1 Local injection of endogenous gowth factors for Achilles tendon pathology

Mikel Sánchez, Eduardo Anitua, Juan Azofra, Roberto Prado, Fracisco Muruzabal, Isabel Andía

Introduction

Current therapies to treat tendinopathies involve long-term conservative and palliative treatments that do not change the intrinsically poor healing potential of a tendinopathic tendon. Under these circumstances, the development of new biological tools could enhance and accelerate repair and remodeling of tendon, providing new opportunities for improving clinical outcomes and reducing costs. The last decade has seen the development of platelet-rich (PR) therapies in different medical fields (1). The application of platelet-rich preparations has revolutionized the field of regenerative medicine in part for the repair capacity of the growth factors (GFs) and proteins secreted by platelets (2, 3). Sánchez et al. (4) were the first to report the use of PR-therapies for orthopedic-related problems. Furthermore, they conceived the "in situ" preparation of fully integrated bioactive grafts for anterior cruciate ligament reconstruction using the now-traditional paradigm of tissue engineering (5). Since then, PR-therapies have been further developed and created new opportunities for tissue repair in sports medicine (6), even open the door to novel therapies for the treatment of knee joint pathologies (7).

PR-therapies seek to enhance the process of tissue repair through the delivery of bioactive agents to provide chemotactic, proliferative, and anabolic cellular responses and ultimately tissue function. Among the released growth factors essential for wound repair are platelet derived growth factors (PDGF), vascular endothelial growth factor (VEGF), fibroblastic growth factor (bFGF), epidermal growth factor (EGF) and transforming growth factor (TGF-beta1). Also present in the plasma are insulin-like growth factor (IGF-I) and hepatocyte growth factor (HGF) (2). Several GFs influence tendon metabolism (8). The process of tissue repair is a complex cascade of biological events controlled by many cytokines, proteins and growth factors which provide signals at local injury sites, regulating the mechanisms and pathways that govern tissue wound healing and regeneration. Therefore, the ability to release signaling factors in a spatiotemporal manner mimicking the needs of the injured tissue has become a challenge.

We provide an overview of the potential therapeutic uses of platelet-rich technologies for the release of growth factors in the management of Achilles tendon injury and pathology.

Platelet-rich plasma: different formulations and re-administration techniques

Platelets are known for their role in hemostasis where they help to prevent blood loss at sites of vascular injury. Recent evidence suggests that platelets may also have an additional and previously unsuspected role in tissue repair and vascular remodeling, as well as being active players in inflammatory and immune responses (3, 9-10). Blood platelets are produced in large numbers from megakaryocytes in the bone marrow, with a normal platelet concentration around 150,000-350,000/µl. Anucleate platelets circulate for 7 to 10 days and mediate primary hemostasis. Upon activation, platelets secrete the contents of their dense and alpha granules; the former contain small molecules such as calcium, ADP, ATP, GTP, thromboxane, histamine and serotonin, while the latter include multiple proteins such as PDGF, TGF-beta1, PF4 (Platelet Factor 4), VEGF, endostatins, angiopoietins and thrombospondin-1 (2, 3). Platelet-rich plasma (PRP) releases both angiogenic factors, including VEGF, which is implicated in the promotion of angiogenesis, and anti-angiogenic factors such as PF4 and endostatins. In addition to providing initial cues for local cell activation and homing of precursor cells to sites of injury and differentiation, platelets and fibrin are also potent adhesive substrates for cells.

Recently, centrifugal separation of PRP for therapeutic use has been introduced. In the viewing of the rise of many commercial setups, the question is what distinguishes one PRP system from another. Despite the lack of standardization in the field, two issues are considered crucial: the biochemical characteristics of the product and the procedure of application. The

methods of producing PRPs determine the composition and concentration in terms of leukocytes, erythrocytes and platelets in a given plasma volume. This determines the molecules present in a given preparation, molecular balance and concentration. There are essentially two methods: single and double spinning procedures. When using a single spinning procedure, the platelet yield is 1-3 fold of baseline levels, while double spinning yields 5-8 fold of baseline levels. Double spinning also concentrates leukocytes; hence, these products are more precisely named leukocyte/platelet concentrates. Commercial examples include SmartPReP by Harvest Corp (Plymouth, USA) or GPS (Gravitational Platelet Separation System) by Biomet Biologic (Warsaw, USA). Single-spin products usually adapt blood volume extraction to the patient's needs, and, even if they contain leukocytes, they do not concentrate them as in double spinning methods. These biomaterials are developed from the patient's own blood, hence clearance for marketing was achieved without the need for randomized controlled trials evaluating their clinical efficacy; thus, differences between products have not yet been demonstrated.

