



UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in
Scienze Fisiopatologiche, Neuropsicobiologiche e Assistenziali del Ciclo
della Vita
Ciclo XXVIII

Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche
MED/28

Platelet concentrate and tissue regeneration: does it work? A pre-clinical study

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Anno Accademico 2014 / 2015

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Abstract

During the last decades, the combination of cellular and molecular biology and mechanical engineering, has stimulated the development of new multidisciplinary fields in biomedical sciences, such as tissue engineering and regenerative medicine. The primary goal of these approaches is to provide an alternative to organ and tissue transplantation, by creating viable substitutes for damaged or diseased organs and tissues.

Tissue healing is a complex process that involves a cascade of cellular and molecular events, that are mostly shared by the different tissues of the body. Interestingly, the tissue repair process initiates immediately after a traumatic injury and is mediated and controlled by a wide range of cytokines, proteins and growth factors released from platelets upon activation. Consequently, many growth factors have been considered as therapeutic molecules for the repair or regeneration of a wide range of tissues. Although their role has been only partially elucidated, the potential benefit of most growth factors has been demonstrated. In the last few years, the development of platelet-rich preparations has revolutionized the field of regenerative medicine, due to the repair capacities of the platelet-released growth factors, that stimulate and accelerate both soft and hard tissue healing and regeneration. Today, platelet concentrates are used in a wide range of disciplines such as dentistry, oral surgery, orthopedics, sport medicine, dermatology, and plastic and reconstructive surgery. Currently, different techniques to obtain these hemocomponents are available, each leading to preparations that may differ in platelet concentration, leukocyte and fibrin content. Among them, there is Plasma Rich in Growth Factors (PRGF), a preparation rich in platelets, easily obtained from a small volume of patient's blood, which, being autologous, does not promote any immune reaction and infectious disease transmission. Additionally, leukocytes are excluded from PRGF, so as to avoid any pro-inflammatory effects. Despite the growing craze for platelet concentrates technology, the scientific literature reported controversial results regarding the beneficial effect of these preparations on tissue healing, especially for bone regeneration.

Considering all this information, the aim of our research was to develop a study model in order to gain more information about the biological effect of a platelet concentrate on both soft and hard tissue regeneration.

For this purpose, human adipose-derived stem cells (hASCs), human osteoblasts (hObs) and human dermal fibroblasts (hDFs) cultured in the presence of PRGF, were studied. Usually, cells used for human therapy are expanded in fetal bovine serum (FBS), but this way carries the risk of potential immunogenic residual bovine proteins exposure and possible contamination with infectious agents, likely generating immune responses in patients. Therefore, in this study, FBS was completely substituted by the platelet concentrate for the cells culture. Interestingly, PRGF never affected cell viability and, after a short adaptation period, both hASCs and hDFs grown in the presence of PRGF, increased their proliferation rate compared to standard culture condition, while hObs growth was evident only without any cell's detachment. Furthermore, neither donor nor cell population effect on both cell viability and proliferation was observed. Regarding osteo-differentiation, alkaline phosphatase (ALP) activity was induced in both hASCs and hObs, with a peak at day 7 and 14, respectively.

In parallel, in order to elucidate the clinical benefit of platelet concentrates in hard tissue repair, the combination of a bone graft substitute with PRGF in enhancing a bone defect regeneration performed in a preclinical animal model, was also investigated. In particular, bilateral circular critical lesions were created in the proximal tibia of six New Zealand rabbits, for a total of 12 defects, filled with the bone substitute alone or in association with PRGF. Untreated defects were also included as control. No side-effects have been observed during the 8 weeks follow-up. Both histological and histomorphometric analysis showed a non-significant difference between treatment groups in terms of bone density, even if the addition of PRGF to the bone substitute seemed to induce a higher percentage of newly formed bone.

In conclusion, this *in vitro* and *in vivo* study provided the evidence of the beneficial effect of platelet concentrates in both soft and hard tissue regeneration, thus supporting the application of this technology in clinical regenerative therapies.

Introduction

1. Platelets

Platelets, also called thrombocytes, form in the bone marrow and are cytoplasmic fragments of megakaryocytes with a discoid shape that range from 1 to 3 μm in diameter. These small cells lack a nucleus, but contain a highly organized cytoskeleton, an unique receptor and specialized secretory granules (α , β and γ) [1]. Approximately 140,000 to 400,000 platelets/ mm^3 circulate into the human bloodstream for 7-10 days before removal by macrophages of the reticuloendothelial system [2].

1.1. Megakaryocyte development

Megakaryocytes (MKs) are platelet progenitor cells and the final stage of megakaryocyte development is the platelet production. Megakaryocytes are the largest (50-100 μm) and one of the rarest myeloid cells (~0.01% of nucleated bone marrow cells) that reside primarily in the bone marrow, but are also found in the lung, the peripheral blood and during early mammalian development in the yolk sac, fetal liver and spleen [3]. They are highly specialized precursor cells that possess the role of producing and releasing platelets into the circulation and maintaining appropriate circulating platelets levels [4]. Megakaryocytes develop from pluripotent hematopoietic stem cells that give rise to two types of precursors: burst-forming cells (BFU-Meg) and colony-forming cells (CFU-Meg), both of them expressing the CD34 antigen [5]. The promegakaryoblast is the first morphologically recognizable megakaryocyte precursor in bone marrow, while the megakaryoblast (or stage I megakaryocyte) is a more mature cell with a distinct morphology, 15 to 50 μm in diameter, large kidney-shaped nucleus that has two sets of chromosomes (4N) and a basophilic cytoplasm lacking granules [4]. The promegakaryocyte (or stage II megakaryocyte) is 20 to 80 μm in diameter and has a polychromatic cytoplasm less basophilic also containing developing granules. The development of cells culminates in the formation of megakaryocyte (Figure 1). The principal cytokine required for megakaryocyte maturation is the thrombopoietin (TPO), also known

as the primary regulator of thrombopoiesis [6], that acts in conjunction with Interleukins (IL-3, IL-6 and IL-11) and stem cell factor (SCF; kit ligand) [7].

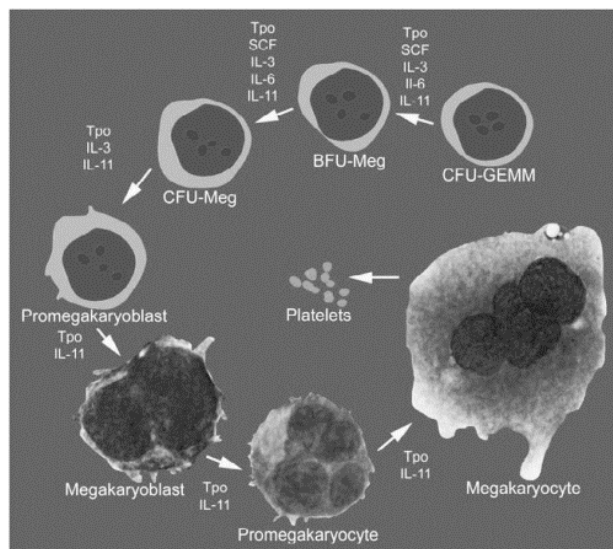


Figure 1. Megakaryocyte maturation and development. Adapted from Platelet, Second Edition, Alan D. Michelson.

Megakaryocytes undergo multiple DNA replications without cell division during endomitosis, a TPO-dependent process by which MKs become polyploidy [8]. An additional maturation event that occurs after the endomitosis completion is the cytoplasmic expansion phase, in which cytoplasm rapidly fills with platelet-specific proteins and organelles, and an elaborated membranous network of cisternae and tubules, called the demarcation membrane system (DMS) in continuity with the plasma membrane, is formed [9]. The DMS is currently known to function as a membrane reservoir for proplatelets formation, the precursors of platelets [10, 11].

1.2. Platelet formation

The final stage of megakaryocytopoiesis is the production of mature MKs able to release sufficient functional platelets to maintain hemostasis. Despite it has been well accepted the importance of platelets and that they derive from megakaryocytes, the mechanism by which platelets are assembled by their precursors is still controversial. To explain how platelets form and release from MKs, three models have been proposed. The first is *cytoplasmic fragmentation via the DMS* and predicts that DMS demarcates predetermined platelet-size fields within the megakaryocyte cytoplasm and that the fragmentation of

cytoplasm along these fracture lines releases platelets [12]. The second model is *platelet budding* from megakaryocytes surface in which platelets pinch off from blebs localized at periphery of the megakaryocyte cytoplasm [13]. The third is *proplatelet formation* and according to this model mature MKs extend long, branching processes called proplatelets into the sinusoidal blood vessels of the bone marrow [14]. Platelets assemble and package their organelles *de novo* within proplatelets and the intracellular components of platelets are sent from their synthesis sites in the MK cell body to the proplatelets. Individual platelets generate from massive fragmentation of the megakaryocyte cytoplasm along DMS fracture lines residing between these fields (Figure 2a). The extruded multi-lobed nucleus remains in the MK cell body, as the rest of the MK is transformed into proplatelets, and undergoes degradation by apoptosis (Figure 2b) [1].

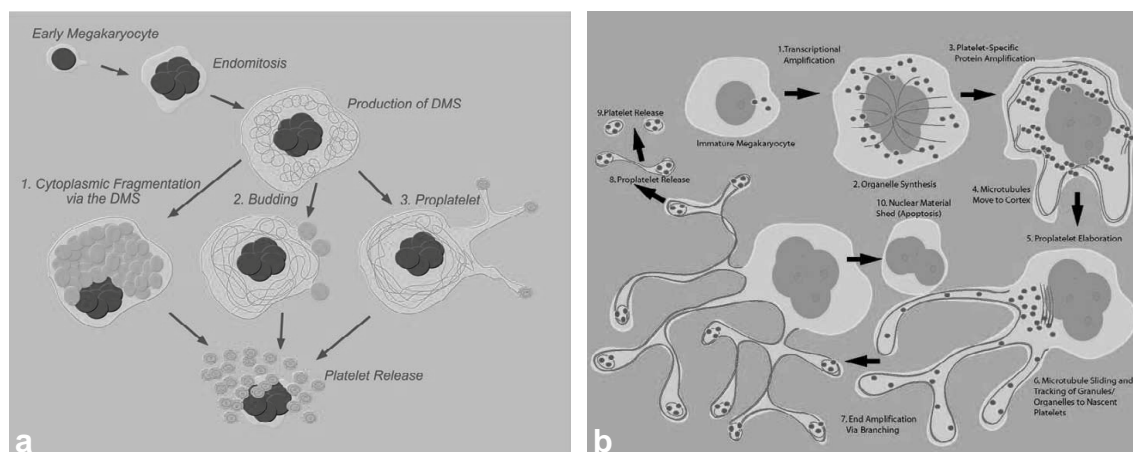


Figure 2. (a) Mechanisms of platelet formation. (b) Cytoskeletal mechanics of platelet biogenesis detail in proplatelet model. Adapted from Platelet, Second Edition, Alan D. Michelson.

1.3. Platelet structure

The platelet plasma membrane has a fine, rugose appearance (Figure 3). These gyri and sulci may provide additional membrane needed when platelet spread on surfaces [15]. Small openings of the surface connected canalicular system (SCCS) are randomly dispersed on the platelet exterior surface connecting the channels and the surface membrane.

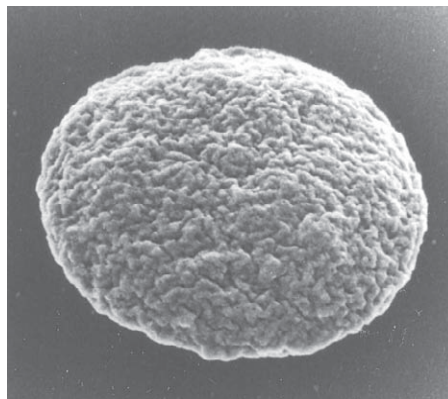


Figure 3. Discoid platelet (magnification x30.000). Adapted from Platelet, Second Edition, Alan D. Michelson.

The platelet plasma membrane has a thicker exterior coat, called glycocalyx, covered with major and minor glycoprotein receptors necessary to facilitate the first contact during the platelet hemostatic response at sites of vessel injury [16-19]. The lipid bilayer of the peripheral zone on which the glycocalyx rests is incompressible and cannot stretch [20]. The submembrane area contains a relatively regular system of thin filaments similar to actin ones that play an important role in the shape change and receptor and particles translocation over the exterior cell surface [21]. In the platelet cytoplasm can be identified small numbers of relatively simple mitochondria that are essential in energy metabolism.

Platelets contain three major types of secretory organelles - α granules, dense bodies (δ granules) and lysosomes - and occasionally multivesicular bodies that serve as sorting stations in the development of such granules [22]. These granules types contain a number of proteins with autocrine and paracrine functions, including adhesive proteins, coagulation factors, chemokines, cytokines, growth factors, proteoglycans, immunoglobulins, proteases and protease inhibitors [23].

- α Granules are the most abundant platelet organelles and their number depends on the platelet size and the presence of other structure that occupy the space. There are approximately 40 to 80 α granules per platelet, but larger and giant cells may contain over 100, ranging from 200 to 500nm in diameter and round to oval shape [24]. α Granules interior structure is divided into zones. They include: the peripheral membrane of the granule, the electron dense nucleoid containing chemokines and proteoglycan, a less electron dense area

near the nucleoid with fibrinogen, and a peripheral electronluscant zone that contains von Willebrand factor [25]. Their function derives from their content that includes both membrane bound proteins then expressed on the platelet surface and soluble proteins released into the extracellular space (Table 1) [26].

- Dense bodies are smaller than α granules, fewer in number (about 1 to 1.4 dense body per platelet) and with a high morphological variability [26]. Some of them have an irregular form, filaments that extend from the dense inner core to the enclosing membrane and contain a granule-like substance that fill the empty space otherwise unfilled (Table 2).

Adhesion molecules	P-Selectin, von Willebrand factor, thrombospondin, fibrinogen, integrin α IIb β 3, integrin α v β 3, fibronectin
Chemokines	Platelet basic protein [platelet factor 4 and its variant (CXCL4) and β -thromboglobulin], CCL3 (MIP-1 α), CCL5 (RANTES), CCL7 (MCP-3), CCL17, CXCL1 (growth-regulated oncogene- α), CXCL5 (ENA-78), CXCL8 (IL-8)
Coagulation pathways	Factor V, multimerin, factor VIII
Fibrinolytic pathway	α 2-Macroglobulin, plasminogen, plasminogen activator inhibitor 1
Growth and angiogenesis	Basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor 1, transforming growth factor β , vascular endothelial growth factor-A, vascular endothelial growth factor- C, platelet-derived growth factor
Immunologic molecules	β 1H Globulin, factor D, c1 inhibitor, IgG
Other proteins	Albumin, α 1-antitrypsin, Gas6, histidine-rich glycoprotein, high molecular weight kininogen, osteonectina protease nexin-II (amyloid beta-protein precursor)

Table 1. α Granules content. Adapted from Platelet, Second Edition, Alan D. Michelson.

Ions	Ca, Mg, P, pyrophosphate
Nucleotides	ATP, GTP, ADP, GDP
Membrane proteins	CD63 (granulophysin), LAMP 2
Transmitters	Serotonin

Table 2. Dense granules content. Adapted from Platelet, Second Edition, Alan D. Michelson.

- Lysosomes are fewer in number, in fact there are no more than three and usually zero to one lysosome per platelet. They are spherical in form, slightly smaller than α granules and contain at least 13 acid hydrolases, cathepsin D and E, the lysosomal membrane proteins LAMP-2 and CD63 [27-29]. The lysosomes appear to have no significant role in platelet function, but just to be vestigial remnants.

Recent proteomic studies suggested that platelets possess an extensive transcriptome and proteome in addition to prestored mediators, and although they lack a nucleus and hence genomic DNA, platelets contain messenger RNA (mRNA) and the translational machinery required for protein synthesis [30, 31].

1.4. Platelet functional role in hemostasis

The major physiological function of circulating platelets is to detect damage to the walls of blood vessels. When damage is detected they respond rapidly, hence platelets provide the first line of defense in stopping the loss of blood following vascular injury by adhering to the site of wound. In an intact system platelets circulate in a quiescent state, near the endothelial cells lining of blood vessels without forming stable adhesions, but after disruption of the integrity of the vascular tissue, platelets are exposed to damaged blood vessels and become in direct contact with subendothelial matrix proteins including von Willebrand factor (VWF), collagen, fibrinogen and fibronectin, normally covered by endothelial cells [32-35]. This interaction lead to platelets aggregation, to the change from a rounded shape to one that includes large, sticky protuberances, or pseudopodia (Figure 4) [36], and to the formation of a platelet plug that reduces or temporarily stops the loss of blood. This process is called platelet activation and represents an essential step in hemostasis.

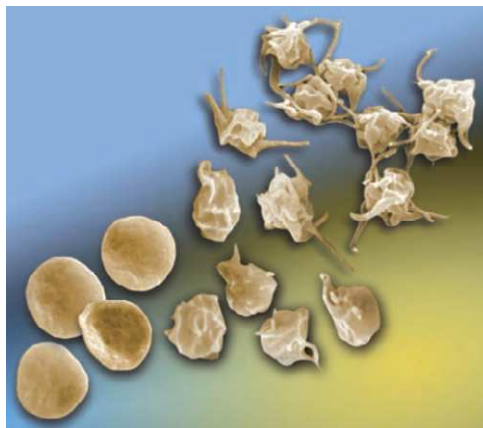


Figure 4. Platelet shape change and aggregation. Resting (left), partially activated (middle) and fully activated (right) platelets. Adapted from *Platelet*, Second Edition, Alan D. Michelson.

