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Operative Techniques in
Orthopaedics

Platelet-Rich Plasma: Preparation and Formulation

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Platelet-rich plasma is a set of autologous platelet products used to accelerate recovery from injury. The basic rationale is to mimic the natural ways of healing, bringing to the injury site a set of molecules that will accelerate the functional recovery of the tissue, trying to regenerate the tissue itself, and not to merely repair with scar tissue. Among the jungle of products in this field, PRGF-Endoret (BTI-Biotechnology Institute, Vitoria, Spain) is a pioneering autologous regenerative technology with multiple therapeutic potentials, present in at least 4 different formulations, depending on the coagulation and activation degree of the samples. PRGF-Endoret technology is safe and has multiple applications and potentials.
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Potential of Plasma Rich in Growth Factors (PRGF-Endoret): Mimicking the Natural Healing

The increasing number of musculoskeletal injuries has produced an increasing number and improvement of different treatments of these lesions, especially in the search for non-operative management modalities.[1] One of these cutting-edge technologies is the use of plasma rich in growth factors (PRGF-Endoret).[2] This type of biological treatment mimics the natural ways of wound healing[3] trying to optimize and reduce healing times. This is achieved driving to the injury site the whole protein array of platelet-rich plasma (PRP) that will be involved in the repair of damaged tissues. In this way, all the proteins necessary for tissue repair are released locally.

The process of tissue repair occurs naturally in a staged fashion,[4] and includes removal of dead cells, proliferation, migration of cells to the injury site, production of new vascular structures, and so on. The organization of all these elements influences the healing of a given injury, preventing fibrotic elements that cause loss of functional capacity of that tissue.[5,6] Growth factors play an important role, coordinating the whole process in an orchestrated fashion in all tissues of the musculoskeletal system, including muscle,[7] tendon,[8] bone,[9,10] and cartilage.[11] Growth factors act on other tissues as well, including skin,[12] oral soft tissue,[13,14] cornea,[15] among others.

The technology of PRGF-Endoret mimics the natural healing mechanisms but with 2 special features: trying to avoid loss of functionality (fibrous tissue) and shortening healing times. This is achieved in part adjusting the PRGF-Endoret formulation and dosage to the type of tissue and injury.

PRGF-Endoret therapy accelerates and improves tissue healing by local delivery of autologous bioactive molecules and contributing with a first-line provisional scaffold.[16] This autologous toolbox consists in the use of platelets as a reservoir and vehicle of a large repertoire of proteins.[17,18]

In the past decade, several systems have been developed to produce a biologically active product, both commercial and homemade, but they differ in the presence of white blood cells, growth factors' concentration, and architecture of fibrin scaffold.[19-23]

For human therapeutic uses, we recommend that only commercial systems are used, although some centers still use homemade products for both basic research and clinical use.

The commercial systems can be certified for various medical applications, but the therapeutic outcome will depend on the type of PRP and the dosage used. Establishing a proper classification of the PRPs and identifying the biological differences

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Table 1 Platelet Protein Classification and Their Biological Role[16]

