN, USA	3 mL WB	2,5 mL	glass fiber calcium chloride 10%	10 – 15 IU	1:4
Petri dish Catharina Hospital	variable PPP/PRP	variable	glass Petri dish calcium chloride 10%	10 – 15 IU	1:4
Thrombin Assessing Device™ Thermogenesis, Rancho Cordova, CA, USA	9,5 – 10,5 mL PPP	8 mL	ethanol 18,8%, ceramic beads calcium chloride 10%	40 – 50 IU	1:3

Table 3. *Autologous Thrombin Processing Kits.*
The ratio AT:PRP refers to the manufacturer's proposed ratio for mixing PRP with thrombin to produce PG. (AT: autologous thrombin; IU: international units; PG: platelet gel; PPP: platelet poor plasma; PRP: platelet rich plasma; WB: whole blood).

Mixing PRP with thrombin and calcium chloride, to antagonize the anti-coagulative effect of the citrate present in the pre-donation blood bag, will result in the activation of the platelet concentrate with the development the viscous PG solution. Thereafter, the PG can be exogenously applied with a syringe or as a solid clotted jelly mass applied to soft tissues, bone or synthetic bone.

From a surgical point of view, an "ideal" PG procedure is often defined as a procedure forming a platelet coagulum within 10 seconds. However, the formation of the coagulum is merely a function of the activated fibrinogen concentration, rather that the number of platelets.

Platelet Gel growth factors

The PGF of the PG are peptides that promote cell proliferation, differentiation, and chemotaxis inducing the migration of various cells. Therefore, they play an important role in healing processes, as demonstrated in several studies[47,48]. We can classify growth factors into two groups, morphometric and mitogenic. The morphometric growth factors, involved in bone growth, can turn undifferentiated multipotent mesenchymal stem cells (MSC) into immature and mature osteoprogenitor cells through the presence of the so called bone morphogenic proteins (BMP)[49]. These BMP growth factors belong to the TGF-β super family, a growth factor which is also present in PRP.

Most of the PGF in the PRP have mitogenic actions which increase the population of healing cells by mitogenesis. However, the action depends on the presence of further differentiated MSC.

PGF receptor binding

After PG has been applied to tissues and the clot has retracted and degranulated, PGF will be deposited in the extracellular matrix. Thereafter, during matrix degradation, growth factors are released that interact and bind with the platelet tyrosine kinase receptor (TKR), present in the cell membranes of tissue cells (ligand-receptor interaction). The actual binding site is on the outer surface of the cell membrane. The TKR is a membrane spanning protein that extends into the cytoplasm of the cell. After growth factor interaction with the external part of the TKR, activation of (inactive) messenger proteins in the cytoplasm occurs. The activated TKR cytoplasmic tail now serves as a binding site for the messenger proteins. An activated protein is generated via a signaling cascade that is capable of entering the cell nucleus where it triggers the genes responsible for controlling cell division. Subsequently, transcription of messenger RNA is induced, producing a biological response that initiates the cascades that induce tissue repair and regeneration (Figure 6)[50,51].

Due to the unique modes of action, growth factors are capable of inducing effects on multiple cell types, and may therefore provoke a series of cellular functions in different tissues[52,53].

The next paragraph gives some background information on two of the most well described platelet growth factors, and on a more recent evaluated growth factor present in PRP. In Table 4, a synopsis of PRP growth factors is provided, along with a description of the growth factor source and their specific function[16,54-63].

