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EXPERIMENTAL STUDY

Plasma rich in growth factors (PRGF-Endoret) stimulates tendon and synovial fibroblasts migration and improves the biological properties of hyaluronic acid

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Abstract
Purpose Cell migration plays an essential role in development, wound healing, and tissue regeneration. Plasma rich in growth factors (PRGF-Endoret) technology offers a potential source of growth factors involved in tissue regeneration. Here, we evaluate the potential of PRGF-Endoret over tendon cells and synovial fibroblasts migration and study whether the combination of this autologous technology with hyaluronic acid (HA) improves the effect and potential of the biomaterials over the motility of both types of fibroblasts.
Methods Migration of primary tendon cells and synovial fibroblasts after culturing with either PRGF or PPGF (plasma poor in growth factors) at different doses was evaluated. Furthermore, the migratory capacity induced by the combination of PPGF and PRGF with HA was tested.
Results PPGF stimulated migration of both types of cells but this effect was significantly higher when PRGF was used. Tendon cells showed an increase of 212% in migratory ability when HA was combined with PPGF and of 335% in the case of HA + PRGF treatment compared with HA alone.
Conclusions PRGF-Endoret stimulates migration of tendon cells and synovial fibroblasts and improves the biological properties of HA.

Keywords Tendon fibroblasts · Synovial fibroblasts · Growth factors · Hyaluronic acid · Migration

Introduction

Cell migration plays an essential role in many physiological and pathological processes due to its implication in development, wound healing, angiogenesis, and matrix deposition. The appropriate regulation of cell migration is critical for the maintenance of integrity of the tissues and self-renewal of the cell population. After an injury, almost all stages of tissue repair and remodeling are controlled by endogenous growth factors (GFs), proteins and cytokines that act as local regulators of basic cell functions [16, 34]. Most of these biologically active molecules are secreted to the local environment from the serum and degranulating platelets.

Plasma rich in growth factors (PRGF-Endoret) technology offers a potential source of a myriad of autologous growth factors and proteins involved in tissue regeneration that can be locally applied at the injury site. This technology permits the replacement of a natural blood clot with different formulations enriched in GFs that stimulate wound healing and promote tissue regeneration including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-I), hepatocyte growth factor (HGF), angiopoietins (Ang), platelet

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factor-4 (PF-4), and thrombospondin-1 (TSP-1) among others [1, 2].

On the other hand, hyaluronic acid (HA) is a natural component of the extracellular matrix and responsible for the viscoelasticity of joint synovial fluid. HA has a crucial role in development, inflammation and tissue repair, including wound healing and it is involved in cell migration and cell-to-cell communication through the receptor CD44 [10]. As a consequence, HA has been used as a scaffold to allow tissue repair and regeneration at the sites of injury.

Anterior cruciate ligament (ACL) injuries are very common among active young population and require a surgical intervention named ACL reconstruction (or ACL tissue engineering). The biological aspects of the reconstruction of this surgery involve intrasynovial remodeling and tendon-bone integration [28, 29]. Tendon healing requires the migration of tendon cells to the repair site, followed by the proliferation and synthesis of the extracellular matrix. There is also a recruitment of precursor cells from synovium with a great potential to repopulate the area.

The purpose of this study is to evaluate the potential of PRGF-Endoret technology over tendon cells and synovial fibroblasts migration and to elucidate whether the combination of PRGF or PPGF (plasma poor in growth factors) with HA improves the effect and potential of the biomaterials over the motility of both types of fibroblasts.

Materials and methods

Isolation and culture of primary fibroblasts

Human fibroblast cells were obtained from synovium and tendon biopsies removed during routine joint surgery. Tissue samples were harvested with the informed consent of the patients of the study, approved by our Institutional Review Board. The study was performed following the principles of the Declaration of Helsinki. Cells were isolated following our standard protocol [4] and cultured with complete culture medium (Dulbecco's Modified Eagle Medium: Nutrient mixture F-12 (DMEM/F12) (Gibco by Invitrogen) supplemented with 15% fetal bovine serum (FBS, Biochrom AG, Leonorenstr, Berlin, Germany), 2 mM L-Glutamine (Sigma–Aldrich), antibiotics and antimycotics) and maintained in a humidified atmosphere at 37°C with 5% CO_2. The culture medium was changed twice a week. Cell viability was tested by Trypan Blue dye exclusion (Sigma–Aldrich). Cells between 3 and 5 passages were employed for all experiments.

Characterization of cell cultures

Fibroblasts obtained from the two anatomical localizations were characterized by immunofluorescence. Expression of three fibroblast markers was analyzed: Collagen type I (Chemicon-Millipore, Billerica, MA, USA), vimentin (Sigma-Aldrich), and CD90 (BD Biosciences, Bedford, MA, USA); also CD105 and CD34 (BD Biosciences) expression was evaluated as markers of endothelial, macrophages, and hematopoietic cell lineages.

