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Healing or Not Healing

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Abstract: Healing process might be considered as a byproduct of the mechanisms underlying the biological defense system consisting of hemostasis and clotting, the innate immune system, and fibrogenesis. But there is no biological process that does not potentially entail high costs through trade-offs with other life-history parameters and that might be seen as collateral damage. Depending on the balance among the robust and flexible modular defense system, which will be deployed in many different arrays, the structural outcome of the healing process will not resolve with a unitary outcome. Drawing on the regenerative potential of platelets, plasma biomolecules and fibrin matrix, several systems of producing autologous platelets-and plasma derived products (APPDPs) have been developed and aimed at enhancing the natural in vivo tissue regenerative capacity of damaged tissues. Despite the care with which the medical staff elaborate and apply autologous platelets-and plasma derived products, some pitfalls arise regarding the composition of autologous plasma-and platelet derived products, the modalities of their application, and the *in vitro* versus *in vivo* evaluations, all of which can deeply influence tissue healing – a process which is already unpredictable, without a unitary mechanism that might be deployed in many different structural and functional arrays which culminate in the tissue repair. A biological approach to the application of autologous platelets-and plasma derived products is crucial to obtaining optimum functional healing outcomes in addition to avoiding poor clinical results and reaching misleading conclusions.

Keywords: Biological defence system, Inflammation, Platelets, Macrophages, Growth factors, Fibrin, Healing, Fibrosis, Innate immune system.

1. INTRODUCTION

In the animal kingdom, the struggle for existence frequently brings about wound trauma and/or infection. These two conditions, which have challenged the survival of living beings with a closed circulatory system, account for the primary life-threatening emergencies, namely, bleeding and microbial invasion [1]. Through natural selection and conservation, evolution has shaped the biological defence system of vertebrates (Fig. **1**) to cope with bleeding and microbial invasion by systems consisting of several interlinked modules such as hemostasis and clotting, the innate immune system, and fibrogenesis [1, 2].

Once tissue damage and/or pathogen invasion is detected, organisms mount a systemic and a local host defence response [2, 3]. The systemic response, that is not in the scope of this paper, primarily stems from the influence of locally produced prostaglandin E2 (PGE2) and other inflammatory mediators [4] on the central nervous system, and known as sickness behaviour (chiefly fever, anorexia, fatigue, and sleepiness) which appear to have only one purpose: survival [3, 5, 6]. The local response encompasses procoagulant and proinflammatory mechanisms which, together with the activation of platelets, endothelial cells, tissue-resident macrophages, recruitment of circulant monocytes and neutrophils, release inflammatory mediators in a quick autocrine and paracrine reaction (seconds to a few hours) [6-8]. The former cells manage to halt the bleeding process through platelet aggregation, thrombin generation, and fibrin clot formation [9] while the latter prevent the assault of microbial germ lines by killing them, thereby sterilizing the damaged area [8, 10]. Both processes of hemostasis and inflammation are triggered by a life-preserving program.

Immediately after this cascade module, where the pivotal players are platelets, leukocytes and fibrinogen, a dramatic switch from killing to healing occurs in the damaged area known as the resolution of inflammation [6, 11, 12]. As a continuum process, the biological defence program will later address reconstruction of the damaged area, which will require not only resolution of the inflammatory stage, but also the coordinated incorporation and action of other cell effectors such as macrophages, lymphocytes, fibroblasts, EPCs and structural biomolecules aimed at promoting angiogenesis, parenchymal and stromal cell proliferation and differentiation, and extracellular matrix (ECM) synthesis, [7, 8, 10] where the new formed fibrin clot acts as a transient scaffold (Fig. **2**) [13, 14].

Drawing on the regenerative potential of platelets, plasma biomolecules and fibrin matrix [13, 15-17] several systems that produce autologous platelets- and plasma-derived products (APPDPs) have been developed, aimed at triggering and

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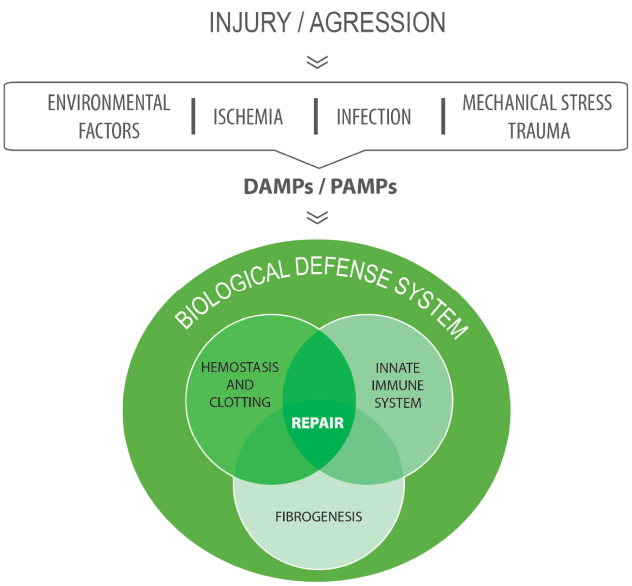

Fig. (1). Biological defense system. Tissue repair is an open and condition-sensitive process ruled by microenvironment cues. Depending on the balance of hemostasis and clotting, innate immune system, and fibrogenesis, parenchymal and stromal cell processes such as myogenesis, osteogenesis, angiogenesis, and neurogenesis will be deployed in different arrays 1, 2, 17.

enhancing both the natural *in vivo* tissue morphogenesis and the regenerative capacity of damaged tissues. Autologous platelets- and plasma-derived products (APPDPs) are platelet concentrates within a plasma suspension whose composition is determined by the method used to obtain it. APPDPs include plasma and twofold-or-more increases in platelet concentrations above baseline levels, and the concentration of leukocytes and erythrocytes varies widely, from a complete absence to a high concentration of them [18]. The therapeutic potential of APPDPs [19-21] and PRGF for *in situ* regenerative medicine has yielded extremely promising clinical and surgical outcomes in musculoskeletal system pathologies such as osteoarthritis and cartilage repair, [17, 21-27], in oral and maxillofacial surgery [17, 19], as well as in the treatment of diabetic ulcers [19].

