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New insights into and novel applications for platelet-rich fibrin therapies

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The therapeutic use of autologous platelet-rich plasma constitutes a relatively new biotechnology that has been a breakthrough in the stimulation and acceleration of soft-tissue and bone healing. The efficiency of this process lies in the local and continuous delivery of a wide range of growth factors and proteins, mimicking the needs of the physiological wound healing and reparative tissue processes. Consequently, the application of platelet-rich plasma has been extended to many different fields, including orthopedics, sports medicine, dentistry, cosmetic and periodontal medicine and cosmetic, plastic and maxillofacial surgery. This article highlights the use of this technology and discusses some of the obstacles and challenges that need to be addressed to maintain progress in this field.

Introduction: demand for musculoskeletal care

Musculoskeletal disorders are a major cause of morbidity throughout the world. They have a substantial impact on quality of life, including disability, with widespread economic consequences [1]. The severity of this problem is illustrated by the increasing number of patients suffering from articular diseases and osteoporotic fractures [2]. In addition, musculoskeletal injuries derived from sports activities and road traffic accidents also contribute to the escalation of social and financial costs [3]. As a result of this, the period between the years 2000 and 2010 has been named the decade of bone and joint, as an international initiative to promote and advance research on prevention, diagnosis and treatment [4].

Although a wide range of therapeutic options are available, several important drawbacks need to be addressed. For example, in the case of bone defects and lesions, although autologous bone autografts are the preferred treatment, the supply of suitable bone is limited and its collection is painful and carries a risk of infection, nerve damage and hemorrhage [5]. With articular injuries, such as osteoarthrosis, treatments other than surgery are palliative and do not modify the progression of the disease. Finally, in the case of muscle lesions the RICE (rest, ice, compression and elevation) principle is initially applied in combination with non-steroidal anti-inflammatory drugs but, unfortunately, there is still a high rate of injury recurrence [6]. Considerable research and clinical efforts are being made to accelerate tissue healing and to improve the therapeutic treatments for these musculoskeletal disorders.

Growth factors and healing mechanisms

It is generally accepted that growth factors have an essential role in the healing process and tissue formation [7]. In fact, all stages of the repair process are controlled by a wide variety of cytokines and growth factors acting locally as regulators of the most basic cell functions, using endocrine, paracrine, autocrine and intracrine mechanisms. Growth factors influence many of the processes common to both tissue repair and disease, including angiogenesis, chemotaxis and cell proliferation; they also control the synthesis and degradation of extracellular matrix proteins. Their mode of action is to bind to the extracellular domain of a target growth-factor-receptor that, in turn, activates the intracellular signal-transduction pathways [8,9]. The elucidation of some of the functions of growth factors in tissue repair has led to the conclusion that their controlled temporal expression is crucial following surgical interventions and in the treatment of musculoskeletal disorders, including bone fractures, cartilage defects and muscle and tendon lesions (Figure 1) [10].

These findings have led to a significant research effort aimed at testing different growth factors and cytokines as therapeutic molecules for the repair or regeneration of a wide range of tissues [11]. The molecules that have been most intensively investigated in orthopedic research include bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF). However, despite promising results from the early animal [12,13] and small-scale clinical studies [14], few large-scale clinical trials have been performed [15]. Recently, the field of bone repair has seen several

