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Morphogen and proinflammatory cytokine release kinetics from PRGF-Endoret fibrin scaffolds: Evaluation of the effect of leukocyte inclusion

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Abstract: The potential influence of leukocyte incorporation in the kinetic release of growth factors from platelet-rich plasma (PRP) may explain the conflicting efficiency of leukocyte platelet-rich plasma (L-PRP) scaffolds in tissue regeneration. To assess this hypothesis, leukocyte-free (PRGF-Endoret) and L-PRP fibrin scaffolds were prepared, and both morphogen and proinflammatory cytokine release kinetics were analyzed. Clots were incubated with culture medium to monitor protein release over 8 days. Furthermore, the different fibrin scaffolds were morphologically characterized. Results show that leukocyte-free fibrin matrices were homogenous while leukocyte-containing ones were heterogeneous, loose and cellular. Leukocyte incorporation produced a significant increase in the contents of proinflammatory cytokines interleukin (IL)-1β and IL-16 but not in the platelet-derived growth factors release (<1.5-fold). Surprisingly, the availability of vascular endothelial growth factor suffered an important decrease after 3 days of incubation in the case of L-PRP matrices. While the release of proinflammatory cytokines was almost absent or very low from PRGF-Endoret, the inclusion of leukocytes induced a major increase in these cytokines, which was characterized by the presence of a latent period. The PRGF-Endoret matrices were stable during the 8 days of incubation. The inclusion of leukocytes alters the growth factors release profile and also increased the dose of proinflammatory cytokines. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2014.

Key Words: platelet-rich plasma, leukocytes, growth factors, inflammation, fibrin scaffold

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INTRODUCTION

Different strategies have been developed for the stimulation of tissue regeneration and function recovery of injured tissue. These strategies vary from the use of biomaterials to the application of tissue engineering technology. Recently, a biological approach to provide pleiotropic morphogens and a biodegradable three-dimensional (3D) fibrin scaffold to the injured tissue has been proposed.[1,2] This approach consists of the use of blood-derived biomaterials to create different therapeutic formulations that adapt to the needs of various biomedical fields.

At present, many types of musculoskeletal injuries are treated with this autologous technique, such as tendinopathies,[3,4] ligament and tendon ruptures,[5,6] joint diseases,[7–9] and muscle injuries,[10,11] among others. A recent review has also highlighted the therapeutic applications of plasma rich in growth factors (PRGF-Endoret), an autologous and specific pure platelet-rich plasma (P-PRP), in the regeneration of hard and soft tissues in oral and maxillofacial surgery, the treatment of chronic ulcers, and in the development of tissue-engineered approaches.[12,13]

PRGF-Endoret technology is characterized by a moderate platelet concentration, absence of leukocytes (P-PRP), and the use of calcium chloride for platelet activation.[1,14] The absence of leukocytes in this formulation is supported by the fact that they synthesize matrix metalloproteinases (MMPs), oxygen and nitrogen reactive species (free radicals), and catabolic cytokines, which may not be an optimal milieu for the regeneration of damaged tissue.[15–18] Additionally, in several clinical studies leukocyte-enriched PRPs (L-PRPs) have not improved the clinical outcomes in comparison to placebo or no treatment.[19–22]

Interestingly, plasma rich in growth factors once activated can be viewed as a local protein and morphogens delivery system as it creates a fibrin scaffold enriched in biologically active molecules[2] that are then progressively released. However, the kinetic release process of protein release from this fibrin scaffold is fairly unknown as it is the potential impact of having leukocytes and proinflammatory cytokines in this fibrin matrix.

To address this, the release of platelet derived growth factors [platelet-derived growth factor AB (PDGF-AB), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), and EGF], plasmatic growth factors

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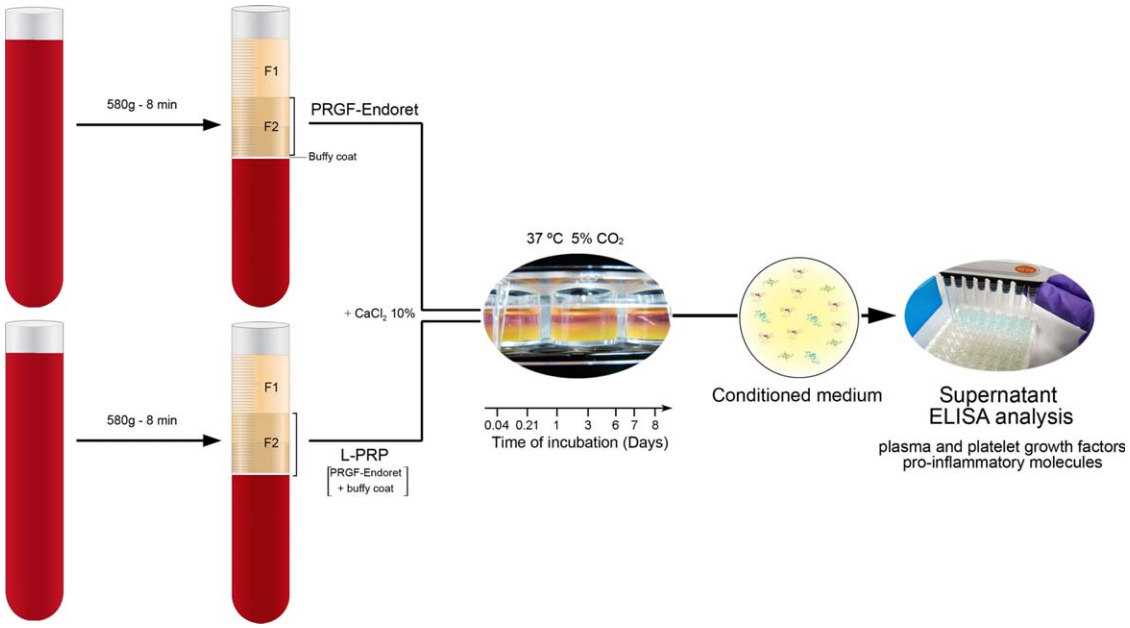

