

# Tissue-engineered blood vessel substitute by reconstruction of endothelium using mesenchymal stem cells induced by platelet growth factors

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**Background:** Cardiovascular diseases remain leaders as the major causes of mortality in Western society. Restoration of the circulation through construction of bypass surgical treatment is regarded as the gold standard treatment of peripheral vascular diseases, and grafts are necessary for this purpose. The great saphenous vein is often not available and synthetic grafts have their limitations. Therefore, new techniques to produce alternative grafts have been developed and, in this sense, tissue engineering is a promising alternative to provide biocompatible grafts. This study objective was to reconstruct the endothelium layer of decellularized vein scaffolds, using mesenchymal stem cells (MSCs) and growth factors obtained from platelets.

**Methods:** Fifteen nonpregnant female adult rabbits were used for all experiments. Adipose tissue and vena cava were obtained and subjected to MSCs isolation and tissue decellularization, respectively. MSCs were subjected to differentiation using endothelial inductor growth factor (EIGF) obtained from human platelet lysates. Immunofluorescence, histological and immunohistochemical analyses were employed for the final characterization of the obtained blood vessel substitute.

**Results:** The scaffolds were successfully decellularized with sodium dodecyl sulfate. MSCs actively adhered at the scaffolds, and through stimulation with EIGF were differentiated into functional endothelial cells, secreting significantly higher quantities of von Willebrand factor (0.85  $\mu\text{g/mL}$ ;  $P < .05$ ) than cells cultivated under the same conditions, without EIGF (0.085  $\mu\text{g/mL}$ ). Cells with evident morphologic characteristics of endothelium were seen at the lumen of the scaffolds. These cells also stained positive for fascin protein, which is highly expressed by differentiated endothelial cells.

**Conclusions:** Taken together, the use of decellularized bioscaffold and subcutaneous MSCs seems to be a potential approach to obtain bioengineered blood vessels, in the presence of EIGF supplementation. (J Vasc Surg 2013;■:1-9.)

**Clinical Relevance:** The reported outcomes and techniques on this paper may be employed to constitute decellularized heterologous blood vessel banks. These specimens may be seeded with the patients' own mesenchymal stem cells, which can be differentiated into functional endothelial cells that secrete von Willebrand factor. This is done through a novel approach using a concentrate of growth factors obtained from the patient's own platelets, also described in this article. Extra steps would be the seeding of smooth muscle cells and adventitial fibroblasts, which also could be differentiated from the patient's mesenchymal stem cells. Finally, an autologous blood vessel substitute can be obtained for cardiovascular peripheral disease graft treatment. These grafts will not induce tissue rejection, as the donor cells are completely removed and replaced by the patient's cells.

Recent reports have aimed to provide better alternative biocompatible options for vascular conduits such as human umbilical veins grafts, biopolymers, and seeding of endo-

thelial cells in polymers.<sup>1-3</sup> Tissue engineering has provided a new kind of conduit using different techniques,<sup>4-6</sup> often employing three-dimensional (3D) scaffolds followed by cell seeding.<sup>7,8</sup> These can be differentiated cells previously expanded in vitro, or mesenchymal stem cells (MSCs),<sup>9</sup> followed by the differentiation to the desired cell.<sup>10,11</sup> By using MSCs, one can rapidly obtain high cell counting, faster than differentiated cells are able to.<sup>12</sup> Moreover, several tissues may be used as the harvesting site. Among them, adipose tissue is a preferred choice due to the high concentration of MSCs present at the stromal-vascular fraction in this tissue (which can be collected in a low invasive procedure that offers equal differentiation capabilities of other tissue sources).<sup>13,14</sup>

To obtain a functional endothelium from MSCs, these cells require direct stimulation with growth factors that induce endothelial differentiation.<sup>15</sup> Given that the two principal growth factors that modulate this differentiation, the platelet-derived growth factor<sup>16,17</sup> and vascular

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endothelial growth factor,<sup>18</sup> are readily obtained from platelet's granules,<sup>19-21</sup> in physiological concentrations, we developed a novel way to differentiate MSCs into endothelial cells.

Researchers have inferred that it is possible to deploy cells at synthetic scaffolds such as biodegradable polymers<sup>1</sup> or organic tissues that were previously decellularized, obtaining in vitro tissue specimens.<sup>22-24</sup> It is, therefore, possible to yield cardiovascular conduits, with equivalent quality such as in the native artery, thus lowering the risk of tissue rejection and improving patency rates. Moreover, the production of organs employing heterologous decellularized scaffolds is already under preclinical and clinical trials<sup>25</sup> and have demonstrated lower rejection rates.<sup>26</sup>

This study reports the production of a blood vessel substitute through tissue engineering, using a 3D bio-scaffold from a decellularized rabbit vena cava, followed by the reconstruction of the endothelium, with adipose tissue-derived MSCs and platelet-derived growth factors.

