



Infiltration of plasma rich in growth factors enhances *in vivo* angiogenesis and improves reperfusion and tissue remodeling after severe hind limb ischemia

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ABSTRACT

PRGF is a platelet concentrate within a plasma suspension that forms an *in situ*-generated fibrin-matrix delivery system, releasing multiple growth factors and other bioactive molecules that play key roles in tissue regeneration. This study was aimed at exploring the angiogenic and myogenic effects of PRGF on *in vitro* endothelial cells (HUVEC) and skeletal myoblasts (hSkMb) as well as on *in vivo* mouse subcutaneously implanted matrigel and on limb muscles after a severe ischemia. Human PRGF was prepared and characterized. Both proliferative and anti-apoptotic responses to PRGF were assessed *in vitro* in HUVEC and hSkMb. *In vivo* murine matrigel plug assay was conducted to determine the angiogenic capacity of PRGF, whereas *in vivo* ischemic hind limb model was carried out to demonstrate PRGF-driven vascular and myogenic regeneration. Primary HUVEC and hSkMb incubated with PRGF showed a dose dependent proliferative and anti-apoptotic effect and the PRGF matrigel plugs triggered an early and significant sustained angiogenesis compared with the control group. Moreover, mice treated with PRGF intramuscular infiltrations displayed a substantial reperfusion enhancement at day 28 associated with a fibrotic tissue reduction. These findings suggest that PRGF-induced angiogenesis is functionally effective at expanding the perfusion capacity of the new vasculature and attenuating the endogenous tissue fibrosis after a severe-induced skeletal muscle ischemia.

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1. Introduction

A remarkable clinical motivation for building microvessels arises from the need to reestablish or improve blood flow and tissue function in compromised or damaged tissues. The latter occurs in many pathological

Abbreviations: BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic proteins; CXCR4, chemokine receptor type-4; ECs, endothelial cells; EGF, epidermal growth factor; FGF, fibroblast growth factor; GF, growth factors; HGF, hepatocyte growth factor; hSkMbs, human skeletal myoblasts; HUVECs, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; PBS, phosphate buffer saline; PDGF, platelet-derived growth factor; PF4, platelet factor 4; PRGF, plasma rich in growth factors; SC, satellite cells; SDF-1, stromal cell-derived factor; TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor

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conditions including chronic wound healing, arterial obstructive syndromes and coronary artery and peripheral arterial diseases among others [1–3]. As an approach to the challenge of optimizing tissue protection and regeneration through promotion of angiogenesis and minimization of the formation of fibrotic scar, several different strategies have been developed by using a sustained delivery of growth factors (reviewed in [4]). The application of endogenous intramuscular infiltrations of plasma rich in growth factors (PRGF) [5] is an innovative biological approach grounded in the regenerative potential of platelets, fibronectin, plasma biomolecules, and fibrin scaffold [6–8]. There is an increasing body of evidence that points towards growth factors as instrumental in angiogenesis and muscle regeneration (reviewed in [9]). Once PRGF is activated, it polymerizes *in situ* into a three-dimensional fibrin scaffold that releases progressively a pool of proteins, growth factors, cytokines and even exosomes, acting as a local growth factor delivery system [7], whose release kinetics has been recently described [10]. This transient fibrin scaffold presents

important features: (i) heparan sulfate binding domains, that may sequester and then release heparin-binding growth factors including PDGF, FGF, HGF, and VEGF and (ii) cell adhesion motifs that may contribute to cell survival [11–14].

PRGF muscle infiltrations are aimed at recruiting, activating and mobilizing mainly myogenic progenitor cells and resident macrophages which contribute to muscle reparation processes by cell signaling soluble factors, in addition to the already activated endothelial cells, macrophages, and platelets in the injured area [15].

The aim of the current study was to explore and assess the angiogenic and myogenic effect of PRGF on *in vitro* experiments with endothelial cells (ECs) and skeletal myoblasts as well as on *in vivo* mouse subcutaneously implanted matrigel and on limb muscles after a severe ischemia.

2. Materials and methods

2.1. PRGF preparation and characterization

For the preparation of PRGF, 27–36 mL of peripheral venous blood was withdrawn from 3 healthy donors into 9 mL tubes containing sodium citrate (3.8%). 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. PRGF was obtained using the Endoret protocol (BTI-Biotechnology Institute, Vitoria, Spain). Briefly, blood was centrifuged at 580 g for 8 min at

room temperature (PRGF System IV, BTI-Biotechnology Institute, Vitoria, Spain). The upper plasma volume (F1) was drawn off and deposited in a collection tube, whereas the 2 mL plasma fraction (F2) located just above the buffy coat was collected in other tube (Fig. 1A). All of the assays were performed with F2. Measurements of hematologic parameters of both the peripheral blood and the PRGF were performed (ABX Pentra 60 analyzer, Horiba ABX Dagnostics, Montpellier, France). Platelet enrichment factor was calculated and compared to the average platelet concentration in peripheral blood.

For *in vitro* cell culture experiments PRGF releasate (supernatant) was prepared by adding PRGF activator (10% wt/vol calcium chloride; BTI-Biotechnology Institute, Vitoria, Spain) to samples at a final concentration of 22.8 mM and posterior incubation at 37 °C for 1 h. Clots were allowed to retract and the released supernatant was collected, filtered through a 0.22 μm filter membrane, and then frozen at –80 °C until used.

On the other hand, for *in vivo* matrigel and ischemic hind limb assays, just activated PRGF liquid was applied for *in situ* scaffold formation. In this way, PRGF activator was added to the liquid PRGF aliquots (50 μL of CaCl₂ /mL of PRGF) just before application (Fig. 1A).

