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Disclosures:

Financial disclosure statements have been obtained, and no conflicts of interest have been reported by the authors or by any individuals in control of the content of this article.

0894-9115/10/8910-0854/0
American Journal of Physical Medicine & Rehabilitation

DOI: 10.1097/PHM.0b013e3181f1c1c7

REVIEW & ANALYSIS

Platelet-Rich Plasma in Muscle Healing

ABSTRACT

Borrione P, Di Gianfrancesco A, Pereira MT, Pigozzi F: Platelet-rich plasma in muscle healing. Am J Phys Med Rehabil 2010;89:854–861.

The muscle healing process is defined as a complex and dynamic process resulting in the restoration of anatomic continuity and function. This process is characterized by a cascade of events triggered by the tissue injury itself. It is widely accepted that growth factors play a central role in the healing processes by modulating the recruitment, duplication, activation, and differentiation of different cell types. This observation is the basis on which the use of platelet-rich plasma in several circumstances is founded; all of them requiring the activation or the modulation of the tissue repair process. There is an extensive documentation of in vitro and in vivo studies demonstrating the safety and efficacy of growth factors in the muscle healing process. Unfortunately, the precise biological efficacy and the lack of long-term side effects have not been clearly demonstrated. With regard to sports medicine, doping-related issues are still a matter of debate, especially regarding the treatment of muscle injuries. The purpose of this review is to examine the role of growth factors during muscle healing processes and to discuss the implications of platelet-rich plasma in its therapeutic applications. Sports medicine issues are also discussed particularly with regard to antidoping regulations.

Key Words: Growth Factors, Muscle, Healing Process, Platelets

As reported by the World Health Organization, musculoskeletal injuries are the most common cause of severe long-term pain and physical disability, affecting hundreds of millions of people around the world and accounting for the majority of all sport-related injuries.[1] Depending on the trauma mechanism, muscle injuries are classified as direct and indirect. The former is represented by laceration and contusion, whereas the latter class involves complete or incomplete muscle strain. Muscle strains are normally divided into three grades accordingly to severity (Table 1).

The healing process is usually defined as a complex and dynamic process resulting in the restoration of anatomic continuity and function. This process is characterized by a cascade of events triggered by the tissue injury itself. Physiologically, healing progresses in a series of overlapping phases. These stages include hemostasis (stage 1), the acute inflammatory phase (stage 2), the intermediate (repair) phase (stage 3), and the advanced (remodeling) phase (stage 4).

TABLE 1 Muscle strains classification in accordance to clinical severity

Grade	Clinical Manifestation
I	• Tear of a few muscle fibers with minimal swelling and discomfort • Minimal loss of strength with almost no limitation of movements
II	• A greater damage of muscle • Partial loss of strength and limitation of movements
III	• A severe tear across the whole section of the muscle • Total loss of the muscle function

Stage 1 usually starts with the formation of a blood clot and is followed by the local degranulation of platelets, which release several granule constituents, some of them at high concentrations. Stage 2 can last up to 72 hrs and involves a number of inflammatory responses. It is usually characterized by pain, swelling, redness, and increased local temperature. During the first 2 hrs after injury, peripheral muscle fiber contraction occurs. The resulting edema and anoxia led to further cell damage and death within the first 24 hrs. During this phase, the aim of the treatment is to control hemorrhaging and to minimize inflammation and pain. Nonsteroidal antiinflammatory drugs are an almost universally accepted treatment, and the only controversial issue regarding their use is the appropriate timing of administration.[2,3]

Stage 3 lasts from 48 hrs up to 6 wks. During this phase, anatomic structures are restored and tissue regeneration occurs. Several cell types are involved in this phase, and in particular, fibroblasts start to synthesize scar tissue while capillary neoformation occurs to reestablish nutrition to the area. This phase ends with the beginning of the wound contracture. Regular concentric strength exercises can be performed in this phase as soon as the subject has achieved a full range of pain-free motion. Moreover, controlled resistance activities can be initiated to maintain cardiovascular fitness.