The general belief that the regenerative potential of PRP depends exclusively on the amounts of growth factors and cytokines released from platelets could not be demonstrated. The optimal platelet formulation that triggers tissue specific responses for each clinical application should be supported by basic science studies. Anitua et al. (11) varied the traditional plasma formulation and showed that platelet-poor plasma also had the ability to stimulate wound healing-associated cell function. Further tenocyte studies confirmed these results (12). Our recent research (13) reveals that PRPs with a higher platelet concentrate ratio, 2-fold versus 4-fold above baseline levels (whole blood), do not improve the angiogenic paracrine response in tendon cells. In line with these results, Rughetti et al. (14) showed that high concentrations of platelets induce a reversion of angiogenic stimulatory processes such as proliferation, motility and invasiveness of human endothelial cells. Much work lies ahead to determine the most appropriate plasma formulation for each condition. The product characteristics and procedure of application, in conjunction with the precise nature of the injury and the ideal volume and time for injection, may be of paramount importance.

PRGF® by BTI (Vitoria, Spain) emerged in the 1990s as the first of the single spin procedures, addressing some of the limitations of the double spin methods for conventional platelet-rich plasmas. Upon activation, this platelet-based product enables the formation of a three-dimensional biocompatible fibrin scaffold and the local delivery of a myriad of growth factors and proteins that contribute to the accelerated postoperative wound healing and tissue repair. The release of biologically active agents via platelet degranulation is induced by the addition of a standardized dose of calcium chloride, which permits more sustained release of growth factors than thrombin (15, 16). Leukocyte content has been eliminated from PRGF with the aim of avoiding the pro-inflammatory effects of the proteases and acid hydrolases contained within white blood cells (17).

The successful clinical applications also depends on clarification of the optimal re-administration procedure, the volume of PRP used, and the frequency of the applications. Understanding the biology of platelets and coagulation is important for the correct administration of the preparation. Upon activation, platelets provide primary hemostasis by performing five primary functions: adhesion, aggregation, secretion, providing procoagulant surface and clot retraction. When administering PRGF, the goal is to obtain a fibrin scaffold at the injured site. This can be achieved by clotting the plasma ex-vivo, as explained below, or by administering the activated liquid preparation in such a way that fibrin will develop in situ. Developing guides to instruct PRGF preparation and efficient administration for tendon injury and pathology are shown below.

Biological stimulation of tendon cells by autologous growth factors

Most tendinopathy is associated with a failed healing response, which is an active, cell-mediated process involving increased turnover and remodeling, and gradual transformation in the quality and quantity of the extracellular matrix (ECM) that precedes tendon rupture. Tenocytes have a central role in the repair and maintenance of ECM, synthesizing new proteins and producing the enzymes that degrade them. The process of matrix turnover is normally in balance, and changes in this activity in response to micro-trauma and hypoxia might precede any lesion. This activity is likely to be influenced by external factors such as GFs and cytokines released from platelet-rich preparations. The growth factor modulation begins when growth factors bind to specific cell-surface receptors. This triggers the process of signal transduction, during which the occupied receptor initiates a chain of intracellular signaling that results in protein synthesis.

PRGF deliver a complex pool of growth factors and other molecules essential to natural wound repair and tissue promotion at the required time and level, mimicking the physiological wound healing process. To

translate this platelet-rich therapy into clinical practice it is necessary to evaluate the potential role of the growth factors released from the newly developed fibrin matrix in tenocyte proliferation and metabolism and to confirm the biological effects in vivo. Initially, we decided to evaluate the effects of the pool of growth factors released from PRGF on primary tenocyte cultures. Tenocytes, the dominant cell type in tendons, are primarily responsible for the tendon's physiologic and pathologic changes. Human tenocytes increased their proliferation rate and were stimulated to release VEGF and HGF (11, 18). The former is a potent proangiogenic factor, the focus of many strategies to promote therapeutic angiogenesis. HGF (also known as scatter factor) is a potent endothelial mitogen, motogen and morphogen, although in contrast to VEGF, these effects are not limited to endothelial cells. The combination of VEGF and HGF results in a much more robust endothelial proliferative and angiogenic response than either growth factor alone (19). HGF is also a potent anti-fibrotic agent, which could reduce scar formation around tendon tissues.

The angiogenic potential of PRGF is an advantage from a therapeutic point of view, and neo-vascularization is a key process in tissue regeneration (20). Moreover, tendons heal poorly because they, or at least certain parts of them, have a poor blood supply (21). This may explain why the mid-portion of the Achilles tendon is so vulnerable and has such a good healing response to the application of PRGF injections. Recently, we demonstrated the angiogenic potential of PRGF after the infiltration of the biological preparation in the Achilles tendon in sheep. Tendons injected with PRGF showed an increase in cellularity and a change in cell morphology compared with saline injection and untreated control tendons (22). When PRGF was injected, tendon cells with an ovoid shape appeared aligned along the collagen fibers, showing organization along lines of tension. The latter contrasted with tendons injected with saline, where disorganized and disordered cells accumulated in defined limited areas. Additionally, in tendons treated with PRGF, endotenons were more prominent through an accumulation of round cellular elements. Cells found in the endotenon and epitenon surrounding the main fiber bundles possess a greater proliferative capacity and different matrix synthesizing activities compared with the tenocytes within the fibers, and they are the first cells to respond after acute tendon injury. Kajikawa et al. (23) reported the importance of preserving blood supply because cell migration through blood flow contributes to the healing process. Recently, they found an increase in circulation-derived cells in tendons treated with PRP, suggesting that PRP locally injected in tendons is a useful activator of chemotaxis, favoring the early phase of the healing process (24).