2.2. *In vitro* cell proliferation and apoptosis inhibition assays

Early passages (passages 2–3) of primary human umbilical vein endothelial cells (HUVECs) and human skeletal myoblasts (hSkMbs) were isolated from human umbilical cords and muscle biopsies respectively

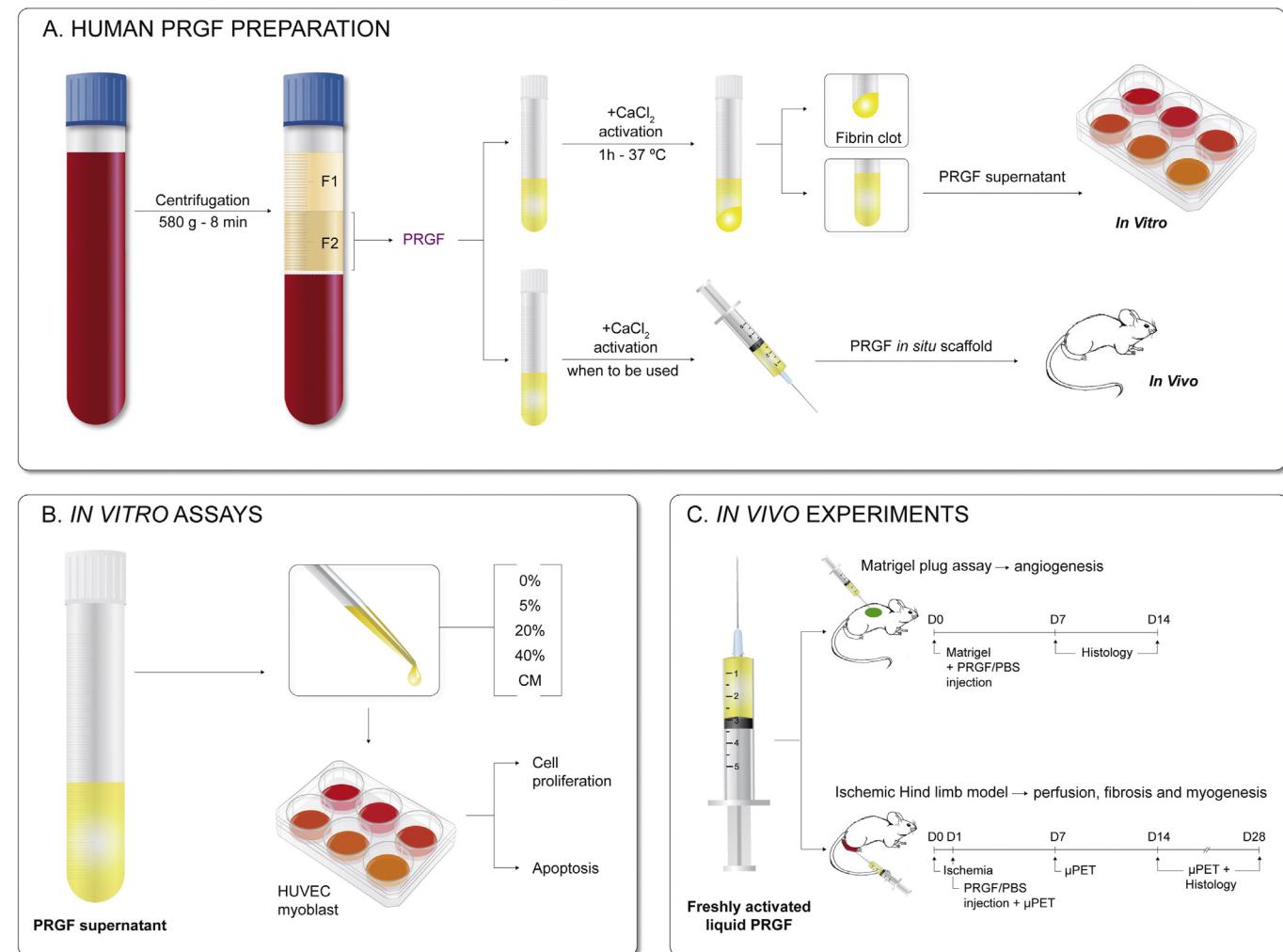


Fig. 1. Experimental design. PRGF preparation process, *in vitro* and *in vivo* experiments are shown. The different evaluations as well as the experimental times are indicated.

and expanded in complete media (CM) (for HUVEC: Ham's F12 basal media (Gibco), 10% fetal bovine serum (FBS), 0.1 mg/mL heparin, 300 µg/mL Endothelial Cell Growth Supplement (ECGS) (BD Biosciences) and 1% penicillin/streptomycin (P/S) (Gibco); for hSkMb: DMEM high glucose basal media (Gibco), 20% FBS, 10 ng/mL basic-FGF (Gibco) and 1% P/S, as previously described [16,17]. For proliferation assays (Fig. 1B), cells were plated in 96-well plates at a density of 2×10^3 cells/well in CM and 24 h later, media were replaced by F12 basal media and 6% FBS plus heparin (for HUVEC) or DMEM basal media and 10% FBS (for hSkMb) in the presence or not of PRGF at several doses (5–40%). Cells grown in CM were also included as positive cell growth control. After 48 h (HUVEC) or 72 h (hSkMb), the number of viable cells was quantified by a luminescent cell viability assay (CellTiter-Glo™, Promega, USA) following the manufacturer's protocol. Three independent experiments were performed and every cell type was seeded in quadruplicate for each condition.

For apoptosis assays (Fig. 1B), endothelial and skeletal cells were seeded in 96-well plates at a density of 5×10^3 and 10×10^3 cells/well respectively, and cultured in CM. After 24 h, media were replaced by basal media (non serum or cytokines added) in the presence or not of PRGF doses (5–40%) and cells cultured under hypoxic conditions (1% O₂) during 48 h (HUVEC) and 72 h (hSkMb). A group of cells kept under normal cell culture conditions (20% O₂ and CM) were included as positive control. The degree of cell apoptosis was measured by an ELISA kit that quantifies the presence of oligonucleosomal fragments (Roche Applied Science, Barcelona, Spain). The apoptosis control corresponds to cells cultured under hypoxia and non-serum with 5% PRGF as when no PRGF was added all the cells died. Under this condition apoptosis was clearly detected and considered 100%. The rest of the samples was referred to that value, in order to determine the degree of "protection" conferred by the PRGF at the different doses. Three independent experiments were performed and every cell type was seeded in quadruplicate for each condition.

2.3. In vivo animal studies

2.3.1. Matrigel plug assay

For the *in vivo* matrigel plug assay (Fig. 1C), 10-week-old immune-competent BALB/c mice were injected subcutaneously in the back with 0.5 mL cold growth factor-reduced matrigel (BD Biosciences) containing 150 µL of PRGF. Matrigel with 150 µL of PBS was injected in the control group. Ten-fourteen mice were included in each treatment group. Seven and 14 days after injection, animals were perfusion-fixed and matrigel plugs were removed and processed for paraffin or OCT embedding. Tissue sections were examined and photographed under a fluorescence microscope (Zeiss) or a confocal microscope.