FIGURE 1. Graphical representation of sample preparation process. After blood centrifugation F1 and F2 PRGF-Endoret can be obtained over the buffy coat. To prepare L-PRP white cells must be collected with F2 fraction. Contrarily, the buffy coat is avoided in the obtaining of F2 PRGF-Endoret. The experimental design is also represented. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

[hepatocyte growth factor (HGF), insulin-like growth factor I (IGF-I)], and proinflammatory cytokines [interleukin (IL) 1β, IL-16] over 8 days has been studied. Furthermore, structural analysis of the fibrin hydrogel has been performed to highlight differences in the architecture of leukocyte-free and leukocyte-enriched platelet hydrogels.

MATERIAL AND METHODS

Preparation of platelet-rich plasma formulations

Human venous whole blood from three healthy volunteers was withdrawn into 22 vacuum tubes of 9 mL containing 3.8% (w/v) sodium citrate as anticoagulant. None of the donors had taken medication in the last week that could alter the hematological parameters. This study was conducted following the ethical principles for medical research contained in the Declaration of Helsinki amended in 2008.

All samples were processed immediately after collection. For each donor, half of the tubes were processed according to the PRGF-Endoret protocol and the other half were used to prepare L-PRP. In brief, according to the PRGF-Endoret protocol,[14] 11 blood tubes from each donor were centrifuged for 8 min at 580*g* (centrifuge PRGF-Endoret system IV; BTI Biotechnology Institute, Vitoria, Spain). PRGF-Endoret was composed of two fractions: fraction 1 (F1) had a similar platelet concentration to the peripheral blood while fraction 2 (F2) had twofold to threefold higher platelet concentration than the peripheral blood. For this study, the 2 mL of plasma just above the buffy coat (F2) was collected to prepare the leukocyte-free PRP (PRGF-Endoret hydrogel). For obtaining L-PRP, the same protocol was followed with the other half of tubes, but in addition to the 2 mL of plasma, the buffy coat was also collected (Fig. 1).

Characterization of PRP formulations

The number of platelets and leukocytes was determined in peripheral blood, PRGF-Endoret and L-PRP, using a standard hematological analyzer (ABX MICROS 60; Horiba Medical, Montpelier, France).

In order to characterize the 3D-structure of the gels by conventional microscopy and scanning electron microscopy (SEM) techniques, one aliquot of each type of PRP was activated with 10% $CaCl_2$ (PRGF-Endoret activator; BTI Biotechnology Institute, Vitoria, Spain).

For optical microscopy analysis samples were fixed in formaldehyde 4% at room temperature, dehydrated in a graded series of alcohols and embedded in paraffin. Next, 5-μm-thick sections were cut, stained with hematoxylin and eosin, and observed under microscopy (DMLB; Leica Microsystems, Wetzlar, Germany) equipped with a digital camera (DFC300FX; Leica Microsystems). For SEM evaluation, samples were fixed with 2% glutaraldehyde in 0.1 *M* cacodylate buffer. Then, samples were postfixed with osmium tetroxide (1% OsO_4 in 0.1 *M* cacodylate) and finally dehydrated through ascending alcohol concentrations. Next, the hydrogels were subjected to critical point drying (Autosamdri 814; Tousimis, Rockville) and sputter coated with 5 nm of gold (E306A; Edwards, Crawley, UK) before examination in a electron microscope (S-4800; Hitachi, Japan). The 3D structure of both types of matrix was qualitatively evaluated by two independent observers in terms of the presence of cells and structure of the fibrin network.

Characterization of the release of morphogens and proinflammatory cytokines

The characterization of the bioactive release from hydrogels was performed according to Anitua et al.[1] Briefly, 55 μL of

TABLE I. Platelet, Leukocyte, and Erythrocyte Measurements in PRGF-Endoret and L-PRP From Three Donors

	Donor 1		Donor 2		Donor 3	
Cell Type	PRGF-Endoret	L-PRP	PRGF-Endoret	L-PRP	PRGF-Endoret	L-PRP
Platelets ($\times 10^3/\mu L$)	434 (3.0×)	449 (3.1×)	562 (2.6×)	650 (3.0×)	279 (2.0×)	349 (2.5×)
Leukocytes ($\times 10^3/\mu L$)	0.3 (0.05×)	5.7 (0.97×)	0.2 (0.05×)	2.2 (0.52×)	0.1 (0.02×)	1.1 (0.2×)
Lymphocytes ($\times 10^2/\mu L$)	2.90	44.75	1.78	17.36	n.d.	9.75
Monocytes ($\times 10^2/\mu L$)	0.07	5.76	0.21	3.50	n.d.	0.89
Granulocytes ($\times 10^2/\mu L$)	0.03	6.50	0.01	1.14	n.d.	0.36
Erythrocytes ($\times 10^6/\mu L$)	0.04	0.06	0.02	0.02	0.00	0.01

The change relative to the values of peripheral blood is indicated in brackets (n.d., not determined).

10% calcium chloride solution was added to 1.1 mL of liquid PRP into a 12-well culture plates to form the hydrogels. After clot formation, 1.6 mL of osteoblast cell medium without growth supplements (ObM; ScienCell Research Laboratories, Carlsbad, CA) was added. Samples were then maintained in a cell incubator at 37°C and 100% humidity. The incubation medium was collected after 1, 5, and 24 h and 3, 6, 7, and 8 days of incubation. The experiments were performed in triplicates for each hydrogel and for each time point. After each period of incubation, the incubation medium was centrifuged at 400 g during 10 min at room temperature. The supernatant obtained was distributed in aliquots and stored at −80°C until use.