Stage 4 lasts from 3 wks to 12 mos. This phase is characterized by collagen remodeling, leading to the increase in the functional capabilities of the injured tissues. The regeneration of the injured muscle does not fully restore muscle tissue to its previous level. In fact, it is well known that the two processes of healing and fibrosis compete with each other, impairing complete anatomical regeneration. Muscle stretching can begin in this phase as well as strengthening exercises.

The healing process is controlled by complex and dynamic molecular mechanisms involving local and systemic factors interacting with many different cell types recruited to the injured site from the surrounding tissues or circulation or both. Many signaling molecules involved in the previously described healing phases can be categorized under three different groups: proinflammatory cytokines, transforming growth factor-beta (TGF-β) superfamily members, and angiogenic factors. Each of these molecules shows different biological activities, mainly promoting the interactions among different cell populations at different stages of maturation. Skeletal muscle regeneration after injury is characterized by the proliferation and differentiation of muscle precursor cells. The fusion of such differentiated precursor cells leads to the formation of new young multinucleated myotubes. Similar to skeletal muscle development during embryogenesis, precise control of proliferation and differentiation during regeneration is critical to the generation of functional tissue with the correct number and types of cells.[4] Notably, experimental data have shown that recovery of contractile function after injury by a single, large-strain lengthening contraction involves the repair of the damaged sarcolemma with minimal myogenesis. On the contrary, recovery from multiple, small-strain lengthening contractions requires myogenesis.[5] Abundant evidence suggests that growth factors (GFs) may play a significant role during the muscle regeneration processes involving myogenesis.[4–6]

GFs are proteins secreted by cells acting through specific cell surface receptors on the appropriate target cell. Normally, the effect of each GF is related to its concentration and to receptor sensitivity, although some experimental studies have shown that at higher concentrations the GF effect may be reversed.[6] With regard to their activity, GFs are usually divided into three groups: mitogen, chemoattractant, and transforming factors. Although the roles of all the GFs involved in the healing process are only partially known, the efficacy of many of them has been extensively demonstrated. This hypothesis is the basis on which the use of platelet-rich plasma (PRP) in several circumstances is founded; all of them requiring the activation, modulation, speeding up, or amelioration of the tissue repair process.[6]

Platelets are small, nonnucleated cell fragments in the peripheral blood known primarily for their hemostatic role. Normal platelet count ranges from 150,000/μl to 400,000/μl. Alpha granules are storage units within platelets, which contain prepackaged GFs in an inactive form. The main GFs contained in these granules are PDGF (platelet-derived growth factor), TGF-β, FGF (fibroblast growth factor), IGF-I and IGF-II (insulin-like growth factor I and II), VEGF (vascular endo-

TABLE 2 Activity of the growth factors contained in the platelets α-granules

Growth Factors	Function	Stimulation of Myoblasts Proliferation	Stimulation of Myoblasts Fusion
Transforming growth factor-beta	• Stimulates mesenchymal cell proliferation[13] • Regulates endothelial cells and fibroblasts mitogenesis[13] • Promotes extracellular matrix production[13,15,16] • Stimulates endothelial chemotaxis and angiogenesis[12,13] • Inhibits macrophage and lymphocyte proliferation[12,13] • Inhibits satellite cell proliferation and differentiation[14]	No	No
Fibroblast growth factor	• Stimulates fibroblasts proliferation[19] • Promotes satellite cells proliferation[17–19] • Inhibits satellite cells differentiation[17–19] • Stimulates the mitogenesis of mesenchymal cells[21]	Yes	Yes
Platelet-derived growth factor	• Stimulates the mitogenesis of mesenchymal cells[9] • Stimulates fibroblasts chemotaxis and mitogenesis[8,9] • Stimulates satellite cells proliferation[11] • Inhibits myoblast terminal differentiation[11]	No	No
Epidermal growth factor	• Stimulates endothelial chemotaxis and angiogenesis[32] • Regulates extracellular matrix turnover[32] • Stimulates fibroblasts migration and proliferation[31,32] • Inhibits satellite cells apoptosis[32]	No	No
Vascular endothelial growth factor	• Stimulates endothelial cells mitogenesis[30] • Stimulates endothelial cells migration[30] • Increases vessel permeability[30] • Stimulates myoblast migration[28,29] • Inhibits myoblast apoptosis[28,29]	No	No
Insulin-like growth factor I	• Promotes the mitogenesis of mesenchymal cells[26] • Promotes collagen synthesis[24] • Stimulates fibroblast chemotaxis and mitogenesis[24] • Stimulates the proliferation and fusion of myoblasts[25] • Inhibits myoblast apoptosis[24]	Yes	Yes