Serum, plasma and PRGF-Endoret preparation

Blood from one young healthy donor was collected in 9-ml tubes with 3.8% sodium citrate (w/v) to obtain plasma preparations after informed consent. The tubes were separated in two groups. To prepare PRGF-Endoret, blood was centrifuged at 580*g* for 8 min at room temperature (PRGF-Endoret System, BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) and plasma column was collected. The other group of tubes was centrifuged at 3,000*g* for 20 min at room temperature to precipitate platelets and obtain the PPGF. In both cases, care was taken to avoid the buffy coat containing the leukocytes. Number of platelet and leukocytes was counted using a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France); the results are shown in Table 1. The two plasma preparations were incubated with PRGF Activator (BTI Biotechnology Institute) at 37°C for 1 h and were then centrifuged at 4,500*g* for 10 min at 4°C. Finally, PRGF and PPGF preparations were separated into aliquots and maintained at −80°C until utilization. To obtain autologous human serum (HS), blood from the same donor was collected into 9-ml tubes with clot activator. To help the coagulation process, blood was maintained at 4°C overnight, and the following day, it was centrifuged at 1,000*g* for 10 min at 4°C. Serum aliquots were stored at −80°C. The quantification of relevant GFs implicated in cell migration process was performed by ELISA kits (R&D Systems, Minneapolis, USA). PDGF-AB, TGF-β1, VEGF, HGF, and IGF-I concentrations were determined in serum and plasma preparations following the manufacturer's recommendations.

Cell migration assays

In order to quantify the migratory potential of tendon and synovial fibroblasts, they were plated in culture inserts (Ibidi, GmbH, Martinsried, Germany) placed on a 24-well plate at high density and were grown with complete culture medium until confluence. After carefully remove the inserts, two separated cell monolayers leaving a cell-free gap of approximately 500 μm thickness were created. The cells were washed with PBS and incubated with the

Table 1 Description of plasma and serum preparations

Blood preparations	Leukocyte count ($\times 10^6$/ml)	Platelet count ($\times 10^6$/ml)	Growth factors concentrations				
			TGF-β1 (ng/ml)	PDGF-AB (ng/ml)	IGF-I (ng/ml)	VEGF (pg/ml)	HGF (pg/ml)
PRGF	<0.02	580	39.7 ± 0.9	23.1 ± 1.1	94.6 ± 0.4	330.6 ± 12.9	348.8 ± 20.5
PPGF	0.0	57	3.8 ± 0.3	2.3 ± 0.1	88.3 ± 2.2	22.8 ± 3.3	238.1 ± 66.4
HS	6.2*	258*	42.0 ± 1.1	24.3 ± 0.0	106.0 ± 1.1	235.2 ± 1.6	921.5 ± 49.1

Table shows the platelet and leukocyte counts and the concentrations of the most relevant growth factors for our study. *PRGF* plasma rich in growth factors, *PPGF* plasma poor in growth factors, *HS* human serum

* For HS, the measurements correspond to hematological counts in peripheral blood

corresponding treatments: (1) 0.2% FBS, (2) 25% PPGF, (3) 25% PRGF, (4) 50% PPGF, (5) 50% PRGF, (6) 25% hyaluronic acid (HA, 1% sodium hyaluronate, Euflexxa, Ferring Pharmaceuticals, St-Prex, Switzerland), (7) 25% PPGF + 25% HA, (8) 25% PRGF + 25% HA and (9) 25% HS in triplicate for 24 h. After this period, treatments were removed and cells were incubated with 1/500 Hoechst 33342 in PBS for 10 min. To quantify the number of migratory cells, phase contrast images of the central part of the septum before treatment and phase contrast and fluorescence photographs after the treatment time were captured with a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB, Leica Microsystems). The gap area was measured and the number of migratory cells found in this gap after the 24-h treatment was counted using the Image J Software (NIH, Bethesda, Maryland, USA). The results corresponded to means of three independent assays and they were expressed as number of cell migrated per mm^2 of area. Nevertheless, they will be discussed as the percentage of increase in response to each treatment comparing with control group.

Statistical analysis

In order to control the influence of possible confounding factors and effect modifying variables, an adjustment was carried out by applying analysis of variance with repeated measures (general linear method), where the within-subject variable included was the concentration and tissue as the inter-subject factor. Comparison of variables adjusted in efficacy between the two treatment groups was performed by determining the confidence intervals for 95%.