Quantification of PDGF-AB, TGF-β1, VEGF, HGF, IGF-I, EGF, IL-1β, and IL-16 was performed using available ELISA kits and according to the manufacturer's protocol (Invitrogen Corporation, Camarillo, CA, for IL-1β and R&D Systems, Minneapolis, MN, for the rest of molecules).

Study of remaining adherent cells

For samples incubated during 8 days, the plate surface was observed through phase-contrast microscopy (DM IRB, Leica Microsystems) after removing both the fibrin matrix and supernatant. In case that cells are detected, nuclear staining with Hoechst 33342 (Molecular Probes-Invitrogen, Grand Island, NY) was performed, and microphotographs taken with a digital camera (DFC300 FX; Leica Microsystems).

Statistical analysis

Shapiro–Wilk test was applied to verify whether the data followed a normal distribution. Then paired Student's t test was selected to analyze the statistical significance of the differences between PRGF-Endoret and L-PRP hydrogels at each time point and for each measured protein. Nonparametric Wilcoxon test was selected if the data did not follow a normal distribution. The statistical significance was set at $p < 0.05$. All the statistical analyses were performed using the SPSS v15.0 for Windows statistical software package (SPSS, Chicago, IL).

RESULTS

Characterization of plasma rich in growth factor preparations

The platelet concentration obtained in PRGF-Endoret hydrogel is shown in Table I. Platelet concentrations were 434, 562, and 279 $\times$ 10^3 platelets/μL for donors 1, 2, and 3; while the enrichment with respect to the peripheral blood was 3.0-, 2.6-, and 2.0-fold, respectively. Table I showed that platelet concentrations in the L-PRP hydrogel for donors 1, 2 and 3 were 449, 650 and 349 $\times$ 10^3 platelets/μL, accounting for an enrichment factor of 3.1, 3.0, and 2.5, respectively.

In relation to the white blood cells, PRGF-Endoret fibrin hydrogels of the three donors were almost leukocyte free, with a content less than 0.3 $\times$ 10^3 leukocytes/μL (Table I). In contrast, L-PRP hydrogels from donors 1, 2, and 3 had values of 5.7, 2.2, and 1.1 $\times$ 10^3 leukocytes/μL, respectively. Thus, the leukocyte-containing preparation (L-PRP) protocol resulted in a leukocyte-enrichment of PRGF-Endoret by a factor of 19, 11, and 11, respectively, when compared with leukocyte-free PRGF-Endoret.

A morphological characterization of the 3D structure of fibrin scaffolds of both PRGF-Endoret and L-PRP was performed. The photonic microscopy revealed the virtual absence of leukocytes and erythrocytes in the fibrin scaffold prepared from PRGF-Endoret [Fig. 2(A)]. SEM analysis showed the presence of a consistent and homogeneous network of fibrin [Fig. 2(B)] with the absence of cellular elements that might disrupt the mesh. At higher magnification, only platelet aggregates were observed scattered throughout the fibrin [Fig. 2(C)]. In contrast, the presence of leukocytes and erythrocytes was evident in the fibrin prepared from L-PRP [Fig. 2(D)]. In the SEM study the disturbance of the fibrin network was evident. The presence of erythrocytes and scattered leukocytes [Fig. 2(E)] resulted in an irregular three-dimensional network [Fig. 2(F)].

Characterization of the release of morphogens and proinflammatory cytokines

Growth factor release from PRGF-Endoret fibrin scaffolds was monitored during an observational period of 8 days.

Release of platelet-derived growth factors. The released dose of PDGF-AB from PRGF-Endoret fibrin scaffold varied between donors being the highest for donor 2 followed by donor 1 (Fig. 3). PRGF-Endoret fibrin scaffold from donor 1 released about 5201, 5377, and 6948 pg/mL of PDGF-AB after 1, 5, and 24 h of incubation, respectively. This amount was 5729, 7093, and 8916 pg/mL for donor 2 and 2354, 2818, and 3596 pg/mL for donor 3. A steady state release