To enhance tendon tissue organization in the short term and enhance mechanical properties, PRPs should be combined with appropriate loading regimes as tendon is a mechanoresponsive tissue. Hence, Virchenko et al. (25) reported that injections of PRP one week postoperatively increased tendon regenerate strength after four weeks if combined with early physiotherapy.

An imbalance in matrix turnover may be implicated in tendinopathy (26) as in other degenerative conditions such as osteoarthritis (27). It has been hypothesized that matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) are involved in remodeling the extracellular matrix of tendons and are up- or downregulated in tendinopathy (28). After acute tendon rupture, Karousou et al. (29) observed changes in gene expression of TIMP-1, TIMP-2 and gelatinolytic activity attributed to MMP-2. Thus, a balance between MMPs and TIMPs is probably necessary to maintain tendon homeostasis. The mechanism of activation of MMPs, however, is poorly understood, and their precise role in tendinopathy is still unclear (30). While PRPs have an anabolic effect on matrix synthesis, they do not influence MMPs synthesis (31). Schnabel et al. (32) have cultured tenocytes with PRP and found enhanced gene expression of the collagen matrix molecules COL1A1, COL3A1 and cartilage oligomeric matrix protein (COMP), with no concomitant increase in the catabolic molecules MMP-3 and MMP-13.

Management of tendinopathy with PRGF

The senior author developed the above-described protocols for PRGF-assisted management of Achilles tendon injuries and pathology. Medical imaging has an irreplaceable role in the diagnosis and treatment of tendon disorders. Ultrasonography and magnetic resonance imaging (MRI) are not only required to verify the diagnosis and exclude other disorders but also crucial to develop novel non-invasive PR therapies for tendon pathology.

PRGF preparation

For the preparation of PRGF from patients, peripheral venous blood was withdrawn into 9-ml tubes containing 3.8% (wt/vol) sodium citrate. Percutaneous injections require about 54 ml (6 tubes), while, for surgical applications, 72 ml (8 tubes) of citrated blood is with-

drawn from the patient before inducing anesthesia. PRGF is prepared by centrifugation at 580g for 8 min at room temperature (PRGF® System, Vitoria, Spain). The roughly 2-ml plasma fraction located at the top of the tube is collected in a glass dish and used to prepare the dense fibrin membranes used in surgical procedures or in the treatment of skin complications. For this purpose the plasma is clotted ex vivo by adding calcium chloride and incubating the mixture for at least 40 minutes. The 2 ml of plasma located just above the sedimented red cells, but not including the buffy coat, is collected in other tubes. After the addition of 10% (wt/vol) calcium chloride, the plasma is promptly injected intratendinously and peritendinously.

Repeated percutaneous injections of PRGF

Initially, we recommended percutaneous injections for patients with painful tendinopathy in whom other conservative management had proven ineffective. We apply PRGF to either mid-portion or insertional areas of tendinopathy. In general, we perform 2-3 PRGF injections at weekly intervals on an outpatient basis. Ultrasonographic monitoring drives the clinical decision of whether or not to perform additional PRGF injections. Because the procedure is not very painful, we do not administer anesthesia; instead, ice is applied for about ten minutes after the PRGF injection.

Technical description: The patient lies with ankles resting on a pillow; the end of the operating table is lifted, and the feet are hanging over the end of the operating table. Skin preparation is performed with an antiseptic solution in the usual fashion. The patient is clinically examined to correctly identify and mark the area of maximum tenderness and swelling. A sterile longitudinal 7.5 MHz transducer is then used to confirm and image the precise location of the degenerated area.

For the percutaneous injection, the goal is to control the activation degree of the preparation. Therefore, PRGF should be injected using a luer-lock syringe through 21G needle, shortly after $CaCl_2$ addition, within the focus of altered tendon substance. The target volume of the PRGF injection is the maximum volume that can be injected intratendinously within the area of degeneration, commonly between 3-5 ml. The injected biomaterial does not wash away, since platelet-rich fibrin develops within the target site, releasing growth factors into pathological and adjacent areas such as the pre-Achilles fat and the space between the tendon and the paratenon, aimed at impacting tenoblasts and mesenchymal cells in these areas. The principles of PRGF injection are restoring vascularity, initiating cell responses such as cell migration and proliferation, and achieving structural recovery by initiating the synthesis of extracellular matrix.