This review article is aimed at both providing new insight into the potential role that autologous platelets-and plasma-derived products (APPDPs) may play in underpinning the biological processes of tissue healing, and shedding light on some pitfalls in the application of these products as enhancers of tissue repair.

BIOLOGICAL DEFENCE SYSTEM: CONNECTING THE DOTS AMONG HEMOSTASIS AND CLOTTING, IMMUNE SYSTEM, AND FIBROGENESIS

The presence of damaged and pathogen-associated molecular patterns (DAMPs or PAMPs) released as a result of necrotic and apoptotic cell death and damaged microbial and (ECM) host products, produce a complex cascade of mechanisms affecting hemostasis and clotting, the innate immune system, and fibrogenesis, which are triggered in a sequential and intertwined spatiotemporal manner [10, 28, 29]. DAMPs and PAMPs are damage signals which are recognized by transmembrane toll-like receptors (TLRs) expressed by several tissue cells that act as sensor elements including platelets, mast cells, macrophages, epithelial and endothelial cells, and neutrophils (Fig. **3**) [30-33]. The interaction of DAMPs and PAMPs with TLRs brings about, in these sentinel cells, the activation of a highly conserved intracellular signalling pathway known as NFkB. Its activation will end up inducing the gene expression of growth factors and cytokines, which will govern inflammation through the interplay between soluble factors and cells [6, 34]. This acute storm of pro-inflammatory cytokines such as interleukin 1beta (IL-1B), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-a), interferon gama (IFN-j), transforming growth factor beta (TGFB), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and connective tissue growth factor (CTGF) mainly coming from activated platelets, endothelial cells, pro-inflammatory macrophages, fibroblasts and mast cells, will attract, recruit, and activate circulating cells such as platelets, neutrophils and monocytes into the injury site [6]. From the beginning, platelets adhere to exposed collagen and tissue factor from damaged ECM, aggregate and facilitate blood clot and thrombin formation and polymerization of fibrinogen to fibrin, thereby halting the bleeding process [9, 13]. In addition to activating platelets and inducing pro-inflammatory cytokines from immune and endothelial cells [1, 35], thrombin performs other important roles as a potent endothelial permeability enhancer, chemoattractant of monocytes and neutrophils, inducer of expression of adhesive molecules and promoter of the degranulation of platelets, as well as influencing cell cycles [1, 15, 36]. Simultaneous with platelet actions, neutrophils generate a cytotoxic microenvironment [8] as a result of both degranulation of anti-microbial molecules, proteases and metalloproteinases (MMPs), highly reactive oxygen and nitrogen species (ROS and RNS), and by respiratory burst, inducing microbial death and sterilizing the damaged area (Fig. **2**) [8, 10]. Neutrophils also attract circulating monocytes to the injured site, and activate dendritic cells and macrophages. These resident and migratory mononucleated cells take on a pro-inflammatory phenotype (M1) releasing nitric oxide (NO) and citrulline, ROS, MMPs and a tissue inhibitor of metalloproteinases (TIMPs). Moreover, macrophages express IL-1, IL-6, IL-12, and TNFa, induce Th1 cell infiltration and activation, and phagocytose apoptotic/necrotic cells and other ECM breakdown products, thereby paving the way for the resolution of inflammation [6, 28, 37, 38]. This first cell-and bacterial-killing, and matrix destroying microenvironment stems from both the initial noxious agents and the collateral damage inflicted by the inflammatory effectors of the innate immune system which can wreak havoc if the inflammatory process is too intense or too persistent [6, 12]. In addition, platelets release Stromal cell-derived factor (SDF-1), CTGF, TGFB, Platelet factor 4 (PF4) and VEGF, which together with fibrin adhesion cell receptors may well contribute to the resolution of inflammation by recruiting endothelial progenitor cells (EPCs) [39], preventing monocyte apoptosis, promoting trophic macrophages (M2) [40] and activating resident mesenchymal fibroblasts precursor cells [41, 42]. In doing so, platelets somehow favour fibrogenesis [30] and generate both a trophic microenvironment, and tissue angiogenesis [43]. Simultaneously, the formed transient fibrin