Results

Primary culture characterization

Tendon cells and synovial cells exhibited the typical spindle morphology of fibroblasts as shown in Fig. 1. CD90 antigen, vimentin, and collagen type I expression were homogenously detected in cells of both primary cultures (Fig. 1) confirming their fibroblastic nature. Moreover, isolated cultures were negative for the hematopoietic, epithelial, and endothelial markers tested (data not shown).

Quantification of growth factors in plasma and serum preparations

The platelet enrichment of PRGF-Endoret® preparation was 2.25-fold over peripheral blood. PRGF treatment did not contain leukocytes. The growth factors measured derive exclusively from plasma and from platalets. In the case of HS, GFs measurements corresponded to the releasates from leukocytes and platelets. Concentration of platelet factors TGF-β1, PDGF-AB, and VEGF on PRGF-Endoret preparation was about 10 times higher than on PPGF preparation and similar to HS. IGF-I levels were similar in three treatments. HGF measurements were threefold higher in HS compared with PRGF (Table 1).

Dose-dependent effect of plasma preparations

To decide the appropriate dose of plasma preparations, synovial cells were chosen and cultured with 25 and 50% of PPGF and PRGF treatments. Interestingly, cell migration observed in cultures treated with 25% PPGF was similar to that in 50% PPGF, and when we compared 25 and 50% of PRGF preparation in treatments, the results showed that cell motility decreased in a 66.5% when synovial cells were incubated with 50% PRGF (Fig. 2). Therefore, a percentage of 25% of plasma and biomaterial preparations was the percentage chosen for following experiments.

Effect of PRGF-Endoret preparation on cell migration

Tendon cells treated with 25% PPGF increased their motility in 168.2% compared with NS (0.2% FBS) (Fig. 3). This pro-migratory effect was highly intensified by the

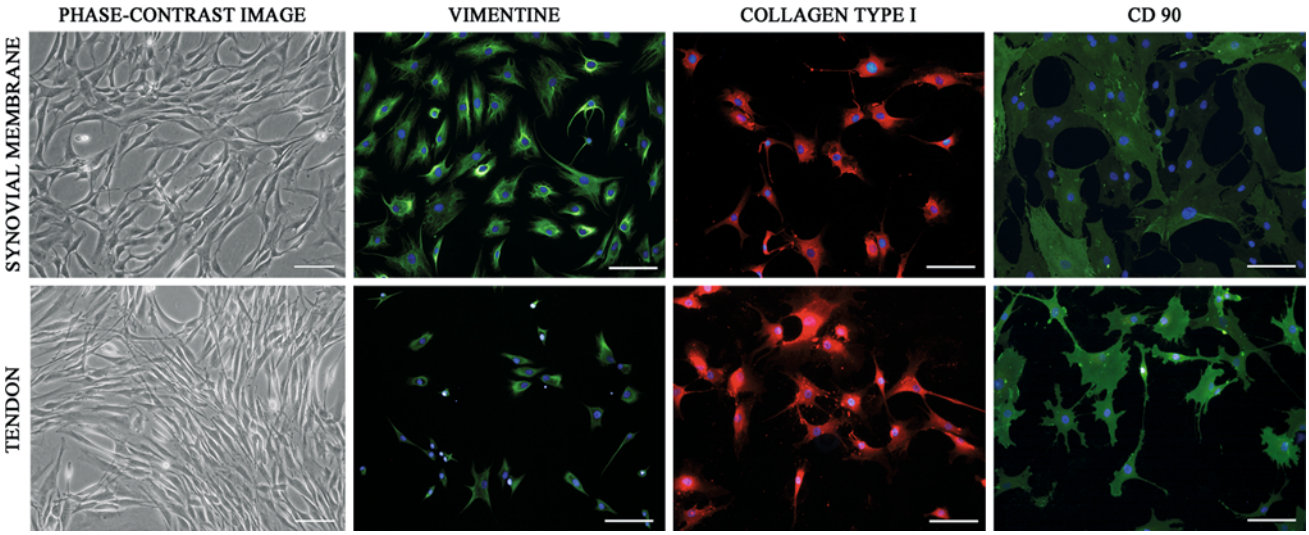

Fig. 1 Fibroblasts isolated from synovial membrane and tendon. Representative phase contrast photomicrographs showing the typical shape of both types of fibroblasts cultured in vitro and immunofluorescence microscopy confirming that fibroblasts were uniformly positive for vimentin, collagen type I and CD90. *Blue* Hoechst, *green* vimentin and CD90, *red* collagen type I. *Scale bar* 100 μm