2.3.2. Hind limb ischemia models

Severe hind limb ischemia (Fig. 1C) was induced under anesthesia (100 mg/kg ketamine–10 mg/kg xylazine) in 12–16 weeks-old male immune-competent BALB/c mice by ligation/transection of the left iliac artery as previously described [18]. Twenty-six animals were randomly distributed in the 3 treatment-groups. Two doses of PRGF (6 µL for PRGF^{low} and 18 µL for PRGF^{high}), or 18 µL of PBS as control, were injected in 4 equal fractions in the adductor and quadriceps region of the left limb, 24 h after surgery.

2.3.3. Live imaging and assessment of limb perfusion

Limb perfusion assessment was performed by microPET as described by our group [18]. Briefly, mice were anesthetized with 2% isoflurane in 100% O₂ gas for ¹³N-ammonia injection (75 MBq) in a tail vein and were kept under such condition during the entire study. MicroPET imaging was performed 10 min after ¹³N-ammonia injection 15 and 30 days after surgery. For quantitative analysis and comparisons among subjects, evaluation of perfusion in both hind limbs was carried out as follows: regions of interest (ROIs) were drawn on coronal 1-mm thick

microPET images over the hind limbs, and activity concentration per area unit calculated as a measurement of perfusion. The ratio between left (ischemic) and right (non-ischemic) hind limbs was used in all cases for comparisons and obtained data were exported to the PMOD software package (PMOD Technologies Ltd., Zürich, Switzerland) for quantification. Fourteen and 28 days after injection, animals were perfusion-fixed and limbs and muscle weight and caliber measured.

All experiments were performed in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, and published by the National Academy Press, revised 1996. All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals.

2.3.4. Histological and histomorphometric analyses

Both matrigels and muscles were fixed in 10% neutral buffered formalin for 24 h, paraffin-embedded and sectioned at 5 µm thickness. For histological evaluation, the following antibodies were used: rabbit polyclonal anti-CD31 (phospho-Y713) antibody (ab62169, Abcam), mouse monoclonal anti-α-Smooth Muscle Actin (α-SMA) clone 1A4 (Sigma-Aldrich) and rabbit monoclonal anti-desmin (Y266, Abcam).

For matrigel vascularization analysis, a double immunohistochemical staining for CD31 (fast red)/α-SMA (DAB) was performed to identify vessels. The number of vessels was quantified in three highly vascular hotspots identified at 10× magnification. Subsequently, a counting of vessels was performed at 20×/40× magnifications. For each sample, up to 0.92 mm² were evaluated. Two independent measures, the number of capillaries (CD31⁺/α-SMA⁻) and the number of arterioles and arteries (CD31⁺/α-SMA⁺), were obtained.

Myocytes were identified by staining with desmin. The analysis of the muscle fibrosis was performed on sections stained with picrosirius red (Polysciences, Warrington, PA, USA) and data were expressed as a percentage of the fibrotic area (red) vs. the total tissue area. For histological evaluation, images at low and high power magnifications were obtained with a digital camera Leica DFC 300 FX (Leica Microsystems, Wetzlar, Germany) coupled to a light microscope Leica DMLB. For each sample, 0.92 mm² were evaluated.

2.4. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Differences between experimental groups and time-points were determined by ANOVA general linear model approach. Statistical significance between groups was accepted for p-values lower than 0.05. Statistical analyses were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. PRGF characterization

The hematological results were consistent with the PRGF standardized protocol. A description of relevant parameters (platelets and leukocytes) in peripheral blood and PRGF, as well as the platelet enrichment efficacy is indicated in Supplementary Table 1.

3.2. PRGF prevents apoptosis and induces muscle and endothelial cell proliferation *in vitro*

Primary human umbilical vein ECs (HUVECs) and skeletal myoblasts (hSkMbs) were incubated with different doses of PRGF and proliferated in a dose-dependent manner in response to PRGF (Fig. 2A). Endothelial cells cultured with 20% and 40% of PRGF showed higher proliferation ratio than the ones cultured with complete medium (CM). Similarly,