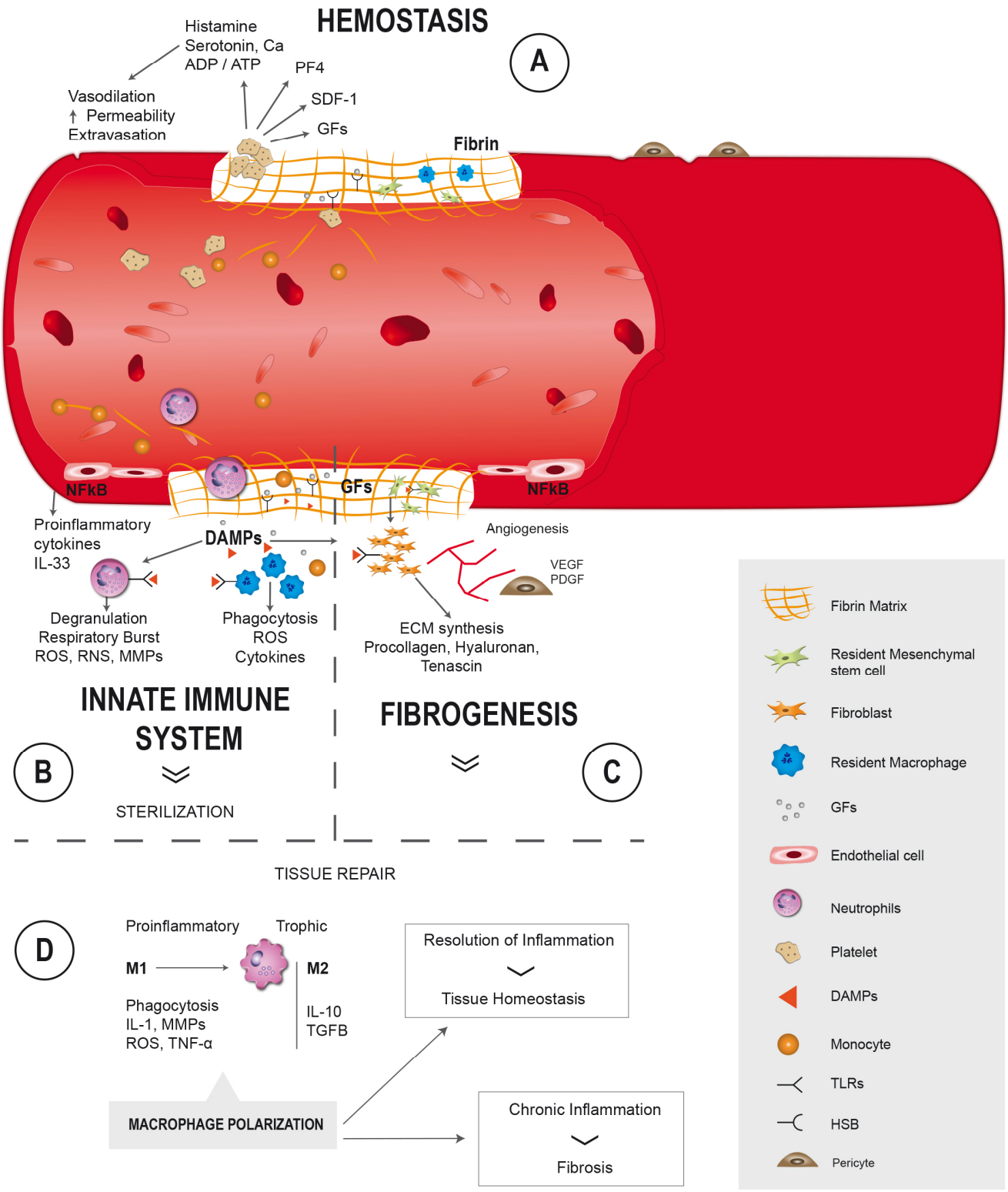

Fig. (2). Connecting the dots among the different modules of the biological defense system. Tissue reconstruction might be considered a by-product of the mechanisms underlying the biological defense system 23, 42.

matrix traps several growth factors and cytokines, promotes adhesion of immune cells, and is chemotactic for phagocytic leukocytes. Fibrin natural scaffold mediates cellular attachment through P-selectin and integrins binding on platelets, monocytes, macrophages, neutrophils, adult stem and hematopoietic progenitor cell antigen (CD34) progenitor cells [1, 13, 14, 43-45], and provides mechanical support and plastic-elastic stiffness which have a drastic impact on the fates of

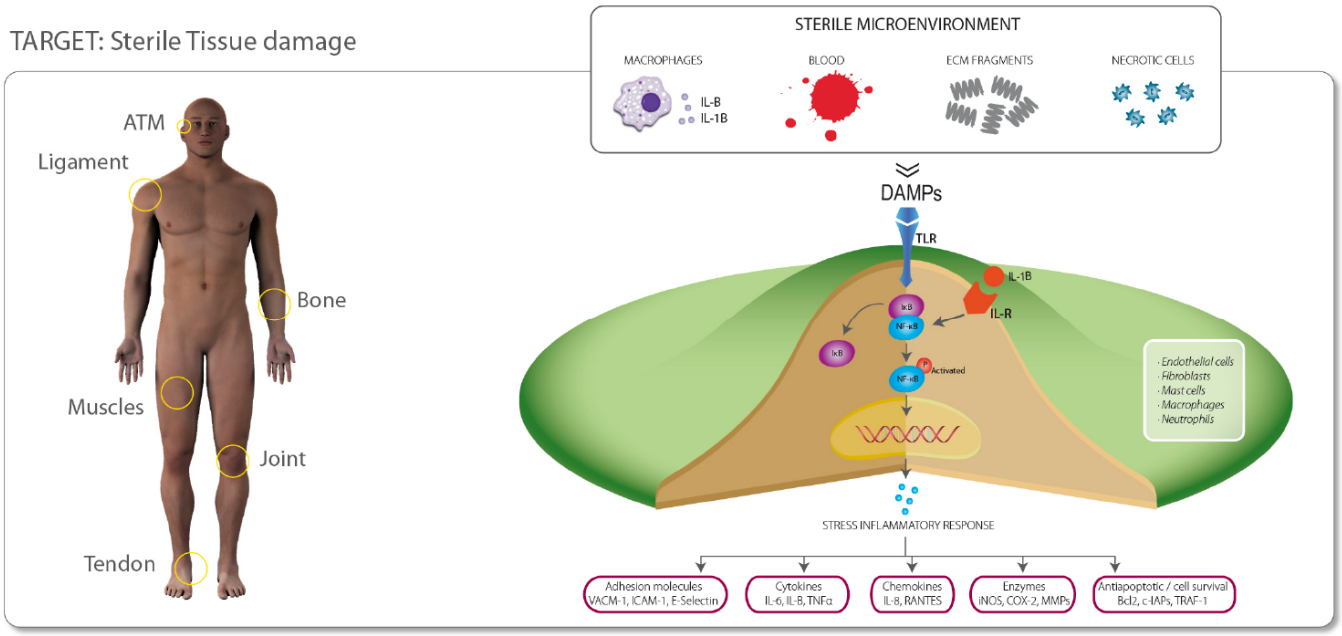

Fig. (3). Tissue repair in sterile conditions. Different types of signals reporting injury such as damage-associated molecular patterns (DAMPs) as well as toxins, minerals, crystals, chemical and antigens can trigger sterile inflammation 8, 20.