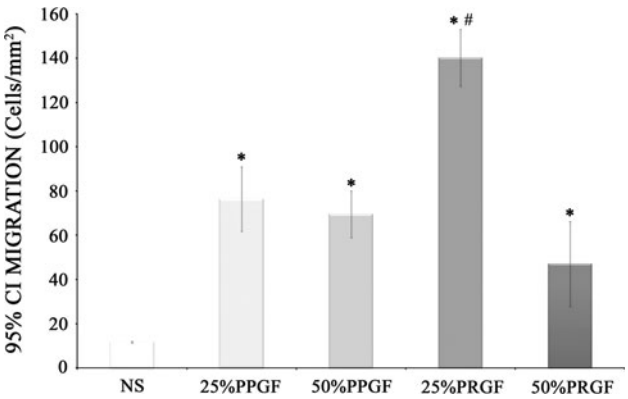

Fig. 2 Synovial cells migratory response to different percentages of plasma preparations (PPGF and PRGF). The results represent means ± SD of three replicates. *NS* non-stimulatory situation, *PPGF* plasma poor in growth factors, *PRGF* plasma rich in growth factors. *Significant respecting to NS; #significant respecting to the other treatments (95% confidence interval in both cases)

activity of platelet-released GFs present in PRGF preparation. In this way, tendon cells treated with 25% PRGF increased their migration rate in 231.8%. In addition, plasma treatments augmented significantly the migration of synovial cells compared with NS. In fact, the migratory cell count was increased by 212.6 and 380.7% after adding PPGF and PRGF to the cultures, respectively (Fig. 4). It should be highlighted the weak stimulation detected after 25% HS treatment in both cell lineages, specially in tendon cells, despite the similar GFs concentrations found in HS and PRGF preparations (Figs. 3, 4; Table 1).

Effects of HA-plasma combinations on cell migration

To analyze the synergic effect of HA and plasma preparations, isolated cell cultures were incubated with 25% PPGF + 25% HA and 25% PRGF + 25% HA during 24 h, and the results were compared with 25% HA alone. Tendon cells showed an increase of 211.9% in their migratory ability after the incubation with the mixture of HA + PPGF and of 334.4% in the case of HA + PRGF treatment (Fig. 5). In the same way, PPGF or PRGF addition to HA treatment induced a great increment in synovial cell migration compared with the response to HA alone (93.7 and 368.4%, respectively) (Fig. 6). Moreover, the significantly highest migratory effect was detected after PRGF + HA incubation in both tissue analyzed.

Discussion

The most important contribution of the present study deals with the potential advantages that the use of PRGF-Endoret® Technology provides in terms of promoting cell migration, a pivotal process of tissue regeneration. The different formulations under study are 100% autologous, versatile, safe and effective in promoting tissue healing and regeneration [2, 3, 27]. In this paper, we studied the effect of two plasma preparations (PRGF and PPGF) over the migratory potential of tendon cells and synovial primary fibroblasts. PPGF stimulated both types of cells migration but this effect were significantly increased when platelet-