Classification	Protein	Biological Effects
Adhesive proteins	Von Willebrand factor (vWF) propeptide, Fibrinogen, Fibronectin, Vitronectin, Thrombospondin 1 (TSP-1), laminin-8 (alpha4- and alpha5- laminin subunits), signal peptide-CUB-EGF domain containing protein 1 (SCUBE 1)	Cell contact interactions, homeostasis and clotting, and extracellular matrix composition
Clotting factors and associated proteins	Factor V/Va, Factor XI-like protein, multimerin, protein S, high-molecular-weight kininogen, antithrombin III, tissue factor pathway inhibitor	Thrombin production and its regulation
Fibrinolytic factors and associated proteins	Plasminogen, Plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator (uPA), alpha2-antiplasmin, histidine-rich glycoprotein, thrombin activatable fibrinolysis inhibitor (TAFI), alpha2-macroglobulin (α2M)	Plasmin production and vascular modeling
Proteases and antiproteases	Tissue inhibitor of metalloprotease 1 -4 (TIMPs 1 -4), metalloprotease-1, -2, -4, -9, A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), tumor necrosis factor-alpha-converting enzyme (TACE), protease nexin-2, C1 inhibitor, serpin proteinase inhibitor 8, alpha1-antitrypsin	Angiogenesis, vascular modeling, regulation of coagulation, and regulation of cellular behavior
Growth factors	Platelet-derived growth factor, transforming growth factor beta1 and beta2, epithelial growth factor, insulin-like growth factor type I, vascular endothelial growth factor (A and C), basic fibroblastic growth factor (FGF-2), hepatocyte growth factor, Bone morphogenetic protein (BMP)-2, -4, -6, connective tissue growth factor (CTGF)	Chemotaxis, cell proliferation and differentiation, and angiogenesis
Chemokines, cytokines, and others	Regulated upon Activation - Normal T-cell Expressed, and Secreted (RANTES), Interleukin-8 (IL-8), Macrophage inflammatory protein-1 (MIP-1) alpha, Epithelial Neutrophil-Activating Peptide 78 (ENA-78), Monocyte chemotactic protein-3 (MCP-3), Growth regulated oncogene- alpha (GRO-alpha), angiopoietin-1, IGF-1 binding protein 3 (IGF-BP3), interleukin-6 soluble receptor (IL-6sR), Platelet factor 4 (PF4), beta-thromboglobulin (bTG), platelet basic protein, neutrophil-activating protein-2 (NAP-2), connective tissue-activating peptide III, high-mobility group protein 1 (HMGB1), Fas ligand (FasL), Homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus (HSV) glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes (LIGHT), Tumor necrosis factors (TNF)-related apoptosis-inducing ligand (TRAIL), Stromal cell-derived factor-1 (SDF-1) alpha, endostatin-I, osteonectin-1, bone sialoprotein	Regulation of angiogenesis, vascular modeling, cellular interactions, and bone formation
Antimicrobial proteins	Thrombocidins, Defensins	Bactericidal and fungicidal properties
Others	Chondroitin 4-sulfate, albumin, immunoglobulins, disabled-2, semaphorin 3A, Prion protein (PrPC)	

Table 1 shows a set of proteins present in platelets and its physiological role in the regeneration of tissues.

among them is absolutely necessary to understand some of the controversial results obtained with these types of technologies so far.[21] In terms of composition, different types of plasma rich in platelets differ in their platelet count enrichment (greater or less than 5x), leukocyte content (greater or less than 1x), and whether they are activated or not. On this basis, it can be classified into 8 different types. These variables influence tissue biological response and thus the treatment efficacy.[22]

Understanding the Properties of PRP Products

Several key biological mediators are present in a PRP. The more studied growth factors contained in PRP that are important during tissue repair include insulin-like growth factor type I (IGF-I), transforming growth factor beta type 1 (TGF-β1), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), epithelial growth factor, and basic fibroblastic growth factor among others (Table 1).[24,25] Some of them (IGF-I and HGF) are plasmatic proteins, and their concentrations do not depend on the platelet enrichment. However, most of the growth factors are indeed platelet proteins, both synthesized and adsorbed, and thus, their quantity does depend on the platelet concentration.