During activation, the α granules fuse with the platelet plasma membrane and release to the surrounding their protein and small molecules content that accelerate and increase platelet plug formation and initiate the tissue repair process [37]. Adenosine diphosphate (released by activated platelets), thrombin and adrenaline are other factors that mediate platelet activation [35]. In case of small vascular damage, this platelet plug may be able to stop blood loss, but if the defect is large, the generation of a blood clot may be necessary. The coagulation cascade is initiated by one of two pathways, called the extrinsic and intrinsic pathways [35].

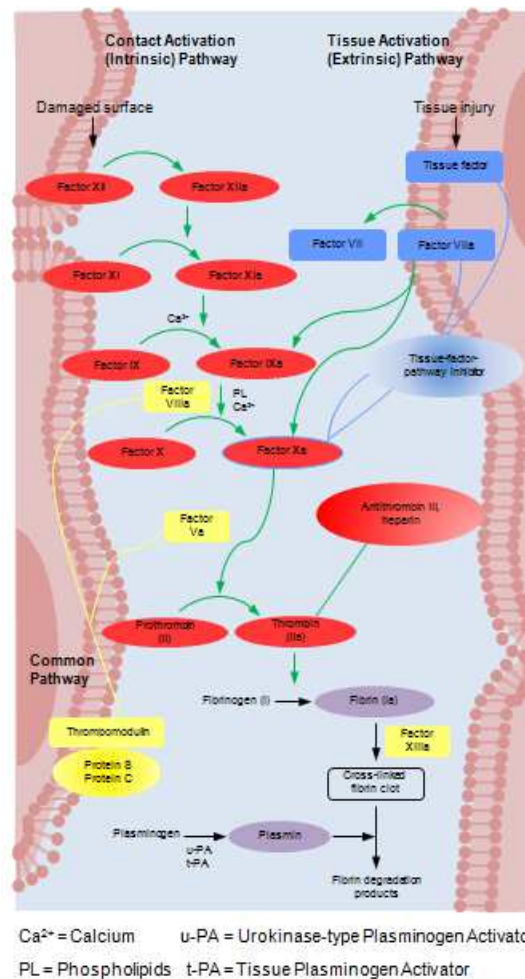


Figure 5. Coagulation cascade. The intrinsic and extrinsic pathways converge at the factor IX activation level.

The extrinsic pathway begins after the contact between a tissue factor located in the tissue adventitia and the blood after vascular injury, whereas the intrinsic pathway is initiated by damage or alteration of the blood. Despite the two pathways begin differently, they converge and share many of the latter steps in the cascade reaction (Figure 5). Both pathways involve a cascade of events by which inactive factors become active that in turn catalyze the formation of other products from precursors, leading to the final formation of a fibrin clot. Calcium ion is required for the completion of the reaction, while platelets participate at multiple levels in the fibrin threads generation and are part of the final clot which is formed by a fibrin mesh, the activated platelet aggregate, and red and white blood cells within them. To further close the blood vessel, the clot retracts by means of the platelet actin-myosin fibers contraction by 20 minutes to 1 hour after clot formation [38]. Hemostasis is also facilitated by local vasoconstriction

in response to thromboxane and serotonin release from the platelet aggregate [39].

1.5. Platelet functional role in wound healing

The repair process starts immediately after tissue damage and hemostasis can be considered to be the first stage of wound healing [40]. Traumatic injury to the skin initiates a cascade of events characterized by three overlapping stages known as inflammation, new tissue formation and tissue remodeling, that, as result, lead to at least partial reconstruction of the damaged area (Figure 6) [41]. Inflammation is the initial response to tissue injury and aims to provide a rapid hemostasis and the formation of the blood clot, as previously described. The clot has two functions: it represents a barrier that temporarily protects the denude tissue against invading microorganisms, and it works as a provisional matrix to maintain the regenerative space and provide a scaffold for cell migration and proliferation [41, 42]. Within a few hours after injury, inflammatory cells invade the clot (early stage of inflammation).

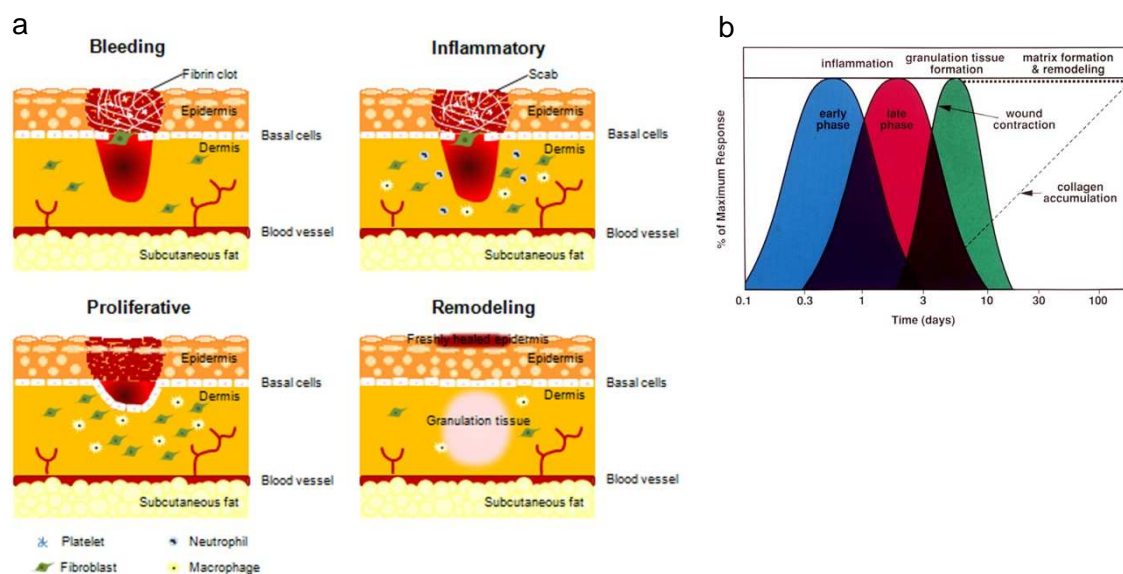


Figure 6. (a) Schematic representation of different stages of wound healing. (b) Phases of wound repair. Adapted from Polimeni *et al.*, *Periodontol 2000* 2006;41:30-47.

Neutrophils arrive first within a few minutes followed by monocytes and T lymphocytes, and clean the wound of bacteria and tissue debris through phagocytosis and the release of enzymes and reactive oxygen products. Next,

monocytes differentiate to macrophages and become the predominant cell type. Within 3 days, the inflammatory reaction moves into the late phase where macrophages migrate into the wound area, assist neutrophils in their function and secrete polypeptide mediators targeting cells involved in the wound healing process [41, 42]. Macrophages secrete also growth factors and cytokines that initiate the proliferative stage of wound repair. The latter begins with the migration and proliferation of keratinocytes and dermal fibroblasts into the wound area, where they deposit large amounts of extracellular matrix. Blood vessel endothelial cells also proliferate and form new capillaries that extend into the injured site, beginning a massive angiogenesis process [43]. The resulting connective tissue, called granulation tissue for the pink, soft, granular appearance, undergoes maturation and remodeling. During this third and final phase of wound healing, the newly generated tissue reshapes and reorganizes in order to repair (scar formation) or regenerate the injured tissue closely resembling the original one. Fibroblasts produce a new collagen-rich matrix and approximately 1 week after injury, some of them transform into myofibroblasts and express α -smooth muscle actin responsible for contraction [44, 45]. This final stage of healing can require years for completion[43]. Soft tissue heals by scar tissue formation that differs from normal one as it consists primarily of fibroblasts and matrix, and may restore integrity but not form and function. On the other hand, bone typically heals without scar, in fact, the new formed tissue cannot be distinguished from the existing bone [43].

2. Platelet growth factors

Wound healing and tissue repair require a sequence of events, including undifferentiated cells recruitment, cell division and differentiation, modulated by a wide variety of different growth factors (GFs) and cytokines, primarily stored in the α granules of platelets and released upon activation [46]. Growth factors are polypeptides that transmit signals to modulate cellular activities. In fact, they have the potential to stimulate, support, enhance or inhibit growth, differentiation, migration, adhesion and gene expression of various cell types towards specific phenotypes, which leads to accelerated tissue regeneration [47]. Many cell types can produce the same growth factor and the same growth

factor can act on many cell types (pleiotropism) with the same or different effects. In addition, different GFs can share the same biological effect (redundancy) and can influence the secretion and action of other growth factors (antagonize or enhance). Platelet growth factors act binding to the extracellular domain of a specific GF-receptor on the surface of target cells. Depending on the proximity of their synthesis site to the action ones, growth factors binding through autocrine (target cell is the same that secreted the growth factor), paracrine (target cell is nearby), endocrine (target cell is distant), juxtacrine (target cell is apposed to the growth factor/receptor complex), and/or intracrine (growth factor/receptor complex is internalized) mechanisms, which, in turn, activate the intracellular signal-transduction pathways: a second messenger transmits the signal into the cell, eliciting a physiological response (Figure 7) [48]. Growth factors usually exist as inactive or partially active precursors that require proteolytic activation, and may need to bind to matrix molecules for activity or stabilization [49]. Hundreds of growth factors have been identified, characterized and, based on structural homologies, grouped into several families and super-families [50]. Among them there are: platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor- β (TGF- β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF). Furthermore, growth factors possess a short biological half-lives [51]. Although several studies have demonstrated a beneficial effect of many of these growth factors on the healing process both in animal models and also in patients [52, 53], the suggested function of these endogenous molecules have been only partially elucidated, and in most cases it is based on descriptive expression studies and/or functional cell culture data. Therefore, the *in vivo* roles of many growth factors remain largely unconfirmed [46].

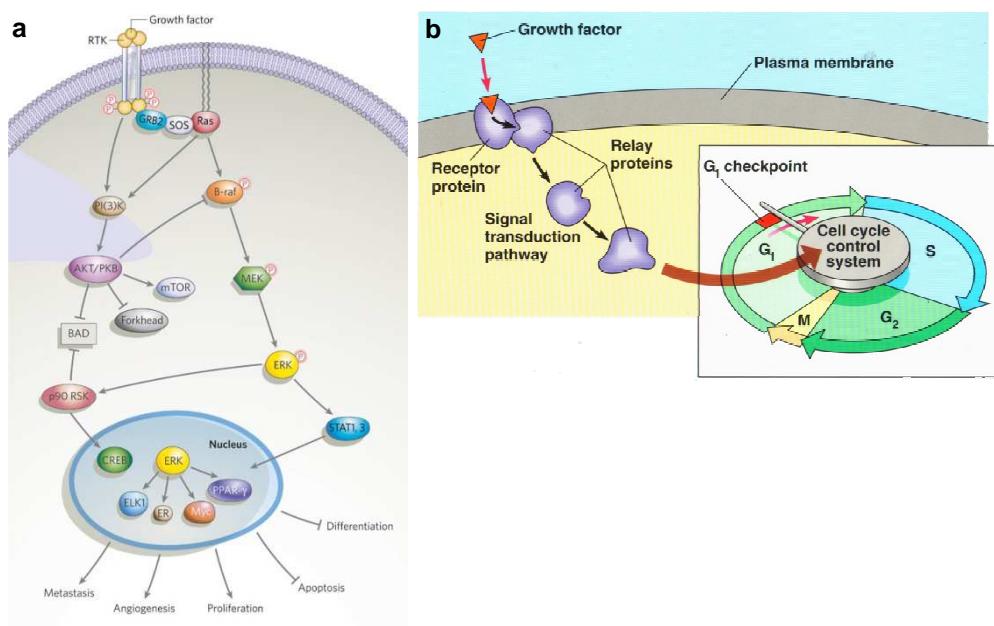


Figure 7. Growth factors' signaling pathway. (a) Central growth factor signaling pathways that drive pleiotropic cellular responses. Adapted from Sebolt-Leopold *et al.*, *Nature* 2006;441:457-462. (b) How a growth factor signals the cell cycle control system. Copyright © 2003 Pearson Education, Inc., publishing as Benjamin Cummings.

2.1. α Granules growth factors

The principle growth factors contained in the platelet α granules are:

a. Platelet-derived growth factor (PDGF)

PDGFs comprise a family of homo- or heterodimeric growth factors, including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD, that exert their function by binding to three different transmembrane tyrosine kinase receptors, which are homo- or heterodimers of an α - and a β -chain [54]. PDGF is the first growth factor shown to be chemotactic for cells migrating into the healing skin wound, such as neutrophils, monocytes, and fibroblasts. In addition, it enhances proliferation of fibroblasts and the production of extracellular matrix by these cells, it stimulates fibroblasts to contract collagen matrices and it induces the myofibroblast phenotype in these cells [55]. Thus it has long been suggested to be a major player in wound healing. Furthermore, PDGF was the first growth factor to be approved for the treatment of human ulcers [56]. Endogenous PDGF was considered to play an important role in the repair process, since

early after injury it is released in large amounts from degranulating platelets [57], and it is present in wound fluid [58]. On the other hand, augmented PDGF production might be involved in the pathogenesis of hypertrophic scars and keloids as suggested by the potent effect of PDGF on fibroblast proliferation and extracellular matrix production by these cells, the presence of enhanced levels of this growth factor in hypertrophic scar tissue [59], and the increased responsiveness of keloid fibroblasts to PDGF [60].

b. Fibroblast growth factor (FGF)

FGFs comprise a growing family of structurally related polypeptide growth factors, currently consisting of 22 members [61] that transduce their signals through four high-affinity transmembrane protein tyrosine kinases, FGF receptors 1-4 (FGFR1-4) [62], which bind the different FGFs with different affinities. Most FGFs bind to a specific subset of FGF receptors, FGF1, binds to all known receptors, while FGF7 specifically interacts with a splice variant of FGFR2, designated FGFR2IIIb [63]. One characteristic feature of FGFs is their interaction with heparin or heparan sulfate proteoglycans that is essential for the activation of the signaling receptors [64] and that stabilized FGFs to thermal denaturation and proteolysis, strongly limiting their diffusibility. Most members of the FGF family stimulate the proliferation of a broad variety of mesodermal, ectodermal, and also endodermal origin cells, with the only exception of FGF7 (keratinocyte growth factor, KGF), which seems to be specific for epithelial cells, at least in the adult organism [65]. In particular, FGFs are mitogenic for several cell types present at the wound site, including fibroblasts and keratinocytes [66]. In addition to their mitogenic effects, FGFs also regulate the migration and differentiation of their target cells, and some of them have been shown to be cytoprotective and to support cell survival under stress conditions [65]. Furthermore, FGF1 and FGF2 were shown to stimulate angiogenesis in various assay systems [67]. FGFs are therefore candidates for contributing to the wound healing repair.

c. Epidermal growth factor (EGF)

The epidermal growth factor (EGF) family comprises several members, including EGF, transforming growth factor- α (TGF- α), heparin-binding EGF (HB-

EGF), amphiregulin, epiregulin, betacellulin, neuregulins, the recently discovered epigen, as well as proteins encoded by Vaccinia virus and other poxviruses [68, 69]. In addition, more distantly related proteins known as neuregulins (heregulins, neu differentiation factors, NDF 1-4) can also bind to some EGF receptor family members [70]. All these growth factors exert their functions by binding to four different high-affinity receptors, EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Overexpression of these receptors, in particular of HER2, is often found in human cancers and is likely to have a causative role in tumorigenesis. In addition, EGF, TGF- α and HB-EGF have demonstrated a positive effect on wound repair in a series of experimental and clinical studies, suggesting that these endogenous growth factors are also involved in the healing process [71].

d. Vascular endothelial growth factor (VEGF)

The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PLGF) that exert their biological functions by binding to three different transmembrane tyrosine kinase receptors, known as VEGFR-1, VEGFR-2, and VEGFR-3. The biological functions of VEGF-A and its receptors VEGFR-1 and VEGFR-2 have been characterized in most detail and it has been identified as the major regulator of vasculogenesis and angiogenesis during development, indicating that VEGF might also be involved in the regulation of angiogenesis during wound healing [72].

e. Insulin-like growth factor (IGF)

IGF-I and IGF-II stimulate mitogenesis and survival of many different cells types, through the type I IGF receptor, a tyrosine kinase that resembles the insulin receptor. IGF-II also binds to the IGF type II/mannose-6-phosphate receptor, resulting in the internalization and degradation of IGF-II [73]. The availability of free IGF for interaction with the IGF-I receptor is modulated by six IGF-binding proteins (IGFBPs) that have also been shown to have IGF independent effects on cell growth [74]. IGFs exert their functions in an autocrine, paracrine, or endocrine manner. In addition, several studies have revealed a beneficial effect of exogenous IGF-I on wound healing, in particular in combination with other growth factors [75].

f. Nerve growth factor (NGF)

Nerve growth factor (NGF) is the prototype for the neurotrophin family of polypeptides, which are essential for the development and survival of certain sympathetic and sensory neurons in both the central and peripheral nervous systems [76]. It plays a key role in the initiation and maintenance of inflammation in various organs, therefore NGF has been suggested to be also involved in cutaneous wound repair, since exogenous NGF was shown to accelerate wound healing in normal and healing-impaired diabetic mice [77, 78], and to promote the healing of pressure ulcers in humans [79].

g. Transforming growth factor- β (TGF- β)

The TGF- β superfamily comprises a wide range of proteins, many of which play important roles during development, homeostasis, disease, and repair. The structurally related but functionally distinct mammalian members include TGF- β 1, - β 2, and - β 3, bone morphogenetic proteins (BMPs), Mullerian inhibiting substance, nodals, inhibins, and activins [80]. Their biological effects are mediated by heteromeric receptor complexes, which activate intracellular signaling cascades [81]. The three mammalian TGF- β isoforms are synthesized as latent precursors, usually secreted as a complex with latent TGF- β -binding protein, then extracellularly removed via proteolytic cleavage. Active TGF- β s exert their biological functions via binding to a heteromeric receptor complex, consisting of one type I and one type II receptor, both of which are serine-threonine kinases. In addition, they bind with high affinity to a nonsignaling type III receptor, which functions mainly to present TGF- β to the type II receptor [82]. *In vitro*, the three TGF- β isoforms have been shown to be mitogenic for fibroblasts, but they inhibit proliferation of most other cells, including keratinocytes. Furthermore, they are very potent stimulators of the extracellular matrix proteins and integrins expression [83]. Thus, they are among the most studied molecules in the wound healing scenario.