diverse cell types such as muscle stem cells [46, 47]. These cellular and tissular events give rise to the resolution of inflammation, which is mainly driven by the plasticity and polarization of macrophages from phagocytic and pro-inflammatory phenotype (M1) to anti-fibrotic and anti-inflammatory ones (M2) [2, 6, 11, 12]). In an uninterrupted process, the biological defence program will intensify and address the already ongoing reconstruction of the damaged area, which will require not only resolution of the inflammatory stage, but also the coordinated action of other cell effectors such as trophic macrophages (M2), lymphocytes, activated fibroblasts, EPCs and structural biomolecules aimed at promoting angiogenesis, parenchymal and stromal cell proliferation and differentiation, and ECM synthesis, [10, 28, 48]. As a result of local activation of the tissue plasminogen activator/plasminogen system, fibrinolysis will release the growth factors and cytokines previously trapped in the fibrin network through the cell surface heparan sulphate-binding domains [45, 49] such as SDF-1, PDGF, VEGF, hepatocyte growth factor (HGF), Brain-derived neurotrophic factor (BDNF), Fibroblast growth factor (FGF) previously released by platelets, macrophages, endothelial cells, and newly activated fibroblasts [13, 14, 43-45]. This gradual release of growth factors [45] from fibrin controls morphogen gradients at the repair scenario [50] and facilitates vascular, epithelial and mesenchymal reconstruction (Fig. **4**) [13, 14, 45]. Inflammatory macrophages (M1) will turn, after the phagocytosis of apoptotic neutrophils, lymphocytes or inflamed parenchymal and stromal cells, into an anti-inflammatory phenotype and express pro-inflammatory cytokines, including TGF B1, IL-4, IL-10, and PGE2 [12]. These cytokines are involved in immunoregulatory functions as well as in the resolution or progression of fibrogenesis [28, 37] which, along with PDGF, and TGFB1, are released from fibrin matrix [14, 45], leading the switch in the injured area from tissue breakdown to tissue reconstruction [12, 30, 38]. These cytokines allow circulating fibrocytes, perivascular pericytes, and resident mesenchymal cells to differentiate first into fibroblasts and later into alfa-smooth muscle actin myofibroblasts (αSMA) (Fig. **2**) [31, 37, 41, 45]. Both fibroblasts and myofibroblasts are highly synthetic and secretory cells that will address the loss of tissue in the injured area by synthesizing fibrillar ECM components such as collagen, elastin, fibronectin, tenascin-C, and hyaluronan, and releasing mitogenic and motogenic cytokines which, together with IL-33 released from the dying cells [51] modulate cells involved in mesenchymal and parenchymal healing response, and which are known as part of the Th2 response [30, 51, 52]. In fibrogenesis (Fig. **2**), besides the chemical signalling pathways coming chiefly from profibrotic macrophages, the biomechanichal signalling will strongly influence the myofibroblast activity and fate, mainly through the presence of CTGF [10, 41].

Depending on the balance among these modules of a robust and flexible biological defence system, which will be deployed in many different arrays, the structural outcome of the healing process will not resolve with a unitary outcome [53]. Therefore, the repair process might be considered as a byproduct, or epiphenomenon of the mechanisms underlying the biological defence system [15, 54] and the resolution of inflammation and fibrogenesis [28, 55].

The hemostatic-inflammatory period will heavily influence the ensuing healing process as a secondary outcome of the biological defence system, [55] and often the newly formed tissue presents several structural and patterning differences from the original one, the fibrotic scarring being the most unsuccessful and nonfunctional secondary outcome [10, 31, 56]. The resolution of the trophic or reparative period will be followed by the remodelling stage provided that