To understand the properties of PRP products, it is necessary to detail the different roles of molecules that contain the following:

- IGF-I: This protein circulates in plasma as a complex with binding proteins. This determines the bioavailability and regulates the interaction between this IGF-I and its receptor.[26,27] IGF-I is involved in keratinocyte migration and wound healing,[28,29] stimulates bone matrix formation and maintenance[30] by promoting preosteoblast proliferation,[31,32] and is also involved in striated muscle myogenesis.[33] Furthermore, knockout mice for IGF-I receptor (IGF-IR) in muscle exhibited impaired muscle regeneration and deficient myoblast differentiation.[34]
- TGF-β1: The role of TGF-β family proteins in wound healing has been recently reviewed.[35] TGF-β has different effects, depending on tissue and cell type.[5] The release and posterior bioactivation of latent TGF-β contributes to the early cellular reparative responses, such as migration of cells, neovascularization, and angiogenesis[36] into the wound area. In bone, TGF-β1 induces osteogenic differentiation of mesenchymal cells of the bone marrow, upregulating osteoblast differentiation markers.[37]
- PDGF: This growth factor is a mitogen and chemotactic factor for all cells of mesenchymal origin. It is important in the repair of joint tissue, including cartilage and me-

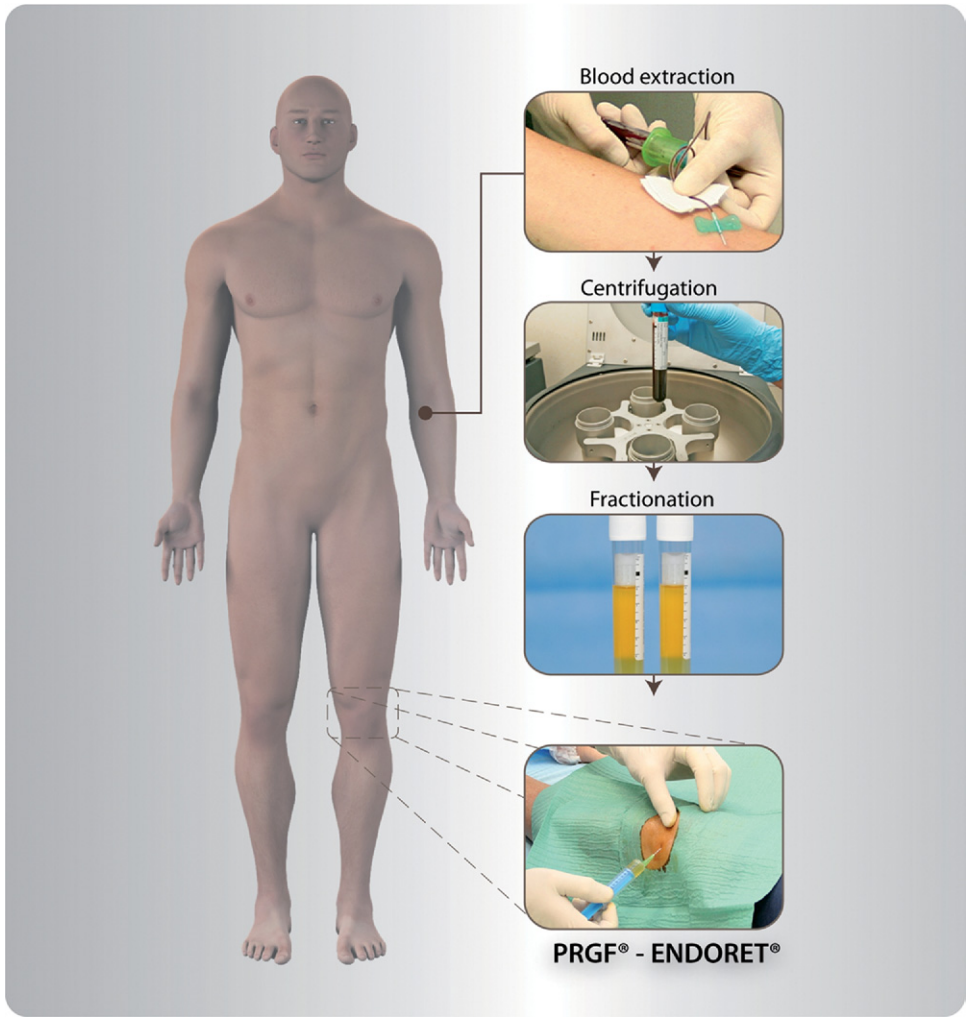

Figure 1 PRGF-Endoret technology overview. PRGF-Endoret aids in the preparation of different autologous therapeutic formulations from patient's own blood.