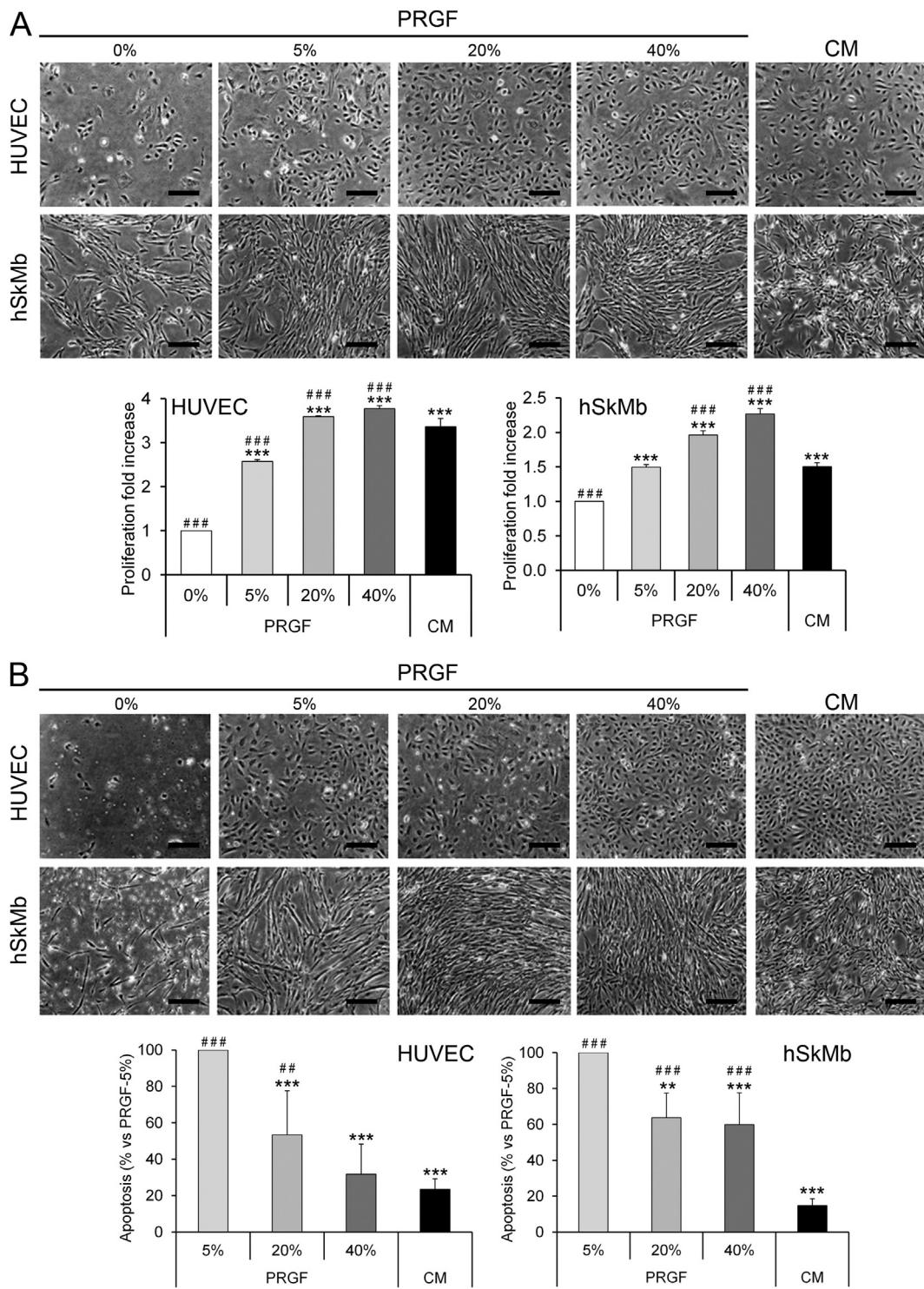


Fig. 2. PRGF *in vitro* mechanisms: cell proliferation and survival: (A) representative images of HUVEC and hskMb cultures grown with different percentages of PRGF and quantitative analysis of cell proliferation of HUVEC and hskMb. Cell proliferation is shown as fold increase (F.I.) in comparison with proliferation in non-treated group (0% PRGF). *** Indicates $p < 0.001$ when compared with 0% PRGF and ### indicates $p < 0.001$ when compared with the complete media (CM). (B) Representative images of HUVEC and hskMb cultures grown with different percentages of PRGF under pro-apoptotic conditions. Quantitative analysis of apoptosis of HUVEC and hskMb. ** Indicates $p < 0.01$ and *** indicates $p < 0.001$ when compared to 5% PRGF; ## indicates $p < 0.01$ and ### indicates $p < 0.001$ when compared to CM. Data from 3 independent experiments in quadruplicate. Scale bars for all micrographs: 200 μ m.

the skeletal myoblast proliferative response when incubated with 20% and 40% was significantly higher compared with the proliferation shown by myoblasts cultured with CM (Fig. 2A).

To explore the anti-apoptotic effect of PRGF on HUVEC and hSkMb cultures, we ascertained the degree of apoptosis present at cultures

with 5% PRGF as the apoptotic reference value of 100%, due to the fact that in absence of PRGF all the cells died. HUVEC cultures incubated with 20% and 40% PRGF showed a statistically significant reduction of apoptosis with respect to 5% PRGF, with the response being dose-dependent (Fig. 2B). A similar protective response was detected in

hSkMb culture in the presence of PRGF, with a significant reduction of apoptosis in both cases, with 20% and 40% PRGF (Fig. 2B).

3.3. PRGF induces vascular formation in an *in vivo* matrigel assay

A matrigel plug was mixed with PRGF or PBS as control and thereafter, subcutaneously injected. After 7 days, a significant higher number of new vessels was observed in the PRGF matrigel plugs compared with the PBS-matrigel plug formulation (Fig. 3A). The infiltration of new capillaries showed an early and strong angiogenic response in the PRGF matrigel plug significantly greater than in the control group. In this way, at days 7 and 14 after the implantation, the number of capillaries ($CD31^+/\alpha SMA^-$) in the PRGF-matrigel plugs was significantly higher than in the control group. Also, a significant increase was detected at days 7 and 14 at the level of arterioles and arteries ($CD31^+/\alpha SMA^+$) (Fig. 3B). No statistically significant differences were found in the number of vessels between days 7 and 14, neither in the control group, nor in the PRGF-treated group.

3.4. PRGF induces an improvement in perfusion and muscle recovery after severe ischemic hind limb ischemia

To determine the *in vivo* efficacy of PRGF treatment in a more clinically relevant model, BALB/c mice underwent a complete ligation of the iliac artery. Twenty-four hours after hind limb ischemia, mice

underwent either PRGF muscle infiltration or control treatment with PBS. Evaluation of limb perfusion was performed by microPET using ^{13}N -ammonia (Fig. 4A). No differences in perfusion recovery were observed 7 and 14 days after treatment in both groups. However, at 28 days after arterial ligation, the blood perfusion ratio in the PRGF^{low} treated mice limb reached $54.3 \pm 2.4\%$ whereas in the untreated mice it was $31.6 \pm 0.7\%$, with statistically significant differences ($p < 0.001$). No statistically significant differences were found with the PRGF^{high} dose (Fig. 4B).

In addition, at the macroscopic level (Fig. 4C), the loss of adductor muscle weight (Fig. 4D) at day 28 was lower in the PRGF treated groups than in control group, although the statistical significance only was reached in the PRGF^{high} group ($p < 0.01$). Similarly, the caliber of the muscles at day 28 was higher in the PRGF groups than in the control, but without achieving statistical significance (Fig. 4E).