We injected 22 tendons in 21 patients using this procedure. Of the 22 tendons, 19 had been diagnosed with chronic Achilles tendinopathy, and had initially been managed with conservative therapy.

Four out of the 19 patients in whom conservative management with percutaneous injections failed required surgical management. Two of the four patients presented with insertional tendinopathy with concomitant retrocalcaneal bursitis and bony edema. Another case presented with bilateral Achilles tendinopathy; while the left side became asymptomatic with PRGF injections, the right tendon had to be operated. In contrast, three older women of this group presenting with chronic rupture, who could be expected to have poorer healing, recovered well (normal gait, return to previous activities and no discomfort) with PRGF injections but without surgery. Although chronic ruptures are usually treated operatively, PRGF injections may represent an alternative management approach, especially for patients with low functional demands.

Intratendinous and peritendinous injections of PRGF seem to have a marked effect on pain. The remarkable reduction in pain along with anti-inflammatory and antibacterial effects is mainly attributed to the presence of platelets in these preparations (33-35). Although improved MRI images are evident, the hypoechoic image does not disappear completely. Mild to moderate changes were frequently observed in the involved Achilles tendon after treatment, but the occurrence of these changes was asymptomatic. The thickness of Achilles tendon decreased significantly, and the tendon structure looked more normal on ultrasonography.

Open tenotomy with removal of abnormal tissue and PRGF application (Figure 1)

In patients that failed percutaneous injections, longitudinal tenotomy was performed.

The objective of surgery is to excise fibrotic adhesions and remove areas of degenerated tissue. Representative examples of the prominent degenerative changes in the Achilles tendon are shown in Figure 2. Histologically, tendinopathy does not present a single histological picture: hypoxic degeneration, mucoid or mixoid degeneration, hyaline degeneration, fatty degeneration, fibrinoid degeneration, fibrocartilaginous metaplasia, bony metaplasia, fiber calcification or combinations of these can all be present. The tendinopathic process affects tendon cells as well as the ma-