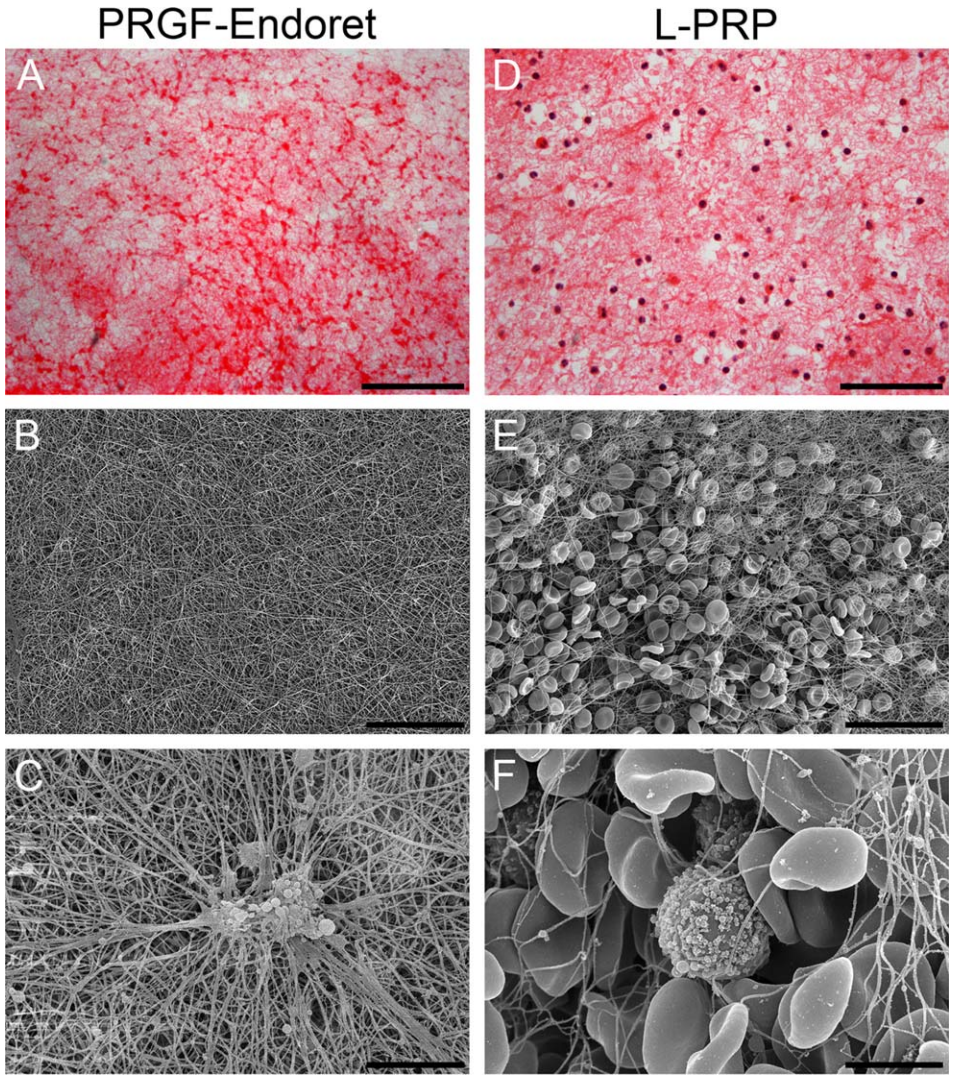

FIGURE 2. Microscopic characterization of the fibrin scaffold obtained from PRGF-Endoret (A–C) and from L-PRP (D–F) scaffolds. Samples were observed by staining with H&E (A and D) and by SEM at low (B and E) and high (C and F) magnifications. Scale bars: (A) and (D), 50 μm; (B) and (E), 25 μm; and (C) and (F), 5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was observed after 3 days, reaching an average value of 7845 ± 69.58, 9611.5 ± 245.06, and 4146.5 ± 195.64 pg/mL, for donors 1, 2, and 3 (Fig. 3).

PDGF-AB release from L-PRP fibrin scaffolds showed a similar pattern that those obtained from PRGF-Endoret (Fig. 3). The dose of PDGF-AB released was the highest for donor 2 and the lowest for donor 3. In addition, a steady state release was reached after 3 days of incubation at an average value of 7148 ± 510.2, $11{,}272 \pm 266.4$, and 5547 ± 332.1 pg/mL was obtained for donors 1, 2, and 3, respectively (Fig. 3).

Interestingly, the release of VEGF was markedly different between the two types of fibrin scaffolds (Fig. 3). PRGF-Endoret fibrin scaffolds showed a VEGF release profile similar to that of PDGF-AB. It was characterized by a burst release during the first 24 h and a steady state release after 3 days of incubation (Fig. 3). VEGF released from fibrin scaffold in donor 1 was the highest, reaching 279.61, 319.45, and 422.65 pg/mL during the first three time points of the observation period. A plateau region was reached at average values of 547.89 ± 31.04, 160.77 ± 5.92, and 61.70 ± 2.95 pg/mL for donors 1, 2, and 3, respectively.

The incorporation of leukocytes in the scaffold provoked a modification on the VEGF release (Fig. 3). Thus, the release of VEGF was increasing during the first 24 h (for donor 3) and the first 3 days (for donors 1 and 2), after which a progressive decrease in the amount of VEGF in the incubation medium was observed. VEGF was almost absent from the incubation medium after 7 days of incubation (Fig. 3).

Figure 4 shows the results of TGF-β1 and EGF release from PRGF-Endoret and L-PRP fibrin scaffolds. PRGF-Endoret fibrin scaffolds released TGF-β1 at an increasing dose during the first 6 days of incubation before reaching a steady state release. After 24 h, TGF-β1 release reached to 12,650, 13,267, and 6980 pg/mL of TGF-β1 for donors 1, 2,