3.5. PRGF attenuates fibrosis after hindlimb ischemia favoring myocyte recovery

The fibrotic outcome in limb muscles after severe ischemia was evaluated by picrosirius staining (Fig. 5A). At day 14, a lower presence of fibrosis in the PRGF-treated groups with respect to the PBS-group was detected. These differences became statistically significant at day 28 ($p < 0.05$ for PRGF^{low} group and $p < 0.01$ for PRGF^{high} group), partially

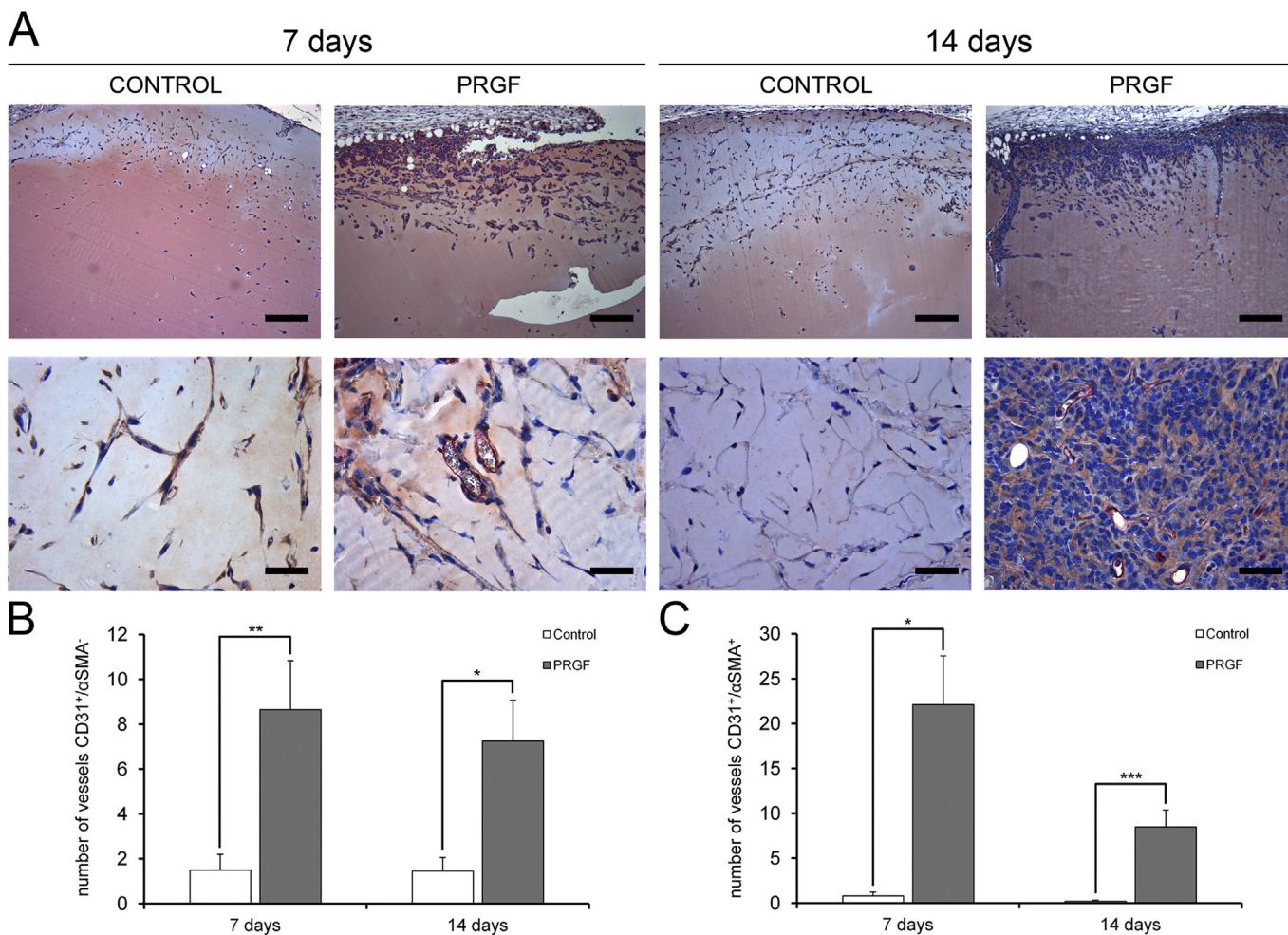


Fig. 3. *In vivo* matrigel angiogenesis assay: (A) representative microscopic photographs of representative matrigel plugs stained with CD31 (fast red) and α -SMA (DAB) for identification of vessels. The top row shows microphotographs at low magnification ($10\times$) and the bottom row at high magnification ($40\times$). Quantitative analysis of the number of vessels found in the different experimental groups: (B) capillaries ($CD31^+/\alpha SMA^-$) and (C) arterioles and arteries ($CD31^+/\alpha SMA^+$). * Indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$; $n = 4-5$ mice/treatment/time-point were included for *in vivo* matrigel angiogenesis assay. Scale bars: 200 μm for top row images and 50 μm for bottom row ones.