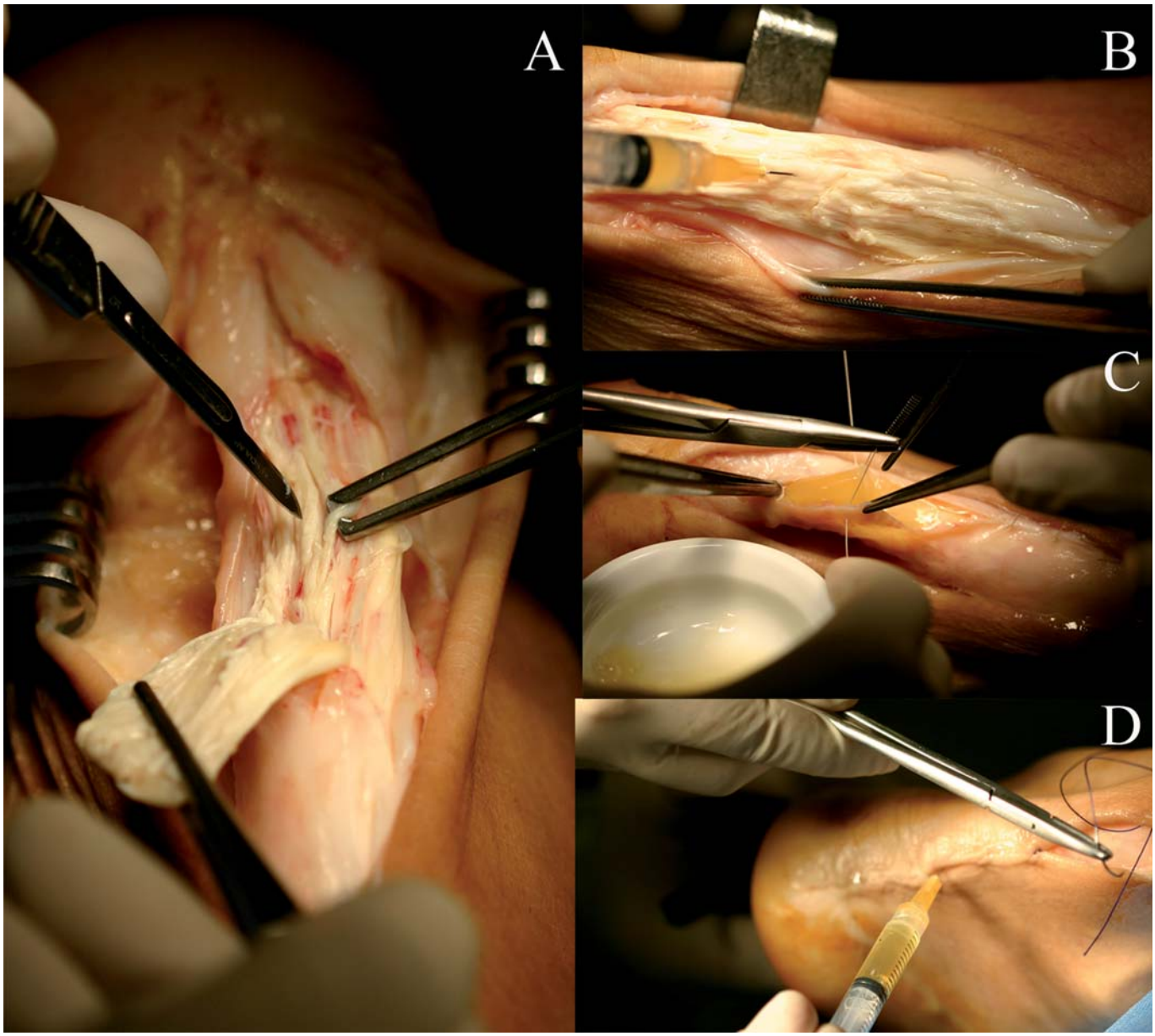

Figure 1 Open tenotomy assisted with PRGF: (**A**) longitudinal tenotomy to remove fibrotic adhesions and areas of degenerated tissue. (**B**) Injection of activated liquid PRGF within tendon fascicles. (**C**) Application of PRGF scaffold before closing the paratenon. (**D**) After suturing the overlying skin, liquid-activated PRGF is injected into the subcutaneous tissue.

trix components (Fig. 2). Although the pathways that lead to tendon degeneration are not well understood, tissue hypoxia is probably a critical factor. Tendons suffering from hypoxia show vascular proliferation with connective tissue accumulation. Deficient blood flow in the mid-portion of the Achilles tendon may initiate the lesion, decreasing the viability of tendon cells, which could in turn result in the initiation of degenerative changes in the tendon tissue.

PRGF injection after tenotomy seeks to stimulate the restoration of well ordered vascularity and possibly stimulate the remaining viable cells to initiate cell matrix response and healing. Recent studies show that multiple longitudinal tenotomies trigger neoangiogenesis of the Achilles tendon, with increased blood flow. This would result in improved nutrition and a more favorable environment for healing. The use of PRGF in this context might also be focused on restoring physiological vascularity and normal tissue composition, while avoiding further further progression of the failed healing response. By covering the area with a PRGF membrane, we may facilitate extrinsic repairing mechanisms so that fibroblasts can move easily from the peripheral paratenon or external tissue