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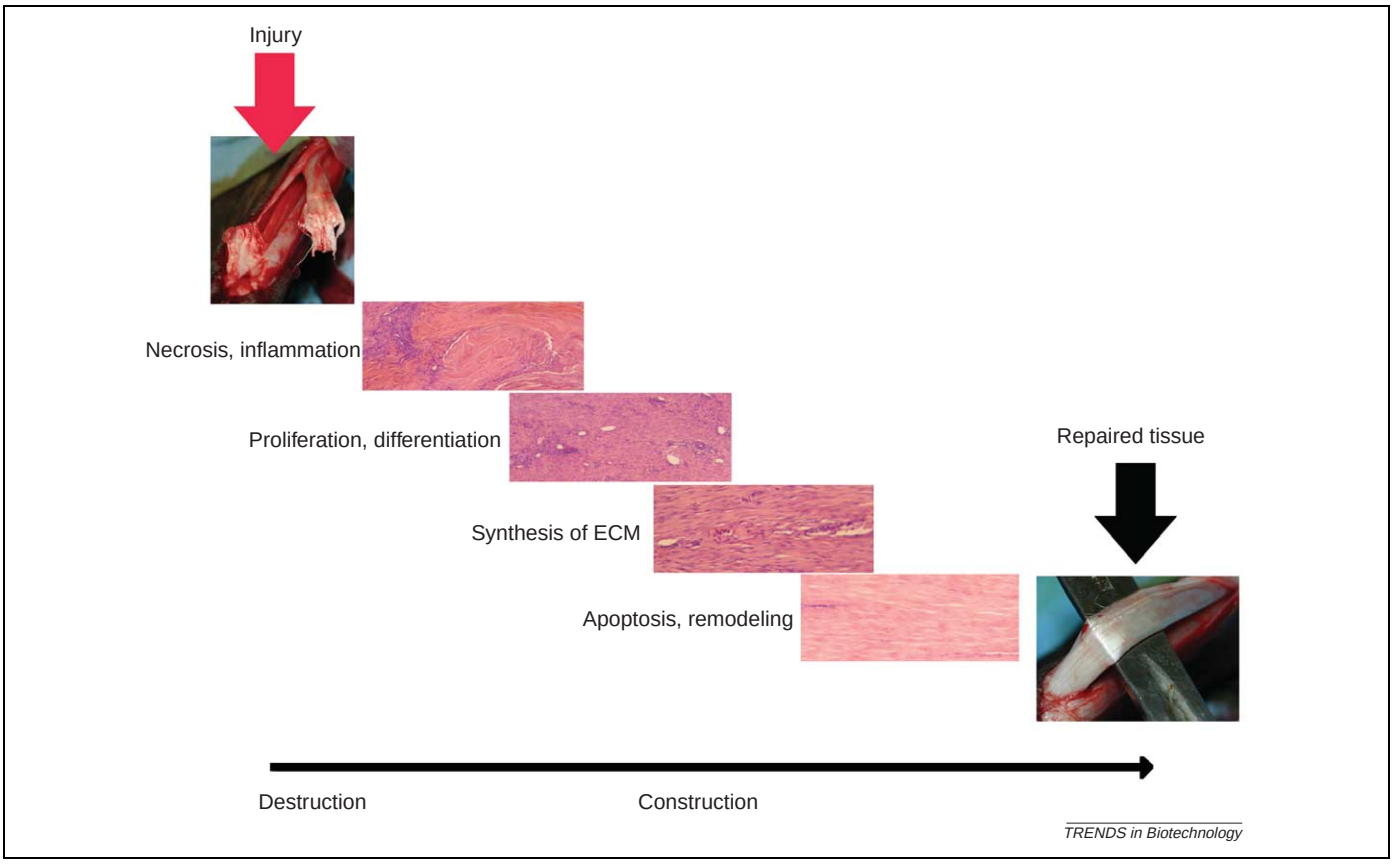

Figure 1. Injured Achilles tendon in a sheep model. Histological sections illustrate the different stages of the healing process, where growth factors are crucial regulators of cell functions. A platelet-rich fibrin scaffold enhances the natural process because it permits the progressive and balanced release of a large pool of molecules, including growth factors and other cytokines, mimicking the physiological process of repair.

firsts, namely, two BMP-containing products, incorporated in a collagen sponge, have been approved by several federal agencies for the treatment of long bone fractures. Unfortunately, besides these two products, no other growth factor is, at present, commercially available for clinical applications in the enhancement of bone regeneration [10].