A multitude of cytokines and chemokines are also stored and released by platelet α granules [46, 84].

2.2. Growth factors biologic effects

Tissue regeneration is a complex biological process that involves a vast number of molecules and a broad cascade of events [46, 85], including cell proliferation and differentiation, modulated by a number of cytokines and growth factors that provide local signals at sites of injury [86]. Determining the roles that growth factors play in tissue repair and regeneration is important in order to design, develop and apply suitable formulations that release them with a spatiotemporal control for the functional and accelerated repair of the tissue [87].

a. Hard tissue regeneration

Bone tissue, which consists of mineralized matrix and cells, [88] originates from mesenchymal stem cells, and its growth and remodeling are regulated by cytokines, growth factors and hormones [88-90]. In bone healing humoral and cellular events cooperate in order to re-establish revascularization, matrix synthesis and mineralization, and hematopoiesis restoration [91]. Fracture repair is a complex process with initial stages similar to those observed in healing after soft tissue injured, but although both healing responses involve new tissue formation at the injured site, soft tissues heal by replacing the injured tissue with a fibrous scar, whereas bone heals by regeneration of the normal osseous anatomy. Fractured bones heal by a cascade of cellular events in which mesenchymal cells respond to unknown regulators by proliferating, differentiating, and synthesizing extracellular matrix, and different steps in this cascade may be regulated by growth factors [92]. Bone healing begins immediately after injury, when growth factors are released into the fracture hematoma by platelets and inflammatory cells. At later times, growth factors in the callus, presumably regulate additional steps in the fractured bone repair, suggesting that these molecules are central regulators of cellular proliferation, differentiation, and extracellular matrix synthesis during fracture repair. Furthermore, abnormal growth factor expression has been implicated as causing impaired or abnormal healing in other tissues, suggesting that altered growth factor expression may also be responsible for abnormal or delayed fracture repair [93].

However, there is a significant lack of consensus regarding the biological effect of GFs on primary human osteoblasts, as several studies reported success in

promoting bone regeneration [94-96], while others referred negative findings [97, 98]. Furthermore, in literature the investigation of GFs is described mainly in humans or animals [97, 99], while the amount of *in vitro* studies for basic research is limited [100].

b. Soft tissue regeneration

Angiogenic growth factors have been demonstrated to enhance soft tissue healing accelerating epidermal regeneration in cutaneous wound. There are four major families of angiogenic growth factors: fibroblast growth factor (FGF), transforming growth factor β (TGF- β), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF), and each of them has the ability to induce soft tissue vascular proliferation in microgram quantities, offering a promising potential in soft tissue wound healing [101]. Compared with any other soft tissue sites, growth factors have been studied most extensively in skin, since its poor wound healing is a common clinical problem derived from many reasons, including irradiation, chronic venous stasis, diabetes, burns, and pressure necrosis. Furthermore, skin wounds are much more accessible for growth factors applications observation and for wound-healing measurements. For a normal healing, skin injure requires adequate collagen/matrix turnover, angiogenesis and epithelialization, but if one component is delayed, normal healing is significantly hindered, leading to patient morbidity and mortality. Several clinical trials studying the effects of growth factors on chronic skin wounds, from diabetes to venous stasis ulcers have been performed over the last 10 years. Some of these preclinical and clinical studies have shown promising results [102-105], whereas others [106, 107] did not find positive effects. What primarily emerged from these investigations is that the state of a healing wound not only depends on its growth factor milieu, but also on other variables such as wound care, tissue oxygen level, bacterial count, and nutritional status of the patient [108]. Most clinical trials have been conducted by topical growth factors application to the wound and by testing the effect of a single growth factor on dermal injures. Some researchers believe that the administration of growth factors in combination could be more effective to improve wound healing, than the use of a single agent alone. This was

suggested by the demonstration that many growth factors can synergistically interact with each other [109].

3. Platelet concentrates

Platelets represent a potential source of multiple autologous growth factors and proteins involved in tissue regeneration. In order to improve healing on a surgical site, many biological products trying to mimic the natural wound healing process, have been developed. This effort dates back many years ago with fibrin glue, and recently turned into platelet concentrates technology [110]. Platelet concentrate (PC) is a blood-derived product with a platelet concentration above baseline, produced by a centrifugation procedure in order to concentrate and collect most platelets from the harvested blood, and designed for the local release of platelet growth factors essential to enhance healing [111]. The initial rationale of platelet-rich products was the replacement of blood clot with a preparation enriched in platelets that once activated, could secrete a large pool of proteins and factors driving the tissue regeneration mechanism. Eliminating erythrocytes and, in some cases, leukocytes, the preparation would take full advantage of the concentrated platelets and the stored growth factors, enabling an accelerated wound healing and tissue regeneration [112].

3.1. History of the medical use of platelet concentrates

The development of devices that may decrease bleeding, reduce operative time and improve the quality of surgical tissue management is a rapidly expanding area of research and clinical use. Thus, topical hemostats, sealants and adhesives are important elements for surgical practice [113].

a. Fibrin glue

Fibrin glue is the first product introduced for medical purpose and the only one approved by the Food and Drugs Administration (FDA) for indications in all three of these categories. Its use was first described by Bergel in 1909 [114] and Grey in 1915 [115] as a hemostatic agent, while during World War I, fibrin patches were used to stop bleeding from parenchymatous organs. In 1940

Young and Medawar [116] applied fibrinogen as an adhesive for peripheral nerves. The combination of fibrinogen and bovine thrombin was employed in 1944 by Tidrick and Warner [117] in the attempt to accelerate the fibrin clot formation and to improve skin grafts. In the 1970's Matras and colleagues [118] introduced a concentrated form of fibrinogen and the first commercial, multidonor fibrin sealant product became available in Europe. Dresdale and coworkers [119] first described the combination of autologous cryoprecipitates and bovine thrombin to create fibrin glue in 1985. Fibrin sealant is now approved in a broad variety of surgical and especially cardiovascular procedures, including neurosurgery, plastic, thoracic and oral surgery, gynecologic procedures and a large number of additional clinical applications [120, 121]. Fibrin glue is a mixture of components involved in blood clotting that mimics the last phase of the physiological coagulation cascade, through the conversion of fibrinogen to fibrin by thrombin, leading to the formation of a fibrin clot. Fibrin glue consists of two components usually contained in separate syringes, a fibrinogen with factor XIII solution and a calcium ions-rich-thrombin solution. The mixing of the two components leads the thrombin to the cleave of the fibrinogen into fibrin and, in the presence of ionized calcium, to the simultaneous activation of factor XIII, which then cross-links fibrin to form an organized clot [122]. Fibrinogen could be isolated from either pooled blood (blood from several donors) or single donor blood (autogenous or allogenic) using centrifugation in combination with either cryoprecipitation or precipitation using different agents (ethanol, ammonium sulphate or polyethylene glycol) [123]. Before solubilization for reconstitution the precipitate undergoes viral inactivation using solvent/detergent or dry heat, sterilization and freeze-drying [124]. Fibrin glue is formulated for topical use only and not for systemic administration, and is probably one of the most complex human plasma derivatives both in terms of composition and clinical uses that, the latter, depends on the mode of application and the physical properties of the resulting clot. This biologic product can be applied in different ways depending on the specific indication and the surface area being treated. For small localized areas sequential application (fibrinogen followed by thrombin solution) or simultaneous application of the 2 solutions to the repair site by a dual-syringe delivery device is possible (Figure 8). In this system, separate syringes

containing the two components enable a single plunger to dispense equal volumes of the fibrinogen and the thrombin concentrates passively mixed at the end of the delivery needle. For covering larger body surfaces sequential spray application (thrombin solution is sprayed on a surface previously sprayed with fibrinogen ones) may be used. A double syringe system made of multi-channel catheter connected to a pressurized gas source is also available: the two components are injected simultaneously into the channels and are dispersed by the continuous gas jet producing a thin fibrin film, thus ensuring homogeneous glue application on the wound surface. Fibrin glue can also be applied by collagen fleece, sponge, absorbable or nonabsorbable mesh and with or without the help of an endoscopic delivery device [125, 126].

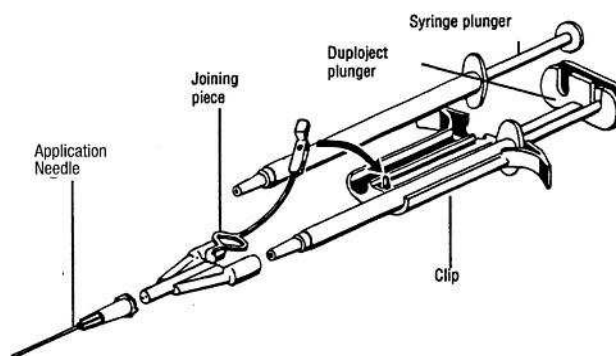


Figure 8. Dual-syringe system for fibrin glue application. Adapted from Brennan M., *Blood Rev* 1991;5:2040-244.

To avoid patient's allergic reactions and the hypothetical risks of infectious agents transmission, today are available commercial fibrin glues with human and recombinant thrombin in place of bovine origin.

Wound repair involves a complex interaction between biochemical amplification cascades, circulating platelets and monocytes, locally acting growth factors, and skin and connective tissue cells, therefore fibrin glue imitating the last steps of the coagulation cascade is only a part of the wound healing process [127]. To avoid the risk of infectious disease transmission from commercially available fibrin glue systems, the development of different autologous techniques has been proposed, but the request of patient interaction with the blood bank few days before the surgery, clerical error by blood bank and the failure to meet the blood bank's criteria, disqualify autologous fibrin glues usage [128]. Furthermore, these autologous preparations were only used as fibrin tissue

sealants rather than healing stimulators. In fact, platelets were only supposed to support the fibrin polymerization and growth factors and healing properties were not considered. Also, due to the limited tensile strength and the lack of growth factors, fibrin glue has been recognized as not being ideal for wound and tissue healing [129], therefore, the use of the circulating platelet as a source of autologous locally acting growth factors has been introduced. These platelet-rich products were named platelet gels and were considered an autologous modification of fibrin glue [130]. Platelet gel is prepared by mixing platelet-rich plasma derived from an empirical 2-steps gradient density centrifugation of autologous whole blood (collected immediately preoperatively), with thrombin and calcium chloride. The first clinical application was described by Knighton *et al.* in 1986 [109] for the treatment of chronic non-healing cutaneous ulcers, and a few years later by Whitman *et al.* [128] for oral and maxillofacial surgery.

b. Platelet-rich plasma (PRP)

Platelet concentrates technologies were originally introduced for the treatment and prevention of hemorrhage due to severe thrombopenia. The standard platelet concentrate for transfusion has been named “platelet-rich plasma” (PRP) and although the term PRP was first used in 1954 by Kingsley [131] during blood coagulation experiments, its use truly started with Marx *et al.* [132] in a study about bone graft reconstruction in maxillofacial surgery. PRP is a concentration of platelets in a small volume of plasma and differs from both fibrin glue and platelet gel [133]. The growing popularity of this product led to the development of a wide range of preparation protocols, kits and centrifuges, but all these commercial PRP techniques have some points in common. Blood is collected with anticoagulant just before or during surgery and is immediately processed by two-steps centrifugation: the first centrifugation (also called soft spin) separates the blood into three layers, red blood cells at the bottom, a buffy coat layer rich in leukocytes and platelets in the middle, and acellular plasma (platelet-poor plasma, PPP) at the top. PPP, PRP and few red blood cells are transferred to another tube without anticoagulant and centrifuged again. The second step (hard spin) differs between the numerous protocols, but it aims to separate the platelets from the PPP, including or not white blood cells (Figure 9). The resulting platelet concentrate is then applied to the surgical site together

with thrombin and/or calcium chloride (or similar factors) in order to activate platelets and trigger fibrin polymerization [134].

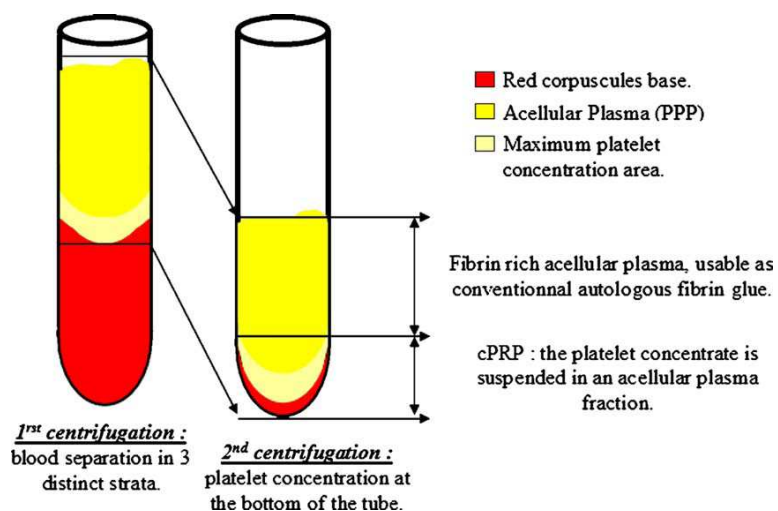


Figure 9. PRP preparation using a two-steps centrifugation procedure.

Adapted from Dohan Ehrenfest *et al.*, *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2006;101:e37-e44.

c. Plasma Rich in Growth Factors (PRGF)

In 1999, Anitua [135] described a manual version of the original PRP technology, using a one-step centrifugation process to obtain the three typical blood layers (red blood cells, buffy coat and acellular plasma) and several pipetting steps to entirely collect the final product named PRGF (Plasma or Preparation Rich in Growth Factors) (Figure 10). This platelet concentrate contains no leukocyte and lower platelet and growth factor concentrations than other products [136] and could be activated with 10% calcium chloride to induce fibrin polymerization and PRGF gel formation.

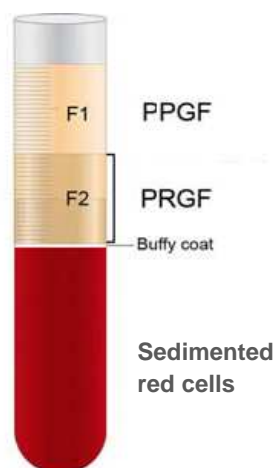


Figure 10. PRGF preparation using one-step centrifugation procedure.

Adapted from Anitua *et al.*, *J Biomed Mater Res A*. 2015;103:1011-1020.

d. Platelet-rich Fibrin (PRF)

The latest development of these protocols was introduced by Choukroun *et al.* in 2001 [137]. The PRF (Platelet-Rich Fibrin) technique was considered a second-generation platelet concentrate technology that differed from the other products for several characteristics: blood is collected without anticoagulant and immediately centrifuged. In the absence of anticoagulants and activators (i.e. thrombin or calcium chloride), platelet activation and fibrin polymerization are triggered immediately, leading to the natural formation of a strong PRF clot in the middle of the tube, red blood cells (RBC) in a base layer and an acellular plasma at the top (Figure 11) [110]. The PRF clot is a strong fibrin matrix in which most of the platelets and leukocytes are concentrated [138, 139] and that does not dissolve quickly after application, but is slowly remodeled similarly to a natural clot.

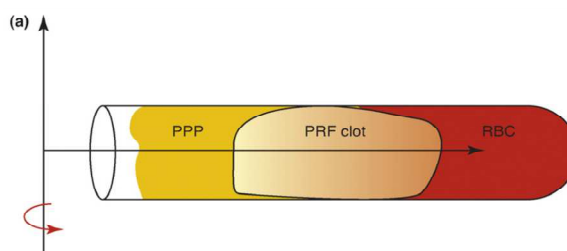


Figure 11. PRF preparation. Adapted from Dohan Ehrenfest *et al.*, *Trends Biotechnol* 2009;27:158-167.

3.2. Classification

To date, several techniques for platelet concentrate preparation are available and, although each method leads to a different product in term of biological properties, most of these products were initially called PRP. This term was the same as the original transfusion platelet concentrates, but it does not permit to make a distinction between the numerous commercial systems and protocols necessary to make more informed decisions regarding platelet concentrate application in the clinical setting. In order to clarify between the extreme variety of biological preparations, together with a deriving plethora of terms, often very similar to each other, a full classification system for all platelet concentrates has been proposed in two main publications [134, 140].

According to Dohan Ehrenfest *et al.* [134] three main sets of parameters are necessary for the clear classification of platelet concentrates. The first set regards preparation kits, centrifugation parameters (number, speed and time of centrifugations), system type (automatized or manual), and costs; the second one relates to the content of the concentrate (platelet and leukocytes collection efficiency), and the third set concerns the fibrin network (fibrinogen concentration and fibrin density, and fibrin polymerization process). These parameters enable to classify all the available methods into four main categories on the basis of their leukocyte content and fibrin architecture: leukocyte-poor or pure platelet-rich plasma (P-PRP) without leukocytes, leukocyte- and platelet-rich plasma (L-PRP) with leukocytes, leukocytes-poor or pure platelet-rich fibrin (P-PRF) without leukocytes and leukocyte- and platelet-rich fibrin (L-PRF) with leukocytes [134].

DeLong *et al.* [140] defined the “PAW classification system” an acronym that stands for Platelet, Activation, and White cells, to identify the PRP preparations based on three components: the absolute number of platelets (P), the manner in which activation occurs (A), and the presence or absence of white blood cells (W).

The Dohan Ehrenfest’s classification is probably the easier and more accurate, even if few clarifications are still necessary in order to find a consensus terminology on platelet concentrate technologies, as in literature the lack of standardization and consistency makes it difficult for investigators to compare results between articles or to replicate published data.