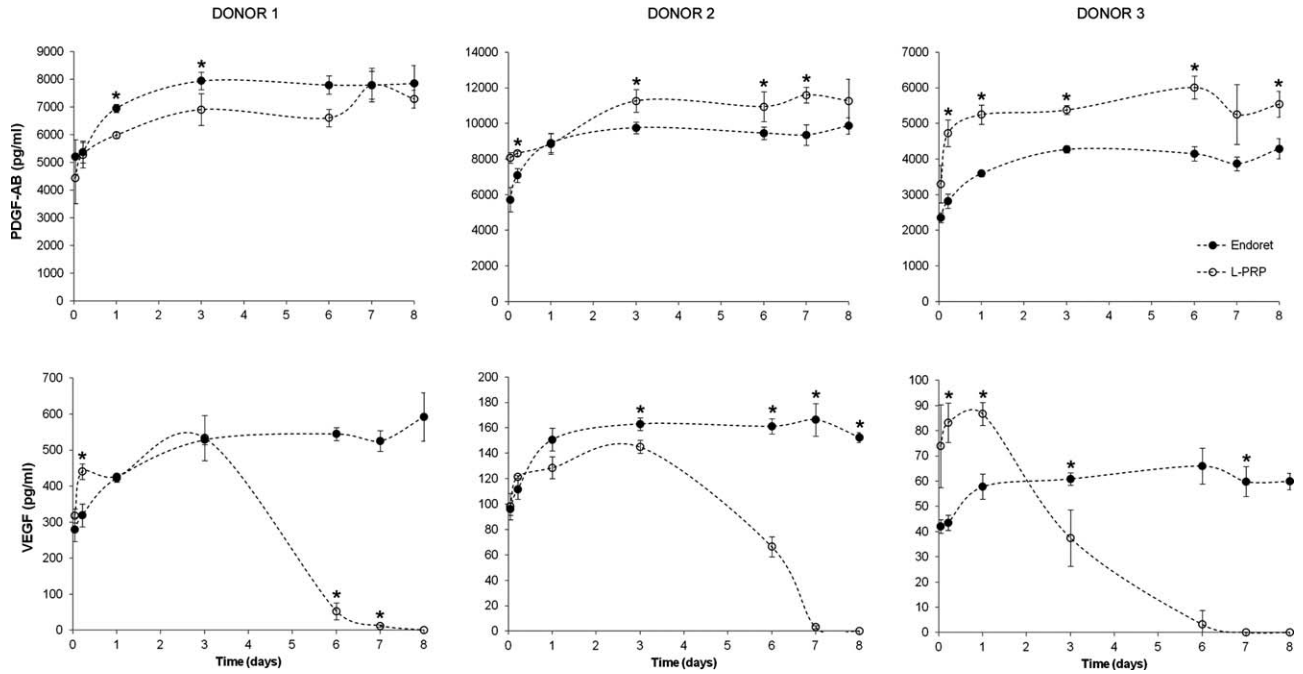

FIGURE 3. PDGF-AB and VEGF release from fibrin scaffolds obtained from PRGF-Endoret and from L-PRP during an observation period of 8 days. *: $p \leq 0.05$.

and 3, respectively. This amount increased up to 19,200, 19,333, and 9361 pg/mL for donors 1, 2, and 3, respectively, after 6 days of incubation.

Leukocyte enrichment of the fibrin scaffold increased the amount of TGF-β1 released to the incubation medium (Fig. 4). This increase was continuous during the observation period for donors 2 and 3 while a steady state release was reached for donor 1. This increase was statistically significant for donor 1 (except at 1 h) and donor 3, but was not statistically significant for donor 2.

In the case of EGF release, PRGF-Endoret fibrin scaffolds from the three donors showed a similar release profile (Fig. 4). Leukocyte inclusion did not significantly alter EGF release from fibrin scaffolds in donors 1 and 2 (Fig. 4). The increase in EGF released was statistically significant at 5 h and days 1, 3, and 6 for donor 1. In the case of donor 2, these differences were only statistically significant at 5 h and day 1, meanwhile the increase was significant at 5 h, and days 1, 3, 6, and 8 for donor 3.

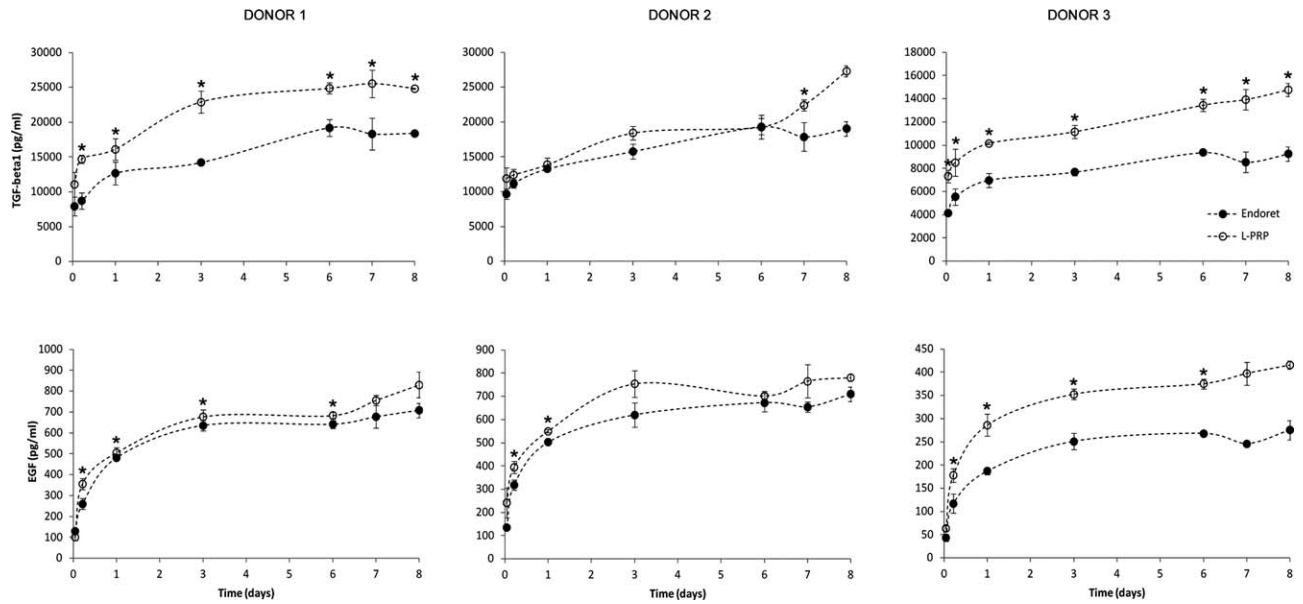

FIGURE 4. TGF-β1 and EGF release from PRGF-Endoret and L-PRP fibrin scaffolds during an observation period of 8 days. *: $p \leq 0.05$.