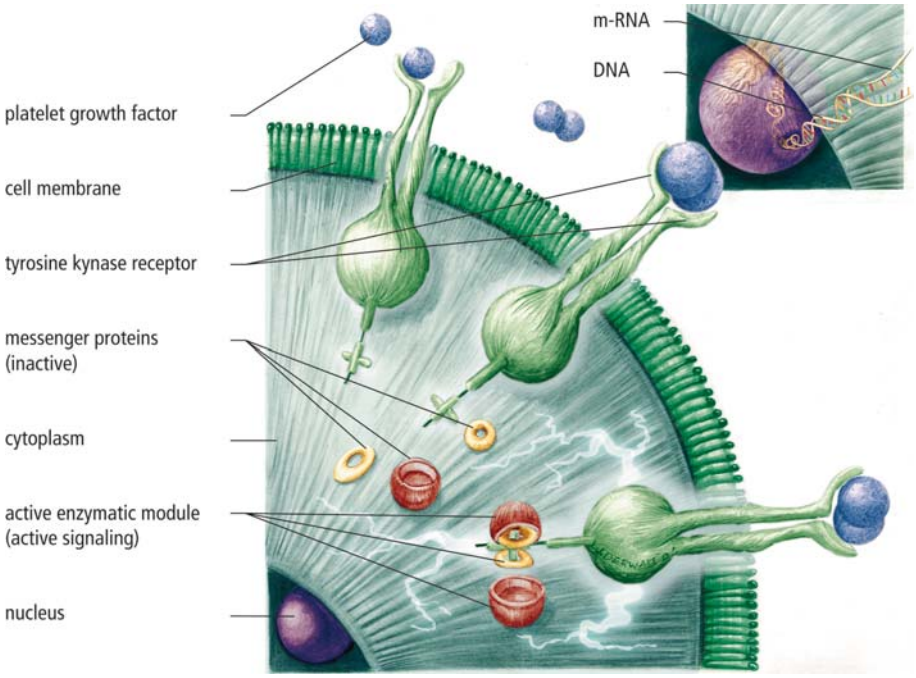

Figure 6. *Diagram demonstrating the mechanism by which platelet growth factors binds to the tyrosine kinase receptor. Ultimately, extra cellular platelet growth factor-receptor binding results in intracellular signaling and transmission to the cell nucleus. (DNA: Deoxyribo Nucleic Acid; mRNA: messenger Ribo Nucleic Acid).*

Platelet-derived growth factor, PDGF

PDGF is a glycoprotein with a molecular weight of approximately 30 kD, with two disulphide-bonded polypeptides, referred to as A and B chains. There are three isoforms, PDGF-AA, -BB and -AB[57,59]. PDGF is not only found in the dense α-granules of the platelet but is also synthesized and secreted by macrophages and the endothelium. PDGF appears to be the first growth factor present in a wound and initiates connective tissue healing through the promotion of collagen and protein synthesis. Furthermore, PDGF enhances the proliferation of bone cells and increases bone regeneration through osteoblastic mitogenesis. After the adhesion of PG to wound sites, PDGF will emerge from the degranulating platelets nd receptor activation will be initiated as described above[52,64,65]. The most specific function of PDGF includes mitogenesis (attraction of cells to the wound site), angiogenesis (endothelial mitosis into

functioning capillaries), macrophage activation (biological wound debridement, and they act as a secondary source of growth factors). Bowen-Pope *et al.*, studied the production of PDGF and concluded that there are approximately 0.06 nanograms of PDGF per 10^6 platelets, or about 1200 molecules per platelet[66]. Therefore, one might assume that PG with a platelet count in excess of 3 to 5 fold the baseline level would have a profound effect on both wound healing and bone regeneration.

Transforming growth factor beta, TGF-β

TGF-β is the name given to a group of proteins with a molecular weight of approximately 25 kD. They are involved in the formation and development of many tissues[67]. TGF-β is part of a super family to which BMP also belongs. In humans, three subtypes of TGF- β are present, but TGF-β_1 and TGF-β_2 appear to be the most important with regard to general connective tissue repair and bone regeneration[68,69]. TGF-β is found predominantly in platelets which account for 95% of the total, while some is also found in macrophages, in a latent form. TGF-β has an inhibitory effect on cell growth of many tissues, except for MSC where proliferation is enhanced. The other functions of TGF-β are to promote chemotaxis and mitogenesis of fibroblasts and osteoblastic precursor cells. Later, they will differentiate into mature osteoblasts, and they also stimulate osteoblasts deposition at the collagen matrix of the tissue wound-healing and bone matrix regions[70]. TGF-β acts both in an autocrine and paracrine fashion, making it a growth factor with long term healing and bone regeneration capabilities[71]. Some concern on the use of TGF-β has been muted by Dieudonne and co-workers, who studied its effect of on osteoclastic bone resorption in an experimental setting. They concluded that low concentrations have a stimulatory effect on bone cell proliferation, whereas at higher concentrations proliferation is suppressed[72].