Limiting factors in the current efforts are related to both the mode of growth factor delivery and the requirements for multiple signals to drive the regeneration process to completion. The latter is essential, assuming that no single exogenous agent can mediate, effectively, all aspects needed for tissue repair. Thus, delivery of a wide range of biological mediators is required if complete tissue engineering is to be achieved. Furthermore, the way these growth factors are made available is of paramount importance. Ideally, they should be delivered locally, following specific and distinct kinetics, to mimic, as far as possible, the requirements of the injured tissue during the different regeneration phases *in situ* [16]. The development of easy, non-toxic, safe and cheap therapeutic alternatives, which might result in the local release of growth factors for the treatment of musculoskeletal disorders and mimic natural expression patterns, is becoming the 'holy grail' of orthopedic, medical and pharmaceutical researchers.

Platelet-rich plasma

Platelets contribute to haemostasis by preventing blood loss at sites of vascular injury, and they contain a large number of growth factors and cytokines that have a key role in bone regeneration and soft-tissue maturation. In the past two decades, an increased understanding of the physiological roles of platelets in wound healing and after tissue injury has led to the idea of using platelets as therapeutic tools. Indeed, after fibrin glue was introduced in the early 1990s as a biomaterial with haemostatic and adhesive properties, the strategic modification of the fibrin to include platelets was reported [17]. The source of the new preparation, known as platelet-rich plasma (PRP), consists of a limited volume of plasma enriched in platelets, which is obtained from the patient. Once the platelet concentrate is activated by way of thrombin generation with calcium, a three-dimensional and biocompatible fibrin scaffold is formed (Figure 2), and a myriad of growth factors and proteins are released, progressively, to the local environment, contributing to the accelerated postoperative wound healing and tissue repair [18]. Furthermore, this preparation promotes rapid vascularization of the healing tissue and, because it is autologous, it eliminates concerns about immunogenic reactions and disease transmission [19]. Consequently, the use of autologous PRP as a novel therapeutic alternative opens new avenues in other fields such as orthopedics, sports medicine, dentistry, periodontal surgery and plastic and maxillofacial surgery.

However, there are controversies in the literature regarding the potential benefits of this procedure. In fact, although some authors have reported significant