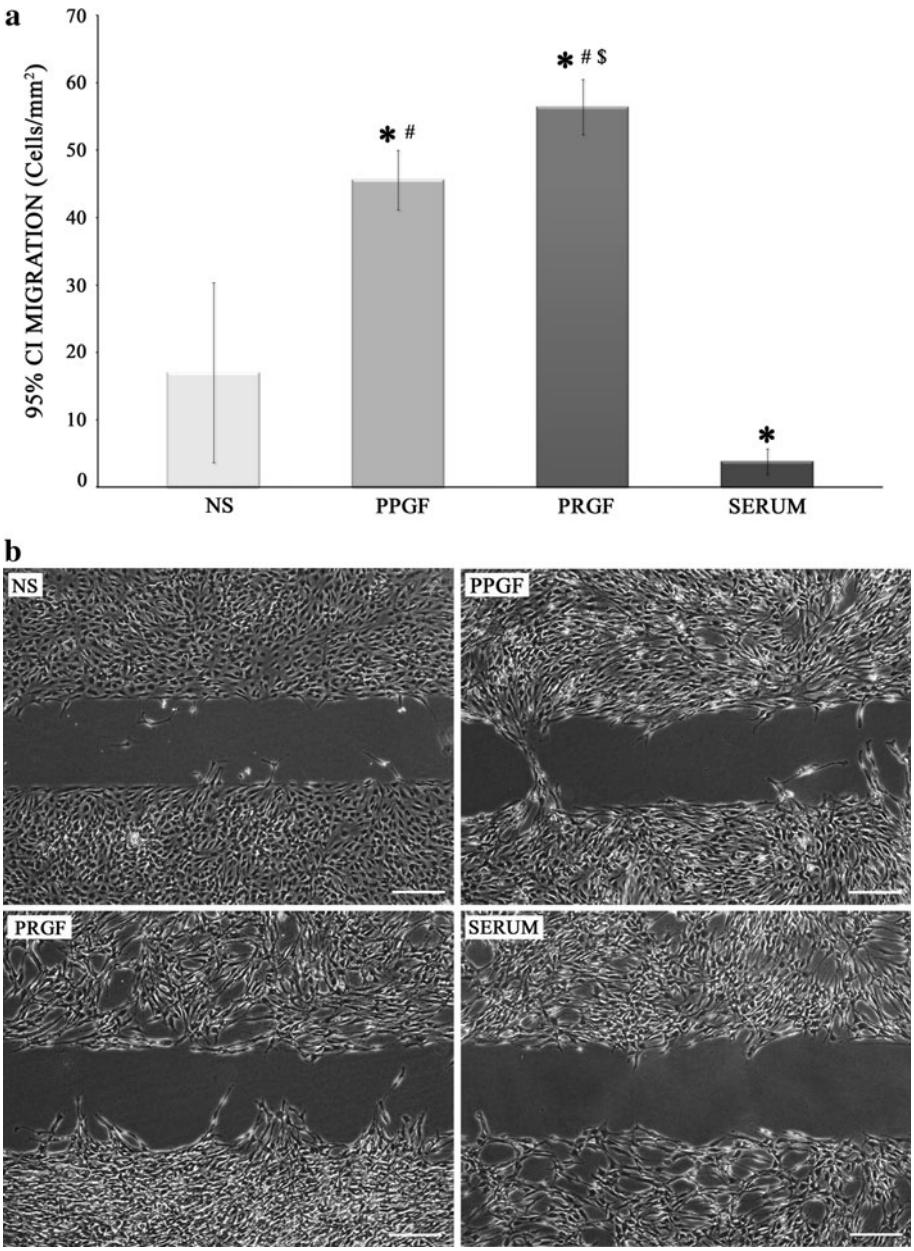

Fig. 3 Tendon cells migration induced by 25% autologous blood preparations. **a** Graph showing means ± SD of three replicates of the migratory response. *Significant respecting to NS; #with significance to serum; $Significant respecting to PPGF (95% confidence interval in all the cases). **b** Phase contrast microphotographs showing representative migratory responses of tendon cells to the different treatments. *Scale bar* 300 µm. *NS* non-stimulation, *PPGF* plasma poor in growth factors, *PRGF* plasma rich in growth factors, *Serum* autologous serum

secreted growth factors were included (PRGF). Previous results from other groups point out the inductive effect of platelet lysate over the motility of myoblasts promoting wound healing [25] and the modulation of PRGF over the migration of gingival fibroblasts, [8] osteoblasts [9, 11] and endothelial cells [26]. Tendon cells and synovial fibroblasts showed a weak migration after stimulation with human serum. In fact, in the case of tendon cells, motility was lower than in non-stimulatory situation. This may result surprising because the content and concentration of growth factors in serum is very similar to the one in PRGF. One possible explanation may be due to the presence of leukocyte-derived factors that could inactivate the pro-migratory effect of the growth factors from platelets. Englesbe et al. [12] demonstrated that interleukin 1β (IL-1β), which is actively released by the leukocytes, was able to inhibit the migration of muscular cells after stimulation with PDGF-BB. PRGF was capable to induce the highest stimulatory response. In fact, both types of fibroblasts migrated significantly more with PRGF than with PPGF. The effect of platelet alpha granules content over chemotactic activity of fibroblasts was already studied by Senior et al. [31]. Moreover, it was described the inductive role of the pool of growth factors present in the platelets and therefore in the PRGF, over the migration of several cell phenotypes. For example, PDGF is responsible for the in vitro capacity of migration of human fibroblasts and bone marrow stem cells [21, 30], whereas IGF-I induces