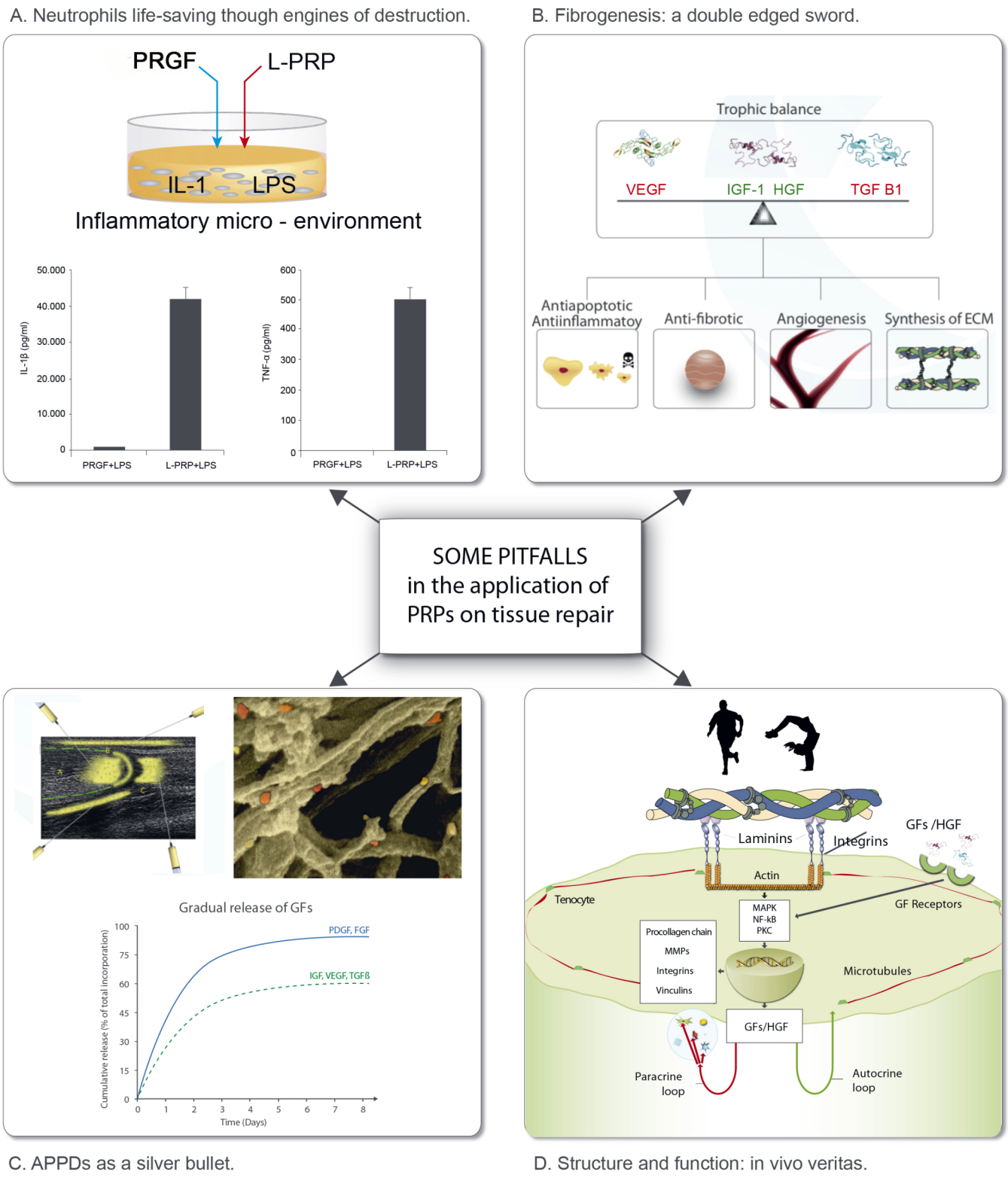

Fig. (4). Some pitfalls in the application of PRPs on tissue repair: composition of autologous plasma-and platelet derived products (APPDPs), fibrogenesis and pleiotropism of several growth factors, the modalities of their application, and the *in vitro* versus *in vivo* evaluations 15, 33, 70, 81, 84, 91, 94.

the apoptotic clearance of myofibroblasts by regulatory macrophages is carried out, thereby eliminating the stimuli inducing TGF- ß1 and other profibrotic factors which otherwise would lead to a persistent fibrotic microenvironment [10, 57].

THE SCIENTIFIC RATIONAL BEHIND THE THERAPEUTIC USE OF AUTOLOGOUS PLATELETS – AND PLASMA DERIVED PRODUCTS (APPDPs)

Pivotal players in the biological defence system modules are platelets, leukocytes and fibrinogen, located within a connective tissue, namely, the blood, which acts as an incessantly dynamic and renewing kind of container in mammals. In addition to the biological defence function, the blood plays a central role in other physiological processes such as the transport of gases by erythrocytes, caloric energy transport and body temperature regulation by water, communication of body systems by hormones, and transport of waste products, among others functions [58]. Determination of the functional roles of the many components of blood has been a daunting research task pursued in very diverse realms of the biosciences. In consequence of such successful research, we no longer think of blood as an indivisible tissue-body whose functions reside in the blood as a whole, but rather as a tissue with cellular and acellular elements that carry out a myriad of interactions and specific functions [58, 59].

Mammal platelets are circulating monitors, trackers and surveyors of the integrity of the vascular system and of the internal milieu as well as carriers of cytokines, chemokines and growth factors, coordinating coagulation, inflammation and repair processes as the core of the biological defence system [36, 60]. Platelets appear to be crucial in post-embryonic morphogenesis, and their activation in a biological context is carried out by factors such as thrombin, serotonin, or other tissue constituents such as DAMPs [1]. These molecules activate the platelets thereby releasing, by degranulation, growth factors and cytokines which, along with the formation of thrombin, trigger cell migration and proliferation, regulating angiogenesis, chemoattracting circulating progenitor cells, and guiding tissue remodelling and restoration of function [9, 15, 36, 61].

In addition to many bioactive mediators, (α-granules: TGFB, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF-1), hepatocyte growth factor (HGF), bone morphogenic proteins (BMPs), brain-derived neurotrophic factor (BDNF) and the dense granules: Histamine, Serotonin, Calcium (Ca) and ATP/ADP), there are other constituents in the plasma of APPDPs, namely IGF-1, HGF, prothrombin, fibrinogen, fibronectin and other proteins which, together with adhesive proteins expressed by activated platelets, play a central role in the cell signalling pathways which are involved in both tissue injury recognition and in repair of damaged tissues [9, 61]. For instance, several studies have reported an important HGF-mediated anti-inflammatory effect of platelet-rich plasma on tenocytes, macrophages and chondrocytes [62-64] by attenuating the transactivating activity of NF-kB [62] a highly conserved intracellular signalling pathway whose activation induces tissue inflammation [34]. HGF, a plasmatic key growth factor within APPDPs, has been shown to have a remarkable anti-inflammatory and anti-fibrotic effect on different tissues [65-67].