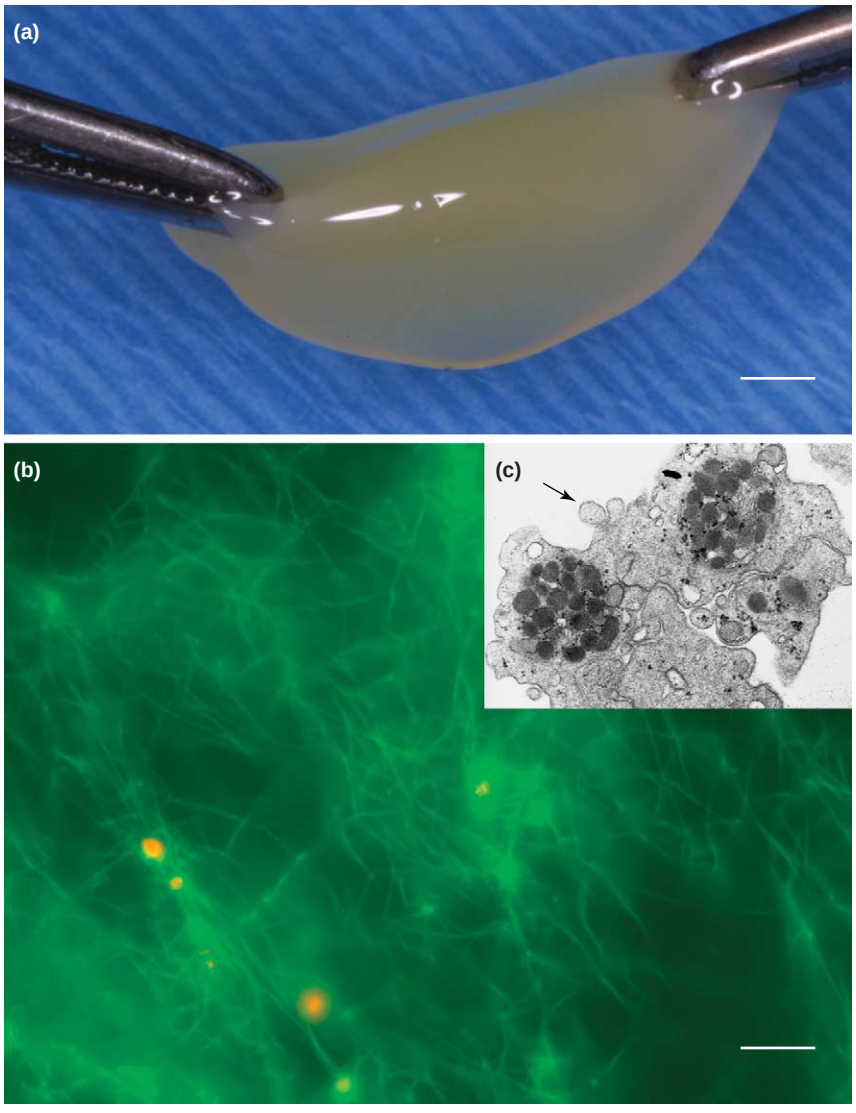

Figure 2. Activation of platelet-rich plasma with calcium brings about thrombin generation and platelet aggregation and the development of a fibrin scaffold. **(a)** Photograph showing the platelet rich fibrin scaffold (bar=5 mm). **(b)** Structure of the platelet-rich fibrin clot as seen by fluorescence microscopy showing a network of fibrin strands (green fluorescence) and platelets aggregates (red–yellow fluorescence) (bar=40 μm). **(c)** Transmission electron micrograph of a platelet aggregate showing signs of activation, including the centralization of granules and pseudopod extrusion (arrow).

improvements in tissue healing and bone formation using platelet-rich plasma [20–22], others failed to observe improvement [23–25]. Such discrepancies are probably related to the lack of suitable standardization and definition of the different PRP preparations; the protocols and biological and surgical techniques used in the elaboration and administration of the PRPs differ widely [26,27]. Variations in some key properties of the PRPs, including the platelet concentration, the type of clot activator, the leukocyte content and the time that the fibrin scaffold is put into place around the tissue after clotting has started can influence the different biological effects markedly.

For more than a decade our group has studied the characteristics and the potential impact of the different variables on the use of platelet-rich preparations in healing. These efforts have given rise to an optimized and safer product known as 'preparation rich in growth factors' (PRGF), which circumvents many of the limitations of other reported PRPs [21]. For example, sodium citrate and calcium chloride are used as an anticoagulant and a clot activator, respectively. Addition of calcium chloride promotes the formation of native thrombin, mimicking the physiological clotting process and enabling a more sustained release of growth factors, which might be crucial to proper tissue repair and wound healing [28]. Moreover, this procedure obviates immunological reactions and the risk of disease transmission associated with the use of exogenous bovine thrombin [29]. PRGF contains a moderately elevated platelet concentration of $\sim 6 \times 10^5$ platelets/μl, which has been reported to induce the optimal biological benefit [30]. In fact, lower platelet concentrations can lead to suboptimal effects, whereas higher concentrations might have an inhibitory effect [30]. Finally, in the quest for a safer and more effective product, PRGF does not contain leukocytes, thus improving the homogeneity of the product and reducing donor-to-donor variability [26]. The former is particularly

relevant because neutrophils express matrix-degrading enzymes, such as matrix metalloproteinases-8 (MMP-8) and MMP-9, and release reactive oxygen species that destroy all surrounding cells, whether injured or healthy [31].