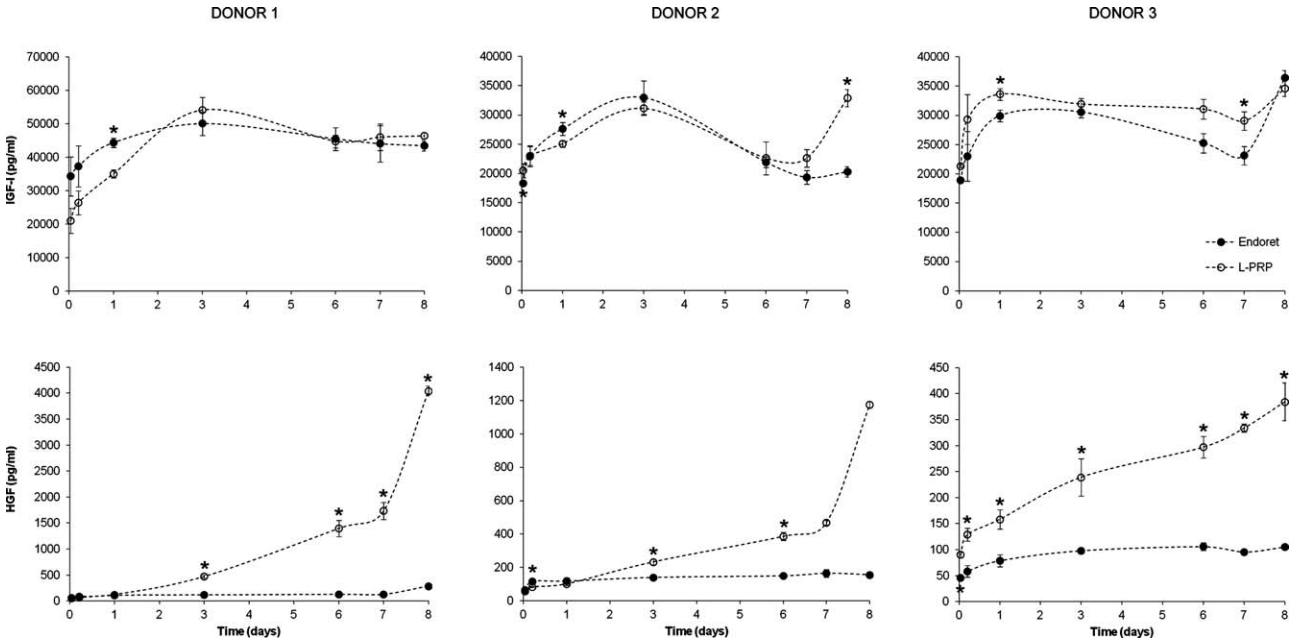

FIGURE 5. IGF-I and HGF release from PRGF-Endoret and L-PRP fibrin scaffolds during an observation period of 8 days. *: $p \leq 0.05$.

Release of plasma-derived growth factors. Release of plasmatic growth factors (IGF-I and HGF) from both types of fibrin scaffolds was analyzed. Figure 5 shows that the highest IGF-I released was obtained in PRGF-Endoret fibrin scaffolds of donor 1 while similar amount was delivered in the case of patients 2 and 3. The peak of IGF-I release was observed at day 3, being of 50,080, 32,990, and 30,570 pg/mL for donors 1, 2, and 3, respectively. A steady state release was then obtained with values ranging from 20,517 to 44,333 pg/mL (Fig. 5). Results show that the dose of IGF-I released was significantly higher in PRGF-Endoret fibrin scaffolds at day 1 for donors 1 and 2. Meanwhile, these differences were significantly higher in L-PRP fibrin scaffolds at 1 h and day 8 for donor 2 and at days 1 and 7 for donor 3. Finally, inclusion of leukocytes in the fibrin matrix significantly increased the dose of HGF released in all the donors (Fig. 5).

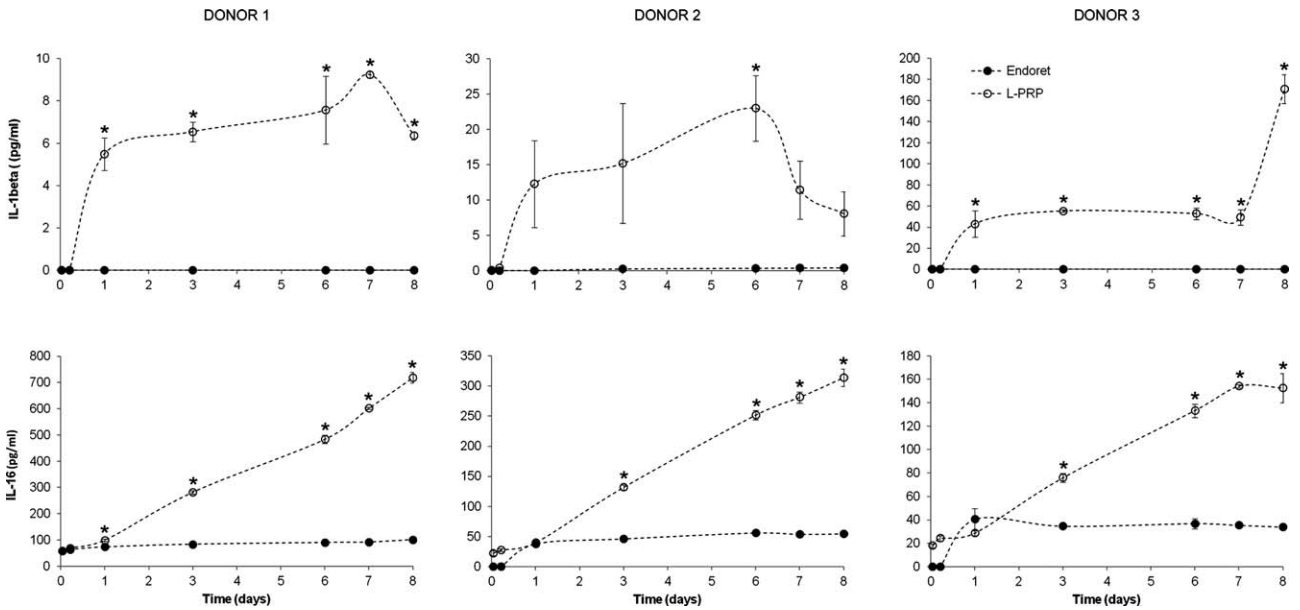

FIGURE 6. Proinflammatory cytokine (IL-1β and IL-16) release from autologous fibrin scaffolds (PRGF-Endoret and L-PRP) during an observation period of 8 days. *: $p \leq 0.05$.