There are a wide range of APPDPs obtained by different blood-spinning preparation protocols (number of centrifugations and centrifugation speeds, the type of anticoagulant and clot activation) [18]. The preparation protocols heavily influence the composition of the final products (platelet concentration, the presence of pro-inflammatory leukocytes and erythrocytes, the level of activation) and thereby their biological effects [18].

PRGF (Plasma rich in growth factors) which is included in APPDPs, conveys growth factors, cytokines, and morphogens contained in the platelets, as well as fibrinogen and other plasmatic proteins in a biologically balanced aggregate, managed and delivered in a pharmacological manner [18, 21] which might account for two special features: the resolution of inflammation and avoidance of fibrosis [68]. In addition to conveying GFs, PRGF provides the damaged tissue with a transient biological fibrin scaffold which stems from the polymerization of fibrinogen. This pleiotropic blood protein regulates coagulation, inflammation, and tissue regeneration [14, 16, 61]. The three-dimensional fibrin network, formed either *in vitro* as a clot or *in situ* as an extracellular matrix, contains binding sites for cell adhesion as well as proteins such as thrombospondin-1 (TSP-1), α-1-antitrypsin fibronectin, acute phase proteins or proteins related to lipid metabolism [14, 16, 69]. This fibrin-scaffold formed as a provisional EC serves as a highway for mechanical energy to transit from the environment to the cell, bridges cell-to-cell tissue transition, promotes multi-cellular assembly, provides mechanical support and plastic-elastic stiffness which has a drastic impact on the fates of diverse cell types such as muscle stem cells [46, 47, 70], and endows tissues with a suitable microenvironment for biological restoration [9]. In addition, fibrin matrix, by heparin-binding domains, may sequester growth factors such as PDGF, FGF, HGF, BBNF, and VEGF [14, 45, 71] to gradually release them later.

SOME PITFALLS IN THE APPLICATION OF AUTOLOGOUS PLATELETS-AND PLASMA DERIVED PRODUCTS ON TISSUE REPAIR

Despite the care and seriousness with which the medical staff elaborate and apply APPDPs in different medical fields, the poor standardization in APPDPS therapies, the modalities of their application, and the *in vitro* versus *in vivo* assessments are elements that somehow are hampering advancement as well as drawing misleading conclusions about their clinical efficacy. We will try to shed some light on their biological underpinning, bringing into focus some pitfalls regarding the composition of autologous plasma-and platelet derived products (APPDPs) since this composition is crucially dependent on different blood-spinning preparation protocols, and the modalities of their application. They all can deeply influence tissue healing — a process which is already unpredictable — without a unitary mechanism (Fig. **4**) [53].

A. Neutrophils as Engines of Destruction Although Life-Saving

A first concern regarding the APPDPs, easily understandable though controversial, is leukocyte concentration. In a repair scenario, leukocytes may aggravate tissue damage and promote a pro-inflammatory microenvironment by re-

leasing TNF-α, IL-6, IFN-γ cytokines which induce the over-expression of MMPs, elastase and cathepsin G among others, thereby breaking down the ECM [8, 72, 73]. The release of reactive oxygen species (ROS) by neutrophils facilitates the removal of necrotic tissue in the repair stage but may exacerbate the initial lesion [74, 75]. *In vitro*, neutrophils injure cultured myotubes, and, in addition, can cause further muscle injury and disrupt some of the processes involved in skeletal muscle healing [76]. For instance, due primarily to a continual presence of offending elements in the damaged area, leukocyte infiltration may give rise to a nonresolving inflammation [28] thereby preventing the polarization of macrophages and triggering myofibroblast proliferation. These secretory cells may persist over time, leading to an excessive accumulation of ECM, ultimately producing a pathological and non-functional scar tissue (Fig. **2**) [31, 77]. Anitua *et al*. [78] reported that the inclusion of leukocytes in a fibrin scaffold obtained from APPDPs, both peaked the amount of two molecules involved in inflammation, IL-1 and IL-16, and produced a de-structured and heterogeneous aspect of the fibrin mesh [78]. In the same work and as a way of mimicking pathological conditions in a fibroblast culture exposed to pro-inflammatory cytokines, the addition of leukocytes to an autologous platelet- and plasma derived supernatant triggered over-expression of TGF-β1 and down-regulation of VEGF, an imbalance which may favor the formation of fibrosis. This phenomenon is not observed with an autologous platelet- and plasma-derived product supernatant which is leukocyte-free [78]. The presence of leukocytes in autologous platelet- and plasma-derived product formulations tips the balance of matrix synthesis towards catabolism [79] which may very well lead to a nonresolving inflammation [28]. Filardo and colleagues [22] compared the efficacy and safety of intra-articular injections of a leukocyte-free APPDP with a leukocyte-APPDP in the management of osteoarthritis. Patients treated with the former had fewer side effects than those treated with Leukocyte-APPDP, who presented more pain and more swelling. Tissue injury and microbial infection appear to represent distinct stresses to the host and much of the collateral damage inflicted by neutrophils and macrophages during the hemostatic-inflammatory period might be unnecessary to repair sterile injuries [80] such as musculoskeletal injuries (Fig. **3**).

Although a less common compound in APPDPs, erythrocytes too might be detrimental to healing, since their phagocytosis by macrophages may promote inflammation, oxidative stress, and promote the persistence of myofibroblasts, thereby leading to fibrosis [57].