3.3. Clinical and therapeutic applications

The ability to prepare autologous platelet concentrates enjoyed a great increase in popularity, therefore the use of this technology spread in many different medical fields including dentistry, oral implantology, orthopedics, ulcer treatment and ophthalmology among others.

Despite the great interest regarding the properties of platelet concentrates, reflected also in the large amount of published articles in this regard, the indications that their usefulness is fully demonstrated are few. Such conflicting data in literature could not be necessarily due to the absence of positive evidence, but frequently because of the particular animal model used (a higher concentration of platelets may aid in human wound healing, whereas other animal species may heal extremely well even without additional platelets, making it more difficult to show a benefit of platelet concentrate in these models), the different techniques, preparation protocols, kits and centrifuges used to concentrate platelets and prepare the PC (some isolation techniques may contribute to early and premature platelet degranulation), the post-operative follow-up period (long-term data are preferred as PC exerts a direct influence upon only the initial phase of osseous healing, but physiological mechanisms still continue to promote osseous repair throughout the entire period of osseous maturation), the specific regenerative material combined with the platelet concentrate, and the clinical design employed (when platelet concentrates are used in combination with other components, their effect is masked). All these reasons may influence the clinical success of platelet concentrate.

a. Oral and maxillofacial surgery and oral implantology

In the dentistry and oral and maxillofacial surgery area, platelet concentrates have been used for the treatment of many clinical conditions (i.e. periodontal defects and post-extraction socket treatment, oral implant osseointegration, maxillary sinus augmentation and maxillofacial skeletal reconstruction). In 1994 Tayapongsak *et al.* [141] introduced the novel idea of adding autologous fibrin adhesive to cancellous bone during mandibular continuity reconstruction, whereas the application of PRP to oral surgery was first described by Whitman *et al.* [128] and Marx *et al.* [132]. Whitman and coworkers in their 1997 article

suggested that platelet's activation within the gel could enhance wound healing, while in 1998 Marx *et al.*'s study showed that the combination of PRP with autogenous bone for the treatment of mandibular continuity defects, resulted in a faster maturation and a denser bone regeneration. To date, divergent results that ranged from the significant enhancement of bone formation [142] to a limited or null effect of platelet concentrates [143], have been reported.

b. Orthopedics and sports medicine

Platelet concentrates have significant potential in the treatment of pathologic conditions of cartilage, tendon, ligament and bone including elbow, Achilles and patellar tendinopathies, rotator cuff injuries, fractures and their complications (delay consolidation and nonunion), anterior cruciate ligament injury and total joint arthroplasty. Clinical benefit of platelet concentrates in this medical field remains controversial, since their application in trauma and orthopedic procedures still lacks the support of randomized controlled clinical trials [144, 145]. Therefore, additional studies with higher levels of evidence are required to support the use of this technology in routine clinical care of this area.

c. Skin lesions, esthetic and plastic surgery

Chronic wounds can be classified into three categories: venous, diabetic and pressure ulcers, and one of the most common complications of diabetes in the lower extremity is the diabetic foot ulcer. Several clinical studies treating small-sized non healing diabetic ulcers with platelet concentrate are available, however controversies exist in the literature regarding the added benefit of this technology. Some authors [146-148] found that platelet concentrates had improved healing rates over standard care, while others [149, 150] showed no major difference in healing outcome of leg ulcers. Kazakos *et al.* [151] reported evidence of effectiveness of platelet concentrates for the treatment not only of chronic or non-healing wounds, but also for the management of acute trauma lesions. For large, deep ulcers a combination of platelet concentrates with skin grafting may be required.

Platelet concentrates have also been used as an adjuvant in cosmetic surgery, and more specifically, in facial plastic surgery. Man *et al.* [152] reported positive results in cosmetic surgeries, Adler and Kent [153] in facelift surgeries, Abuzeni

and Alexander [154] in cosmetic dermal fat grafts, and Schade *et al.* [155] in skin grafts. Platelet concentrates have been used as filler in scar management [156-158], while in the treatment of burns their relevance remains unclear [159]. Recently, the application of autologous platelet concentrates has emerged as a new treatment modality in a wide range of dermatological indications ranging from hair restoration to acne scarring. In patients receiving platelet concentrate's subcutaneous injection alone [160, 161] or previous hair implants [162] a significant increase on the hair density, transplanted follicular and new hair growth were detected. Positive effects were also observed in skin rejuvenation [163]. More often platelet concentrates are mixed with adipose tissue in procedures requiring autologous fat transfer, as breast fat graft or reconstruction and abdominoplasty, but the value of their use in this field remains unclear [164].

d. Other medical fields

Another interesting application of platelet concentrates is in ophthalmology field, where the development of autologous eye-drops can be used in the treatment of many eye disorders (i.e. symptomatic dry eye, corneal ulcers) with positive effects observed [165, 166]. In addition, the use of this technology in peripheral nerve regeneration in rats [167-169] and in perianal fistula [170] has been evaluated.

4. Tissue engineering

End-stage organ failure and tissue loss or damage due to aging or pathological conditions is one of most devastating problem in medicine, since this incurs a human health care cost and work days lost [171]. Unfortunately, organ and tissue transplantation are imperfect solutions as they are limited by a number of factors, including the discrepancy between the available organs and the number of patients needing transplants, the lifelong immunosuppression regimens required for transplantation recipients associated also with the increased risk of infection, disease transmission, tumor development, donor site morbidity, and unwanted side effects [172]. Additionally, the replacement with mechanical devices (i.e. joints and heart valves) or artificial organs is also limited by an

increased risk of infection, thromboembolism and finite durability [173]. Recently, a new alternative therapy has become available to clinicians, called tissue engineering (also known as regenerative medicine). Tissue engineering, an emerging field in biomedical sciences, has been described as “an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” [171] in order to “replace diseased living tissue with living tissue that is designed and constructed to meet the need of each individual patient” [174]. Tissue engineering concept dates back to 1933 when Bisceglie [175] encased mouse tumor cells in a polymer membrane and placed them in the abdominal cavity of a pig, showing that cells were not killed by the immune system. In 1975, Chick and colleagues [176] reported that pancreatic-islet cells encapsulated in semipermeable membranes improved glucose control in diabetes, while by the early 1980s other studies attempted the replacement of the skin with techniques consisting of fibroblasts cells seeded onto collagen scaffold to guide regeneration, which are currently used in clinical practice [177]. The primary goal of tissue engineering is to restore the function by means of the delivery of living elements that integrate into the patient (Figure 12) [174].

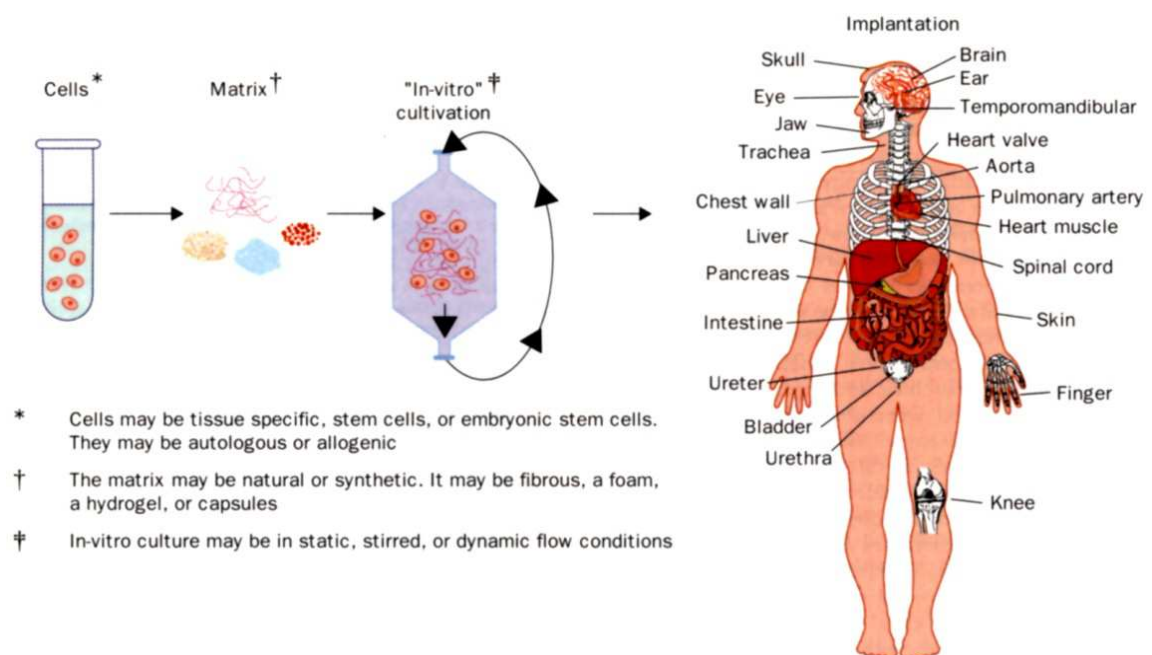


Figure 12. Tissue engineering process. Adapted from Vacanti and Langer, *Lancet* 1999;354:s32-s34.

Currently, tissue engineering requires three essential elements: a cellular component able to give rise to new structural tissue, a biocompatible and mechanically stable matrix scaffold to provide a substrate for cell attachment, proliferation and differentiation, and a bioactive component consisting of growth and differentiation factors to guide the proper development of the cellular component [178]. Three basic approaches have evolved over the last few years in tissue engineering to form new tissues. The first strategy is factor based and stimulates endogenous cells to induce neo-tissue formation via delivery of biological factors that promote cell migration, proliferation and differentiation of desired cell type from the surrounding tissues [179]. The second one is a cell based approach that involves semipermeable membrane encapsulated cells transplantation to augment limited biochemical functions of a tissue or organ [180]. The cellular component should consist of healthy, viable cells that are accessible, manipulable, and nonimmunogenic. The matrix based therapy is the most widely used now, and in this third strategy cells are placed on or within matrices in order to create new tissues capable to integrate with the host one, thus replacing all the functions of deficient tissue [181]. The carrier component has a dual role, acting as both a delivery vehicle and a matrix scaffold. The injection of cells alone and the use of cells transplanted on matrices are the most common, since isolated cells or cell substitutes avoid potential surgical complication and allow cell manipulation (such as gene therapy) before injection or infusion, although the possibility of rejection or loss of function [171].

4.1. Essential components

The three essential elements for tissue engineering are scaffold, cells and *in vitro* cell culture system.

a. Scaffolds for tissue engineering

Biomaterials represent a way to enhance the *in vivo* efficacy of growth factors by facilitating the release of these bioactive molecules over an extended time period incorporating them into a polymer carrier. Initially, scaffolds used for tissue engineering were derived from surgical materials, but it is desirable that the scaffolding biomaterial can be degraded as cells go through the supportive extracellular matrix (ECM) forming process [182]. Scaffolds function as an

artificial ECM for cells, mimicking the functional and mechanical properties of the native extracellular matrix [183], and can be engineered to deliver growth factors, signals and cells or to direct the three-dimensional cell orientation. Biomaterials facilitate cells proliferation, differentiation and confinement to the desired destination in the body, and define a three-dimensional space guiding the new tissue generation with appropriate structure and function [184]. Delivery systems have been designed in a variety of geometries and configurations (i.e. reservoirs, matrices) and are produced from diverse types of natural materials such as collagen, fibrin, hydroxyapatite, glycosaminoglycans, or alginate, [174] and synthetic polymers (degradable, non-degradable) such as polyglycolic acids (PGA), polylactic acid (PLA), copolymers of glycolic and lactic acids, polyurethanes, polyhydroxybutyrate (PH4B), polyanhydrides and polyortho esters. Natural materials may closely mimic the native cellular environment as they are often extracellular matrix components for cells, and possess natural interactive properties like cell adhesiveness, while synthetic materials allow for a better control material properties, including strength, degradation time, porosity and microstructure. Hydrogels such as pluronic F-127 (a copolymer of 70% ethylene oxide and 30% propylene oxide) are based on water soluble components and are a class of biomaterials with a great scaffolding potential in many tissue engineering applications [185]. The polymer's chemical modification or its coating can improve cell attachment and, additionally, growth factors can be incorporated into the matrix. Ideal biomaterials should possess good mechanical characteristics and defined shapes and sizes, in order to maintain the polymers' structure during new tissue formation, and must be biocompatible and bioadsorbable, nonimmunogenic, support cell growth, and be able to induce angiogenesis to promote cellular interaction and new tissue development [174]. Biodegradable and bioresorbable biomaterials elude the inflammatory complication of foreign body reactions deriving from their long-term presence inside the body [186]. The scaffold degradation is controlled by different physical and chemical parameters changes, in order that the non-toxic degradation products remain within tolerable limit and are removed from the body through normal metabolic pathways [187].

b. Cells for tissue engineering

Another important consideration in tissue engineering is the cell source and the ability to control cell proliferation and differentiation. Cells can be harvested from numerous sources, including primary tissue and cell lines, and primary cells derived from patient's own healthy tissues (autologous cells) are the first obvious choice, since they avoid the risk of adverse host response and disease transmission [188]. In most cases, these cells are however not readily available in sufficient quantities for immediate use, therefore primary cells derived from normal donors of the same (allogenic cells) or different species (xenogenic and syngeneic cells) are available. The latter offer the advantage of cryopreservation, but disease transmission and rejection by the host's immune system have to be considered [188]. Ideally, cells should be nonimmunogenic, highly proliferative, easy to harvest, and capable to differentiate into the appropriate cell type [189]. However, certain cell types poorly proliferate in culture or not at all. For this reason, stem cells source, more rapidly proliferating and giving rise to a variety of cell types in the body under appropriate culturing conditions, provides probably the most promising alternative source for tissue engineering to solve the cell-source difficulty [190]. The first step in tissue engineering is the isolation and propagation of cells to increase their number and reach a large cell population, but this multiplication process may led to cell characters lost by a process of dedifferentiation [191]. Therefore, the second step aims to induce cell differentiation by means of novel cell carriers and matrices, thus allowing cells tissue specific features expression [192]. Also, cell anchorage to substrates [193], controlled growth factors deliver matrices [179], culture medium composition and media electrolyte concentrations [194] have shown to play a role in cell proliferation, differentiation and new tissue formation. The use of cell-matrix constructs involves either an open or a closed system. An open system consists of *in vitro* culture of isolated cells, which are seeded onto a synthetic or natural scaffold and then the cell-matrix construct is implanted into the host after an appropriate cultivation time (Figure 12). On the contrary, in a closed system cells are isolated from the body by a permeable membrane, allowing exchange of nutrients and waste, but protecting the cells from the immune response [171].

c. Cell culture systems for tissue engineering

Once the scaffold material and the cell source are selected, and cells undergo a differentiation process, it becomes necessary to develop more advanced procedures for growing cells in large quantities, and for the synthesis of extracellular matrix proteins and neogenesis of tissue structures. The most widely used culturing technique in tissue engineering is static culturing, but this system is often characterized by non-homogeneous cell distribution on the scaffold surfaces, that results in an inhomogeneous distribution of the *in vitro* generated extracellular matrix [195]. Several novel approaches such as continuous perfusion culture systems, consisting basically on growth chambers, have been developed in order to overcome this limitation [196]. These systems, so-called bioreactors, are equipped with stirrers and sensors that regulate flow and mixing within them, in order to set the appropriate amount of nutrients, oxygen, gases, metabolites, regulatory molecules, and waste products, essential for the control of the size and structure of the forming tissue and also for the prevention of deleterious effects on cell viability and function [197]. Furthermore, bioreactors, can provide mechanical regulatory signals (i.e. directly applied compression) to stimulate specific biomolecules cells production [174].

4.2. Mesenchymal stem cells

Stem cell based therapies that integrate tissue-engineering technologies and biomaterials, are fundamental pillars of the science of regenerative medicine for the replacement or function restoration of damaged tissues and organs. Consequently, the choice of the right stem cell is fundamental for obtaining favorable results in tissue engineering. In the last decades, the use of adult mesenchymal stem cells (MSCs) in regenerative surgical procedures is steadily growing. Mesenchymal stem cells are non-hematopoietic, stromal cells that have the potential to differentiate into diverse tissues, including bone, cartilage, adipose tissue, tendon, muscle and other tissues of mesenchymal origin, and possess also the ability to generate cell types specific for these tissues, like chondrocytes, osteoblasts, adipocytes, fibroblasts and marrow stroma (Figure 13). In the human body MSCs function seems to be a reserve of reparative cells with no tissue specific characteristics, but responsive to different signals, including tissue damage (such as trauma, fracture, inflammation, necrosis and

tumors) [198], chemotaxis [199] and the local microenvironment [200], that can direct the cell to mobilize and differentiate into specific cell lines.

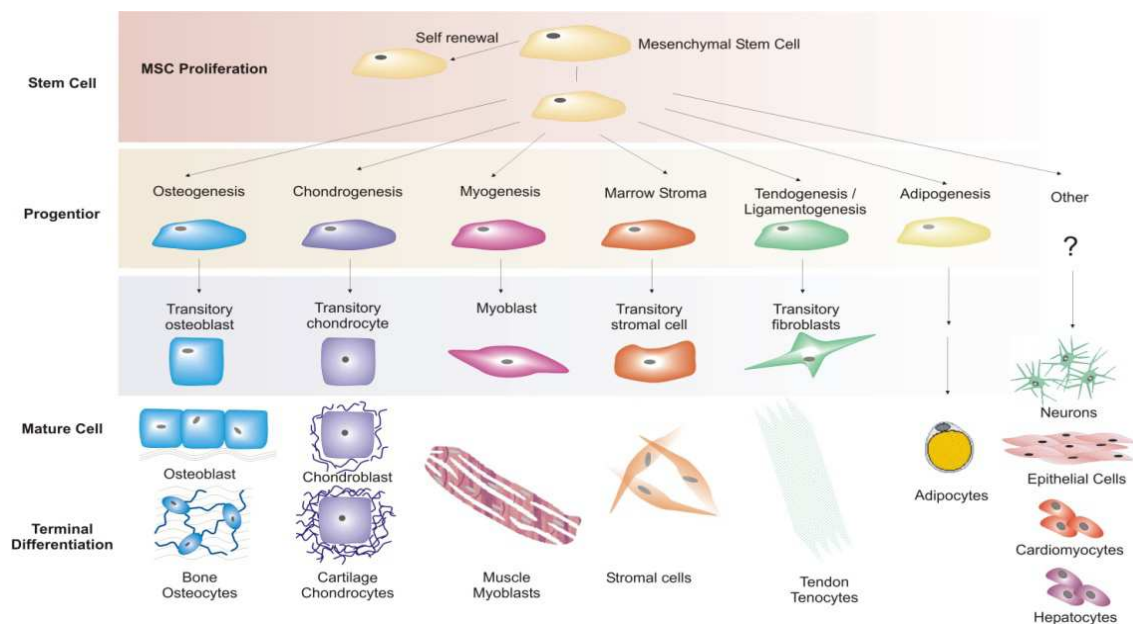


Figure 13. MSCs multilineage differentiation potential. Adapted from Caplan and Bruder, *Trends Mol Med* 2001;7:259-264.