Release of proinflammatory cytokines. The release of proinflammatory cytokines from the fibrin scaffolds was characterized by following the release of IL-1β and IL-16. Figure 6 shows that the IL-1β was absent from the incubation medium of PRGF-Endoret hydrogels throughout the observation period for the three donors. Meanwhile, leukocyte inclusion into the fibrin scaffolds significantly increased the release of IL-1β. Interestingly, the IL-1β profile indicated the presence of latent period. The latter occurred during the first 5 h of incubation (Fig. 6). Then, the released dose of IL-1β was 5.49, 6.55, 7.57, 9.24, and 5.78 pg/mL after 1, 3, 6, 7, and 8 days of incubation for donor 1, respectively. These amounts were about 12.27, 15.19, 23.01, 19.92, and 8.06 pg/mL for donor 2 and 43.08, 55.40, 52.92, 49.37, and 170.94 pg/mL for donor 3.

Similarly, inclusion of leukocytes significantly enhanced the dose of IL-16. However, the release profile of IL-16 was different among donors of L-PRP. The rate of IL-16 released was about 87, 41, and 21 pg/mL per day for donors 1, 2, and 3, respectively. The released dose after 8 days of incubation was about 719, 314, and 152 pg/mL, respectively.

Study of remaining adherent cells

None of the PRGF-Endoret wells had adherent cells, however all L-PRP samples contained cells adhered to the cell culture well. These cells had a morphology consistent with macrophage-derived multinucleated giant cells as shown in Hoechst 33342 staining [Fig. 7(A)] and in more detail [Fig. 7(B)].

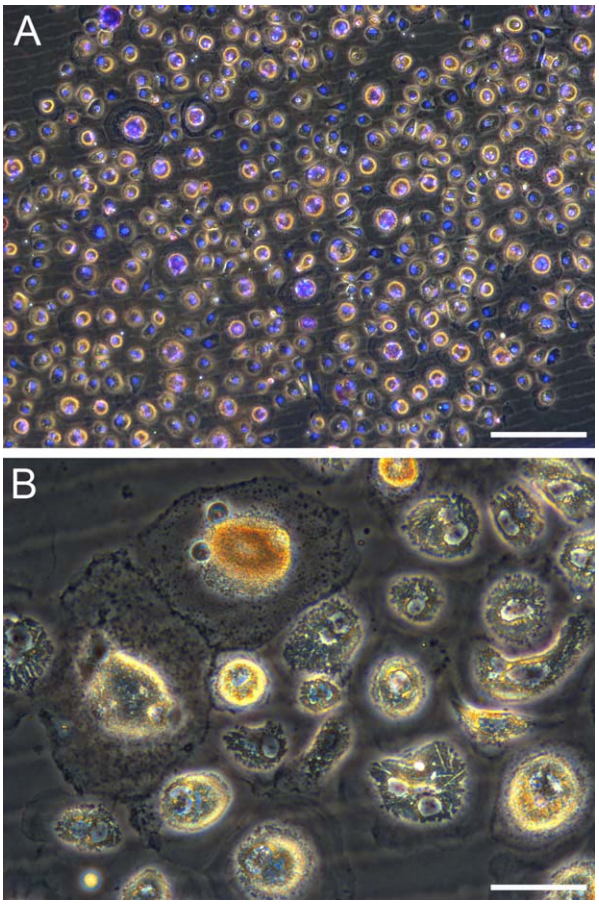

FIGURE 7. Images of cells adhered to the cell-culture well after removing a fibrin (L-PRP) after 8 days of incubation. A: Photomicrograph at low magnification (phase contrast combined with Hoechst 33342 nuclear staining). Note the presence of multinucleate cells. B: High-magnification image showing a detail of the cells found in the culture well. Scale bars: (A) 200 μm; (B) 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

The cellular components of PRP products are a differentiating factor that may influence the clinical effectiveness of their administration for the treatment of a disease and/or the regeneration of damaged tissue. Several studies support the use of P-PRP in a large number of pathologies of the musculoskeletal system.[15,23–25] Other clinical studies have reported the occurrence of adverse effects after the administration L-PRP.[24,26] These adverse effects are related to the capability of leukocytes to trigger immunological response and increase the concentration of proinflammatory cytokines.[24,26] Besides, this cellular composition can affect the structure of fibrin network produced by the polymerization of fibrinogen into a cross-linked fiber mesh.[12]

In this study, the structural characterization of fibrin mesh has clearly shown a more heterogeneous network due to the incorporation of leukocytes and particularly erythrocytes into the mesh, and it has also shown lesser fiber density throughout the network. These morphological modifications may reduce the clot strength and affect the viscoelastic properties of fibrin scaffold.[27,28] Gersh et al.[28] have shown that erythrocytes incorporation into fibrin clot at 5–10% (vol/vol) caused heterogeneity in the fiber network while higher concentration of erythrocytes resulted in loose arrangement of fibers around the cells. Erythrocytes were also capable to modify the viscoelastic properties of the fibrin clot by increase the ratio of viscous modulus (G'') to elastic modulus (G').[28]

Recently, it has been suggested that fibrin scaffold of PRGF-Endoret was dissolved after 5 days of incubation in a culture medium, and that signs of damage were observable at day 3.[29] However, the results of the present study conclude that the stability of PRGF-Endoret fibrin scaffolds was maintained for more than 8 days of incubation. Previously, it was reported that 3D PRGF-Endoret fibrin scaffolds cultured with tenocytes were stable for up to 6 days.[30] On the other hand, it has been shown that the addition of polymorphonuclear (PMN) leukocytes to PRP worsens its viscoelastic properties.[31]

The increase in growth factors release from L-PRP fibrin scaffolds could be related to the higher platelet enrichment in L-PRP and the leukocyte contribution in the synthesis/release of certain growth factors, such as TGF-β.[32] This increase could be related to the proliferation of leukocytes within the fibrin hydrogel, as it was observed the presence of macrophages adhered to the cell culture well.