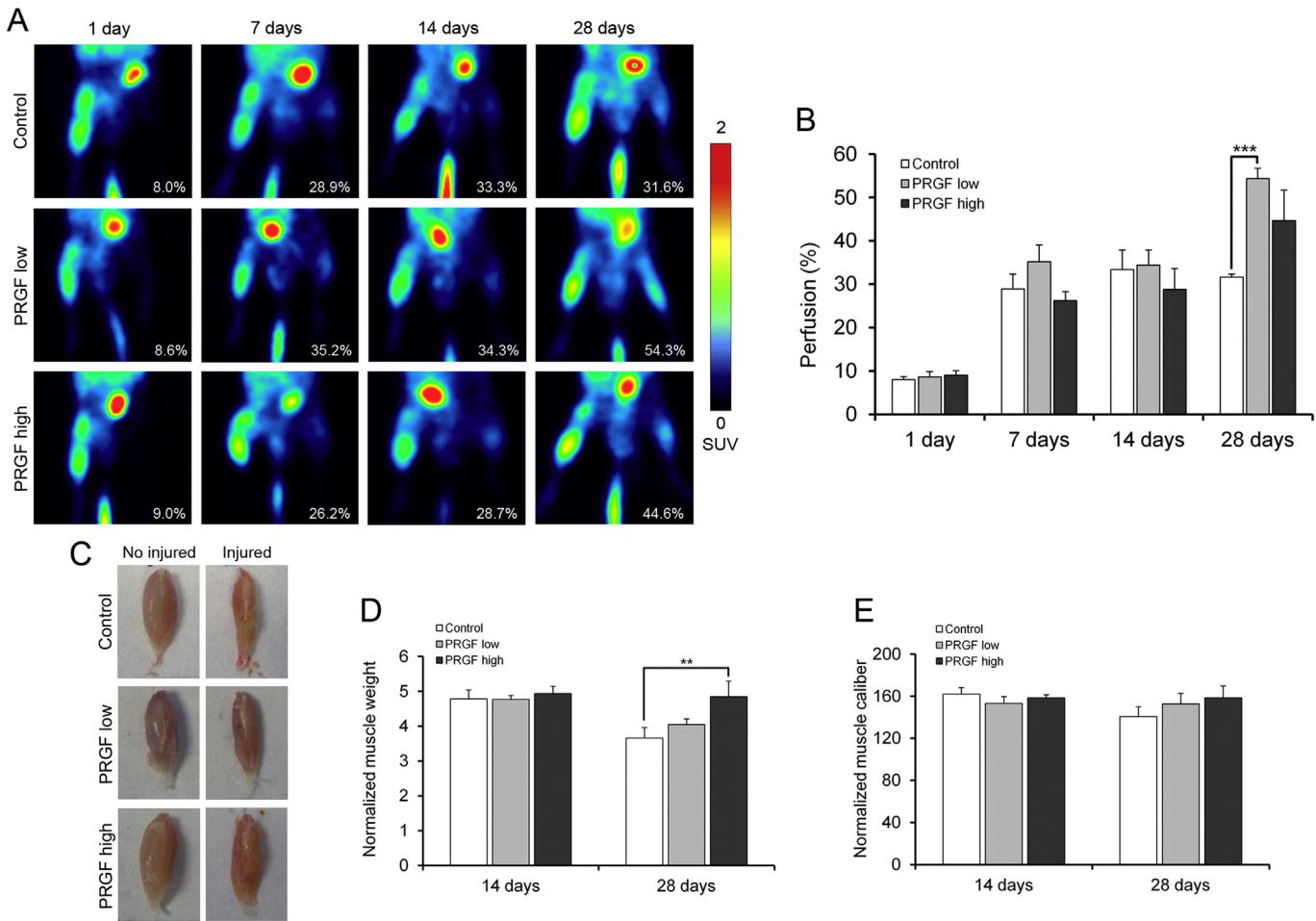


Fig. 4. Muscle reperfusion analysis: (A) representative images of the evolution of perfusion in three experimental groups, control, PRGF^{low} and PRGF^{high}. The color scale (standardized uptake value, SUV) illustrates the perfusion variations from minimal (dark blue) to maximal (red) values. (B) Perfusion quantitative analysis of the PRGF-treated (PRGF^{low} and PRGF^{high}) and control groups. (C) Representative macroscopic images of muscles at day 28. Normalized muscle weight (muscle weight / mouse weight) (D) and normalized muscle caliber (muscle caliber / mouse weight ($\text{mm/g} \times 10^3$)). (E) Quantitative analysis at 14 and 28 days. ** Indicates $p < 0.01$, and *** indicates $p < 0.001$; ($n = 8\text{--}9$ mice / treatment group at day 14 and $n = 3\text{--}5$ mice/treatment group at day 28, were included for PET analysis; $n = 4\text{--}5$ mice/treatment/time-point were included for muscle measurements). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

due to an increment of fibrosis in the control group whereas the PRGF-groups did not substantially changed (Fig. 5B).

Furthermore, after fourteen days of artery ligation, the myocytes present in the ischemic tissue were identified by desmin antigen (Fig. 6A). At 14 days, the presence of myocytes was more notable in the PRGF-groups than in the control group, although the difference was only statistically significant ($p < 0.05$) between PRGF^{high} and control groups. At 28 days, the number of myocytes became similar between groups, without statistically significant differences but always greater number in the PRGF-groups (Fig. 6A).

4. Discussion

To date, the biological benefits [19,20] and the therapeutic applications of PRGF have yielded promising clinical and surgical outcomes in musculoskeletal system pathologies [21] as well as in other medical fields [22]. In addition to many bioactive mediators present in the platelet α -granules (TGF- β , PDGF, VEGF, FGF, EGF, IGF-1, HGF, BMPs, BDNF) and dense granules (histamine, serotonin, calcium and ATP/ADP), there are other plasma constituents, namely, IGF-1, HGF, prothrombin, fibrinogen, fibronectin, vitronectin and other proteins which are active ingredients of PRGF. These molecules exert a central role in the cell signaling pathways involved in both tissue injury recognition and in repair of damaged tissue [8]. The strong and dose-dependent mitogenic response shown *in vitro* by the HUVEC and hSkMb when treated with

PRGF, might be attributed to VEGF and HGF respectively together with PDGF, bFGF and IGF-1 [23,24]. Studies conducted in our laboratory on human fibroblasts [19,20] and osteoblasts [25] showed the same proliferative dose-dependent response in cell cultured with PRGF supernatant. This response can typically be adjusted to a Gaussian distribution function [25], with slight variations depending on the cell type. Also, the anti-apoptotic effect of PRGF observed *in vitro* is consistent with some evidence reporting the protective effect of platelet-rich plasma on several cell types, including stem cells, in different hostile microenvironments [26,27], like stem cell transplantation [28]. Recently, it has been shown that PRGF also enhances cell proliferation and survival in primary neuronal cultures and reduces degenerating neurons in an *in vivo* model of Alzheimer's disease [29]. VEGF, IGF-1 and HGF are among the myriad growth factors within PRGF that appear to have a significant influence on cell survival [30]. Moreover, *in vivo* early angiogenic response has been demonstrated in our study after PRGF injection in matrigel plugs. *In vivo* angiogenesis is an exploratory multistep complex process crucial for tissue homeostasis and regeneration that encompasses the development of endothelial sprouts, their transformation into vessels, and maturation of the capillary network. These processes rely on the participation of multiple angiogenic growth factors including VEGF, PDGF, FGF, IGF-1 and SDF-1 in a carefully orchestrated spatiotemporal manner, which might account for the early and significant sustained angiogenesis observed in matrigel plugs infiltrated with platelet-rich plasma [31,32]. It has been widely evidenced that the