Additionally, MSCs can be easily isolated and expanded in the laboratory under standard culture condition, and there are no limitations to their practical use related to ethical or religious considerations. The MSCs proliferation and differentiation abilities, and the multipotency have suggested them to be potential for therapeutic application in regenerative medicine. Originally, such adult stem cells have been discovered and characterized by Friedenstein *et al.* [201] in the bone marrow and stroma (BMSCs) of spleen and thymus, but MSCs reside in a multitude of tissues, including cartilage, periostium, synovium, synovial fluid, muscle, tendons, adipose tissue, placenta and umbilical blood [202]. Mesenchymal stem cells have been harvested since long from bone marrow for clinical use, since aspirate of this tissue is considered to be the most accessible and enriched source of MSCs. However, due to its procurement limitations, in the last few years subcutaneous adipose tissue is being considered a convenient alternative source of multipotent adult stem cells, since it exists in large quantities in our body, it contains a large number of progenitors and it can be collected under local anesthesia with lower donor site morbidity [203]. Several *in vitro* studies have shown the similarity between human ASCs

(hASCs) and BMSCs regarding their cellular phenotype, growth kinetics, cell senescence and differentiation efficiency [204]. These mesenchymal stem cells possess the ability *in vitro* to self-maintain and to differentiate towards cells of osteogenic [205], chondrogenic [206], adipogenic [205], myogenic [207] and neurogenic lineage [208]. Based on the regenerative and immunomodulatory features of hASCs, they might become useful tools for cell-mediated therapy [203]. hASCs promote tissue regeneration by secreting multiple cytokines and growth factors, including insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGF- β 1), platelet-derived growth factor (PDGF) and the pro-angiogenic vascular endothelial growth factor (VEGF), that stimulate the recovery of damaged tissue in a paracrine manner [209]. This mechanism is due to the recruitment of endogenous stem cells to the site and the promotion of their differentiation toward specific lineages. Due to these biological characteristics, adipose tissue, previously discarded, has recently become an invaluable stem cell source for clinical application [209].

Aim

Platelet-derived growth factors in recent years assumed a growing importance in promoting wound healing and tissue regeneration in a wide range of disciplines, including oral, orthopedic and reconstructive surgery. Though, several aspects of their biological activity are still unclear.

The purpose of the present study was to gain more insight regarding the biological effect of these molecules on soft and hard tissue healing.

The effect of an activated platelet concentrate (Plasma Rich in Growth Factors - PRGF) on both progenitors and differentiated cells was investigated in terms of key cellular functions associated with tissue repair, i.e. cell vitality, growth, and osteo-differentiation. The *in vitro* model developed consisted of a human primary cell population derived from the fat tissue, known as adipose-derived stem cells (hASCs), and two human terminally differentiated cell lineages obtained from bone and derma, that is osteoblasts (hObs) and dermal fibroblasts (hDFs), respectively, cultured in the presence of PRGF as substitute for fetal bovine serum. The proliferation effect was assessed by means of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test and cell count, while the osteo-inductive ability was evaluated with the alkaline phosphatase (ALP) assay- enzymatic activity.

The clinical benefit of platelet concentrates as reported in the scientific literature is still controversial, especially regarding bone healing and repair. Therefore, the effect of the combination of a bone graft substitute with PRGF in enhancing bone density was evaluated in a rabbit animal model by means of *histological* and *histomorphometric* analysis.

These models may in the future allow for the *in vitro* bioactivity characterization of a patient's platelet concentrate, so as to determine the different individual response to treatment with autologous growth factors, in order to prewise the effectiveness of a regenerative therapy.

Material and methods

1. *IN VITRO* STUDY

1.1. Tissues

Cells used in the *in vitro* study derived from plastic and orthopedic surgery procedures' discarded tissues obtained from healthy donors after their written consent and Institutional Review Board (IRB) approval (IRCCS Galeazzi Orthopedic Institute PQ 7.5.125, version 3, 14.05.2012). All patients were in general good health without any systemic complication (ASA I-II according to the American Society of Anesthesiologists classification) and older than 18 years.

1.2. Isolation, characterization and culture of cells

a. Human adipose-derived stem cells (hASCs)

Isolation

Human adipose-derived stem cells were isolated by liposuction of 15-40ml (28.3±12.6ml) subcutaneous-adipose tissue of 3 female patients (age 22-35 years (mean 29±6.5 years), BMI < 30 Kg/m², no metabolic disease) as previously described [210]. Briefly, after digestion at 37°C with 0.075% type I collagenase (250 U/mg; Worthington, Lakewood, N.J., USA) for 30 minutes, the stromal vascular fraction (SVF) was separated by centrifugation (1200g for 10 minutes), filtered and hASCs were plated (10⁵ cells/cm²) in DMEM (Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS), 50U/ml penicillin, 50mg/ml streptomycin and 2mM L-glutamine (Sigma-Aldrich) (control medium, CTRL).

Proliferation

Plastic culture flasks-adherent hASCs were selected during subsequent passages. The fibroblast-like morphology displayed by the isolated cells was

checked by phase-contrast microscopy and the doubling time was calculated as $(t_2-t_1) \times \ln_2 / \ln(N/N_0)$, where N is the number of counted cells and N_0 represents the number of plated ones.

MTT cell viability assay

Cell viability was tested by means of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test. 3×10^4 hASCs/cm² were plated in 96-well plates, and monitored at day 1 and 7. Then 100µl of MTT (Sigma-Aldrich) (final concentration 0.5mg/ml in DMEM) were added and cells were maintained for 4 additional hours at 37°C. Formazan precipitates were solubilized by 100% DMSO (dimethyl-sulphoxide, Sigma-Aldrich) and absorbance was read at 570nm in a Wallac Victor II plate reader (Perkin Elmer Western Europe, Monza, Italy).

Fibroblast-colony-forming unit assay (CFU-F)

hASCs, were plated in DMEM supplemented with 20% FBS, 50U/ml penicillin, 50µg/ml streptomycin, and 2mM L-glutamine, in 6-well plates by serial dilution starting from 1000 cells/well. After 6 days the medium was replaced and, at day 14 cells were washed, fixed in 100% methanol and stained with 0.5% crystal violet (Fluka, Buchs, Switzerland). The colony-forming unit-fibroblast (CFU-F) frequency was established by counting individual colonies (of at least 20 cells) compared to the number of seeded cells.

Flow cytometry analysis

hASCs (3×10^5) in PBS with 1% FBS/0.1% NaN₃ per sample were incubated for 30 minutes on ice with monoclonal antibodies raised against CD14, CD31 CD34, CD73, CD90, and CD105 (Ansell, Bayport, MN). Specific binding was revealed by either streptavidin-PE- or fluorescein isothiocyanate-conjugated sheep anti-mouse antibody. Samples were characterized immunophenotypically by FACS Calibur flow cytometer (BD Biosciences Europe, Erembodegem, Belgium) and data were analyzed using CellQuest software (BD Biosciences Europe). Cells were CD73, CD90, CD105 and CD271 positive and CD14 and CD45 negative.

Osteogenic differentiation

7.75×10^3 hASCs/cm² at 4th and 7th passages were maintained for 14 days in either control (CTRL) or osteogenic medium (OSTEO, DMEM, 10% FBS, 10nM dexamethasone, 10mM glycerol-2-phosphate, 150 μ M L-ascorbic acid-2-phosphate, 10nM cholecalciferol, Sigma-Aldrich).

To evaluate alkaline phosphatase (ALP) enzymatic activity, hASCs were lysed in 50 μ l of 0.1% Triton X-100 and incubated at 37 $^{\circ}$ C with 10mM p-nitrophenylphosphate dissolved in 100mM diethanolamine and 0.5mM MgCl₂, pH 10.5. Samples were read at 405nm and ALP activity was standardized respect to the sample protein concentration determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL).

To determine collagen production, cells were stained with 0.1% (w/v) Sirius Red F3BA in saturated picric acid (Sigma-Aldrich) for 1 hour at room temperature, and then the samples were extracted with 0.1M NaOH for 5 minutes and absorbance was read at 550nm [211]. Standard curve of known concentration of calf skin type I collagen (Sigma-Aldrich) was used to determine the concentration of secreted collagen.

Extracellular matrix (ECM) calcification was determined on fixed hASCs stained by 40mM Alizarin Red-S (AR-S, pH 4.1; Fluka). Mineral deposition was quantified by incubating the stained sample with 10% w/v cetylpyridinium chloride (CPC; Sigma-Aldrich) in 0.1M phosphate buffer (pH 7.0) for 15 minutes to extract AR-S. Absorbance was read at 550nm with Wallac Victor II plate reader [212].

Adipogenic differentiation

hASCs were induced to differentiate towards the adipogenic lineage as previously described with some modifications [213]. Briefly, 1.5×10^4 hASCs /cm² were plated and cultured in control medium supplemented with 1 μ M dexamethasone, 10 μ g/ml insulin, 500 μ M 3-isobutyl-1-methylxanthine, and 200 μ M indomethacin (Sigma-Aldrich). 14 days later, cells were fixed in 10% neutral buffered formalin for 1 hour and stained by fresh Oil Red O solution (20mg/mL [w/v] Oil Red O in 60% isopropanol) for 15 minutes. Lipid vacuoles were quantified by extraction with 200 μ l of 100% isopropanol for 10 minutes and reading the absorbance of 50 μ l at 490nm with Wallac Victor II plate reader.

Chondrogenic differentiation

5×10^5 hASCs were cultured in micromasses in chondrogenic medium (DMEM supplemented with 1% FBS, 100nM dexamethasone, 110mg/l sodium pyruvate, 150 μ M L-ascorbic acid-2-9 phosphate, 1x insulin-transferrin selenium (ITS) and 10ng/ml TGF- β 1) for 21 days. Glycosaminoglycans (GAGs) production was assessed by dimethylmethylene blue (DMMB) assay as previously described [214, 215]. Briefly, micromasses were digested at 56°C overnight by 100 μ l of 50 μ g/ml proteinase K in 100mM K_2HPO_4 (pH 8.0). After 10 minutes at 90°C to inactivate the enzyme, the samples were spun at 14000g for 10 minutes and each supernatant was collected for GAGs. Samples were then incubated at room temperature in 40mM glycine/NaCl (pH 3) with 16mg/ml DMMB and the absorbance was read at 500nm with the Wallac Victor II plate reader. The amount of GAGs was determined respect to known concentrations of chondroitin sulfate (Sigma-Aldrich).

b. Human osteoblasts (hObs)

Isolation

Human osteoblasts were isolated from cortical bone of two male patients (age 43 and 57 years, with no metabolic disease) undergoing osteotomy. Bone fragments were digested for 60 minutes with collagenase type II (0.003%) at 37°C and hObs were plated in basal medium (DMEM) supplemented with 10% FBS, 50U/ml penicillin, 50mg/ml streptomycin and 2mM L-glutamine. Plastic culture flasks-adherent hObs were selected during culture.

c. Human dermal fibroblasts (hDFs)

Isolation

Human dermal fibroblasts were obtained from a 46 years healthy female patient undergoing abdominoplastic surgery. The tissue was digested for 6 hours in DMEM and 0.1% collagenase I at 37°C and hDFs-1 were separated by centrifugation (1200g for 10 minutes), filtered and plated (10^5 cells/cm²) in complete medium.

Proliferation

The fibroblast-like morphology displayed by the isolated cells was checked by phase-contrast microscopy and the doubling time was calculated as $(t_2 - t_1) \times \ln_2 / \ln(N/N_0)$, where N is the number of counted cells and N_0 represents the number of plated ones.

MTT cell viability assay

Cell viability was tested by means of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test. 3×10^4 hASCs/cm² were plated in 96-well plates, and monitored at day 1 and 7. Then 100µl of MTT (Sigma-Aldrich) (final concentration 0.5mg/ml in DMEM) were added and cells were maintained for 4 additional hours at 37°C. Formazan precipitates were solubilized by 100% DMSO (dimethyl-sulphoxide, Sigma-Aldrich) and absorbance was read at 570nm in a Wallac Victor II plate reader (Perkin Elmer Western Europe, Monza, Italy).

Osteogenic differentiation

1.5×10^4 hDFs/cm² at 4th passages were maintained for 14 days in either control (CTRL) or osteogenic medium (OSTEO, DMEM, 10% FBS, 10nM dexamethasone, 10mM glycerol-2-phosphate, 150µM L-ascorbic acid-2-phosphate, 10nM cholecalciferol, Sigma-Aldrich).

To evaluate alkaline phosphatase (ALP) enzymatic activity, hDFs were lysed in 50µl of 0.1% Triton X-100 and incubated at 37°C with 10mM p-nitrophenylphosphate dissolved in 100mM diethanolamine and 0.5mM MgCl₂, pH 10.5. Samples were read at 405nm and ALP activity was standardized respect to the sample protein concentration determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL).

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Extracellular matrix (ECM) calcification was determined on fixed hDFs stained by 40mM Alizarin Red-S (AR-S, pH 4.1; Fluka). Mineral deposition was

quantified by incubating the stained sample with 10% w/v cetylpyridinium chloride (CPC; Sigma-Aldrich) in 0.1M phosphate buffer (pH 7.0) for 15 minutes to extract AR-S. Absorbance was read at 550nm with Wallac Victor II plate reader.

Adipogenic differentiation

hDFs were induced to differentiate towards the adipogenic lineage as previously described with some modifications [213]. Briefly, 2×10^4 hDFs /cm² were plated and cultured in control medium supplemented with 1µM dexamethasone, 10µg/ml insulin, 500µM 3-isobutyl-1-methylxanthine, and 200µM indomethacin (Sigma-Aldrich). 14 days later, cells were fixed in 10% neutral buffered formalin for 1 hour and stained by fresh Oil Red O solution (20mg/mL [w/v] Oil Red O in 60% isopropanol) for 15 minutes. Lipid vacuoles were quantified by extraction with 200µl of 100% isopropanol for 10 minutes and reading the absorbance of 50µl at 490nm with Wallac Victor II plate reader.

All cells used for the *in vitro* study were maintained in a humidified atmosphere at 37°C with 5% CO₂ until confluence and medium changed every 2-3 days.

1.3. Blood collection and preparation of the activated platelet concentrate

The acellular plasma of a platelet concentrate (PC) was obtained using a manual protocol performed by an experienced technician. Peripheral blood from five young healthy volunteer donors (four females and one male, age 23-29 years (26.2 ± 2.6), in general good health and older than 18 years) was collected in 9-ml tubes (BTI blood collecting tubes®) with 3.8% sodium citrate (m/v) as anticoagulant and centrifuged at 580g for 8 minutes at room temperature in a standard laboratory centrifuge (Sigma 3-16K, Sciquip). Blood cells stratified according to a density gradient into three distinct layers: the bottom fraction contained erythrocytes and the top fraction contained platelets. The two fractions were separated by the whitish buffy coat layer, which contained most of the leukocytes. The 2ml plasma fraction just above the buffy coat, containing the highest platelets concentration, was identified as Plasma Rich in Growth Factors (PRGF) and collected. The upper part of the acellular plasma just

above the PRGF layer, presenting the lowest platelets concentration was identified as Plasma Poor in Growth Factors (PPGF) and discarded (Figure 14). Platelets count was performed with a hematology analyzer (Sysmex, XE-2100, Norderstedt, Germany). After PRGF activation with a CaCl_2 solution, (PRGF-Activator®, BTI Biotechnology Institute, S.L., Vitoria, Spain) it was immediately aliquoted and stored at -80°C until its use. After being thawed at 37°C in a water bath for few minutes, it was incubated at 37°C for 30 minutes, triggering thereby platelet activation and growth factors release, and then used as supplement to the culture medium.