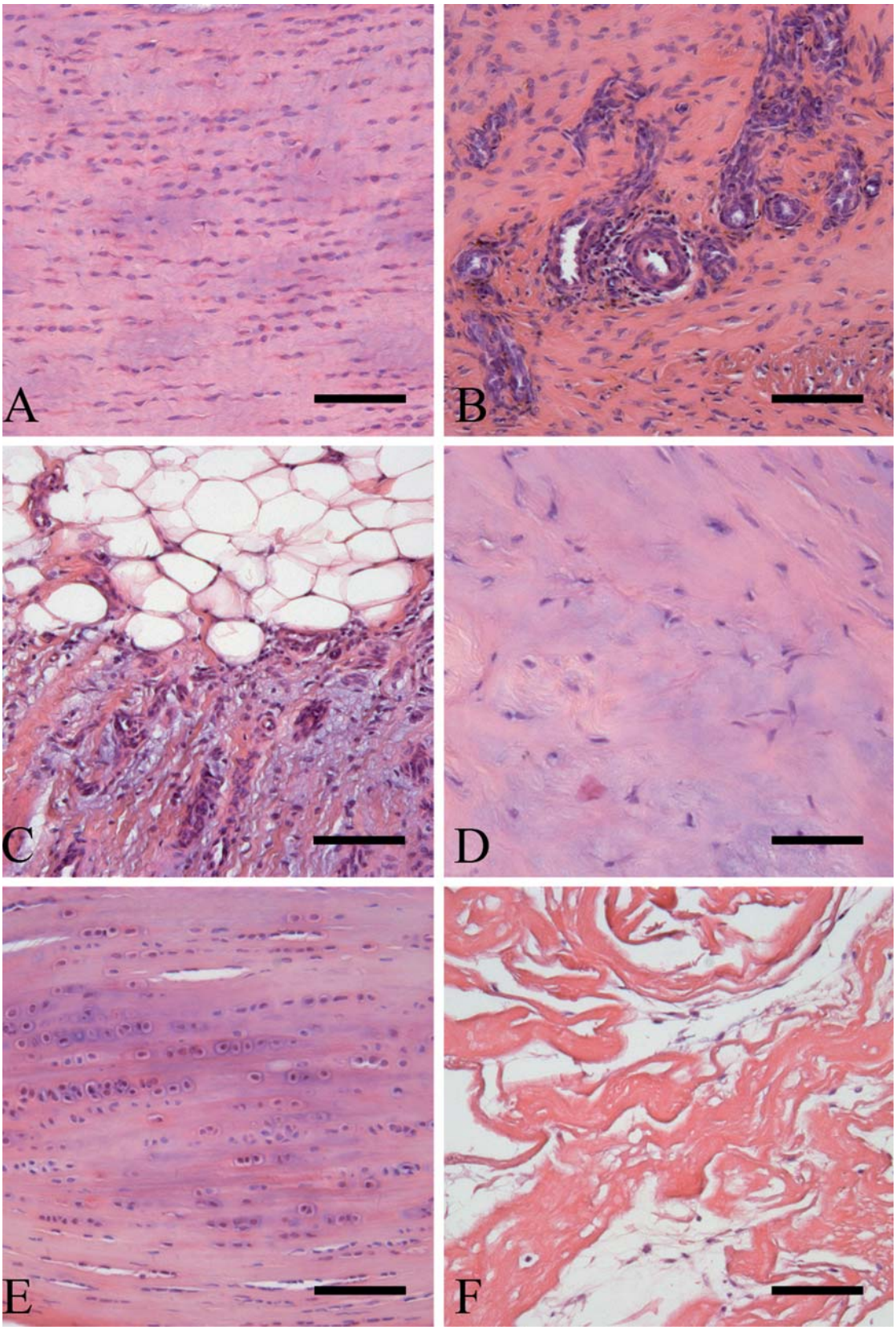

Figure 2 Histopathological changes seen at the ruptured site of the Achilles tendon: (**A**) marked fibroblast proliferation and cellular changes; (**B**) chronic peritendinitis with marked cell density and vascular proliferation with obliterated arteries; (**C**) lipid accumulation, accumulation of glycosaminglycans and proliferation of capillaries; (**D**) mucoid degeneration; (**E**) chondroid metaplasia within the tendon body; and (**F**) fibrinoid degeneration and tissue necrosis. Scale bars: 100 μm

sources to the healing site. This emphasized the importance of structures close to the Achilles tendon and the communication in between and the role of the paratenon as well as the skin barrier (36).

Mini-open repair assisted with PRGF (Figure 3)

Blood is withdrawn from an antecubital vein before administering anesthesia. The patient is placed prone with a tourniquet, and a small 3 cm longitudinal skin incision is made in the midline over the distal tendon stump. Care is taken to prevent damage to the sural nerve. The distal Achilles tendon stump is mobilized, freeing it of all peritendinous adhesions, and a suture is passed from medial to lateral along the distal tendon edge. The proximal tendon stump is freed of peritendinous adhesions, and a fiberwire tape is passed 2 cm above the end of the proximal tendon stump. The proximal end is approximated beneath the intact skin into the distal incision and sutured to the distal stump. After closing the paratenon and before closing the overlying skin, the affected area is covered with the fibrin scaffold prepared as described above.

Surgical reconstruction of Achilles tendon tears (Figure 4)

We have reported satisfactory functional results after PRGF-assisted operative management in Achilles tendon tears (37). In this case-control study, the healing of ruptures localized to the main body of the tendon was enhanced in athletes who underwent PRGF-assisted surgery. Moreover the group receiving PRGF recovered their range of motion earlier and took less time to resume training activities. The same surgical technique was used for degenerative ruptures with minor variations that involved excising fibrotic adhesions and removing degenerated portions of the tendon, followed by end-to-end tendon suture and paratenon repair as previously described (Figure 4). Approximately 4 mL of the activated plasma was injected among the tendon fibers after the tendon was