In PRP, both PDGF and TGF-β are present, implying that a mixture of combinations of growth factors will always be present at tissue sites. This unavoidable effect appears to be beneficial towards tissue healing since various results are reported on the synergistic effect of different growth factors[23,24,50].

Connective tissue growth factor, CTGF

Very recently, Kubota and others described a new PGF known as CTGF[62]. Platelets adhere to CTGF at injured tissue wound sites, where it is over-expressed 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. It was also demonstrated that the CTGF content

in platelets is more than 20-fold higher than any other PGF and that CTGF endorses angiogenetic activity, cartilage regeneration and fibrosis. 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[63]. CTGF might be considered as an important member of the PGF family.

PG STUDIES

Animal studies

There is a large variety of animal studies on PG research in the literature. Table 5 shows some of the more recent experimental studies[2, 73-94]. The results tend to be confusing and the reader might conclude that the animal data on PG studies is conflicting. One concern is that a variety of different animal species has been used and often no information of platelet counts or growth factor numbers in the PRP is provided. Furthermore, methods describing the PRP production are sometimes lacking. Some investigators even used damaged platelets, whereas others did not activate the PRP at all, as most clinicians would do in a clinical

Growth Factor	Source
Transforming Growth Factor-bèta, TGF-β	Platelets, extracellular matrix of bone, cartilage matrix, activated TH1 cells and natural killer cells, macrophages/monocytes and neutrophils
Basic Fibroblast Growth Factor, bFGF	Platelets, macrophages, mesenchymal cells, chondrocytes, osteoblasts
Platelet Derived Growth Factor, PDGFa-b	Platelets, osteoblasts, endothelial cells, macrophages, monocytes, smooth muscle cells
Epidermal Growth Factor, EGF	Platelets, macrophages, monocytes
Vascular endothelial growth factor, VEGF	Platelets, endothelial cells
Connective tissue growth factor, CTGF	Platelets through endocytosis from extracellular environment in bone marrow.

Table 4. *Overview of platelet growth factors, their source, and specific function.*

setting in order to release PGF. Also, "true" autologous PRP is not always achieved in small animals.

It is therefore not surprising that Ranly and co-workers observed a reduced osteoinductivity when human PRP in combination with demineralized bone was mixed and implanted in mice[93].

In summary, the different protocols used in these studies make it difficult to draw conclusions. Therefore, the "no" PG treatment effect and negative outcome following the use of PG in animal studies should be interpreted with caution.

Human clinical studies

Autologous PRP was first used in cardiac surgery by Ferrari *et al*. in 1987, as an autologous transfusion component after an open heart operation, in order to avoid homologous blood product transfusion[95]. Later, in the early 1990's PG was used as a by-product of the sequestration procedure, as an alternative to fibrin sealant, for the control of hemostasis in cardiac surgery[96, 97]. Since that time, an increasing number of institutions have used PG for optimization of soft tissue healing and bone regeneration. However, many case reports or small uncontrolled cases have been presented but only a few have been published[1, 3, 98]. The majority of these clinical studies demonstrated a significantly improved effect on soft tissue healing and bone regeneration when PG was used. Strikingly, in most studies