niscus.[38,39] Bone is also a target of PDGF, influencing its metabolism and acting in repair mechanisms[40,41] including the recruitment of pericytes to stabilize new blood vessels.[42]

- HGF: This growth factor regulates cell growth, migration, and morphogenesis,[43] and plays an important role in wound healing through an epithelial-mesenchymal interaction.[44] The antifibrotic effect of HGF has been shown in various tissues,[45,46] through induction of Smad7, and thus regulates the myofibroblast phenotype, allowing the initial contraction of the wound, but making the myofibroblast to gradually disappear.[47]
- VEGF: This growth factor is a key mediator in wound healing[48] and the main inducer of angiogenesis because it stimulates chemotaxis and proliferation of endothelial cells.[49] Also, VEGF is involved in the regulation of many organ homeostasis, such as brain, heart, kidney, or liver,[50] and its role may be crucial in cell-mediated tissue regeneration.[51]
- Epithelial growth factor: This protein promotes chemotaxis and mitogenesis in epithelial and mesenchymal cells[52,53] by acting on the regeneration of multiple tissues. It has an important role in skin, cornea, gastrointestinal tract, and nervous system.[54-58]
- Basic fibroblastic growth factor: This factor, also called fibroblast growth factor 2, is potent inductor of cell proliferation, angiogenesis, and differentiation.[59,60] Its role in the repair process has been observed in several tissues, including bone,[61-63] tendon,[64,65] and periodontal tissue.[66-68]

Growth factors classically promote several important functions in the regenerative milieu—they are able to stimulate cell proliferation (mitosis), cellular migration (chemotaxis), differentiation (morphogenic effect), angiogenesis, and the combination of several of these effects. These peptides exert the aforementioned functions in the local environment, close to the site of the application.

However, it is difficult to dissect the contribution of each molecule contained in PRP and examine its effect separately, as many have multiple effects, some of which overlap with others. Also, many molecules are activated in the presence of others, such as TGF-β, which is in a latent state[69] and becomes functional after proteolytic activation or in the presence of other molecules, such as thrombospondin-1 or various integrins.

The idea that PRP contains only factors that stimulate angiogenesis and proliferation would be a little simplistic. In fact, another important property of the PRP is the bacteriostatic effect. These antibacterial effects were observed against *Staphylococcus aureus* and *Escherichia coli*.[70] Classically, these properties have been shown in leuko-enriched PRP. However, recently, these antimicrobial properties have been evidenced in PRGF-Endoret,[71] which by definition has no white cells. Specifically, PRGF-Endoret has bacteriostatic effect against Staphylococcal strains. Moreover, the addition of leukocytes to the PRGF-Endoret preparation did not yield greater bacteriostatic potential than it already had. These data raise questions about the role that leukocytes may play in a PRP preparation because they do not improve the bacteriostatic properties, but, on the contrary, they might significantly increase the presence of proinflammatory molecules.

Platelet-rich products act also as anti-inflammatory mediators by blocking monocyte chemotactic protein-1, released from monocytes, and lipoxin A4 production.[72] HGF in PRP inhibits NF-κB, a key nuclear factor implicated in inflammatory responses, by activation of its inhibitor (ikBα). In this same study, it was also observed that PRP reduced the chemotaxis of the monocytic line U937.[73] In addition, serotonin, a neurotransmitter and hormone present in platelets, has been reported to directly mediate liver regeneration.[74]

PRGF-Endoret: A Pioneering Technology

For almost 2 decades, our research group has characterized this technology and has studied its therapeutic potential in tissue repair and wound healing.[75] PRGF-Endoret contains a moderated platelet concentration, a two-third–fold increase compared with peripheral blood, a dosage shown to induce optimal biological benefit.[76] In fact, lower platelet concentrations can lead to suboptimal effects, whereas higher concentrations might have an inhibitory effect.[77] PRGF-Endoret does not contain leukocytes, and activation is performed only with calcium chloride ($CaCl_2$).