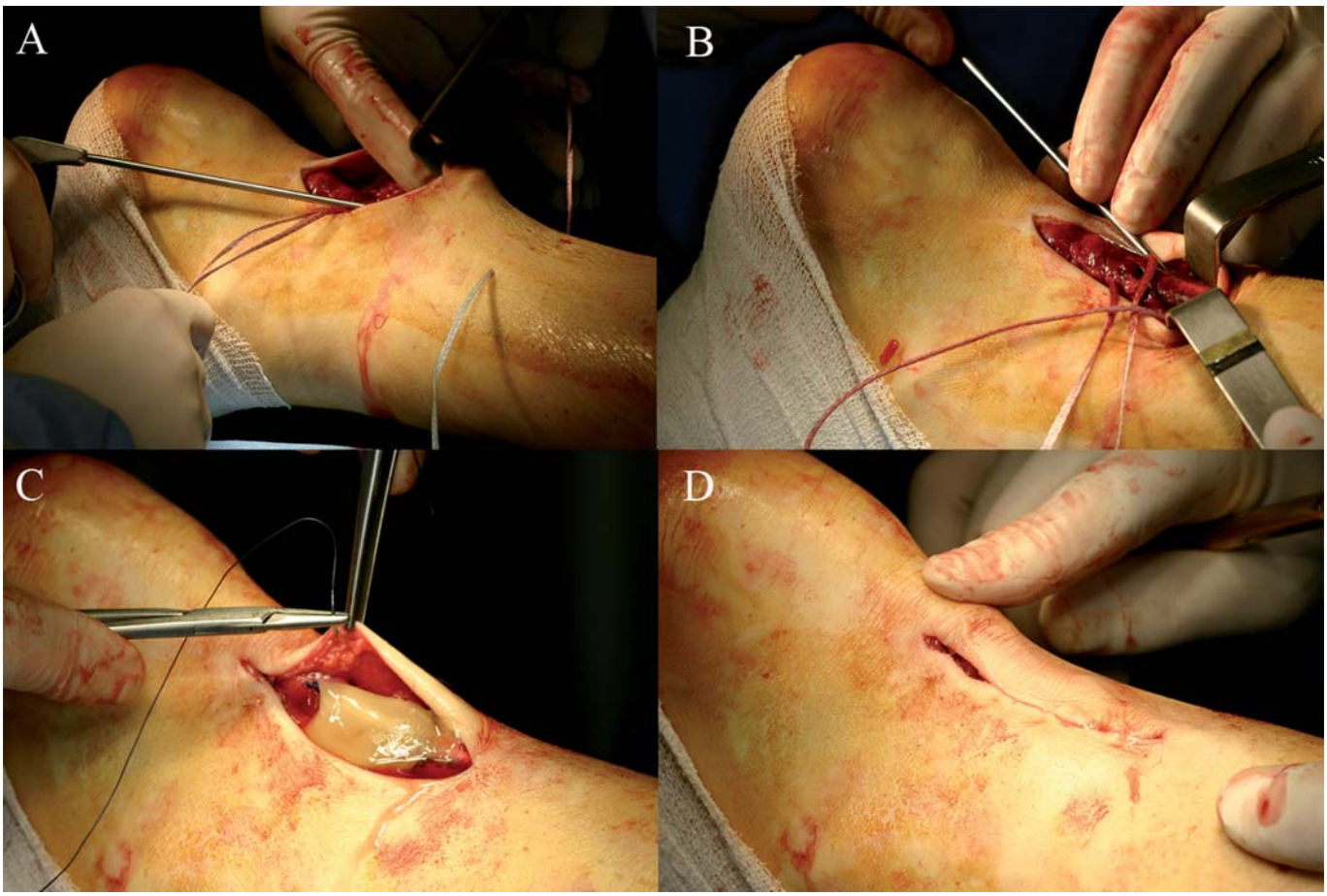

Figure 3 (**A**) A small incision is made over the distal tendon stump, and a suture is passed through the proximal stump. (**B**) The proximal end is approximated beneath the skin and liquid activated PRGF is injected within fascicles. (**C**) Application of the PRGF scaffold. (**D**) Suture of the overlying skin.