Therapeutic applications

The therapeutic administration of PRP extends to the treatment of multiple musculoskeletal disorders and to the regeneration and healing of a wide range of tissues (Figure 3). Below, we summarize recent advances in the field, and address some challenges that need to be considered if progress in the use of this technology is to be maintained.

Bone

More than 6 million bone fractures are reported annually in the USA, of which in the range of 5–10% have impaired healing that causes pain and disability. As a result, scientists are making great efforts both to create bone substitutes and to develop ways of improving bone healing. To succeed, any substitute should be biologically compatible, non-toxic, provide scaffolding for angiogenesis and new bone outgrowth, osteogenic, resorbable, microporous and easy to handle. Furthermore, it should combine osteoconductive and osteoinductive properties.

The use of PRP helps to fulfill some of these requirements, particularly as an aid to bone regeneration. In fact, *in vitro* studies have demonstrated, clearly, that platelet-derived growth factors stimulate the proliferation of both human trabecular bone cells [32] and human osteoblast-like cells [33]. Initial *in vivo* experiments involving PRP were reported in the field of oral-maxillofacial surgery and in dentistry, particularly in periodontal therapy. Marx and co-workers studied the potential effect of autologous PRP on a bone graft reconstruction of mandibular continuity defects, concluding that the combination of PRP and bone grafts resulted in a significantly faster maturation and histomorphometrically denser bone regeneration [20]. In a study involving 20 patients who underwent tooth extraction because of periodontal disease or vertical fractures, we evaluated the effect of PRGF alone and in combination with autogenous bone. Results showed that in most of the patients receiving PRGF, bone regeneration was extensive and the bone tissue was compact with well-organized