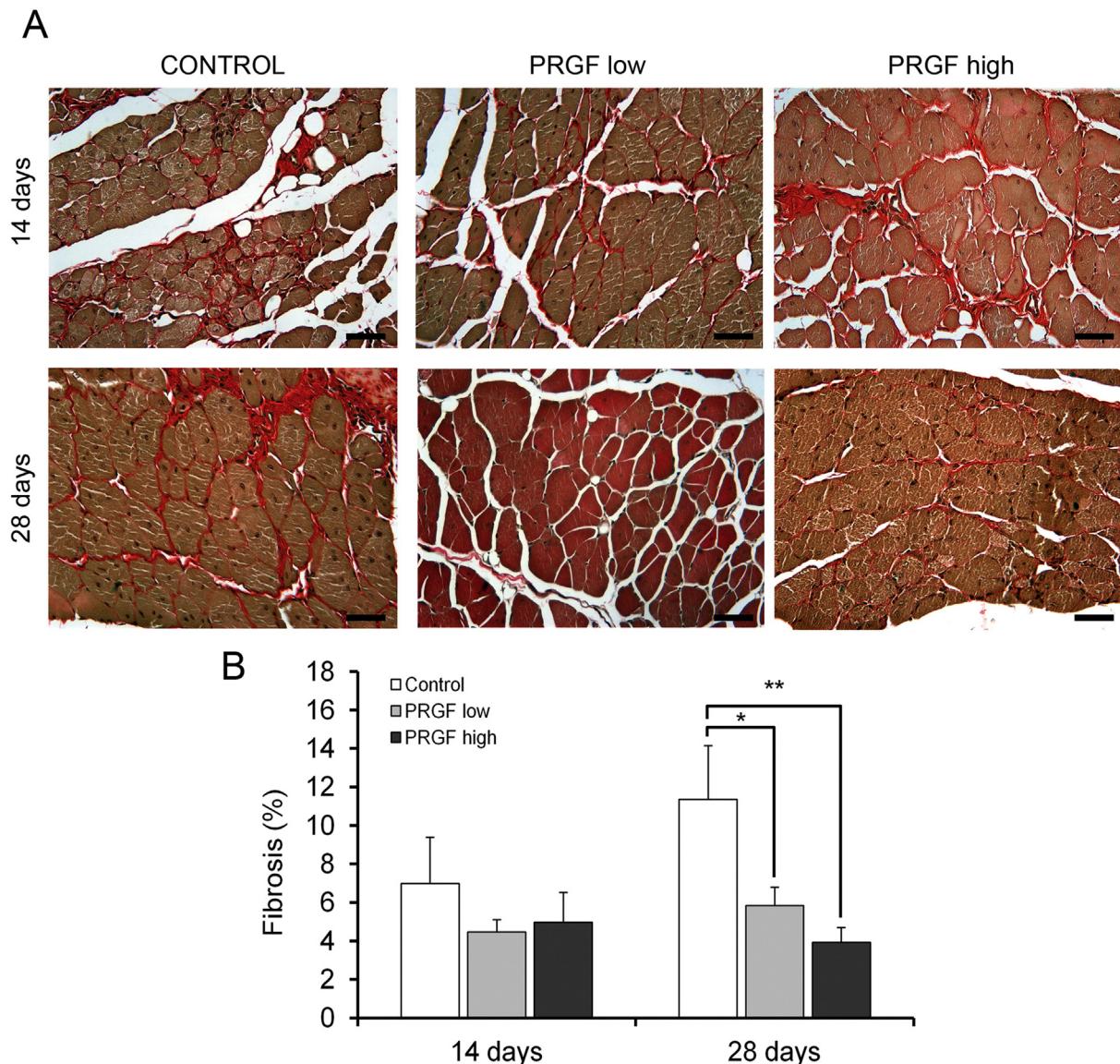


Fig. 5. Analysis of muscle fibrosis: (A) representative micrographs of muscle sections stained with sirius red for identification of fibrotic areas. (B) Quantitative analysis of the percentage of fibrotic area in each study group. * Indicates $p < 0.05$. ** Indicates $p < 0.01$; ($n = 5$ mice/treatment group at day 14 and $n = 4-5$ mice/treatment group at day 28). Scale bars: 50 μ m.

combination of several growth factors is key in the angiogenic induction and not the use of a single molecule [11,33–35]. VEGF, IGF-1, PF4 and SDF-1 are mitogenic and chemoattractant morphogens, present within the PRGF [6,36], leading to homing of endothelial or CXCR4-circulating cells and following maturation into functional endothelial cells [16,36, 37]. In addition, VEGF, PDGF, and FGF promote the proliferation and transformation of EC and surrounding wall cells as pericytes into endothelial sprouts, contributing to further stabilization and remodeling of vessels [31,38].

More importantly, the substantial reperfusion enhancement observed in PRGF^{low} treated ischemic-mice at day 28 could be interpreted as the formation of a functional mature vascular network that ensures the stabilization and remodeling of newly developed vessels [31,38]. Several lines of evidence support the effect of GF and platelet-rich plasma on either neovascularization or restoration of muscle function like the dual local application of FGF2/PDGF or VEGF/IGF-1 on induced hindlimb ischemia mice model [11,34], the tendon engraftment treated with PRGF in human LCA surgery [39], and the myocardial or limb ischemic rats treated with local injection of platelet-rich plasma [1,23] or VEGF [40]. PRGF muscle infiltrations provide the damaged tissue with many GF and cytokines and with a transient fibrin scaffold that, by

heparan sulfate binding domains, may sequester heparin-binding GF such as PDGF, FGF, HGF, BDNF, and VEGF and gradually release them. Also, by cell adhesion motifs might contribute to cell survival [10–12, 41].

In the full reconstruction of muscle tissue, angiogenesis and myogenesis proceed concomitantly, partially due to the anatomical vicinity of survivor endothelial and satellite cells which might, along with macrophages and other myogenic progenitor cells, reciprocally signal through the VEGF and IGF-1 expression [42]. There might be several accounts for the effect of PRGF muscle infiltrations on the muscle function recovery. IGF-1, HGF and VEGF gradually delivered at the dysfunctional damaged muscle may promote myocytes and endothelial survival and proliferation [11,42]. Additionally, IGF-1 and HGF, by inducing an anti-inflammatory phenotype in macrophages may modulate and mute the inflammatory response of ischemic tissue thereby contributing to cell survival of myofibers [11,30,43]. A proteomic characterization study of PRGF fibrin matrix [8] reported a significant representation of acute-phase proteins, a strong network of interconnected proteins linked to the NF- κ B pathway, and an important presence of thrombospondin-1 (TSP-1). Both elements might well cooperate in the early stages of tissue repair, in the resolution of inflammation and