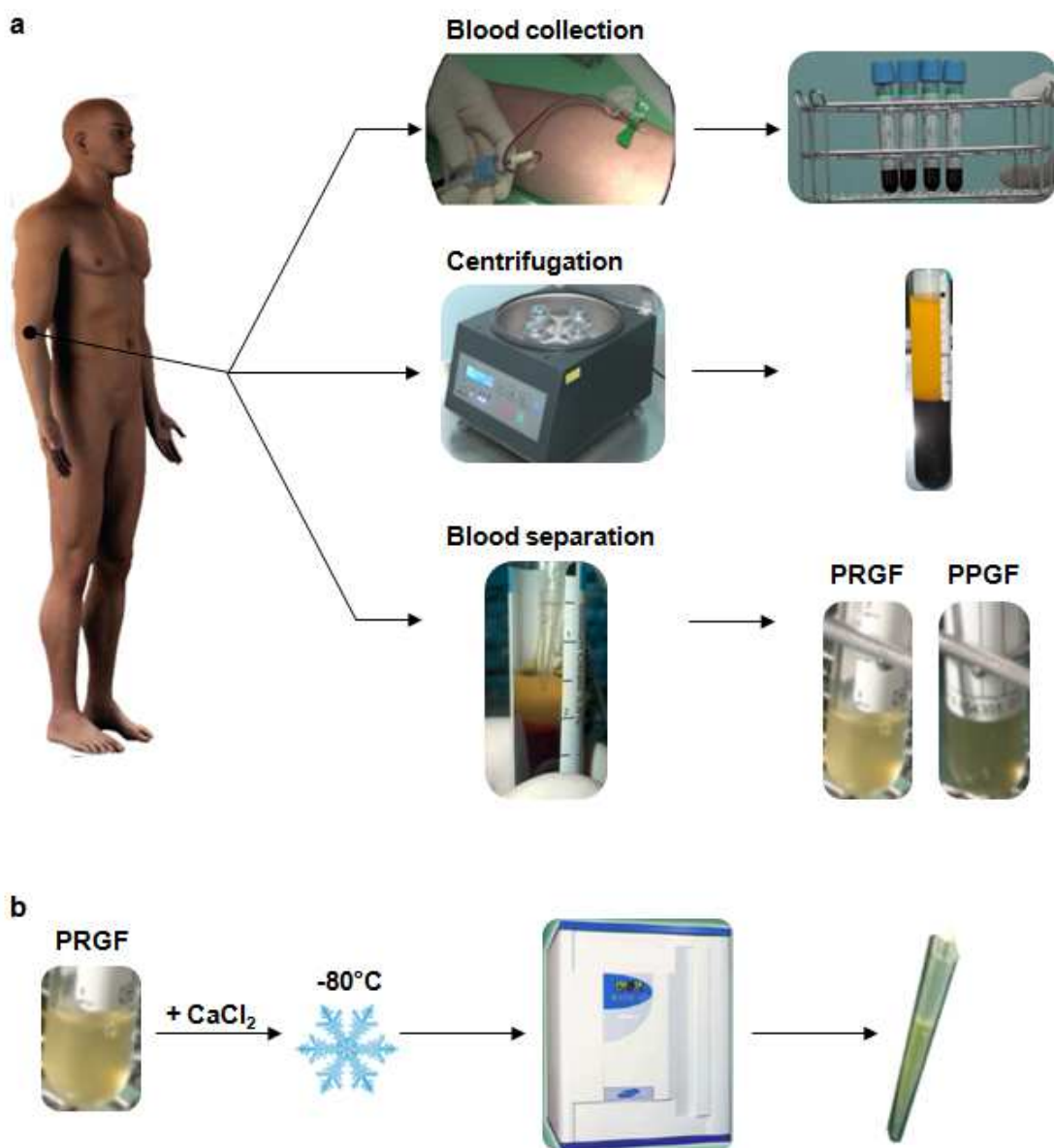


Figure 14. Plasma Rich in Growth Factors protocol. a) Blood collection and processing. b) Activated platelet concentrate's production.

1.4. Cell proliferation assay

From passage 1 to 3 in culture, cells were detached with 0.5% trypsin/ 0.2% EDTA and plated at a density of 8×10^3 cells/cm² in 6-well tissue-culture plates in control medium. After one day cells were starved for 24 hours to allow cell cycle synchronization, then culture medium was replaced by medium supplemented with either 5% PRGF or 10% FBS (CTRL). At day 4, 8, 12, cells were detached with 0.5% trypsin/ 0.2% EDTA (Sigma-Aldrich) and counted in a Burker chamber considering also trypan blue exclusion. All the experiments were performed in duplicates.

1.5. Cell viability assay

Cells derived from the counting assays at day 4, were used for vitality evaluation. From passage 3 to 5 cells were plated at a density of $1,5 \times 10^4$ cells/cm² in 96-well tissue-culture plates in the presence of either 5% PRGF or 10% FBS. After 5 and 12 days in culture, 100µl of 0.5mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) was added and incubated for 4 hours at 37°C. The formazan precipitate was solubilized with 100% dimethylsulphoxide (DMSO; Sigma.Aldrich) and the absorbance read at 550nm with a Celbio plate reader. All the experiments were performed in duplicates.

1.6. hASC and hOb osteogenic differentiation assay

hASCs and hObs at 1st and 3rd passages were plated in 24-well tissue-culture plates at a density of $7,5 \times 10^3$ cells/cm² for 7 days and 5×10^3 cells/cm² for 14 days, and let adhere overnight in control medium. After being washed and cultured for 24 hours in serum-free medium, either 2.5% PRGF or 10% FBS (CTRL) were added. Osteogenic stimuli such as 10nM dexamethasone, 10mM glycerol-2-phosphate, 150µM L-ascorbic acid-2-phosphate and 10nM cholecalciferol (OSTEO; Sigma-Aldrich) were added to the cell culture medium to induce differentiation. Cells were cultured for 7 and 14 days before the biochemical analyses, then they were lysed with 50µl of 0.1% Triton X-100. Cell lysates were incubated with 1mM p-nitrophenylphosphate in alkaline buffer (100mM diethanolamine and 0.5mM MgCl₂, pH 10.5) at 37°C. The reaction was stopped with 1N NaOH and absorbance read at 405nm with a Wallac Victor II

plate reader. Alkaline phosphatase (ALP) assay- enzymatic activity was then normalized respect to each sample protein concentration determined by BCA protein assay (Pierce Biotechnology, Rockford, Ill., USA).

1.7. Statistical analysis

All data are expressed as mean values \pm standard error (SE) and statistical analysis (unpaired Student's *t*-test) was performed by using GraphPad Prism 5.03 (GraphPad Software, San Diego, Calif., USA). Differences were considered significant at $p < 0.05$.

2. ANIMAL STUDY

2.1. Animals

For the *in vivo* study six adult male white New Zealand rabbits 3 months old and weighing 3-3.5 kg were used. All animal experiments were performed at the animal facility of the Department of Veterinary Medicine, University of Milan in accordance with current European Community standards on testing animals and for the treatment of laboratory animals (guidelines 86/609/EEC of the European Union), and approved by the Italian Ministry of Health (DL 116/92) under the Eurostars Project BioBone. The animals were housed in individual indoor cages with food and water provision *ad libitum*. They were clinically examined and acclimatized to their environment for 7 days before the experiment. Blood harvesting and surgery procedures were carried out by veterinary and oral surgeons.

2.2. Preparation of the activated platelet concentrate

Five milliliters of blood were collected immediately before surgery via ear venipuncture in anticoagulated tubes and centrifuged at 460g for 8 minutes at room temperature in a standard laboratory centrifuge. Blood stratified into the three typical layers (erythrocytes at the bottom, buffy coat in the middle and acellular plasma on top) and the 1ml of plasma just above the buffy coat (PRGF) was collected and activated with 10% CaCl₂ solution (PRGF-Activator®, BTI Biotechnology Institute, Vitoria, Spain) at 37°C for few minutes.

2.3. Experimental design and surgical procedure

Defects were randomly divided into 3 groups:

- 1) negative control group (sham), in which the defects was left without any graft;
- 2) Bio-Oss group, in which the defect was filled only with the bone substitute Bio-Oss (particles sized 0.25mm-1mm);
- 3) Bio-Oss+PRGF group, in which the defect was filled with Bio-Oss in combination with the platelet concentrate PRGF.

Each group consisted of 4 samples, for a total of 12 defects.

All experimental procedures were conducted under a clean protocol with the use of sterile materials and equipment. General anesthesia was induced with subcutaneous (SC) administration of Dexmedetomidina (Dexdomitor®) 80µg/kg, Ketamina (Ketavet100®) 15mg/kg and Buprenorfina (Temgesic®) 20µg/kg, and maintained with Lactated Ringer's solution administered with an intravenous catheter placed in the ear's vein. Each animal received 100% oxygen by means of a face mask, throughout the entire surgical procedure until complete awakening. After tricotomy and exposure of the proximal tibia, bilateral circular critical lesions (8x2mm) were made at the tibial crest using a 8mm diameter trephine (BTI, Biotechnology Institute, S.L., Vitoria, Spain). The defects were randomly divided so that the two sides of each rabbit received a different treatment: bone substitute alone (Bio-Oss®, Geistlich Pharma, Wolhusen, Switzerland), Bio-Oss+PRGF, no graft (sham -control) (Figure 15). For animals receiving the bone substitute in association with PRGF, the biomaterial was combined in a ratio 0.5g/ml PRGF.

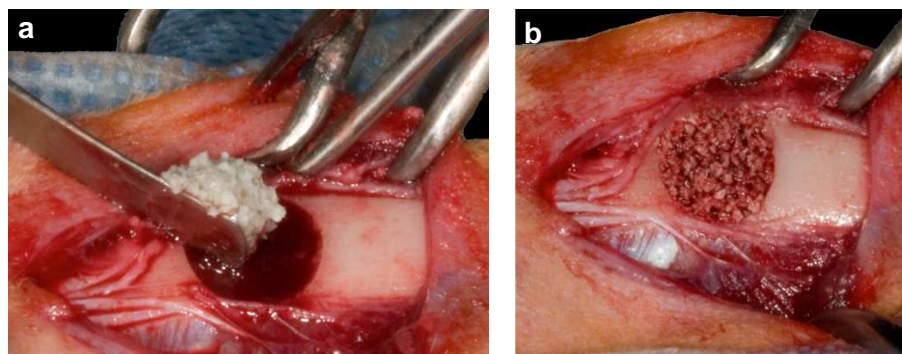


Figure 15. a) Creation of the bone defect. b) Defect filling with bone substitute.

After filling of the defects the overlying soft tissues were repositioned and sutured with an absorbable 2-0 suture (Ethicon®, Johnson & Johnson). The animals were then placed under post-surgical analgesic therapy with Buprenorfin (Temgesic®) 15µg/kg BID SC and Meloxicam (Metacam®) 0,2mg/kg SID SC for 4 days. Antibiotic prophylaxis was also done with Enrofloxacin (Baytril® sol. 2,5%) at 10 mg/kg/die SC and animals underwent periodical controls by veterinary staff until the end of the experiment (week 8 post-surgery). Three days after surgery fracture of one leg in a rabbit occurred, the animal was euthanized and another rabbit undergoing the same treatment (Bio-Oss on one side and Bio-Oss+PRGF on the other side) was used as a replacement.

2.4. Histological and histomorphometric evaluations

After 8 weeks, each animal was anesthetized by subcutaneous (SC) administration of Dexmedetomidina (Dexdomitor®) 80µg/kg, Ketamina (Ketavet100®) 15mg/kg and Buprenorfina (Temgesic®) 20µg/kg and then euthanized with 0.5ml/Kg EV Tanax. Left and right tibiae were removed from each rabbit, dissected free of soft tissue and bone blocks of the surgically involved area were explanted using a surgical bur (outer diameter 4mm, inner diameter 3.4mm) attached to a slow-speed electrical handpiece, and preserved and fixed in 10% neutral-buffered formalin for 48 hours at room temperature. Specimens were labeled so that the histologist was not aware of the group (treated or control) corresponding to the sample. The block sections were decalcified in a formic acid-sodium citrate solution. The solution was changed daily until an assay for free calcium in solution was negative (approximately 3 weeks). The assay for free calcium involved mixing 2ml of the decalcifying sample solution with 0.5ml of saturated ammonium oxalate. If a cloudy milky precipitate did not form within 10 minutes, decalcification was considered complete. After decalcification procedure, samples were rinsed under running tap water for 2 hours, and routinely processed through a graded ethanol series and embedded in paraffin. Serial sections of 5µm thickness of each specimen were prepared, followed by hematoxylin-eosin staining (H-E). Samples were evaluated for ascertaining structural details with a light microscope (Olimpus BX51, Olympus Corporation, Tokyo, Japan). Histomorphometric analysis on

digitalized images was performed by a blinded operator using the Image J software (NIH Image-J v.1.45s; US National Institute of Health, Bethesda, MD, USA). For the determination of the vital bone content, a x25 magnification was used, evaluating the complete section for each case; approximately 7mm² per each sample was examined. The newly formed bone, residual deproteinized bone bovine particles, and soft-tissue areas were measured semiautomatically and expressed as percentage of the total area.

2.5. Statistical analysis

Statistical analysis was performed by using GraphPad Prism 5.03 (GraphPad Software, San Diego, Calif., USA). Unpaired Student's *t*-test was used to compare mean percentage of newly formed bone between groups. All data are expressed as mean values \pm standard deviation (SD) and differences were considered significant at $p < 0.05$.

Results

1. *IN VITRO* STUDY

1.1. Platelet concentration in PC

The mean value of whole blood platelet concentration from the 5 volunteer donors was 248.14×10^3 platelets/ μl (range $219\text{-}291 \times 10^3$ platelets/ μl). After centrifugation, the mean concentration of platelets in the PRGF was 434.43×10^3 platelets/ μl (range $289\text{-}584 \times 10^3$ platelets/ μl), with a mean increase of 1,8% (Table 3).

Variable	number or mean \pm SD
Female/Male	4/1
Age (years)	26.2 ± 2.6
Platelets of blood ($10^3/\mu\text{l}$)	248.14 ± 31.13
Platelets of PRGF ($10^3/\mu\text{l}$)	434.43 ± 108.97

PRGF, Plasma Rich in Growth Factors; SD, standard deviation.

Table 3. Clinical data of the volunteers.

1.2. Cell proliferation

A preliminary *in vitro* study for choosing the ideal PRGF concentration was performed. hASCs were incubated for 4, 8, 12 days in medium enriched with 10% FBS (CTRL) or with 2.5% or 5% platelet concentrate. The percentage of increase or decrease of cell number derived from two independent experiments performed with PC from one donor, was calculated (Table 4). The highest cell count values were obtained when hASCs were cultured in medium supplemented with 5% plasma. After 12 days cells showed an increased proliferation up to 19.8-fold for PRGF respect to the control. 2.5% platelet concentrate induced a lower but comparable to the control cell proliferation. Consequently, the following assays were performed with 5% activated platelet concentrate as substitute for FBS.

PC (%)	4 days		8 days		12 days	
	2.5	5	2.5	5	2.5	5
	PRGF	110	336	-15	1138	-78

PC, platelet concentrate; PRGF, Plasma Rich in Growth Factors

Table 4. Effect of different concentrations of PC on human adipose-derived stem cells proliferation, expressed as percentage as compared to baseline.

Cell proliferation was demonstrated by the trypan blue dye uptake. At day 4, hASCs proliferated faster than in control culture condition (CTRL) medium and the number of cells collected after 12 days was about 10 times higher than the control, suggesting that these progenitor cells adapted easily to the new micro environment (Figure 16a). Human osteoblasts grown in the presence of 5% PRGF showed a proliferation rate either lower or similar to the CTRL at each time point (Figure 16b upper). However, hObs maintained in culture with PRGF for 12 days without any detachment, increased their growth by 1.2-fold respect to the CTRL (Figure 16b lower). A similar trend was observed for hDFs: at early time points cell number did not increase appreciably, while after 12 days of 5% PRGF, cell proliferation rate increased 3-fold as compared to the standard condition (Figure 16c), with a particularly notable growth peak. The incubation with PC determined a non-significant statistical increase between the PRGF stimulation and control during 12-day culture period for all of the three cell types ($P > 0.05$).

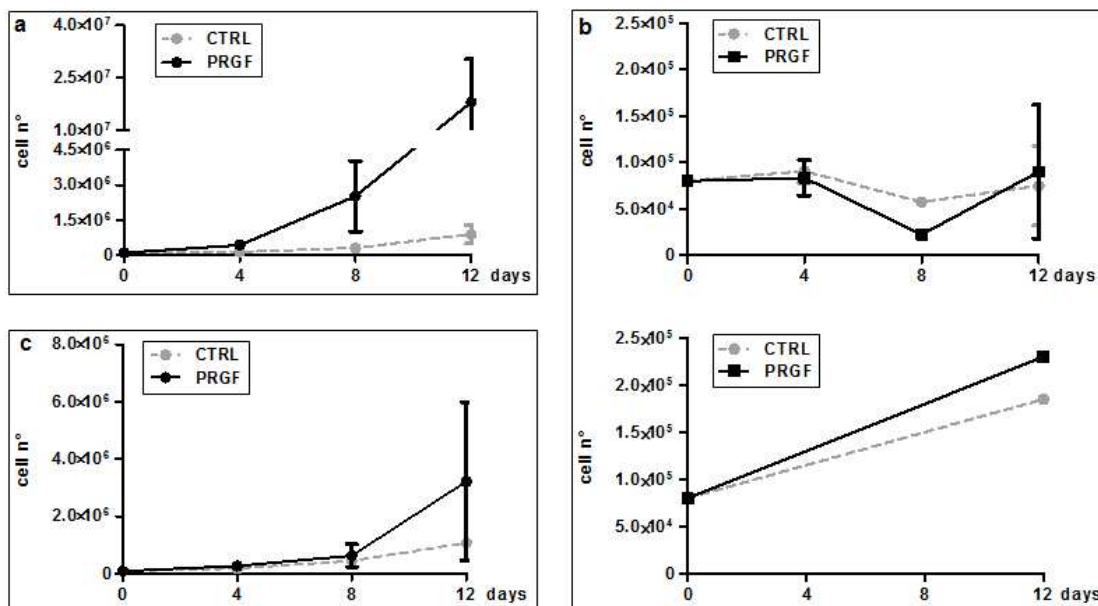


Figure 16. Effect of PRGF on hASCs, on hObs and on hDFs. a) hASC proliferation with PRGF. Data are shown as the means \pm SE (standard error) of three independent experiments performed with platelets concentrate derived from blood of three donors. b) Effect of 5% PRGF on human Osteoblast proliferation. hOb proliferation analyses were performed either after cell detachment every 4 days (upper) or without detaching them (lower). Data are shown as means \pm standard error (SE) of three independent experiments performed with the PRGF of two donors. c) hDFs proliferation after treatment with 5% PRGF. Data are shown as means \pm SE (standard error) of two independent experiments performed with the PRGF from one donor.