thelial growth factor), and EGF (epidermal growth factor)[7] (Table 2).

PLATELET-DERIVED GROWTH FACTOR

The PDGF family is made up of PDGF-A, PDGF-B, PDGF-C, and PDGF-D, forming either homo- or heterodimers. Although the four PDGFs are inactive in their monomeric forms, PDGFs homo- or heterodimers bind to the PDGF receptor-α and receptor-β. These two receptor isoforms dimerize on binding, leading to three possible receptor combinations, namely, -$\alpha\alpha$, -$\beta\beta$, and -$\alpha\beta$. Interaction of PDGF with its receptors causes activation of the receptor tyrosine kinase, which leads to a cascade of biochemical events culminating in myogenesis.[8] PDGF plays a crucial role in the embryonic development, cell proliferation, cell migration, and angiogenesis and is a required element in cellular division for fibroblast. It has been demonstrated that PDGF is a potent mitogen for many mesenchymal cells, including fibroblasts and smooth muscle cells.[9] PDGF can also elicit other biological responses, including increased metabolic rates and chemotaxis for both human inflammatory cells and fibroblasts.[10] Moreover, it has been demonstrated that PDGF can regulate myoblast proliferation and differentiation in vitro,[11] suggesting that PDGF also plays an important role in increasing the number of myoblasts during skeletal muscle regeneration by stimulating myoblast

proliferation or inhibiting their differentiation or both.

Transforming Growth Factor-Beta-1

TGF-β is part of the family of proteins known as the TGF-β superfamily, which includes inhibins, activin, and bone morphogenetic protein. TGF-β is found in at least five isoforms, namely, TGF-β1, TGF-β-2, TGF-β-3, TGF-β-4, and TGF-β-5. Their amino acid sequences display homologies of 70%–80%, and TGF-β-1 is the prevalent form, being almost ubiquitously present, whereas the other isoforms are expressed in a more limited spectrum of cells and tissues. The TGF-β signaling pathway is involved in many cellular processes in both the adult organism and in the developing embryo, including cell growth, cell differentiation, apoptosis, and cellular homeostasis. Depending on cell type, the secretion of TGF-β can be induced by a number of different stimuli, including steroids, retinoids, EGF, nerve growth factor, vitamin D3, and interleukin-1. On the contrary, the synthesis of TGF-β can be inhibited by EGF, FGF, dexamethasone, calcium, retinoids, and follicle-stimulating hormone. It has also been demonstrated that TGF-β may influence the expression of its own gene. With regard to its function, TGF-β stimulates or inhibits the growth of many cell types, depending on the presence of other GFs, and is a potent chemoattractant for macrophages.[12] Experimental data have demonstrated that TGF-β stimulates the proliferation of undifferentiated mesenchymal cells; regulates the mitogenesis of endothelial cells, fibroblasts, and osteoblasts; promotes the production of extracellular matrix; enhances the proliferative activity of fibroblasts; stimulates biosynthesis of type I collagen and fibronectin; supports other GFs, specifically PDGF in the activation of satellite cells; stimulates endothelial chemotaxis and angiogenesis; and inhibits macrophage and lymphocyte proliferation.[13] In response to muscle damage, it has been demonstrated that TGF-β inhibits myogenic satellite cell proliferation and differentiation.[14] In addition to modulating myogenic satellite cell activity, TGF-β is also involved in promoting normal skeletal muscle architecture by regulating local collagen synthesis in tendon-related connective tissue.[15,16] Moreover, it has been demonstrated that measures that can alter the effect of TGF-β1 on its receptors may affect the fibrotic process. Indeed, Suramin, an antiparasitic drug that competes with TGF-β1 for its binding sites to the GF receptor, improved muscle function after experimentally induced muscle strain.[3]