Function	Ref.
Stimulates undifferentiated mesenchymal cell proliferation; regulates endothelial, fibroblastic and osteoblastic mitogenesis; regulates collagen synthesis and collagenase secretion; regulates mitogenic effects of other growth factors; stimulates endothelial chemotaxis and angiogenesis; inhibits macrophage and lymphocyte proliferation	16,53
Promotes growth and differentiation of chondrocytes and osteoblasts; mitogenetic for mesenchymal cells, chondrocytes and osteoblasts	54,55
Mitogenetic for mesenchymal cells and osteoblasts; stimulates chemotaxis and mitogenesis in fibroblast/glial/smooth muscle cells; regulates collagenase secretion and collagen synthesis; stimulates macrophage and neutrophil chemotaxis	16,56
Stimulates endothelial chemotaxis/angiogenesis; regulates collagenase secretion; stimulates epithelial/mesenchymal mitogenesis	57,58
Increases angiogenesis and vessel permeability, stimulates mitogenesis for endothelial cells	59,60
Promotes angiogenesis, cartilage regeneration, fibrosis and platelet adhesion	61,62

data were obtained in oral and maxillofacial surgery, wound care, and cosmetic surgery, mainly because of the availability of histological specimens under these treatment conditions. Advocates of PG cite that it has a beneficial effect on tissue healing and bone growth, and appears to reduce the incidence of infections and postoperative blood loss[28,99-104]. Nevertheless, there are also clinical and experimental data that do not show any effect of PG applications. In Table 6, we summarize a series of 28 clinical human *in-vivo* studies

Authors, ref.	Year	Study animal	Medical area	N =	Outcome Effect
Kim, 73	01	rabbit	M-F: bone	20	+
Kim, 74	02	dog	M-F: peri-implant	12	+
Aghaloo, 2	02	rabbit	cranial: defect	15	-
Fennis, 75	02	goat	M-F: bone	28	+
Kim,76	02	dog	M-F: bone defect	12	
Furst, 77	03	mini pig	M-F: sinus graft	12	+/-
Jakse, 78	03	sheep	M-F: bone	12	+/-
Schlegel, 79	03	pig	Bone implants	15	+/-
Zechner, 80	03	mini pig	Dental implants	12	+
Aghaloo, 81	04	rabbit	Cranial: defect	15	+/-
Choi, 82	04	dog	M-F: soft tissue	8	-
Fennis, 83	04	goat	M-F: bone	28	+
Li, 84	04	pig	SS: bone	10	-
Yazawa, 85	04	rabbit	M-F: bone	10	+
Weibrich, 86	04	rabbit	M-F: bone	24	+
Aghaloo, 87	05	rabbit	Cranial: defect	15	-
Butterfield, 88	05	rabbit	M-F: sinus	12	-
Fennis, 89	05	goat	M-F: bone	6	+
Grageda, 90	05	sheep	M-F: bone	10	-
Kovacs, 91	05	dog	M-F:bone	10	+
Pryor, 92	05	rat	M-F: bone	30	-
Ranly, 93	05	mouse	OS:muscle	30	-
Scalani, 94	05	rabbit	WC: implants	?	+

Table 5. *Summary of animal studies with the use of autologous platelet gel. (M-F: maxillo-facial surgery; OS: orthopedic surgery; SS: spinal surgery; WC: wound care; +: authors conclude a positive effect of PG treatment; +/-, - means respectively a positive, doubtful and negative effect of platelet gel treatment; ?: animal numbers not mentioned)*