Morphological changes were never induced by PRGF, and phase contrast microscopy images after 4 days in culture, revealed a higher number of hASCs, hObs and hDFs when cells were grown in the presence of the platelet concentrate respect to the standard condition (Figure 17).

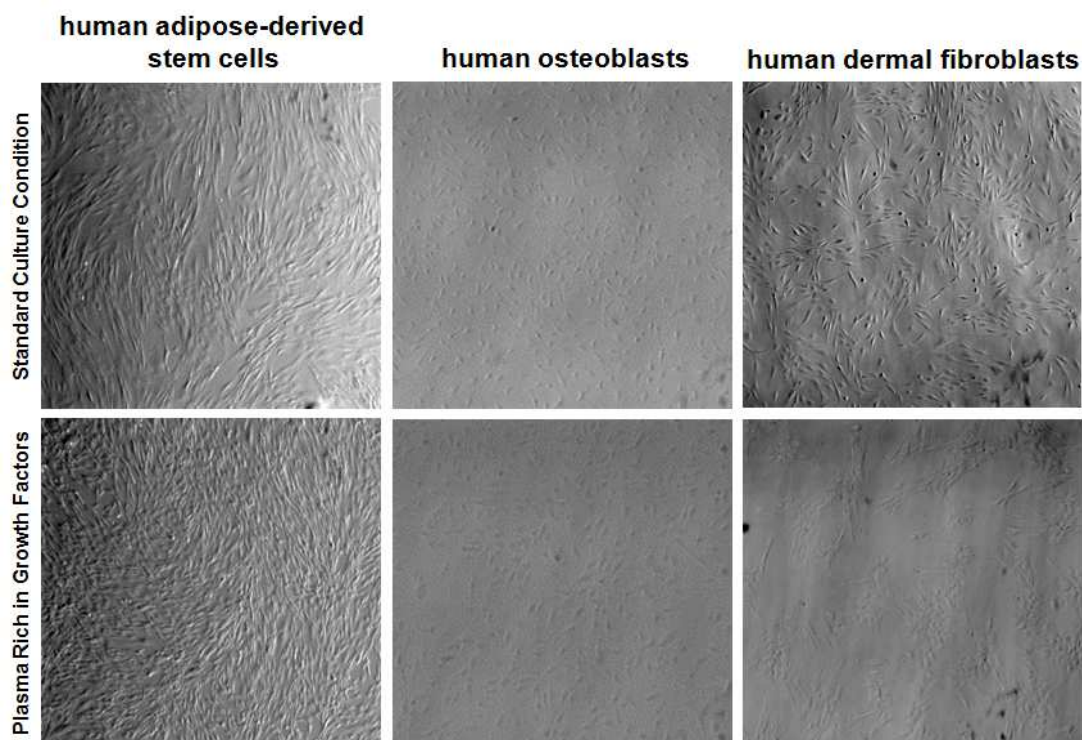


Figure 17. Representative phase contrast photomicrographs. hASCs, hObs and hDFs grown on a tissue culture plate for 4 days in standard condition (CTRL) and in media supplemented with 5% PRGF (40x magnification).

1.3. Cell viability

PRGF never exhibited cytotoxic effects on all of the three cell types. From MTT tests, at day 5, hASC viability was reduced compared to the control, while at day 12 it increased confirming that hASCs required more time to adapt to the human supplement, although this difference was not statistically significant (Figure 18a). hOb vitality improved of 1.8-fold after 12 days, whereas in CTRL condition it increased about 1.9-fold (Figure 18b) ($P < 0.05$). Moreover, hDFs grown for 12 days in medium supplemented with platelet-rich plasma achieved a 7.8-fold cell viability increase compared to day 5, whereas in CTRL condition it just improved of 2.4-fold (Figure 18c).

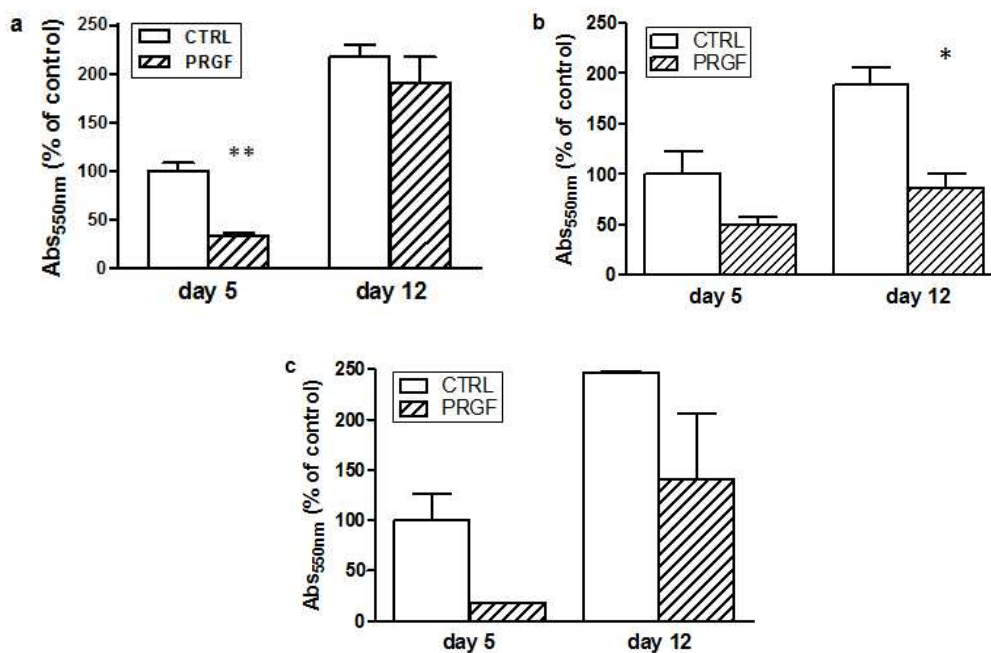


Figure 18. Viability of cells treated with PRGF. a) Evaluation of cell viability (MTT test) with 5% PRGF on hASCs. Data are shown as means \pm SE of three independent experiments performed with platelets concentrate derived from blood of three donors. ** $p < 0.01$ treated vs control value. b) Percentage of absorbance value of hObs with PC compared with that of each control was calculated. Results are expressed as means \pm SE (standard error) of three independent experiments performed with the PRGF of two donors. Significant statistical differences were seen between the PRGF stimulation and untreated control during 12-day culture period ($P < 0.05$). c) hDFs viability after treatment with 5% PRGF. Data are shown as means \pm SE (standard error) of two independent experiments performed with the PRGF from one donor.

1.4. Inter-donor and cell population-origin variability

The effect of PRGF from several donors and on different hASC populations was also analyzed. The inter-donor variability among platelet concentrates and hASC populations was less pronounced at early time points compared to later ones, and the marked effect of PRGF, independently of its donor and of the responder cells (different hASC populations) was confirmed at a later time point (Figure 19).

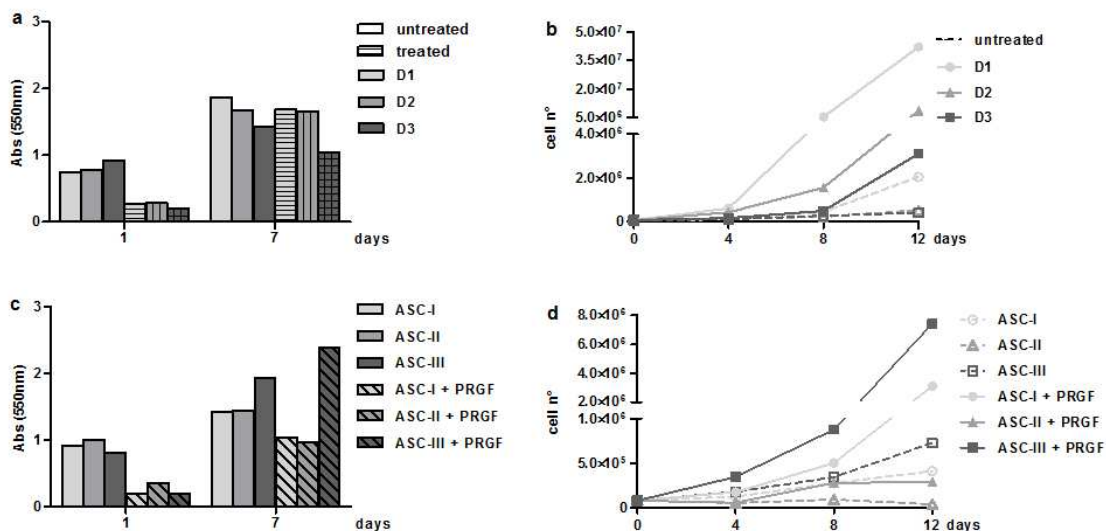


Figure 19. Effect of PRGF from different donors and the same PRGF on different cell populations. a) Cell viability (MTT assay) and b) proliferation (cell count) of a single hASC population (I) cultured with three PRGF samples (D1, D2, D3). c) Viability and d) proliferation of three hASC populations (I, II, III) cultured with a PRGF sample. A representative experiment for each condition is shown.

1.5. hASC and hOb osteogenic differentiation

Alkaline phosphatase activity was determined in two hASC populations (ASC-I and ASC-II) osteo-differentiated for 7 and 14 days in the presence of 2.5% PRGF. After 7 days the enzymatic activity considerably increased when platelet concentrate was present (Figure 20a). Cells cultured with PRGF exhibited higher levels of this marker compared to those cultured with FBS. In addition, in the absence of osteo-differentiative stimuli ALP activity was also induced. At day 14 the effect was reduced respect to day 7, but still present (Figure 20b). ALP activity was also determined in osteoblasts osteo-induced for both 7 and 14 days in the presence of 2.5% PRGF. As shown in Figure 20c, osteoblasts that received PRGF and differentiation medium (OSTEO) slowly increased their ALP activity, with an increase at day 14 (about 4-fold respect to day 7) and the presence of PRGF did not further induced this enzyme. Instead, hObs treated with FBS and OSTEO medium showed the greatest ALP activity increase at day 14.

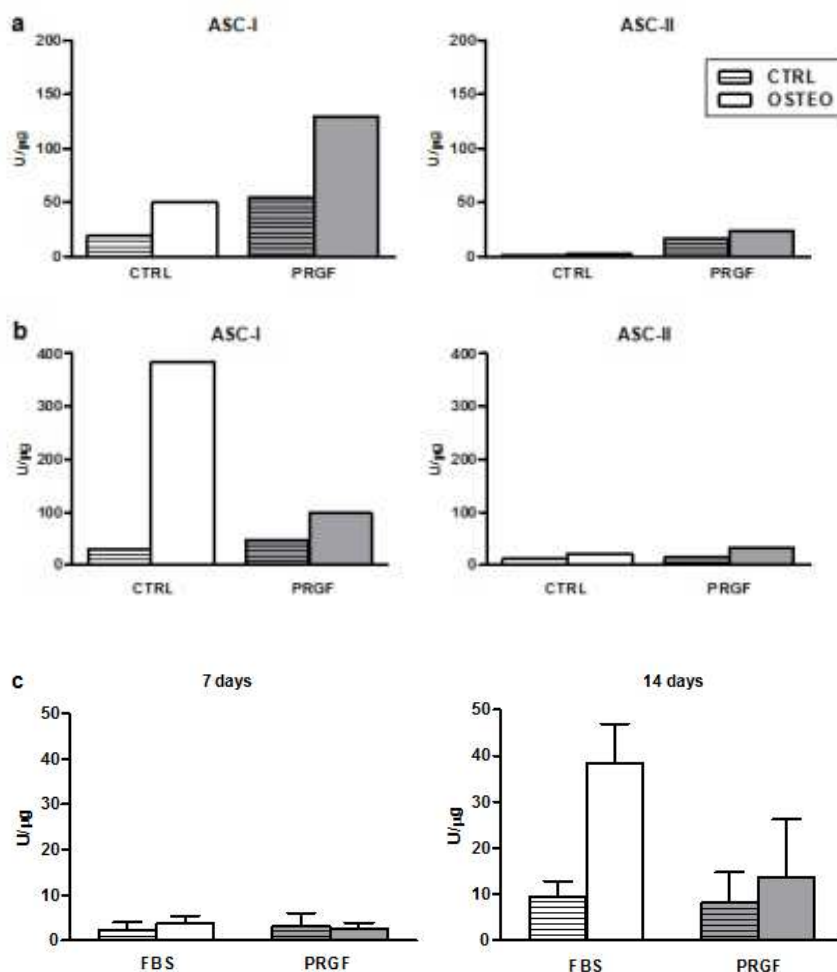


Figure 20. Osteogenic differentiation. a, b) ALP activity of two hASC populations (I, II) osteo-induced in the presence of 2,5% PRGF for 7 and 14 days, respectively. A single experiment for each condition is shown. (c) Differentiation assessment of hObs in the presence of 2,5% PRGF after 7 and 14 days. Data are shown as the means \pm SE (standard error) of two independent experiments. ALP activity is expressed as U/mg proteins.

In parallel, hASC calcium deposition tested by alizarin red staining (ARS) was also increased after 14 days of cell differentiation by the presence of PRGF (Figure 21).

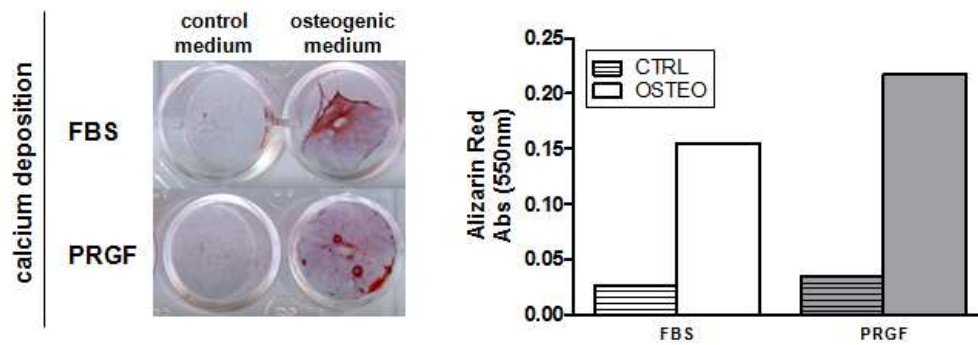


Figure 21. Calcium deposition. Alizarin Red Staining was used to measure calcium deposition after 14 days. Representative images of treated hASCs in plates are shown after staining. A single experiment for each condition is shown.

2. ANIMAL STUDY

2.1. Histological analysis

All specimens showed vital new bone formation. Samples left healing with no biomaterial graft (sham control) showed the presence of compact bone tissue, which lies in between the marrow tissue, parallel to portions of reparative tissue. Near to the latter, some osteoblasts were detectable indicating a regular bone healing process still ongoing (Figure 22). In defects treated with Bio-Oss alone (Bio-Oss group), granules of the biomaterial in contact with compact bone tissue that surrounded the granules were evident (Figure 23). This scenario is compatible with standard bone regenerative process. In the third group (Bio-Oss+PRGF) where Bio-Oss was used in association with PRGF, the biomaterial was evident in contact with compact bone tissue (Figure 24). Furthermore, in the region close to the biomaterial some osteoblasts were visible, indicating an ongoing bone formation process, a scenario compatible with standard bone healing process.

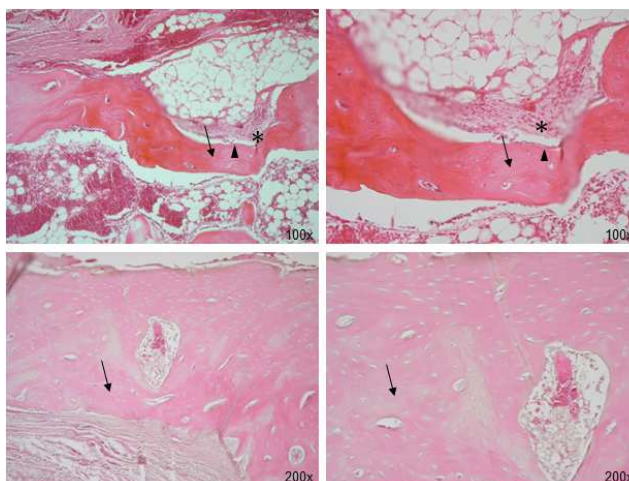


Figure 22. Sham control. Photomicrographs of group 1 after a healing period of 8 weeks revealing a trabecula of compact bone tissue (arrow) which lies in the myeloid tissue, parallel to a portion of reparative tissue (asterisk). Near to the latter some osteoblasts (arrow head) are visible (H-E staining).

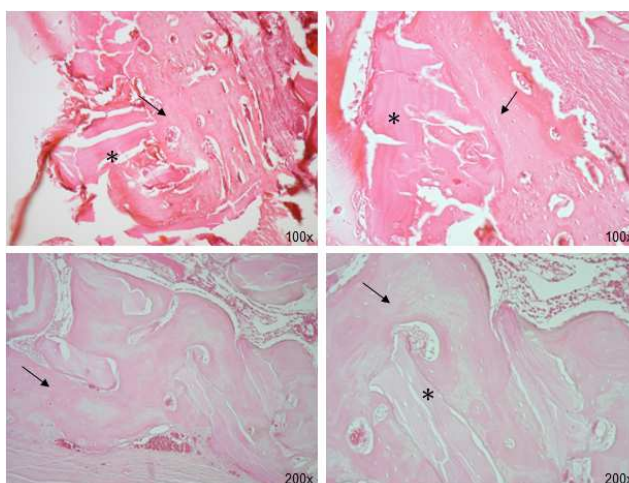


Figure 23. Bio-Oss. Photomicrographs of group 2 after a healing period of 8 weeks revealing the biomaterial (asterisk) in contact with compact bone tissue (arrow) (H-E staining).