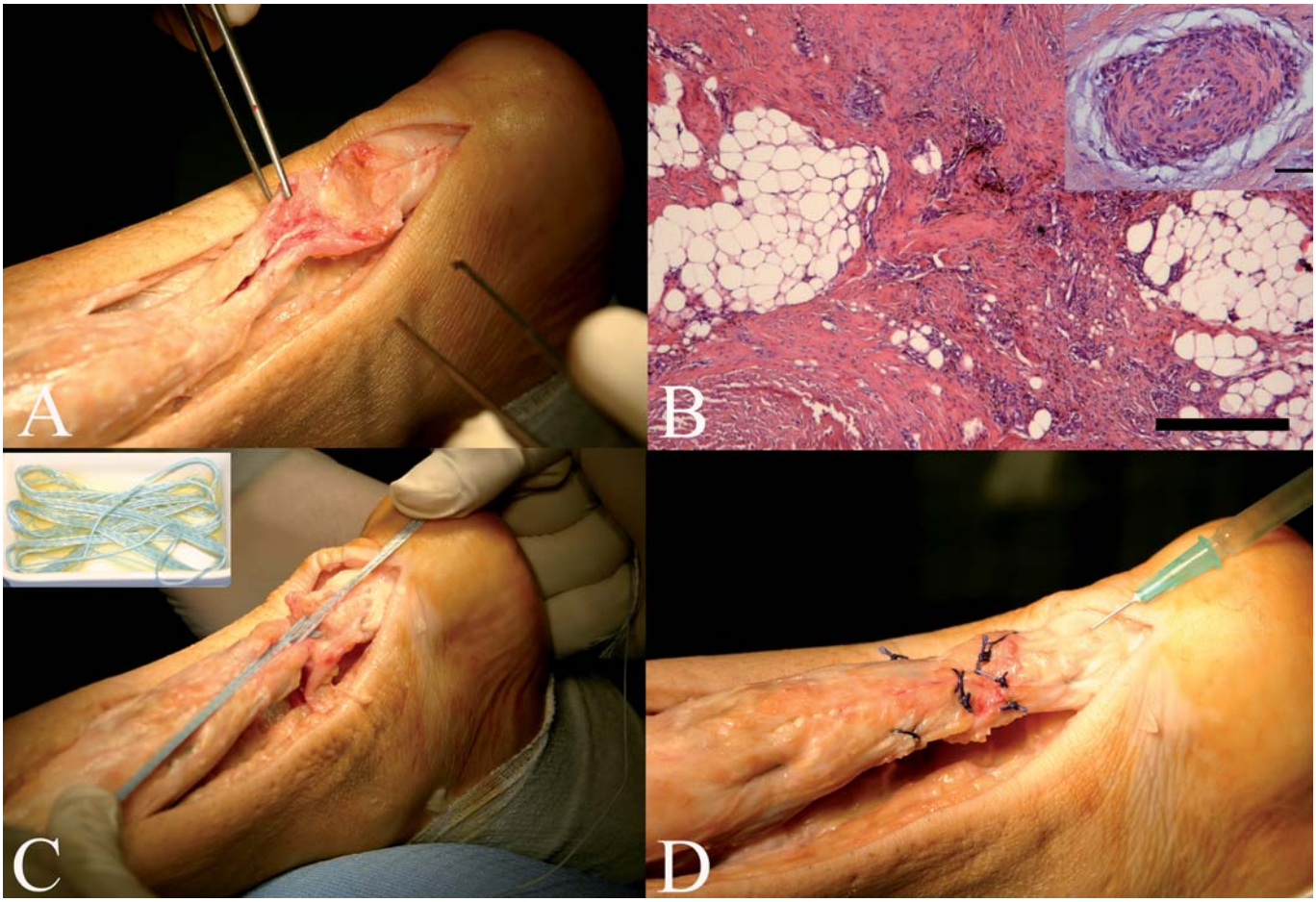

Figure 4 (**A**) Chronic degenerative rupture in the mid-substance of the Achilles tendon is treated with PRGF-assisted surgical reconstruction. (**B**) Histopathological alterations seen at the ruptured site include vascular proliferation with obliterated arteries (scale bar: 50 m) and loss of fiber tendon orientation with fat ingrowth and lipid cells located within the collagen fibers (scale bar: 400 µm). (**C**) After debridement, both stumps are approximated and sutured using PRGF-treated fiberwire tape. (**D**) After closing the paratenon, activated liquid PRGF is injected within the tendon, aiming to restore tissue composition and avoid further degeneration.

sutured. After closing the paratenon and before closing the overlying skin, the affected area was covered with a fibrin scaffold prepared as described above (37).

Management of complications with PRGF (Large tissue loss)

Recently, we reported PRGF-assisted management of major post-operative complications such as infection and Achilles tendon necrosis in two recreational athletes (38). The two cases were challenging due to the critical length of the tendon defects (>8-10 cm), along with detached bone-tendon insertion in one patient. To address this problem, a two-step management was scheduled; first, a surgical debridement to remove degenerated tissue, and secondly a tendon augmentation were performed.

We used PRGF in both stages of surgical debridement and tendon augmentation. In tendon augmentation we injected small volumes of activated PRGF within the fascicles before transplanting the tendon, aiming to add to the scaffold structure the necessary biological cues for cell migration, proliferation, angiogenesis and remodeling.

Wound complications

Achilles tendon surgery and pathology is often associated with complications such as recalcitrant wound healing. Already in 1990, autologous human platelet derived wound healing factors (HPDWHF) were proposed to regulate wound healing of recalcitrant skin ulcers by promoting the formation of granulation tissue in the early healing phase (39). Driver et al. (40) studied a group of 72 patients with non-healing diabetic foot ulcers and showed their efficacy in the setting of peripheral neuropathy and/or vascular complications. We have reported the effectiveness of PRGF

for the treatment of chronic cutaneous ulcers (41). Platelets provided not only autologous growth factors for tissue formation and epithelialization of chronic cutaneous wounds, it was again noted that they provide both infection-fighting and pain-reducing properties (42).

Conclusion

Recent advances in basic research on PR-therapies provide a more detailed understanding of their therapeutic potential for tendon injury and pathology. Many questions remained to be answered regarding the science of signal transduction in platelet-rich plasma applications and the specific needs of tendon tissue. It is necessary to fully characterize and evaluate the plasma products and establish protocols for their application. Our clinical experience using a characterized preparation of PRGF suggests that repeated PRGF injections could be included in the armamentarium of conservative treatments for tendon pathology. Moreover, it could be an alternative to surgery in selected patients with tendinopathic disorders. When conservative treatment fails the surgical treatment with PRGF, as a co-adjuvant, shows enhanced tendon healing while ameliorating the soft tissue covering the tendon. PRGF gives a potential advantage for more predictable healing, suggesting an exciting role for PR-therapies in tendon treatment.

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