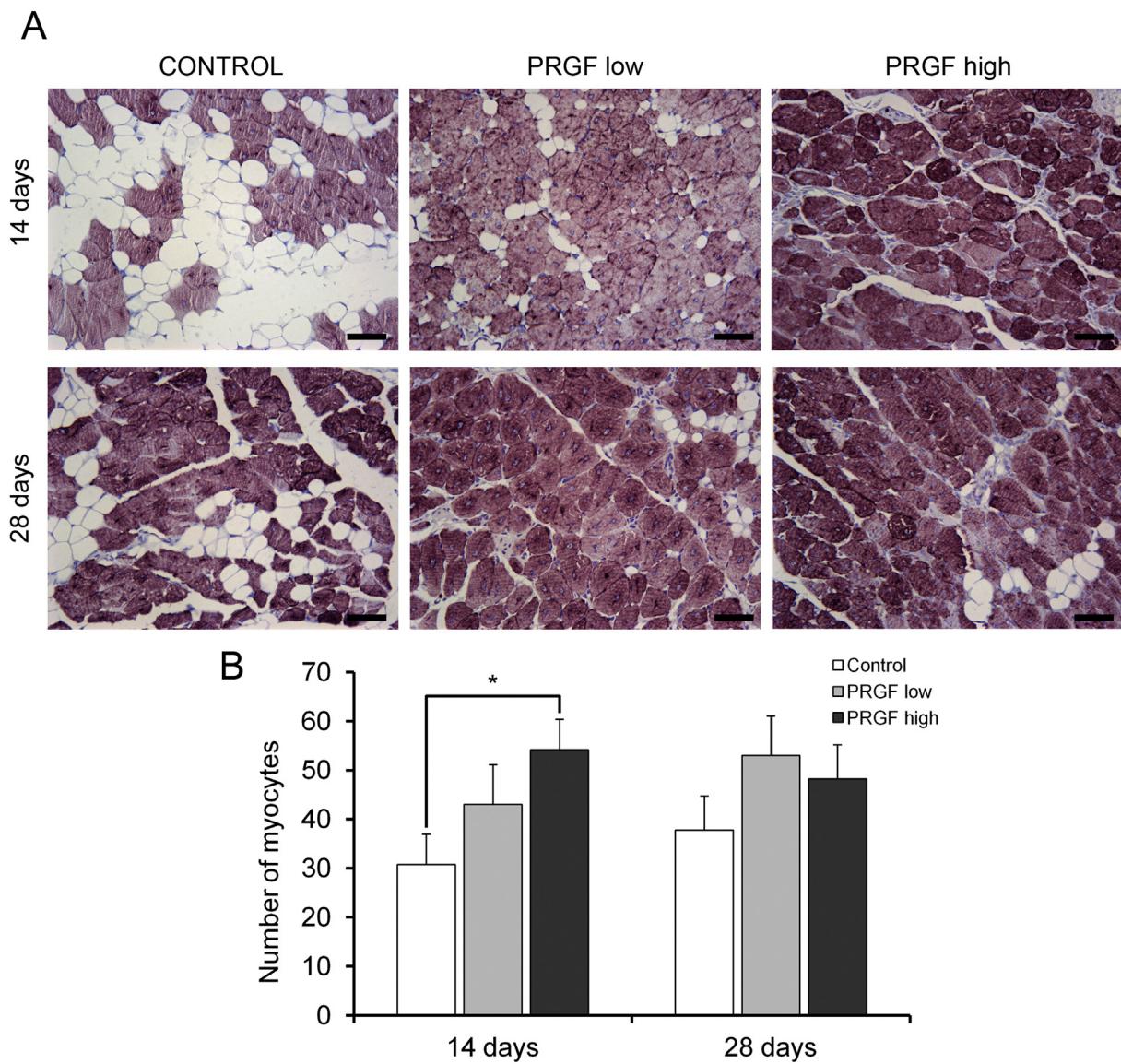


Fig. 6. Analysis of muscle regeneration: (A) representative microscopic photographs of muscle sections stained with anti-desmin antibody for identifying myocytes. (B) Quantitative analysis of the number of myocytes. * Indicates $p < 0.05$; ($n = 5$ mice/treatment group at day 14 and $n = 4-5$ mice/treatment group at day 28). Scale bars: 50 μ m.

in the increase of the half-life of a fibrin scaffold respectively. This protective and trophic effect of PRGF infiltrations is supported by macroscopically observed higher weight and caliber of ischemic muscles compared with endogenous regeneration.

Although a transient fibrogenesis is a crucial process for structural and functional muscle regeneration, one of the most unsuccessful and nonfunctional outcomes of muscle regeneration is the formation of fibrotic scarring [44]. The little fibrosis demonstrated by ischemic limbs treated with PRGF is consistent with previous results obtained with the application of PRGF in damaged tendons which exhibited an absence of scarring [39], or more recently in ocular surface cells exposed to TGF- β 1 [45]. IGF-1, VEGF, and HGF are among the GF within PRGF that have been suggested as playing an important role in preventing tissue fibrosis when applied in a sustained and combined manner [11]. Although the TGF- β family drives fibrogenesis, collagen synthesis and deposition, the concurrent presence of TGF- β 1, VEGF, IGF-1 and HGF within PRGF may render this therapeutic system in an anti-fibrotic product. The anti-fibrotic mechanism could be conducted by either inhibiting TGF- β 1 induced myofibroblast differentiation or by reverting the myofibroblast phenotype, as reported in studies conducted on keratocytes and conjunctival fibroblasts [46].

This study has several limitations, such as a relatively low number of mice per group in the *in vivo* experiments, and the lack of correlation between the function (perfusion) and histology for both PRGF doses. The limited number of animals per group could be one possible explanation for which it has been found a trend and not a statistical significance in the perfusion rate and other variables, such as muscle caliber or myocyte number at 28 days. Additional studies in order to determine the *in vivo* degradation of the PRGF gel in the skeletal muscle and its cytokine release pattern would be interesting for future studies.

Despite these drawbacks, our study provides encouraging results on how the PRGF can help to mitigate the effects of ischemic injury and improve reperfusion.

5. Conclusions

These findings suggest that PRGF-induced angiogenesis is functionally effective not only at providing early vessel growth and maintaining the long-term stability of these new vessels but also at expanding the perfusion capacity of the new microvasculature by generating a suitable cell microenvironment for myogenesis and angiogenesis.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2015.01.029>.

Conflict of interest

E Anitua is the scientific director and R Prado, S Padilla and JJ Aguirre are scientists at BTI-Biotechnology Institute, the company that has developed the PRGF-Endoret technology.

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