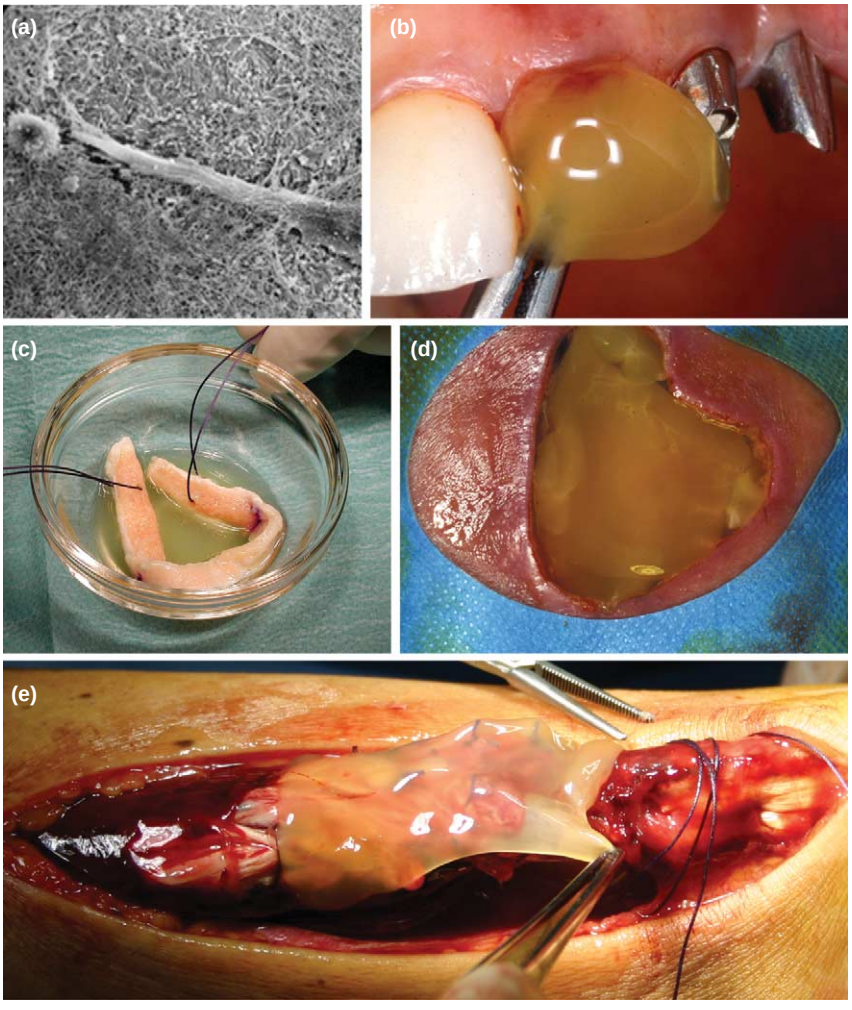

Figure 3. Preparations rich in growth factors (PRGF) are used in a variety of therapeutic applications. **(a)** One of the roles of fibrin is to promote cell adhesion; scanning electron microscopy reveals osteoblast filopodia on the surface of titanium implants bioactivated with PRGF. **(b)** Dental implantology: platelet-rich fibrin applied to a post-extraction alveolus so as to enhance bone regeneration. **(c)** Transfer of autologous growth factors to a tendon graft used to reconstruct an anterior cruciate ligament. **(d)** Treatment of a necrotic skin ulcer with PRGF. **(e)** Surgical repair of an Achilles tendon rupture assisted with PRGF.

trabeculae, whereas in the control group connective tissue and little mature bone were found [21].

One exciting focus of interest lies in the combination of PRGF and bone implants to facilitate the anchorage of dental prostheses. This has been investigated, widely, by our group, both in animal models and in humans, where the surface of the titanium implants are coated with PRGF before insertion in the host tissue [34,35]. Results showed that the extent and quality of bone regeneration around the implant is significantly improved when this PRGF-based strategy is used. In part, this is because the latter bioactivates the implant surface, facilitating the interaction of the implant with the host tissue, thus enhancing both the initial stability of the implants and their subsequent osteointegration. Other groups have also reported substantial benefits after combining the dental implants with platelet-derived preparations [36]. In addition, a single application of PRGF before placement of the implant can be sufficient to increase the percentage of bone-to-implant contact in cortical bone [37].

Once PRGF is activated, a considerable number of growth factors and proteins, with potentially key roles in bone healing and remodeling, are released from platelets, including TGF-β1, PDGF, IGF, bone sialoprotein, thrombospondin and osteonectin. Bone healing is aided by vasculogenesis in the local tissue, and a suitable blood supply during repair is considered essential if optimal bone regeneration is to be achieved [38]. Inadequate or inappropriate blood vessel development is associated with decreased bone formation and bone mass [39]. PRGF is known to release both angiogenic growth factors, including VEGF, which has been implicated in the promotion of angiogenesis and bone repair after injury and in cartilage maturation and resorption [38], and anti-angiogenic proteins, such as PF4 and endostatins. The ratio of these factors under local conditions might determine the efficacy of the treatment. Furthermore, the application of TGF-β1 and PDGF to osteoblasts induces additional VEGF synthesis [40,41]. Given that TGF-β1 contained in PRGF stimulates VEGF synthesis, its application at the time of surgery will enhance the regenerative capacity of bone tissue.

Another therapeutic approach involves the combination of PRP with different bone matrices. There are several review articles describing the use of PRP with both mineral and organic bone matrices [27,42]. One potential benefit of this combination is the improved handling and adaptation of the matrix to the injured tissue because the fibrin acts as a biological glue to hold together the matrix particles. Moreover, early vascular invasion is a key factor in bone allograft or xenograft incorporation, which reduces complications related to slow and incomplete integration. For example, Aghaloo *et al.* evaluated a natural deproteinized bovine bone, known as Bio-Oss®, with and without PRP in rabbit cranial defects and observed significant histomorphometric improvement at one, two and four months with the addition of PRP compared with Bio-Oss® alone [43]. In another study, β-tricalcium phosphate was assayed alone or in combination with PRP in the canine mandible. The histomorphometric results confirmed more intense bone regeneration in the early healing phase following the administration of PRP [44]. Recently, other authors have reported on the benefits of treating intrabony periodontal defects with PRP combined with bone mineral in controlled clinical trials [45,46].