B. Fibrogenesis: a Double-edged Sword

The concern about generating fibrosis has been present since the beginning of the therapeutic application of APPDPs, mainly because of the TGFB1 content in APPDP. The TGFB1 family has been implicated in the development of fibrosis in various tissues [10, 81, 82]. Several studies were conducted both i*n vitro* [83-86] and i*n vivo* [85, 87, 88] to try to tease out the influence of leukocyte-free APPDP on fibrosis. These studies confirmed that, although the TGFB1 family drives fibrogenesis, collagen synthesis and deposition, and it potentially might stimulate the formation of scar tissue [30, 48, 52], the concurrent presence of TGFB1, VEGF, and HGF in the same local environment makes leukocyte-free APPDP an antifibrotic autologous system. In the molecular network of leukocyte-free APPDP, the fibrotic effect of TGFB1 would be either modulated, counterbalanced, or even hindered by the presence and local production of HGF, a remarkable antifibrotic regulator [89] and the VEGF, as shown by our work on cells cultured on fibrin matrices [83-86] thus suggesting the pleiotropic behavior of TGFB1 [6]. For instance, in sheep [86] four intratendinous injections of a pre-clotted preparation of leukocyte-free APPDP into the Achilles tendon fascicles triggered a healing response which stemmed from an increase of cellularity, cell organization and angiogenesis. No signs of fibrosis were observed in the histological examination of the sheep Achilles tendons infiltrated with leukocyte-free APPDP [86, 88]. Likewise, the application of leukocyte-free APPDP fibrin matrices on the surgically repaired Achilles tendon tears on 6 athletes showed no wound complication and significantly shortened by 35% the functional recovery time, compared with the group that underwent the same surgical procedure without leukocyte-free APPDP application [90]. In addition, the cross-sectional area of the repaired Achilles tendon, assessed a few years later by ultrasonography, was significantly greater, while minor complications including 1 superficial infection and 2 keloids occurred in the non-treated group [90]. In another study [87], a comparison was made of the overall arthroscopic appearance and the gross morphology and histology of tendon grafts and of the joints of patients treated with leukocyte-free APPDP infiltration with those not treated during anterior cruciate ligament (LCA) surgery [87]. During the remodelling period (6-24 months) the treated group showed more signs of remodelling, maturation, and a synthesis of new connective tissue which wrapped the infiltrated tendon graft with more and better-oriented cells, more akin to the native LCA than in the non-treated one [87].

Recently two other studies assessed the biological effect of leukocyte-free APPDP on other cell lineages such as keratocytes, conjunctival fibroblasts [91] and gingival fibroblasts [92] and synoviocytes [93]. Leukocyte-free APPDP formulations promote the fibroblast phenotype and revert the myofibroblast phenotype to its original fate by protecting and inhibiting TGF-β1-induced myofibroblast differentiation. In conjunctival fibroblasts, leukocyte-free APPDP inhibits and reverses TGF-β1-induced α-SMA expression of fibroblasts as an expression of myofibroblast differentiation [94], thereby preventing the generation of scar tissue. By modulating gene expression and the gene products of cells as well as their cell cycle, the synergistic action of TGF-β1, VEGF and HGF conveyed by leukocyte-free APPDP might drive cell fates through epigenetic mechanisms [69]. The outcomes of these two studies suggest that leukocyte-free APPDP modulates the fate of myofibroblasts in a way that might be determinant in resolving both inflammation and fibrogenesis, and driving the repair events towards mimicking original tissue, rather than to a tissue-fibrotic outcome [10, 81].

C. APPDPs as a Silver Bullet

A third issue related to APPDPs efficacy in healing is the way they are applied in different fields. It has become commonplace to infiltrate APPDPs in the treatment of

musculoskeletal injuries as a kind of scatter shot instead of adopting a well thought out and executed biological approach. For instance, in a recently published study [95] the authors claim that "*The present clinical trial does not support the use of plasma rich in growth factors in the arthroscopic repair of rotator cuff tears because…*". The application of APPDP to rotator cuff tears is intended to provide the damaged structure with growth factors and cytokines as signalling molecules, one of the three elements involved in the repair process (the two others are cells and 3-dimensional scaffolds). However, it is not enough to add a storm of growth factors to a tendon which for years has been undergoing a degenerative process, and, as a consequence, may have exhausted its healing capacity. There should also be a systematic infiltration of the healthy peripheral tissue surrounding the injury, with the aim of recruiting, activating and mobilizing mesenchymal resident cells to contribute to tissue reparation processes and cell signalling pathways as well as activating endothelial cells and macrophages (Fig. **4**). A procedure has been developed for the arthroscopic repair of rotator cuff tears assisted by leukocyte-free APPDP which involves 5 infiltration sites: into the body and sutured tendon, into the myotendinous junction, into the subacromiodeltoid bursa, into the cancellous bone of the humerus, and finally into the subacromial space. In addition to these intraoperative local applications of leukocyte-free APPDPs, ultrasound evaluation is performed at the third and sixth week after the intervention, during which we proceed to a subsequent ultrasound-guided infiltration of leukocyte-free APPDP into the repaired tendon [96]. In another methodologically well conducted trial evaluating the therapeutic potential of platelet-rich plasma in the treatment of chronic Achilles tendinopathy [97] the authors concluded that "*In this first, to our knowledge, double-blind, block-randomized, placebo-controlled trial on the clinical use of a APPDP injection, there was no benefit on pain and function*." Although the study was elegantly designed and carried out, it is difficult to conclude that a single injection of APPDP, following a local anesthetic injection, could heal a rather degenerated chronic Achilles tendinopathy. A recently published study [98] by Charousset *et al*. showed clinical and radiological improvement of athletes with chronic Patellar tendinopathy who had been treated using three ultrasound-guided leukocyte-free APPDP infiltrations without any sedation or local injected anesthesia [98].

D. Structure and Function: *in vivo Veritas*

Last but not least in the evaluation of the efficacy of PRGF in the process of tissue healing is a big gap to be bridged between the *in vitro* reductionist approach of experimental models and the *in vivo* global approach of experimental models harnessed to tease out APPDPs biological effects and the ultimate mechanisms in virtue of which this biological system acts [99]. The efficacy of leukocyte-free APPDP relies on angiogenesis, innate immune system, innervation and biomechanical stimuli, whose combined presence is pivotal to recovering tissue functionality.