Fibroblast Growth Factor

FGFs constitute a family of heparin-binding proteins involved in angiogenesis, wound healing, and embryonic development, playing a key role in the proliferation and differentiation of a wide variety of cells and tissues. One important function of FGF-1 and FGF-2 is the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures. One of the best-known FGF activities is the stimulation of the proliferation of fibroblasts that give rise to granulation tissue. FGF signaling is mediated through interactions between multiple FGF ligands and transmembrane tyrosine kinase receptors, resulting in activation of a number of signal transduction pathways. Myocytes express FGF ligands and receptors in a coordinated fashion, suggesting that these molecules participate in autocrine signaling in the myocyte. Experimental data have shown that satellite cells in vivo are likely to possess FGF receptors and that isolated satellite cells respond to FGF in culture. In this setting, it has been shown that FGF stimulates proliferation and represses terminal differentiation of satellite cells.[17–19] Moreover, it has been demonstrated that FGF-2 is released from damaged muscle fibers in direct proportion to the severity of the damage, that FGF receptor expression is greater during the early stages of muscle regeneration, and that the availability of FGF receptors plays a key role in the regulation of myogenesis.[20] These data confirm previous studies that showed that FGF stimulates cell proliferation in bovine and chick myoblasts in culture. The mechanism for FGF-stimulated proliferation seems to be the progression of cells from G0 to G1 in the cell cycle.[21] Finally, it has been demonstrated that FGF upregulates IGF-1 receptor expression in muscle cells through the inhibition of IGF-II peptide, thus suggesting the critical role of FGF in the synergistic effect of GFs during the healing process.[22] Unfortunately, although in vitro studies have demonstrated the clear importance of FGF during myogenic satellite cell proliferation, in vivo results find considerably less agreement.[23]

Insulin-Like Growth Factor-1

IGF-1, once called somatomedin C, is a polypeptide hormone with a molecular structure similar to insulin. It plays an important role in childhood growth and continues to exert anabolic effects in adults. Approximately 98% of IGF-1 is bound to one of the six binding proteins. IGFBP-3, the most abundant binding protein, accounts for almost 80% of all IGF binding. IGF-1 action is mediated by binding to its specific receptor, IGF-1 receptor, found on many cell types in many tissues. IGF-1 is one of the most potent natural activators of the protein kinase B (AKT) signaling pathway, a stimulator of cell growth and proliferation and a potent inhibitor of programmed cell death. Experimental data have shown that IGF-1 is chemotactic

for fibroblasts and stimulates protein synthesis.[24] In vitro studies have demonstrated the ability of IGF-1 and IGF-2 to promote myogenic satellite cell proliferation and fusion.[25] Moreover, it has been demonstrated that IGF-2 is produced by skeletal myoblasts during the early stages of myogenic terminal differentiation. Other studies have demonstrated that local administration of IGF to regenerating skeletal muscles in vivo delayed myogenic satellite cells proliferation and differentiation but enhanced muscle fiber enlargement during late regeneration[26] and that skeletal muscle overload and exercises with muscle eccentric contractions increased local IGF-1 in the muscles involved and that this increase was correlated with muscle hypertrophy.[27]