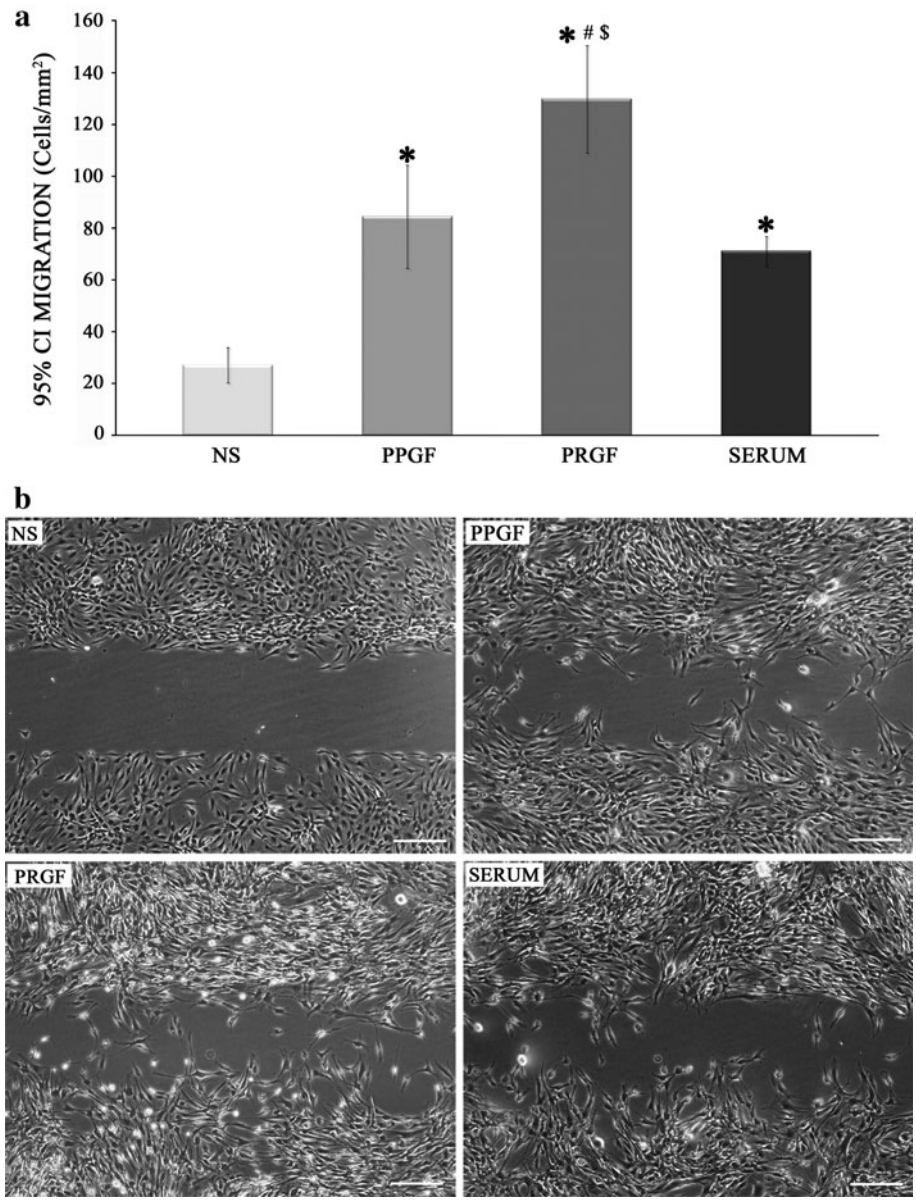

Fig. 4 Synovial cells migratory activity induced by 25% autologous blood preparations. **a** Graph showing means ± SD of three replicates of the induced migration of synovial fibroblasts. *Significant respecting to NS; #with significance to serum; $Significant respecting to PPGF (95% confidence interval in all the cases). **b** Phase contrast microphotographs showing representative images of synovial cells responding to the different treatments. *Scale bar* 300 µm. *NS* non-stimulation, *PPGF* plasma poor in growth factors, *PRGF* plasma rich in growth factors, *Serum* autologous serum

migration of corneal epithelial cells [20]. Another relevant growth factor, TGF-β1 regulates macrophage and hepatocyte migration [5, 19] and basic FGF is involved in the migratory response of cells through different pathways [6]. Epidermal growth factor (EGF) signaling stimulates epithelial cell migration [13, 22] and alters fibroblast migration speed [32] showing a dose-dependent effect [17]. Another interesting aspect is the fact that doubling the percentage of plasma preparations in culture reduces significantly the migration rate of the cells. Some authors have also observed an inhibitory effect of elevated doses of platelet-derived GFs over fibroblasts, osteoblasts, and periodontal ligament cells [15, 18, 33].

After replacing the anterior cruciate ligament (ACL) with extra articular tendon in arthroscopic surgery, there is an important alteration of the inner tendon environment. The synovial fluid from the joint capsule covers tendon and synovial membrane cells and so does the hyaluronic acid present in the synovial fluid. Tendon cells and synovial fibroblasts were incubated with 25% HA simultaneously with 25% PPGF or PRGF. Culture medium with 25% HA served as control of migration and induced the movement of 25.2 ± 3.4 and 35.3 ± 4.3 fibroblastic cells per mm^2 in tendon and synovial tissues, respectively, (Figs. 5, 6). Other research groups achieved an induction over the migratory capability of tendon cells stimulated by different doses of hyaluronan [36] and of synovial cells in culture increasing the velocity of migration [24].