The process to produce PRGF-Endoret is easy, fast, and reproducible (Fig. 1). Blood collection is performed in tubes containing sodium citrate as anticoagulant. Thus, platelets are well preserved. Subsequently, centrifugation is achieved in a specifically designed centrifuge (PRGF System IV, BTI-Biotechnology Institute, Vitoria, Spain). The centrifuge has

Figure 2 The plasma transfer device is a disposable and sterile aspiration system that allows the fractionation of PRGF-Endoret. The device contains an ergonomic button that allows fine control of the suction flow. The suction is performed by the vacuum containing in the fractionation tube. The user accessible needle is a blunt needle to prevent accidental stab injuries. In this way, PRGF-Endoret is obtained directly in a fractionation tube, in which it can be directly activated with calcium chloride.

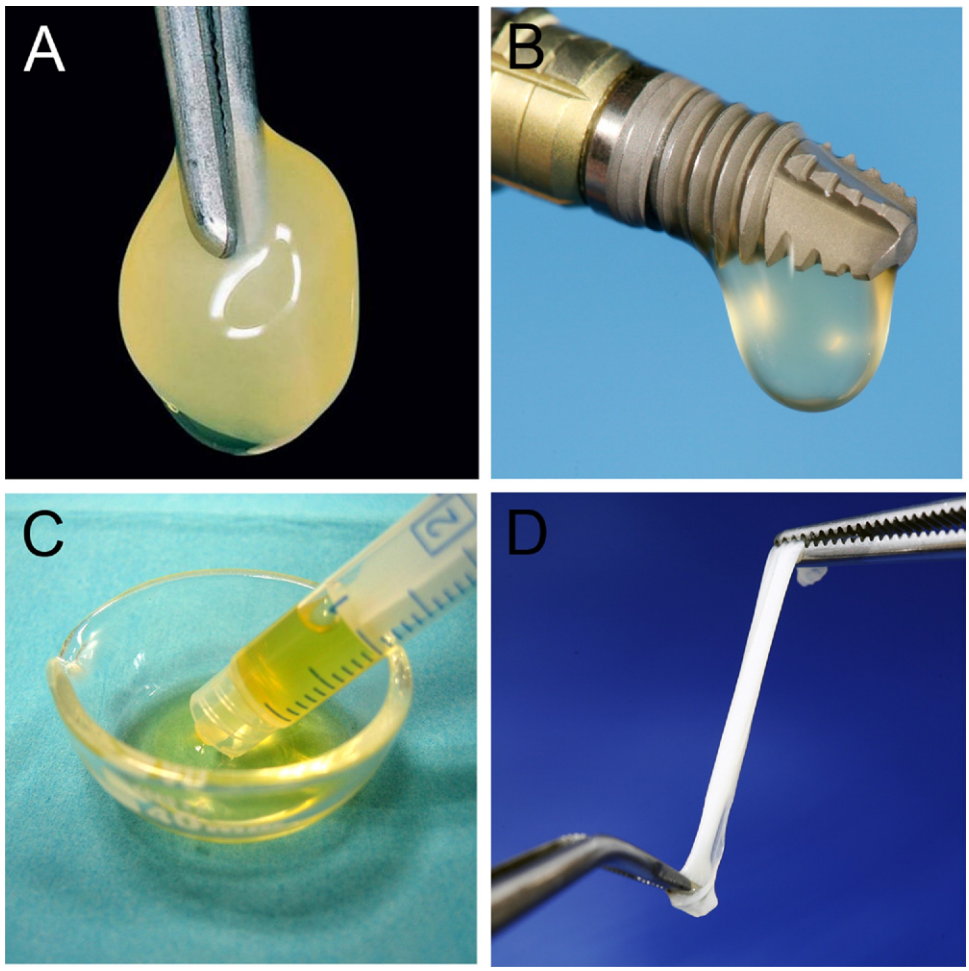

Figure 3 The 4 different formulations of the PRGF-Endoret technology: (A) the 3-dimensional scaffold, (B) the liquid formulation, activated at the moment, on the titanium surface, (C) the PRGF-Endoret supernatant, and (D) the elastic and dense autologous fibrin membrane.