Vascular Endothelial Growth Factor

VEGF is a glycoprotein structurally related to PDGF and stimulates cellular responses by binding to tyrosine kinase receptors (VEGFRs) on the cell surface. VEGF is an important signaling protein involved in both vasculogenesis and angiogenesis, and it has been shown that VEGF stimulates the mitogenesis of endothelial cells and cell migration. It also exerts vasodilator effects and increases microvascular permeability. Experimental data have shown that myoblasts express VEGF, VEGFR-1, and VEGFR-2, and that VEGF administration in vitro stimulates myoblast migration and survival, protects myogenic cells from apoptosis, and promotes myogenic cell growth.[28,29] In normal muscles, VEGF and its receptors are expressed in vascular structures but not in muscle fibers.[30] After experimental muscle damage, VEGF and its receptors are expressed in regenerating muscle fibers, suggesting the presence of an autocrine pathway that may promote survival and regeneration of myocytes.[29] In vivo VEGF administration with recombinant adenoassociated viral vectors injected in normal mouse skeletal muscle resulted in the appearance of a subset of muscle fibers with a central nucleus, a hallmark of muscle regeneration.[30] Moreover, after experimental muscle damage, the delivery of adenoassociated viral -VEGF markedly promoted muscle fiber regeneration in a dose-dependent manner.[30]

Epidermal Growth Factor

EGF is the founding member of the EGF-family of proteins, which also includes heparin-binding EGF-like growth factor (HB-EGF), TGF-á, Amphiregulin, Epiregulin, and neuregulin-1, neuregulin-2, neuregulin-3, and neuregulin-4. EGF acts by binding with high affinity to epidermal growth factor receptor (EGFR), which is expressed in almost all types of tissues. EGF is a common mitogenic factor that stimulates the proliferation of different types of cells, especially fibroblasts and epithelial cells. EGF also influences the synthesis and turn over of proteins of the extracellular matrix, including fibronectin, collagens, laminin, and glycosaminoglycans. To a limited extent, EGF also augments angiogenesis, because it is mitogenic for endothelial cells. EGF is also a strong chemoattractant for fibroblasts and epithelial cells.

Experimental data have shown that EGF and TGF-α induce an equipotent stimulation of fibroblast migration and proliferation and that signaling via EGFR provides an antiapoptotic survival mechanism for satellite cells as they progress to a proliferative state.[31] Some evidence suggests that EGF can promote the growth of satellite cells to a greater extent than TGF-β and that in vitro EGF increases the proliferation of muscle-derived stem cells by increasing the number of mitotically active cells.[32] Moreover, it has been shown that HB-EGF gene expression is induced during myogenesis. HB-EGF is a member of the EGF family and derives its name from the fact that it binds tightly to immobilized heparin. This observation suggests that skeletal muscle cell HB-EGF could interact with EGF receptor and heparin sulfate proteoglycans on adjacent muscle cells to promote cell-cell contact, cell-cell signaling, or cell-cell adhesion, facilitating myotube or myofibril formation.[33]

Platelet-Rich Preparations

The efficacy of all of those GFs should be, in theory, directly proportional to their local concentration, and this hypothesis is the basis for the use of PRP in various circumstances; all of them characterized by the need to activate, modulate, speed up, or ameliorate the process of tissue repair.[6] Indeed, the fact that PRP contains several different GFs, in physiologic proportions, is an appealing characteristic when compared with the use of isolated GFs, because it has been clearly demonstrated that they act synergistically during the different phases of the healing processes. Other advantages of the use of PRP are that this preparation is relatively simple and easy to obtain and that there is little, if any, risk of developing an immune response. It has been demonstrated that PRP applied to muscle cells in vitro results in increased cell proliferation, satellite cells differentiation, and the synthesis of angiogenic factors,[34] and it has also been shown that its application in animal models enhanced muscle repair.[34] Clinically, it has been reported that the application of PRP in muscle injuries can reduce swelling and pain.[35] These studies showed that full recovery of functional capabilities was restored in half the expected time, and echographic images showed full regenerated muscle tissue after PRP treatment.[36]

Despite these reported successes, some researchers have raised concerns that PRP treatment

may induce a fibrotic healing response in muscle tissues. This hypothesis is based on the observed elevation of TGF levels after the injection of PRP into muscle. Indeed, experimental data have demonstrated that TGF stimulates fibrosis in cultured muscle tissue. If this were to be the case, it has to be remembered that fibrotic healing after muscular injury can lead to an increased risk of reinjury.[35] Additional concerns have been raised with regard to the presence of neutrophils in the PRP preparation. In fact, proinflammatory proteases and acid hydrolases released from leukocytes may act as a cytotoxic agent, causing secondary damage to the muscle tissue; the release of reactive oxygen species by neutrophils may act as an aggravating contributing factor.[37] This observation bases the current debate on white blood cells content in different PRP preparations. In fact, some PRP systems increase the white blood cells number, whereas others do not.