Soft-connective-tissue injuries

Soft-tissue disorders, including tendon, ligament and joint capsular injuries, represent 45% of all the musculoskeletal injuries reported each year in the USA, with a high incidence among sport practitioners [47]. The importance of this problem is substantial because the field of sports medicine influences millions of people, from athletes to people who participate in recreational sport or simply people who use exercise to stay healthy and active. It is now estimated that tendon injuries account for between 30 and 50% of all injuries related to sports [48,49] and these often require surgical treatment. In these situations, the application of innovative biological tools, such as platelet-rich therapies, together with the surgical procedure might enable surgeons to enhance and accelerate reconstruction and repair of musculoskeletal tissues, affording new opportunities for improving the outcome and reducing the costs.

To address these issues, our group evaluated the effects of the growth factors released by platelets on tendon cell biology, to test the therapeutic potential of PRGF [50]. The pool of released growth factors increased the *in vitro* proliferation of human tendon cells significantly and stimulated them to produce angiogenic factors such as VEGF and hepatocyte growth factor (HGF) [51]. This might be particularly relevant in the tendon repair process, assuming that the reduced blood supply to the tendon is associated with its low healing capability. Moreover, HGF is a potent anti-fibrotic agent that might reduce the formation of scarring around tendon tissue, which is correlated with inferior repair quality [52]. Using a common strategy, we also cultured tendon cells on autologous fibrin matrices, mimicking the *in vivo* conditions of the cells. Our results show that the use of platelet-rich fibrin matrices is a safe and effective strategy to accelerate tendon cell proliferation, stimulate the synthesis of type I collagen and promote neovascularization both *in vitro* and *in vivo* [53]. Interestingly, although high levels of TGF-β1 are released upon activation of PRGF, no signs of fibrosis were observed in the animal models, perhaps because other biological mediators secreted by the fibrin matrices or the platelets might counteract this effect [53]. Anterior cruciate ligament (ACL) surgery is an example of where current basic research has a clinical application. Here, the injured ligament is removed by arthroscopic surgery and the joint is reconstructed with a tendon graft into which PRGF had been injected [54]. Using this procedure, Sanchez *et al.* reported enhanced healing with less complications and improved fixation of the graft within the bone tunnels in a retrospective clinical trial involving 100 patients [54]. Factors delivered from a PRGF-impregnated graft promote neovascularization [53], thereby favoring the remodeling of the graft that is essential for reproducing the mechanical functions of the native ligament. In our

experience, the surgical management of tendons in combination with autologous platelet-rich therapies has shown promising results. In another retrospective study, PRGF applied during the open surgical repair of the Achilles tendon in athletes showed an enhanced clinical outcome and functional recovery compared with a matched group that followed conventional surgery. Furthermore, the cross-sectional area of tendons in the platelet-rich group was significantly reduced, suggestive of a more physiological repair (M. Sanchez *et al.*, unpublished data).

Tissue engineering and cell therapy

Tissue engineering applies biological, chemical and engineering principles in the development of functional substitutes designed to meet the needs of each individual patient and repair site [55]. In the past few years, several research groups have explored the feasibility of using platelet-rich matrices as tissue engineering scaffolds. In most of these approaches, mesenchymal stem cells (MSCs) have been combined with PRP with the aim of enhancing bone regeneration. One potential advantage of this MSC–PRP mixture is that it is simultaneously osteogenic, osteoconductive and osteoinductive due to the bone-forming capacity of MSCs and the presence of secreted growth factors in the three-dimensional fibrin scaffold. The platelet-rich scaffold also shows excellent biodegradability, commensurate with new bone formation. This makes PRP different from other classical matrices, such as tricalcium phosphate ceramics or coral scaffolds, which virtually do not degrade during the first few weeks after implantation. Additionally, this MSC–PRP mixture is autologous, non-toxic and exhibits excellent plasticity.