Authors, ref.	Year	Study type	Medical field	Patients in study	Outcome Effect
Knighton, 105	90	PR	WC	32	+
Marx, 99	98	PR	M-F	88	+
Anitua, 106	99	case	M-F	20	+
Lowery, 107	99	R-case	SS *	19	+/-
Anitua, 98	01	case	M-F	3	-
Blumenkranz, 108	01	case	ES	121	+
Man, 100	01	case	CS	20	+
Margolis, 28	01	R-case	WC	26.599	+
Petrungaro, 109	01	case	M-F	3	+
Powell, 110	01	PR-B	CS	8	+
Shanaman, 3	01	case	M-F	3	-
Adler, 111	02	case	CS	20	+
Froum, 1	02	case	M-F	3	-
Robiony, 102	02	case	M-F	5	+
Valbonesi, 101	02	case	CS	14	+
Weiner, 4	03	case	SS *	57	-
Castro, 112	04	P-contr	SS *	84	-
Crovetti, 29	04	case	WC	24	+
Giannini, 113	04	case-contr	M-F	5	+
Mazzucco, 27	04	case	WC	22	+
Camargo, 114	05	PR	M-F	18	+
Carreon, 5	05	R-contr	SS *	152	-
Englert, 103	05	R	CTS	30	+
Merkx, 115	05	case	M-F	8	+
Kassolis, 116	05	P-contr-B	M-F	10	+
Savarino, 117	05	PR	OS	10	+
Steigman, 118	05	case-contr	M-F	20	+
Everts, 104	06	PR	OS	164	+

Table 6. *Summary of clinical studies with the use of autologous platelet gel. (PR: prospective randomized; case: consecutive cases; R-case: retrospective case study consecutive cases; PR-B: prospective randomized blinded; case-contr: case study with patient being his/her own control; P-contr: prospective study with controls; R-contr: retrospective study with control patients; P-control-B: prospective consecutive study, single blinded. CS: cosmetic surgery; CTS: cardio thoracic surgery; ES: eye surgery; M-F: maxillo-facial surgery; OS: orthopedic surgery; SS: spinal surgery; WC: wound care. +, +/-, - means respectively a positive, doubtful and negative effect of PG treatment).*

concerning autologous PG application that have been published in peer reviewed journals[1,3-5,27-29,98-118]. However, we excluded abstracts presented at meetings, data obtained from *in-vitro* studies, and results obtained with recombinant (single) growth factors. In seven studies no positive effect of PG was demonstrated. Three of those seven studies were in the maxillofacial surgery field, including a total of only nine patients (3 patients per study).

In the study by Froum *et al.*, the results obtained were from only 3 patients. Moreover, they all were treated with different bone graft materials and synthetic membranes in combination with PG[1]. Shanaman *et al.*, also included only 3 patients in their study, with no statistical analysis possible[3].

Furthermore, the conclusions drawn by these authors are only based on very limited data. The four other studies (*) were conducted as spine surgery, where the PRP was concentrated with a so-called autologous growth factor filter (AGF filter™ Biomet, Warsaw IN, USA)[4,5,107,112]. Kevy *et al.*, observed in an *in-vitro* study that the use of the AGF filter resulted in a significant activation of platelets in the concentrated PRP, and in an unintentional release of PGF before the PG was applied to the tissue. They concluded that platelets were fragmented and bound to the hollow fibers due to repetitive passage of the PRP through the micro porous fiber of the AGF filter[119]. Therefore, the end product of the AGF filter is merely a platelet releasate, rather than a viable PRP product. Normally, PRP contains non-activated platelets until the moment of platelet activation and subsequent tissue application.

It is of concern that based on these considerations, several authors review the results of PRP and PG applications, in human clinical and animal outcome data, side-by-side[120-122]. From a scientific point of view, human and animal trials should to be discussed and reviewed separately. Thus, any conclusions drawn from these reviews, in which human and animal results are combined, should be addressed with caution, especially since there are often no growth factor analysis determination kits available for some animal species. The differences in results obtained in humans versus animals, may therefore be due to the great dissimilarity in species, since PG is a very sensitive autologous biological product and demands specific tissue receptor cells.

What quantities of platelets are required to achieve a positive effect following PG application?