Although experimental data and clinical observation support the use of PRP to enhance tissue healing and although concerns of immunogenic reactions or disease transfer have clearly been eliminated as PRP is prepared from autologous blood, several limitations of this technique should nonetheless be considered. First, it has to be underlined that the review of the literature reveals a lack of standardization in the preparation of PRP, despite the several devices and systems available and the different manual techniques described. This observation may explain the inconsistent clinical and experimental results obtained in different studies. Conceivably, even if all PRP preparations contain a basic set of GFs, the relative concentration of each factor can differ among preparations. Therefore, it should be underlined that PRP preparation must be performed in accordance with the international and national laws regulating blood manipulations and blood product collection and control. It is also required that the asepsis of the whole procedure should always be guaranteed. However, controversies still exist, particularly with regard to the use of local anesthetics and nonsteroidal antiinflammatory drugs.[36] It could be advisable to avoid the use of local anesthetics, where possible, in order not to modify the local pH, which is essential for the stability of several GFs. It could also be of benefit to avoid using nonsteroidal antiinflammatory drugs so as not to reduce the first inflammatory response to injury, which is an essential step in the healing process. The second important limitation is that the majority of human studies have been performed on small samples, usually with a very small number of valuable controls. Therefore, most of the studies do not have enough statistical power to support a precise clinical position.[6] Finally, the lack of long-term side effect has not been clearly demonstrated to date. Indeed, some authors have suggested the possibility of long-term muscle fibrosis after repeated treatment with PRP preparations.

On the basis of the above limitations, it is clear that further studies are required to recommend or discourage the adoption of this approach in routine clinical practice. Moreover, greater knowledge of the biological response to PRP treatment and a greater standardization of the preparative technique are needed.

Sports Medicine Implications

With regard to sports medicine, doping-related issues should be considered when planning the use of PRP in amateur and professional athletes. For instance, in the section S2, entitled Peptide hormones, GFs and related substances of the 2010 World Antidoping Agency (WADA) prohibited list at point 5, it is clearly indicated that "Growth Hormone (GH), Insulin-like Growth Factor-1 (IGF-1), Mechano Growth Factors (MGFs), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factors (FGFs), Vascular-Endothelial Growth Factor (VEGF) and Hepatocyte Growth Factor (HGF) as well as any other growth factor affecting muscle, tendon or ligament protein synthesis/degradation, vascularisation, energy utilization, regenerative capacity or fibre type switching" are prohibited. Moreover, at point 6, it is clearly indicated that "platelet-derived preparations (e.g., Platelet Rich Plasma, 'blood spinning') administered by intramuscular route" are prohibited and that "other routes of administration require a declaration of use in accordance with the International Standard for Therapeutic Use Exemptions."[38] In summary, assuming that this technique, as has been suggested by several studies, is able to ameliorate the muscle tissue repair processes, it is still unclear whether the concentrated amount of GFs injected into skeletal muscles can affect performances. Some authors suggested that local treatment with PRP may have systemic effects, probably influencing GFs and inflammatory cytokines homoeostasis as well as antidoping evaluations.[39] On the contrary, other authors stated that PRP is unlikely to provide athletic advantages particularly because the small amounts of unbound GFs have too short a half-life to produce systemic anabolic effects.[40] Clearly, further studies are required to obtain scientific evidence strong enough to support a precise position. Meanwhile, the use of PRP preparations for the treatment of athletic injuries has to be performed in accordance with the above-reported WADA standards.

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