Based on these principles, Ito and co-workers studied the potential of a MSC–PRP mixture compared with autogenous bone, Bio-Oss® and PRP alone to increase the rate of bone formation in mandible defects of dogs. The combination of cells and PRP provided greater bone maturation and early-stage bone regeneration, as shown by both histological examination and the testing of mechanical properties compared with the rest of the treatments [56]. Yamada *et al.* have previously reported that a MSC–PRP combination induced well-formed mature bone and neovascularization compared with a control group. Furthermore, their data demonstrated that PRP enabled MSC proliferation without deforming cell structure, and that it was an excellent vehicle to hold and deliver cells to correct or reconstruct bone defects in a clinical setting [57]. In a further step, the MSC–PRP combination was used for alveolar bone augmentation, with the simultaneous placement of implants in three human patients. This procedure gave rise to stable implants with minimal invasiveness [58]. The therapeutic potential of this tissue-engineered treatment has also been proven, successfully, in three cases of distraction osteogenesis of the long bones, leading to the conclusion that marrow-derived osteoblast-like cells and PRP could shorten the treatment period and lessen the associated complications by accelerating new bone formation [59].

Interestingly, the soluble factors released from a platelet-rich fibrin scaffold can be a powerful substitute for fetal calf serum (FCS) in the culture of different cells such as MSCs. In fact, the use of FCS should be minimized because it can be a potential source of prion or virus transmission [60,61]. The presence of PRP significantly increases the proliferation of stromal stem cells, reducing the time and cost of culture and improving the safety [62]. This might be advantageous for clinical situations such as the reconstruction of massive bone defects. Furthermore, another advantage of this treatment is that cells return to their normal rate of proliferation once the PRP is withdrawn, which is an obligatory safety requirement if the cells are to be transplanted into humans. The culture potential of PRGF supernatants has also been observed, successfully, by our group for the expansion of mature cells such as tenocytes [50,51].

Other applications of PRP

Ulceration of the lower limbs is a common complication of a wide spectrum of pathologies that cause a negative impact on the quality of life of affected patients and results in >85 000 lower-extremity amputations each year in the USA. Under these conditions, the application of platelet-rich-derived therapies gives ground for optimism. In fact, the material released from platelets has shown efficacy in a retrospective cohort study involving 26 599 patients [63]. Furthermore, the effectiveness and safety of a platelet-rich preparation for the treatment of diabetic foot ulceration has recently been reported [64]. The therapeutic use of PRP has also been extended to other fields, including facial plastic surgery, eye surgery and cosmetic surgery. Because of its haemostatic properties, it has been demonstrated that the platelet-rich preparation is effective in sealing capillary beds during surgery and thereby controlling bleeding [65]. In addition, it decreases postoperative complications and avoids the re-exploration of the patients, which is cost effective. This approach opens a new and emerging field of wound pharmacology that represents an exciting advance in plastic surgery therapeutics.

Conclusion

The use of preparations derived from autologous PRP enables the local and progressive delivery of growth factors and proteins providing unique properties for tissue remodeling, wound healing and angiogenic promotion. Consequently, PRP has been used in many different fields, including orthopedic and maxillofacial surgery, sports medicine, bone reconstruction, tissue engineering and cosmetic and dental implant surgery. However, there are still important challenges that need to be addressed. First, it is necessary to compare the diverse, platelet-rich products available commercially and to determine how differences in their preparation and use affect their final biological efficacy. Furthermore, procedures need to be standardized and additional, well-designed studies and clinical trials are needed to evaluate the potential therapeutic impact of PRP in medicine and surgery and to avoid controversial results [27,42,66]. The ability to address all these challenges will enhance the potential of this technology and extend its therapeutic applications.

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