The question of the actual quantity of platelets required is often put forward by clinicians who need to know the minimal therapeutic PRP platelet concentration

that would result in a significant improved outcome when PG is used, compared to standard treatments. At present, not much data is available to answer this question directly, and only indirect information exists. In 1998, Marx and co-workers performed the first study demonstrating a significant improvement in mandibular continuity defects when PRP was mixed with autogenous bone grafts[99]. Their PRP contained a 3 to 4 times higher platelet count when compared to baseline values, although the average PRP platelet count found in their patients was just below 8 x 10^5/µL, a number which is lower than in most other studies. Nevertheless, they observed a significantly faster radiographic maturation and histo-morphometrically denser bone regeneration. Nowadays, the latest separation devices produce PRP platelet counts in excess of 6 to 10 times the baseline platelet count values. Manufacturers tend to interpret a high platelet concentration as a quality performance indicator of their separation devices, regardless of the fact that these high concentrations may not be necessary, or might even contribute to a negative outcome. Weibrich *et al*., observed an advantageous effect with platelet concentrations of approximately 10^6/µL. Furthermore, they state that higher concentrations might have a paradoxically inhibitory effect[86].

Haynesworth *et al.*, studied the response of PRP on cellular mechanisms of adult human mesenchymal stem cells (ahMSC) *in-vitro*[123]. In soft tissue and bone healing, ahMSC are essential components for the repair processes[124,125]. It was shown that release of PRP growth factors stimulates the migration and proliferation of ahMSCs, in a PRP concentration dependent manner. A significant cellular response occurred with a 4 to 5 fold increase of platelet count, when compared to the baseline platelet count. In another study, Liu *et al.*, showed that the fibroblast proliferation and type I collagen production were augmented by a 4 to 5 fold increase in the PRP platelet count[126].

With these studies it was shown that a PRP platelet count of approximately 10^6/µL is likely to be in the therapeutically effective range. A PRP platelet count with a 4-5 times higher baseline value, appears to be adequate to achieve a significant outcome following PG application, since in most patients a whole blood platelet count between 1.5-3x10^5 µ/L is found.

Safety issues

Patients, who are considered to be candidates for a PG application, must undergo a minor hematological evaluation to exclude blood disorders or platelet dysfunction. The authors feel that the following are relative contra-indications

for PG application: a platelet count less than 10^5 µ/L; a hemoglobin level less than 10 g/dL; presence of a tumor in the wound bed or metastatic disease; and active infections. PRP preparation and PG production is safely executed by certified perfusionists or other health care professionals who have been trained in proper aseptic pheresis and transfusion techniques, complying with generally accepted safety requirements. Any concerns of immunogenic reactions or disease transmission such as HIV and hepatitis which exist with homologous blood products, are eliminated since PRP is produced from autologous blood.

As discussed earlier, the use of bovine thrombin should be reduced, or even better, eliminated, since there are high quality, and safer, alternatives available for activating PRP.

To our knowledge, no wound infections after PG applications have been reported, although the preparation of PG demands many processing steps, and thus theoretically there is the possibility of contamination[119].

Some of the commercial available autologous thrombin kits require the use of ethanol. The safety of using a small amount of ethanol in the PG on nerves was studied in an animal model by de Somer *et al.*[127]. It was concluded that the myelin sheaths were normal in appearance, with no axonal swelling and no collagen necrosis due to the ethanol.

Despite the fact that PGF have mitogenic properties, there is no evidence that these growth factors promote tumor growth, or that they are involved in carcinogenesis[128,129]. Furthermore, Scott and Pawson showed that growth factors act on cell membranes and not on the cell nucleus, and that PGF activate normal, rather than abnormal, gene expression[130]. However, the effect of PG during tumor surgery should be investigated before using it under these circumstances.

Conclusions

Platelets are unique blood elements initiating hemostasis and healing processes. Therefore, the potential of autologous PG growth factor applications are numerous. PRP contains a high concentration of platelets which can be activated to form PG and to release PGF for therapeutic use. Data from human and animal studies provides both direct and indirect evidence that PGF plays a considerable role in tissue regenerative processes. Nevertheless, some uncertainty is present and some clinicians remain skeptical of the clinical benefits of PG and are uncertain about the ideal biological setting (e.g. percentage of vital bone cells, volume of PRP etc.) for the application of the PG. Therefore, randomized

controlled trials are required to investigate the potential of PG applications, and to provide data for sound clinical decision making in the near future.

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