## METHODS

**Animal housing conditions and general plan of investigation.** Fifteen nonpregnant female adult rabbits (New Zealand) were used for all experiments. All procedures were conducted respecting the Ethic Guidelines for Animal Experimentation, after the approval by the Brazilian College for Animal Experimentation (COBEA—Process n° 711). The rabbits were housed in controlled conditions and fed a standard pellet diet and water ad libitum. Median age was 6 months and weight between 2 and 3 kg.

In five rabbits, subcutaneous adipose tissue was excised and MSCs harvested. The cava veins were removed from all rabbits and decellularized. MSCs were then incubated with the decellularized scaffolds and subjected to endothelial cell stimulation using endothelial inductor growth factor (EIGF), which were obtained from human platelet-lysates. Cell adhesion and endothelial differentiation were assessed by general histology, immunofluorescence, immunohistochemistry, and von Willebrand factor (vWF) secretion, as described in the following sections.

**Adipose tissue and cava vein harvesting.** The animals were anesthetized with an intramuscular injection of 20 mg/kg of tiletamine hydrochloride/zolazepam hydrochloride and 4 mg/kg of 2% xylazine chloridate. The areas of tissue harvesting were previously shaved and disinfected with water-soluble iodine polyvinyl pyrrolidone solution. All following procedures were conducted under aseptic conditions.

Adipose tissue samples were surgically removed from interscapular region, from five animal donors. The infrarenal inferior vena cava was removed from each animal and the animals were euthanized using high doses of pentobarbital. Adipose tissue was immediately stored in a sterile cell culture medium (RPMI1640; Invitrogen, Carlsbad, Calif) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 mg/mL amphotericin B (Invitrogen). The vena cava and vessel lumens were washed off with sterile saline solution, supplemented with unfractionated heparin

(1000 UI/100 mL). All tissue samples were stored in the refrigerator at 4°C for 24 hours prior to conducting the experiments.

**Adipose-derived MSC expansion.** Adipose tissue-derived MSCs were obtained as previously described,<sup>27</sup> through digestion reaction with type I collagenase (Invitrogen). Cell culture procedures started with  $2 \times 10^4$  cells/cm<sup>2</sup> seeding and expansion in six-well culture plates (Techno Plastic Products, Trasadingen, Switzerland) with Dulbecco's modified Eagle's medium nutrient F12 mixture medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 25 mg/mL amphotericin B (2 mmol/L L-glutamine; Invitrogen), 1% (v/v) minimum essential medium (MEM) essential amino acids solution ( $\times 50$ ; Invitrogen), and 0.5% (v/v) of 10 mM MEM nonessential amino acids solution ( $\times 100$ ; Invitrogen) until reaching 80% confluence, when the obtained monolayers were detached from culture wells with 0.25% trypsin/ethylenediaminetetraacetic acid (Invitrogen) and seeded at 75 cm<sup>2</sup> culture flasks (Nunc, Roskilde, Denmark). After two more trypsin exposures (passages), cells were cryopreserved with ice-cold FBS supplemented with 10% dimethyl sulfoxide and stored at a liquid nitrogen cylinder.

**MSC characterization.** MSC characterization was conducted as previously described.<sup>28</sup> Briefly, following the third cell culture passage, cells were characterized using specific antibodies designed for flow cytometry. Anti-CD90 antibody (clone OX-7, cross-reactivity with rabbit; Biolegend) was used as a positive marker for MSCs. Anti-CD34 and anti-CD45 antibodies (Biolegend, San Diego, Calif) were employed as negative markers. Anti-mouse-IgG conjugated with fluorescein isothiocyanate was used as the secondary antibody (Molecular Probes, Eugene, Ore). Further analysis was conducted with the FACS Calibur (BD Biosciences, San Jose, Calif) equipment and software to obtain the number of positive CD90 cells. Additionally, the obtained MSCs were investigated for their potential to differentiate into the osteogenic, chondrogenic, and adipogenic lineages, under the recommended differentiation cell culture media (StemPro adipogenesis, chondrogenesis, and osteogenesis kits; Invitrogen) (data not shown).

**Scaffold decellularization.** Scaffolds were decellularized as described,<sup>29</sup> inside a sterile 1% sodium dodecyl sulfate (SDS) solution, over a period of 2 hours. The remaining SDS was removed through repetitive washing with Dulbecco's phosphate-buffered saline (Invitrogen).