specific parameters to maximize the production of platelets and keep the plasma leukocyte free. After centrifugation, the following 3 typical layers are obtained: a yellowish top layer, the plasma, which contains a gradient of platelets, with maximum concentration of those platelets above the buffy coat; the leukocyte layer, or *buffy coat*, is located below of plasma layer; and the bottom layer is the layer containing the red cells. Regarding the plasma volume, it is possible to empirically differentiate between 2 different fractions, depending on the respective concentration of platelets. The upper fraction will contain a similar number of platelets than peripheral blood, whereas the lower fraction will contain 2- to 3-fold the concentration of platelets compared with blood.

With the aim of collecting these plasma fractions from PRGF-Endoret technology, we have recently developed an optimized device—the plasma transfer device (PTD) (Fig. 2). The PTD is a disposable and sterile aspiration system that allows separating the different fractions obtained after centrifugation. In contrast to the traditional pipetting system, the PTD system is faster, avoiding intermediate pipetting steps. In addition, the PTD does not require maintenance of the pipetting system. Depending on clinical needs, the fractionation can be made in 1 or 2 fractions, achieving higher volume—lower concentration of platelets (a single fraction) or lower volume—higher concentration of platelets (2 fractions). After fractionation, PRGF-Endoret can be activated in a controlled way by the addition of $CaCl_2$, providing a clot that mimics its natural structure. Moreover, the coagulation is conducted at a speed that allows controlling the whole process. Activation with $CaCl_2$ avoids the use of exogenous bovine thrombin, a source of possible immunologic reactions.[78-80]

Another important feature of the PRGF-Endoret technology when compared with other PRP systems is the absence of leukocytes, which categorizes it as a safe and homogeneous, because the values of leukocytes are highly variable between donors[81] and, within the same donor, are highly dependent on small perturbation of the body homeostasis.

In addition, polymorphonuclear neutrophils (PMN) contain molecules designed to kill microorganisms, but can seriously damage the body tissues. For example, PMNs are important producers of matrix metalloproteinases (MMP), mainly MMP-8 and MMP-9, which can hamper the regeneration of damaged tissue. PMNs also produce free radicals,

reactive oxygen species and nitrogen, which can destroy not only microorganisms but surrounding cells.[82] Of special concern would be to avoid leukocytes if muscle regeneration is required, as in vivo PMNs increase muscle damage[83] and do not provide extra functionality. Therefore, it is recommended to use leukocyte-free PRP in infiltrations of damaged muscle.[84]

PRGF-Endoret Technology: A Versatile Toolbox with Multiple Formulations

A key point that distinguishes the PRGF-Endoret technology from other PRP products is its versatility. Four different formulations (Fig. 3) with therapeutic potential are obtained from the patient's blood, depending on the coagulation and activation degree of the samples. These formulations may be used for different therapeutic purposes:

1. PRGF-Endoret scaffold. It is a 3-dimensional matrix, encloses autologous growth factors, both plasma and platelet proteins. This scaffold can be used in various applications, such as the treatment of ulcers,[85,86] wound closure, and tissue engineering.[87] The 3-dimensional structure of the fibrin mesh (Fig. 4) allows cell proliferation because, as mentioned earlier, it contains factors necessary for growth and migration of cells. In addition, this formulation can be combined with other materials,[88] such as autologous bone, demineralized freeze-dried bovine bone, collagen among others, adjusting the resulting characteristics of the scaffold.
2. Liquid PRGF-Endoret, activated at the time of use, is used in intra-articular injections,[89,90] surgery,[91,92] treatment of skin disorders,[85,86] skin regeneration,[93] and implant surface bioactivation by producing a biologically active layer on the titanium surfaces.[94]
3. The PRGF-Endoret supernatant contains plasma proteins and platelet releasate, and can be used as eye-drop treatment for dry eye disease[95] and other corneal defects.[96] In both basic and applied studies, this formulation can be used to supplement the cell culture medium.[76,97,98]
4. Autologous fibrin membrane. At the end of the process of coagulation, fibrin scaffold retracts. At that stage, the fibrin membrane can be shaped with tweezers or similar instruments to obtain an elastic, dense, and suturable membrane. It is an excellent tool to seal the postextraction tooth sockets[99] and to promote the full epithelialization of soft tissues.[100]

The autologous platelet products have a high therapeutic potential and can be used in various formulations and in various fields of medicine and tissue engineering. At present, there are over 40 of these products with different characteristics, in terms of enrichment of platelets, presence of leukocytes, kind of activator, and final volume among others. This great variability makes it difficult to standardize protocols and compare results. Furthermore, this large variability can engender confusion among clinicians and researchers.[101] It is, therefore, necessary to reach a consensus and better definition of each product. Our research team has spent more than 15 years developing this technology, which makes PRGF-Endoret one of the best characterized autologous PRP, with multiple and growing therapeutic applications, as result of a continuous research translation to the clinic setting.

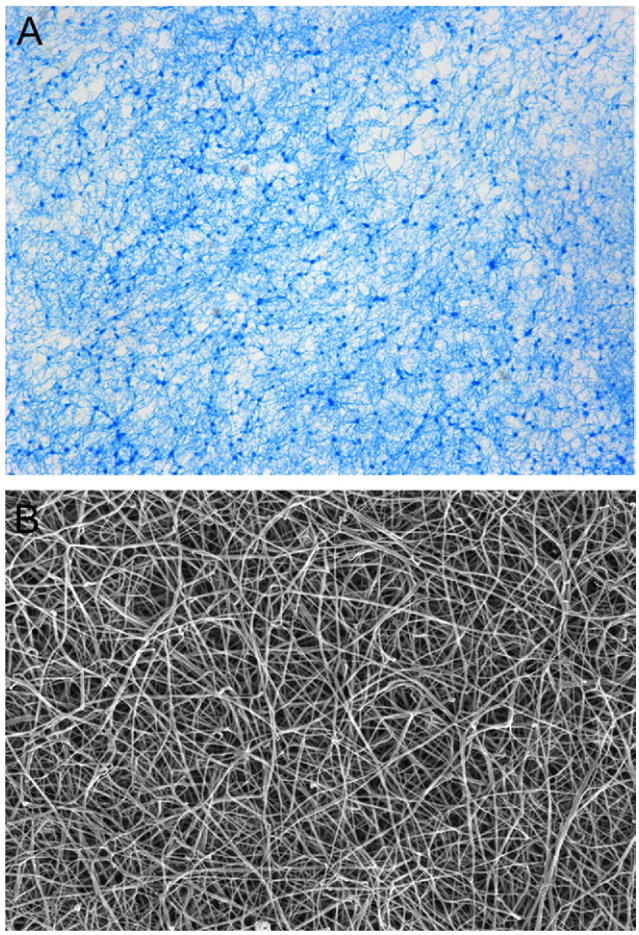

Figure 4 Three-dimensional structure of PRGF-Endoret fibrin network. (A) Network of fibrin and platelet aggregates scattered throughout the network of fibrin. May-Grunwald-Giemsa staining. (B) Detail of the tridimensional structure of the fibrin network. Note the interconnected intact fibrin strands. Scanning electron microscopy. Original magnifications (A, x400; B, x3500).

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