Musculoskeletal tissues are made up of cells that sense mechanical stress. Mechanical stimuli trigger a set of intracellular biochemical signals that will induce gene expression and gene products as a cell response [100-102]. As a paradigm for regenerative biology, mammalian muscle illustrates the significance of innervation acting together with environmental cues such as mechanical tension and tissue stiffness in tissue morphogenesis and repair (Fig. **4**) [103-105].

These events, often absent from *in vitro* studies, are mediated by soluble factors (BDNF, Glial growth factor (GGF), CTGF) which modulates the phenotype and fate of several types of cells such as satellite cells, activated resident fibroblasts (myofibroblasts), endothelial cells, macrophages and Schwann cells [41, 102, 105-108].

Regulating the gene expression products (TGF-β1, IGF-1, IL-10, collagen, fibronectin, tenascin-C) of these cells appears to be essential in the success of regeneration, [56, 81, 109] and mechanical signals coming from the cell environment and chemical signals such as IGF-1 expressed in autocrine and paracrine manner might well complement each other [77, 110, 111].

In the clinical setting, early gradual mechanical loading stimulates gene expression of trophic factors and signals such as COX-1 (cyclooxygenase-1), FGFb, CTGF, HIF-1 (hipoxia-inducible factor1), and BDNF influencing maturation and the correct patterning of myotubes, collagens and tenascin-C [53, 112, 113]. The aforementioned events, which play a crucial role in balancing tissue remodelling versus fibrotic scar in injured muscle imply that what is true for an isolated myofiber is not necessarily true for the entire muscle [114]. After muscle injury, the absence or disruption of innervation is the major cause of poor restoration of tissue, leading to compromised function [53, 112, 114].

Therefore, in musculoskeletal tissue repair, mechanotherapy might be utilized as a clinical application of mechanotransduction, understood as a means by which cells sense mechanical loading and respond to it, thereby harnessing the rehabilitation program in a synergistic application of the healing process, with the goal of full recovery of function [113].

DISCUSSION

Wound healing or tissue reconstruction might be considered a byproduct of the mechanisms underlying the biological defence program that entails a set of overlapping complex phenomena encompassing both the recruitment of competent cells to undergo spatiotemporal phenotype commitment and the patterning of cell gene-products to generate ECM and thereby, new tissues [15, 54]. There is a high degree of self-organization in the regeneration-repair process which could be seen as an open conditon-sensitve process where the environmental cues both biomechanical and physico-chemical, play a crucial role in influencing and modulating cell phenotypes, in the gene expression, and in patterning the new tissue to mimic the one to be replaced [16, 37, 103, 110].

As is the case in TGF- β1, VEGF and HGF, most of the growth factors and cytokines in APPDPs act on a variety of tissues just as they do in any biological system. These proteins exert their regulatory and pleiotropic-biological functions as members of a molecular network linking different modules or systems. Indeed, the results shown in this analysis suggest that applications concerning APPDPs are aptly

framed within Nesse and Dawkins' proposal that it is a mistake to seek single, uni-directional causal agents in biological processes; single specific biological factors do not exist for each function [5]. There are simply biological constituents which, in a particular tissue-cell environment, may act together as inhibitions/activations in so-called "genetic switch activities," and induce the expression of cell phenotypes with various behaviors to promote tissue regeneration [5]. This concept, which has been discussed by Huang, [115] may account for biological findings that would otherwise appear contradictory, such as the fact that the same molecule, for instance TGF-β1 or a given cell type, such as macrophages, may exert diametrically opposed biological functions [6, 116] or that the same adult stem cell may express different cell phenotypes in different microenvironments or tissue niches [117].

Much scientific evidence, together with the foundations of engineering biology, namely, standardization, decoupling and abstraction, [118] has paved the way for several groups to use the blood as a raw material from which to obtain APPDPs as endogenous regenerative technology [19, 119, 120] instead of using the whole blood which conveys a multiplicity of cells and biomolecules whose role in the repair process is negligible or even detrimental [57, 75, 76]. At the same time, there are several crucial differences between autologous whole blood and autologous platelet- and plasma derived products (APPDPs), and therefore it is quite inaccurate to infer clinical results from one product as applicable to the other, or to lump all these blood-derived products together [18, 121-123]. Healing does not mean "regenerating" as repairing does not mean "recovering the function".

FUTURE PERSPECTIVES

A biological approach to the application of APPDPs is crucial to obtaining optimum functional healing outcomes in addition to avoiding poor clinical results and reaching misleading conclusions. Attempting to optimize the degree of functionality by producing a repaired tissue that is intended to be structurally identical to the damaged tissue might be considered an "artificial" goal imposed by specialists. In other words, we are creating new goals and placing new demands on cell-based biological programs (to regenerate rather than repair in quiescent and non-dividing tissues) which were selected and conserved over millions of years in different species with a single goal: survival [1, 36].

As the body of knowledge about the regenerative effects of APPDPs grows, expansion of its applications and new challenges arise. Several unanswered questions remain, some regarding molecular mechanisms that give rise to the clinical benefits and others encompassing dosage aspects such as how many injections would be ideal in a first approach, the interval between them, and whether combining APPDPs with stem cells might enhance the healing power of APPDPs.

The time has come when we should no longer compare the biological and therapeutic efficacy of very distinct products in musculoskeletal orthopaedic surgery by lumping all autologous platelet-and plasma-derived products together.

CONFLICT OF INTEREST

The authors declare the following competing financial interest(s): E. Anitua is the Scientific Director and S. Padilla and G. Orive are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology".

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