In the present study, the presence of hyaluronic acid in the culture medium concurrently with plasma preparations

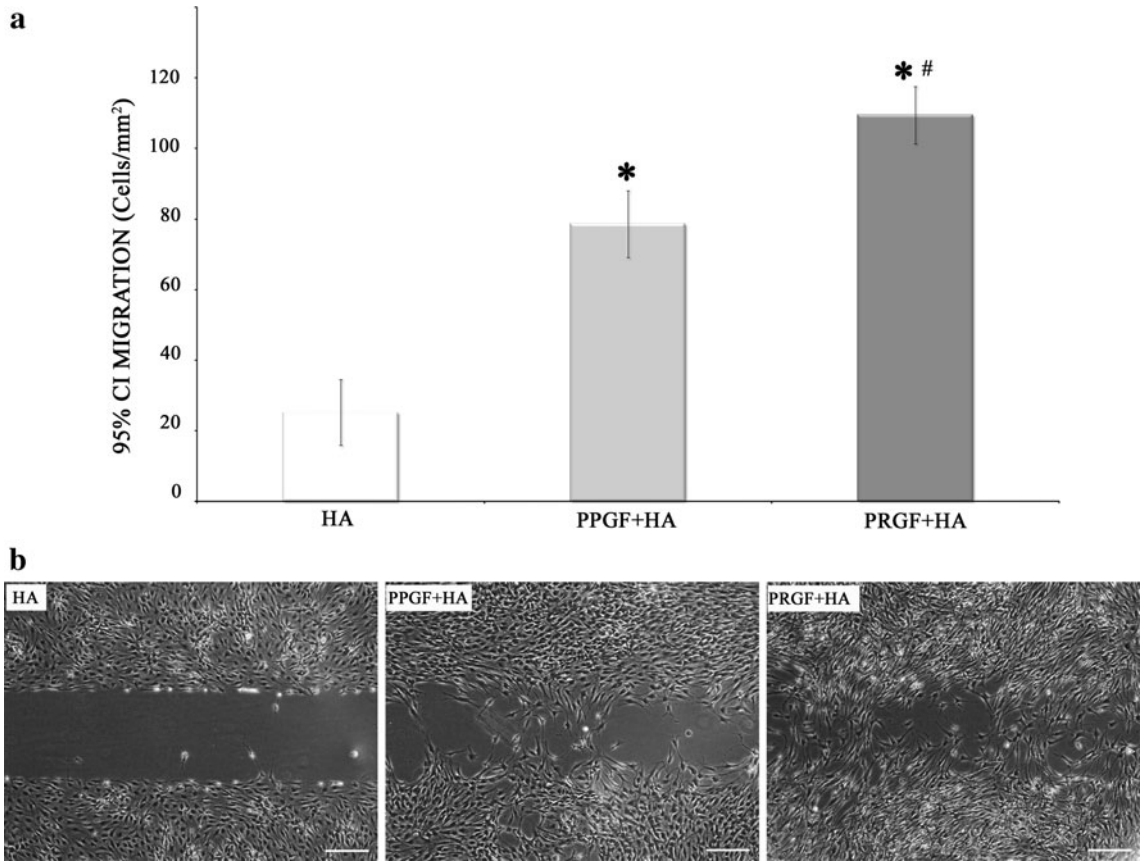

Fig. 5 Strengthening of the inductive effect of 25% hyaluronic acid in combination with 25% plasma preparations over the motility of tenocytes. **a** Means ± SD of three replicates of the induced response. *Significant respecting to HA; #Significant respecting to PPGF + HA (95% confidence interval in both cases). **b** Phase contrast representative images of tendon cells migration. *Scale bar* 300 µm. *HA* hyaluronic acid, *PPGF* plasma poor in growth factors, *PRGF* plasma rich in growth factors

induced an increase in the migratory response of both types of fibroblasts, a difference that was significant in the case of tendon cells. This could be due to a synergism between HA and the growth factor present in the plasma preparations that could involve their respective receptors. The pro-migratory effect of HA is mediated through the interaction with some of their receptors; one of them, CD44 has been implicated in the migratory signal transduction [23] as well as RHAMM receptor (Receptor for HA-mediated motility) in several cell lineages [14]. After culturing mesenchymal stem cells in vitro with PDGF (MSC, mesenchymal stem cell), there is an increase in CD44 and this favors cell migration though the interaction of this receptor with extracellular HA [37]. Furthermore, CD44 acts as a co-receptor for ErbB transmembrane tyrosine kinase family of receptors and for the TGF-β1 serine/threonine kinase receptor [7, 35]. In all these cases, interaction of growth factor with CD44 augments the inner growth factor effect. Our study supports the latter as migratory response is superior in the mixture HA + PRGF because the higher concentration of growth factors in PRGF than in PPGF.

This in vitro paper provides evidence of how the growth factors present in the PRGF promote the migration of tendon cells and synovial fibroblasts. Furthermore, it also shows that PRGF can be used to improve the biological properties of certain biomaterials such as hyaluronic acid. The main limitation of this work is that an in vivo study is lacking to demonstrate all these properties. Animal research could complement the in vitro results and contribute to necessary reliability to extrapolate them to clinical practice. Our research experience in the last 10 years show that this autologous technology can be successfully used in the clinical setting; however, more research is needed to fully understand all the biological mechanisms exerted by the cocktail of proteins released from PRGF.