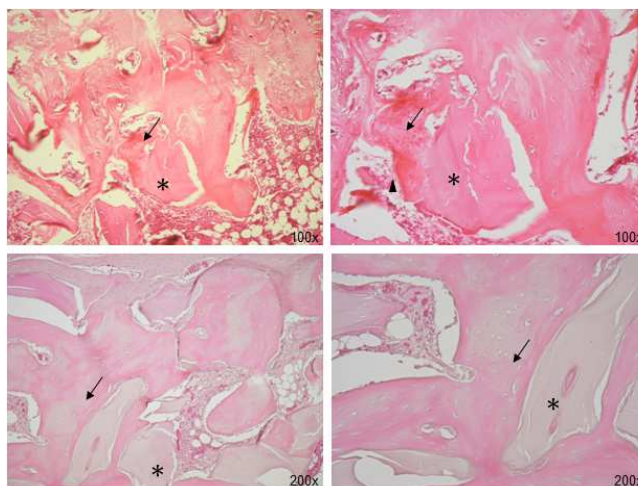


Figure 24. Bio-Oss + PRGF. Photomicrographs of group 3 after a healing period of 8 weeks revealing the biomaterial (asterisk) in contact with compact bone tissue (arrow). In the region close to the biomaterial some osteoblasts are visible (arrow head) (H-E staining).

2.2. Histomorphometric analysis

Data derived from histomorphometric analysis showed that after 8 weeks the mean percentage of newly formed bone was $18.7 \pm 4.0\%$ in the control group and $18.4 \pm 5.4\%$ in the Bio-Oss one, up to $26.0 \pm 2.2\%$ in the Bio-Oss+PRGF group (Figure 25). The differences between groups were not statistically significant ($p = 0.32$ sham vs Bio-Oss; $p = 0.17$ sham vs Bio-Oss+PRGF; $p = 0.09$ Bio-Oss vs Bio-Oss+PRGF).

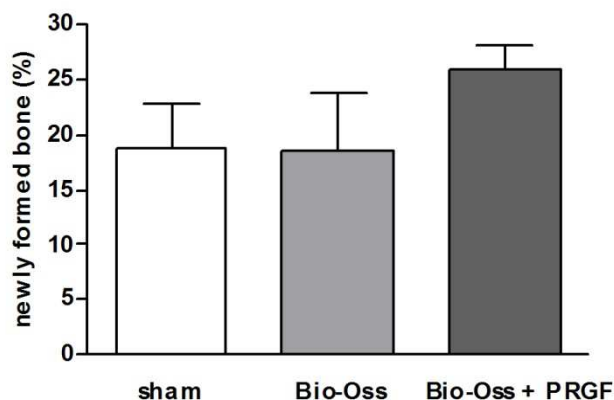


Figure 25. Newly formed bone. Mean percentage values \pm standard deviations (SD) of newly formed bone of each experimental group.

Discussion

Platelets are very important in the tissue healing process since they arrive quickly at the injured site beginning the coagulation cascade, and they release multiple cytokines and growth factors involved in wound healing [216]. Growth factors are thought to contribute to bone regeneration and increased vascularity, and play an important role in both soft tissue and bone repair process [217]. Many of the early phases of both bone and soft tissue reparative process, such as infiltration and proliferation of precursor cells, could be stimulated by a number of GFs in particular PDGF and TGF- β [91, 218, 219]. The use of autologous platelet concentrate has spread in different medical conditions for regenerative purpose [87]. Its effects on tissue regeneration have been shown in dentistry, oral implantology, orthopaedics, sports medicine, and treatment of skin disorders [87]. To date, different techniques to obtain platelet concentrates are available, each method leading to preparations that may differ in leukocyte and fibrin content, and platelet concentration [134]. Plasma Rich in Growth Factors (PRGF) is a modified Pure Platelet-Rich Plasma (P-PRP) introduced by Anitua in 1999 [135] and obtained by means of a manual protocol [220] from a small volume of patient's blood and sodium citrate and calcium chloride as anticoagulant and activator, respectively. The main difference with PRP is that according to the Anitua's protocol, leukocytes are intentionally excluded in order to avoid pro-inflammatory effects of proteases and acid hydrolases contained in white blood cells [87]. In addition, as this source of growth factors is autologous, there is no risk of immune reaction and infectious disease transmission associated with the use of this technology.

In the present study, it was demonstrated the *in vitro* ability of PRGF to induce proliferation of human adipose-derived stem cells, human osteoblasts and human dermal fibroblasts, and osteogenic differentiation of hASCs and hObs, all cell types being critical for wound healing of bone and soft tissues [221]. Such findings may support the clinical use of PRGF in regeneration procedures. Furthermore, the effect of PRGF was compared to control culture condition suggesting that a platelet concentrate could be a valid alternative to the serum commonly used for cell cultures, although further tests are needed to confirm

the biological comparability of platelet concentrate and fetal bovine serum (FBS). Mesenchymal stem cells from adipose tissue were used as a cellular model knowing their role in angiogenesis, growth factors secretion, inflammatory process regulation and differentiation capability [222]. We also included fibroblasts, for their important role in tissue repair [221], and osteoblasts, fundamental in the bone regeneration process. The longest observation time of 12 days was chosen *in vitro* as the end-point for the evaluation of cell proliferation since *in vivo*, after this period, the wound is usually covered with new tissue [46]. In this study cells and blood for the platelet concentrate's preparation were not harvested from the same donors. This choice was supported by a previous study reporting that cells grown in the presence of both human autologous and heterologous sera, increased their proliferation rate compared to standard culture condition [223], and especially to the absence of leukocytes from PRGF with a consequent reduced risk of immune reaction between the PC cells and the cultured ones.

To date, little is known about the *in vitro* effect of platelet concentrates on cell function.

Despite the low number of studies that investigated the effect of platelet concentrates on hASCs, the effectiveness of PCs in combination with hASCs on cell stimulation and tissue healing has been reported [224]. This was also confirmed in our study in which FBS was completely substituted by PC, and although culture media have been supplemented with a lower concentration of platelet concentrate compared to other studies [96], after a short adaptation period, cell viability and proliferation were comparable to the control ones. Most *in vitro* studies on PCs activity reported in the literature have been carried out on either animal cell lineages [225-227] or commercialized ones [94, 100, 228-230], while only few researches used human osteoblast primary cultures [95, 231-233]. This lack can be partially explained by the relative difficulty of obtaining such high-quality lineages and growing them *in vitro* [234] together with the complexity for osteoblasts to express and secrete in culture all the essential proteins for *in vitro* mineralized bone formation [218]. The clinical usefulness of platelet concentrates is still controversial especially for bone healing and repair. Although several animal and clinical studies have suggested that PCs may enhance bone formation [99, 235], a similar number of reports

have failed to demonstrate significant benefit [97, 143, 236-238]. Autologous bone grafts from intra- and extra-oral donor sites, have been regarded as a gold standard in the bony defects regeneration, especially in craniofacial surgery [239], but the disadvantages of donor site morbidity and limited possibility of harvesting autogenous bone, led to the use of bone substitutes to avoid patient discomfort. Currently, several degradable or permanent, mainly osteoconductive bone substitutes like tricalcium phosphate or xenogenic hydroxyapatite ceramics are available [216]. Some clinical trials suggested that the combination of autologous bone graft [132] and bone graft substitutes [240, 241] with growth factors contained in platelet concentrates may be suitable to enhance bone density. Bio-Oss is an inorganic osseous matrix produced after elimination of the medullar bovine bone organic components by means of a thermal treatment. It is a non-resorbable bone substitute [242, 243] that preserves the trabecular architecture and porosity and acts as a osteoconductive material. The physical properties of Bio-Oss permit clot stabilization and revascularization in order to induce osteoblasts migration, leading to osteogenesis [244]. In the present study, the combined effects of PRGF and bone xenograft in treating experimental critical size bony defects created in rabbit tibia were evaluated. Histological evaluation revealed that Bio-Oss is a biocompatible, osteoconductive grafting material with no sign of foreign body reaction and/or severe inflammation after its application. The addition of PRGF to this bone substitute did not affect its biocompatibility, as also reported in previous studies [245-248]. Regarding the amount of newly regenerated bone and remaining biomaterial particles, taken together the data from the histomorphometric analysis showed that the addition of PRGF to the biomaterial caused an increase in bone formation at 8 weeks, in comparison to Bio-Oss alone and control groups, although it was not statistically significant. These findings are in accordance with other studies [248-250] and this could be related to the properties of PRGF that favors the increase of bone metabolism and consequently might accelerate the rate of degradation of the biomaterial [248]. Nonetheless, in the present study, we found similar results when comparing Bio-Oss and sham, as the biomaterial alone could not significantly increase bone formation respect to the control group. These results could be probably attributed to an inadequate sample size, to the defect size (bigger defects could

not be created due to the small tibia dimensions) and to the time period of just 8 weeks that, given the fast metabolism of rabbits, might be sufficient to achieve complete bone healing also in the control sites. On the other hand, replacement of bovine bone mineral with natural bone appeared to be a slow process [245] then, since Bio-Oss is a hydroxyapatite compound, it is expected to have a slower rate of absorption. Therefore, also later findings could be important. Conversely, the addition of PRGF increased the rate of degradation of the biomaterial at 8 weeks. As Marx stated, platelet concentrates work via degranulation of platelets α granules that contain growth factors which, after being secreted, bind to the external surface of cell membranes of cells in the graft, flap or wound. Mesenchymal stem cells and osteoblasts are the principal type of cells expressing these trans-membrane receptors. Therefore, the addition of platelet concentrates to several grafting materials accelerates recruitment of osteoblasts and most probably, mesenchymal stem cells to the grafted area. This stimulates cellular proliferation, matrix formation, and osteoid and collagen production [217]. Accordingly, it can be postulated that platelet concentrates, such as PRGF, accelerate the remodeling process in which osteoclasts arrive at the grafted area more quickly and invade bone substitute's particles more effectively [217]. Additionally, differences in the study design (animal/human), collection efficiency of the platelet concentrates kit, evaluation methods (radiography/histology) and defects shape, size and configuration, may be implicated for the discrepancy in the results of platelet concentrate studies [247].

To date, also the amount of basic research about the effect of PCs on *in vitro* cultures is limited [251] and conflicting [232]. The current study indicated that PRGF technology favored proliferation of both osteoblasts and fibroblasts, in accordance with previous investigations on platelet concentrates [94, 95, 228, 231, 252]. Nevertheless, a number of studies evaluating the *in vitro* influence of PCs failed to demonstrate positive effects [100, 231].

In the current study, PRGF was prepared and utilized adopting the same protocol as in clinical application, except for the freeze-thawing cycle prior to the cell application. The latter, in fact, was demonstrated to have no consequences on PC biological activity [253]. The possibility to store platelet concentrates and the effects of freeze-thawing cycles on platelet activity are poorly understood

and still under debate. Some researchers consider freeze-thawing a procedure that could impair platelet function and lifespan, alter the GFs' release pattern, promote the pyrogenic cytokines accumulation and increase the risk of bacterial proliferation [254], while other authors believe that it does not affect the platelet concentrate's final properties [112]. Despite Zimmermann *et al.* [255] reported that freezing and thawing damaged platelet membranes, causing extensive degranulation and increase of GFs content in plasma, Perut *et al.* [256] showed that this procedure reduced platelet number, but did not affect the GFs release. This result was also confirmed by Roffi *et al.* [253] whose study underlined that although freezing and thawing affected the GFs release kinetics, fresh and frozen PCs did not significantly differ in their ability to induce cell proliferation. This storage option could simplify the management of patients undergoing multiple PC application sessions, keeping however in mind that biological results cannot always be directly translated into clinical outcomes [253]. Another interesting issue is the possibility that a prolonged storage time could have a strong influence on the results of platelet concentrates interactions with cells. Currently, there is a lack of data available about the preservation of biological activity potency of PCs after different storage times, but Anitua *et al.* [257] demonstrated that PRGF eye drops stored at -20°C for 15, 30 and 90 days maintained the same concentrations of the main proteins and growth factors, and did not affect the *in vitro* effects of the eye drops, therefore this point will be of great interest for future investigations.

Despite platelet count and PC growth factors content are likely influenced by the donor's age, gender and thrombocyte count, Weibrich *et al.* [258] did not find substantial variation in individual growth factors content and the donor's biological condition. In fact, our results indicated an improvement of the biological analyzed parameters despite a mild inter-donor variability of the tested PCs.

Aside from proliferation, the study herein presented also examined the PRGF pro-osteogenic activity by alkaline phosphatase activity (ALP) determination, an early osteogenic differentiation marker. Cells were maintained in media supplemented with 2.5% platelet concentrate, indicating that even such low amount of PC was adequate to induce cell survival. ALP activity assay showed that 2.5% PRGF is capable to support *in vitro* differentiation of hASCs

suggesting that platelet concentrates contain *per se* osteo-inductive factors which might activate some early steps of the osteo-differentiative process of mesenchymal cells. Our results are consistent with previous findings that demonstrated that the adjunct of platelet concentrates to culture medium promotes the differentiation of various cell types [95, 256]. Regarding the hOb function in terms of osteoblastic differentiation, our results cannot confirm a substantial contribution of PC to hOb differentiation. Interestingly, different papers indicated stimulation of the osteogenic differentiation potential of PCs [226, 259, 260], while others reported their inhibitory effect [231, 261, 262]. Such contradictory results could be explained by the great variability among PCs preparation methods and consequently their different cell content and growth factors concentration [232]. A higher platelet concentration should be in theory associated with an elevated growth factors content and consequently with an expected major effect on tissue regeneration [228]. Conversely, recent studies showed that cell proliferation is induced by platelet concentrates in a non-concentration-dependent manner. Indeed, higher platelet concentrations may have an inhibitory effect and may decrease the proliferation process *in vitro* [96, 231, 263]. This non direct-correlation between platelet count and growth factors concentration and the unclear relationship between platelet count and cellular response, was also reported by Zimmermann *et al.* [264] and Eppley *et al.* [220]. On the other hand, other authors reported that platelet count positively correlates with the quantity of growth factors released [265]. In principle, several factors should be considered that may affect the establishment of precise correlations. For example, the content of GFs stored in, and the amount of GFs released by each cell may vary. We should keep in mind that platelets derive from fragmentation of larger cells, the megakaryocytes, and this process does not always generate identical cells. Furthermore, platelets activation, which causes discharge of platelets granules content by exocytosis in the extracellular space, may not occur in the same way and simultaneously for all the platelets involved, especially in the *in vivo* situation. The GFs release kinetics may vary according to the interaction of platelets with the surrounding extracellular matrix and/or with other cells and biological mediators involved in early healing events [112]. Another possible explanation is that after platelet activation, not only a variety of GFs but also other proteins such as fibrinogen, β -glucuronidase,

thrombospondin and further coagulation factors, are released [233], that might interfere with the healing process. In particular, thrombospondin-1 (TSP-1) is an important angiogenesis inhibitor that is present in significant quantity in PCs. Cell proliferation reduction could be due to the elevated TSP-1 secretion in concentrated PC that possibly contribute to the antiproliferative effect via cell apoptosis [233]. In addition, Liu *et al.* [266] suggested that the cell proliferation was influenced by the pH as high PC concentrations resulted in pH increase that negatively affected the proliferation process. In particular, the precise PC concentration that would be optimal in promoting wound healing and regeneration is still undetermined. Therefore, more studies are necessary to establish if and how progressively higher PCs concentrations would influence the cell proliferation. A further limitation is that, according to the Anitua's protocol for PRGF production, leukocytes are intentionally excluded in order to avoid pro-inflammatory effects of proteases and acid hydrolases contained in white blood cells [267]. On the other hand, such manual procedure to excluding leukocytes may yield poorly reproducible results. After a low spin centrifugation, most platelets and leukocytes lie in the intermediate buffy coat, leading to a low platelet collection efficiency. Furthermore, the several manipulating steps, needed both to discharge the acellular plasma and to collect the entire PRGF fraction, might be associated with possible handling errors. The PRGF clot formed after activation is unstable and need to be applied immediately [134]. The lack of consistency and standardization in the PCs preparation may influence the research findings and the comparison among different studies [268].

Considering all the information from the literature, the results herein presented indicated that PRGF enhanced simultaneously both cell proliferative and differentiative capacity *in vitro* of all the investigated cell types, suggesting also that the concentrated cocktail of bioactive components present in platelet rich preparations could be a valid alternative to FBS as supplement for culture medium as also suggested by Lucarelli *et al.* [269] and Creeper *et al.* [95]. Additionally, since osteoblasts growth was lower than adipose-derived stem cells and fibroblasts, it can be postulated that this difference in proliferation rate could be due to the difficulty of maintaining hObs in culture and to the lower PC

concentration used to supplement the cell culture medium compared to other studies [94, 96].

An *in vitro* model for the investigation of the PC effects on cell proliferation and differentiation of primary cells was adopted in this study, hoping to determine possible correlations with the wound healing scenario *in vivo*, taking however into account that *in vitro* results could only be partially compared to the *in vivo* condition. In fact, the biological environment of a healing tissue is extremely more complex and undergoes continuous modifications regarding cell composition, tissue pH, oxygen level and soluble mediators content. In fact, *in vivo*, a far greater number of signaling molecules are involved and may interact with platelets and other cells in orchestrating the healing process. Recent proteomics studies showed that more than 4000 soluble factors are contained into and released by the platelet granules, and for most of them the actual role is still unknown [270]. Additional studies focusing on the proteomic analysis of the plasma concentrates with the idea to deplete some factors involved in the healing process, are needed to shed light on the actual role of platelets in the tissue regeneration process. Since large heterogeneity in clinical outcomes has been evidenced by many systematic reviews [271], more standardized clinical studies should be designed.

It can be concluded that PRGF supports the stimulation of wound healing and tissue regeneration in terms of cell proliferation and viability both *in vitro* and *in vivo*. Consequently, we provide evidence to support the clinical application of activated platelet concentrates in healing therapies.

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