Furthermore, fused macrophages (macrophage-derived multinucleated giant cells) were noted, probably due to proinflammatory environment present in the L-PRP.[33]

HGF release was also enhanced in L-PRP fibrin scaffolds. HGF reservoir on the leukocytes surface is created thanks to lower affinity/high-capacity binding site of cell surface-associated heparan sulfate.[34–36] This surface pool of HGF on the leukocytes is rapidly released by the stimulation of coagulation factor Xa[37] and thus may partially account to explain the continuous increase in the amount of HGF released to the incubation medium. Furthermore, Grenier et al.[34] have nicely showed that both secretory vesicles and gelatinase/specific granules of human blood PMN neutrophils contain a mobilizable stock of pro-HGF that is proteolytically processed to mature HGF by neutrophil serine protease during degranulation. This degranulation process is initiated by stimulating agents of PMNs like the IL-1β.[34] We have found that IL-1β was mainly present in the incubation medium of L-PRP hydrogels and thus this cytokine would stimulate the PMNs to release the HGF from the intracellular granules. Interestingly, VEGF levels resulted to be significantly higher at day 8 in the PRGF-Endoret fibrin scaffold, a finding that could be explained by a possible VEGF uptake by its soluble receptor (sVEGFR) synthesized by leukocytes.[38,39] Another plausible hypothesis is that leukocytes release proteases that could either decrease the concentration of growth factors.

One of the most relevant results of this study is the almost absence of proinflammatory cytokines in the PRGF-Endoret fibrin scaffolds, and release of these molecules when leukocytes were included. Of particular concern is the presence of IL-1β in L-PRP hydrogel, since this cytokine triggers a strong inflammatory response because IL-1β can recruit more proinflammatory cells to the site of injury.[40] This cytokine also stimulates catabolic protein production, including MMPs, which breaks the extracellular matrix and inhibits both proteoglycan and collagen synthesis.[41] The negative effect of IL-1β has been investigated in various tissues. *In vitro* studies in tendon cells have shown that the combination of stretching and IL-1β presence produces extracellular matrix degradation.[42] Another study indicates that IL-1β induces the synthesis of catabolic mediators by tenocytes.[43] Cartilage is a particularly sensitive tissue to the presence of catabolic mediators such as IL-1β. This molecule is a major proinflammatory cytokine involved in the pathogenesis of osteoarthritis.[44]

Similarly, the release of IL-16 was significantly increased in the L-PRP fibrin scaffolds and was virtually absent from PRGF-Endoret hydrogels. This cytokine is synthesized and secreted by T and B lymphocytes, monocytes, dendritic cells, eosinophils and mast cells, being chemotactic for all of them.[45–47] It has been suggested that IL-16 can be a key cytokine in both the initiation of inflammation and in its maintenance.[48] High levels of IL-16 have been observed in rheumatoid arthritis,[49,50] which could suggest the cytokine role in joint destruction.

In recent work in equine tendon explants, McCarrel et al.[15] have elegantly demonstrated that a high concentration of platelets does not counteract the increased amounts of proinflammatory molecules synthesized by leukocytes. It was observed that the optimal regenerative environment with the lowest catabolic gene expression was the leukocyte-reduced PRP. On the other hand, previous *in vitro* studies have shown both the antimicrobial properties of PRP,[51,52] and that the inclusion of leukocytes to PRGF-Endoret does not provide an additional bacteriostatic effect.[53] Few clinical trials have been carried out comparing leukocyte-free PRP versus L-PRP. Filardo et al.[24] compared the efficacy and safety of intra-articular injections of PRGF-Endoret against an homemade L-PRP in the treatment of osteoarthritis. Both treatments improved the course of the disease, but patients treated with PRGF-Endoret had fewer side effects than those treated with L-PRP, who presented more pain and swelling events.[54]

There are some limitations in this *in vitro* study. For example, due to biological variability, the enrichment of leukocytes was not homogeneous in the three donors. Furthermore, commercial L-PRP systems have a leukocyte concentration greater than the one obtained in this study,[55] and thus greater differences would be expected when comparing PRGF-Endoret with commercial L-PRPs. It would also be necessary to analyze the biological behavior of various cell lines with these two kinds of formulations, in terms of proliferation, migration, and angiogenesis. Besides these methodological remarks, we should envision the PRP therapy as a biological system that content myriad of balanced bioactive molecules which act in concert with different cell phenotypes rather than reducing or parsing them to a single cause-effect mechanistic approach.

This study has been designed to quantify the release of aforementioned molecules during 8 days. This timing has been chosen in order to get an approximation to an *in vivo* system, because, unlike platelets, leukocytes may continue synthesizing and degranulating different molecules within a fibrin scaffold until this has been degraded.

In summary, the incorporation of leukocytes into PRGF-Endoret fibrin scaffold have resulted in a modification of the architecture of fibrin network and resulted in more heterogeneous and loose mesh. The released amount of growth factors from PRGF-Endoret did not show significant variations in the release profile over time. Overall, leukocyte inclusion into PRP did not enhance the release of platelet derived growth factors, but increased the release of proinflammatory cytokines. Further *in vivo* studies are necessary to study the biological significance of these different protein environments in tissue engineering and regenerative medicine.

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