At present, the most common options in ACL replacement are allografts or autografts. This experimental work presents a great clinical relevance due to the utilization of PRGF-Endoret® technology. This technology may facilitate ACL healing by improving tendon graft ligamentization since cell migration to the regeneration area would be enhanced by local PRGF-Endoret® administration in ACL surgery.

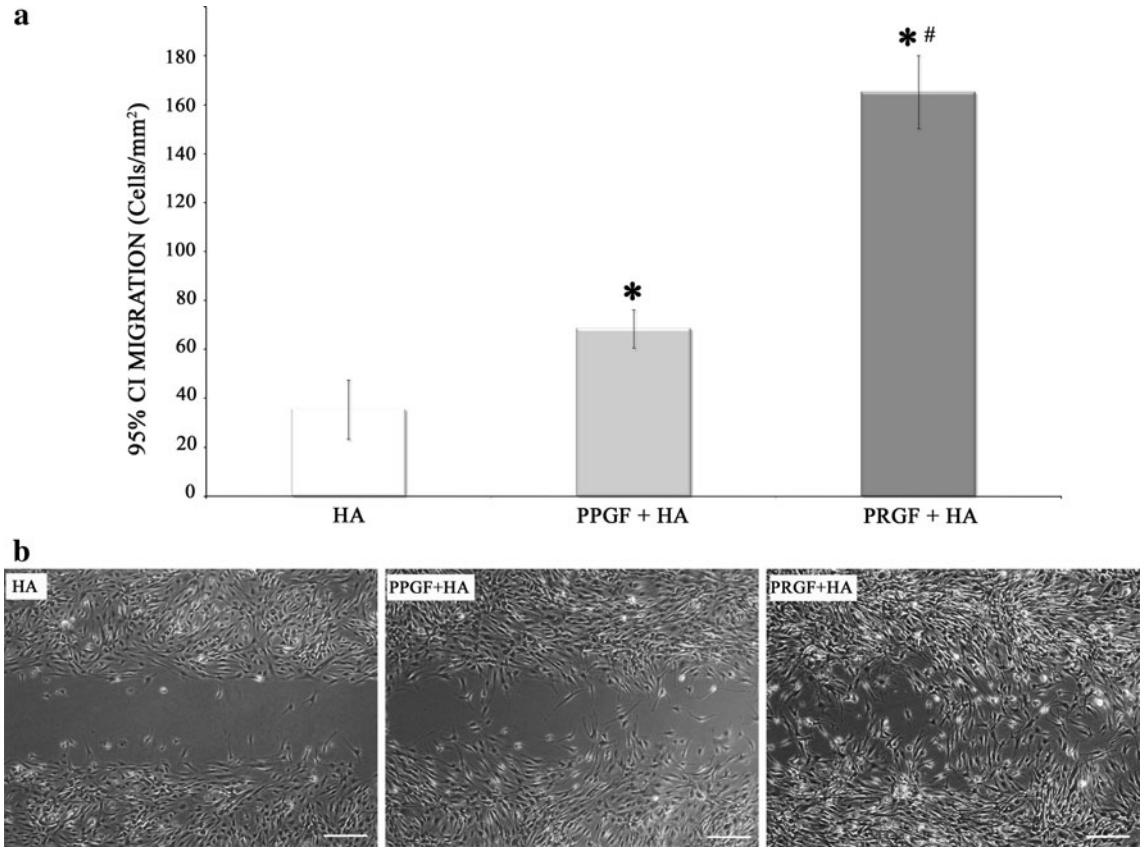

Fig. 6 Intensification of 25% HA effect over synovial cells migration mixed with 25% PPGF or PRGF. **a** Graph illustrating means ± SD of three replicates of synovial fibroblasts response. *Significant respecting to HA; #Significant with respect to PPGF + HA (95% confidence interval in both cases). **b** Phase contrast microphotographs of synovial cells migration. *Scale bar* 300 μm. *HA* hyaluronic acid, *PPGF* plasma poor in growth factors, *PRGF* plasma rich in growth factors

Conclusion

The data showed herein provide evidence of the potential of PRGF-Endoret® for stimulating tendon and synovial fibroblast migration, which can be considered key events in the regeneration process of these tissues. Moreover, we provide insights of the potential of a new biomaterial composed of a mix of autologous growth factors and hyaluronic acid. The new composed biomaterial will help to maintain the PRGF at the injury site while augmenting the migration of cells.

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Conflict of interest The authors declare that they have no conflict of interest.

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