**Cell-to-scaffold adhesion characterization.** To verify the feasibility of the decellularized scaffolds, a cell adhesion test was performed. One vial of cryopreserved characterized MSCs (from one animal donor) was thawed with viability accessed by Trypan Blue staining. Cells were labeled as described,<sup>30</sup> with modifications, with the Qtracker 605 Cell Labeling Kits (Molecular Probes) respecting the manufacturer's instructions. Briefly,  $1 \times 10^6$  cells were mixed in 200  $\mu$ L of culture medium/1.5 mL of prepared Qtracker solution (Life Technologies, Carlsbad, Calif) and incubated at 37°C for 60 minutes. The labeled cells were

washed with D-PBS, diluted with the PuraMatrix peptide hydrogel (BD Biosciences), and applied at the lumens of the decellularized vein scaffolds, with repetitive pipetting (20  $\mu$ L/pipetting), until all the solution was applied. This mixture of PuraMatrix/cells (BD Biosciences) was allowed to polymerize inside the lumen of the decellularized blood vessels. The experiment was conducted in triplicate. These cell-labeled-seeded scaffolds were further incubated for 30 minutes at a 37°C 5% CO<sub>2</sub> incubator with culture medium. These were then fast-frozen in liquid nitrogen for histology/immunofluorescence assessment. Scaffold sections were obtained at the cryostat (Leica, Wetzlar, Germany) and analyzed at a fluorescence microscope (BX41; Olympus, Shinjuku, Tokyo, Japan).

**Production of EIGFs.** The differentiation inductors were obtained from eight human platelet concentrate units from the excess stock of the Botucatu Blood Transfusion Unit that were going to be discarded. The growth factors that are present inside platelets are encoded by highly conserved DNA sequences amongst human and rabbit species, and human growth factors have already proven to induce response in rabbit cells.<sup>31</sup> These units were not submitted to frozen-thawing cycles. The mean platelet counts were obtained by automatic counting hardware (LH 750; Beckman Coulter, Brea, Calif) and the platelets were centrifuged at 5000 rpm for concentration. Supernatant was discarded and the platelet pellet was washed two times with sterile D-PBS. Platelet pellets obtained from the eight units were pooled together, dissolved at sterile injection water (10 mL) and shaken for 5 minutes (500 rpm) to obtain the platelet lysates. After a second centrifugation, the supernatant was collected to obtain the final EIGF.

**Endothelial differentiation cell culture.** Characterized MSCs from four animal donors were seeded at the 2 cm heterologous decellularized vein fragments at  $1 \times 10^5$  cells/fragment and submitted to endothelial differentiation composed of M199 culture medium supplemented with 20% of EIGF (vol/vol), 100 U/mL penicillin, 100 mg/mL streptomycin, 25 mg/mL amphotericin B (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), 1% (v/v) MEM essential amino acids solution ( $\times 50$ ; Invitrogen), and 0.5% (v/v) of 10 mM MEM nonessential amino acids solution ( $\times 100$ ; Invitrogen). Cells were pipetted at the luminal portion of the vein fragments and cultivated at 24-well ultra-low attachment culture plates (Corning; Sigma-Aldrich, St. Louis, Mo) to minimize cell attachment to the culture plate, while maximizing attachment to the scaffolds. Additional experiments were performed as follows: control, composed of scaffolds and M199 culture medium; and culture medium control, composed of scaffolds, MSCs, and regular M199 medium, without endothelial inductors. Eight vein fragments were employed for each experiment. All experiments were conducted in duplicate.

Endothelial cell culture differentiation was performed for 3 weeks, with culture medium being exchanged every 48 hours. At the end of the experiments, fragments were collected for histology and immunohistochemical analysis.

Cell culture was performed in a stress-free environment with no mechanical forces applied.

#### Quantification of vWF on conditioned medium.

After the third week of endothelial differentiation protocol, the conditioned medium (CM) of the different cell cultures were collected for the quantification of vWF, using the respective enzyme-linked immunosorbent assay (Corgenix, Inc, Broomfield, Colo), according to manufacturer's guidelines. Fresh culture medium and fresh culture medium supplemented with 20% of EIGF (vol/vol) were also loaded as controls. Human normal serum was loaded as positive controls. The integrated optical density was accessed at a 450 nm capable spectrophotometer reader, and the obtained values were divided by the values obtained with positive controls. Data was analyzed with INSTANT software (GraphPad Software, San Diego, Calif) using the one-tail Student *t*-test ( $P < .05$ ) to compare the different treatments. The respective concentrations were extrapolated from standards (positive control) and are represented in  $\mu$ g/mL. Values were calculated as mean  $\pm$  standard deviation of the totality of concentrations for the CM of cells cultivated under regular medium and with EIGF supplementation (seven independent samples of each, and the remaining one was employed during the standardization of the technique).

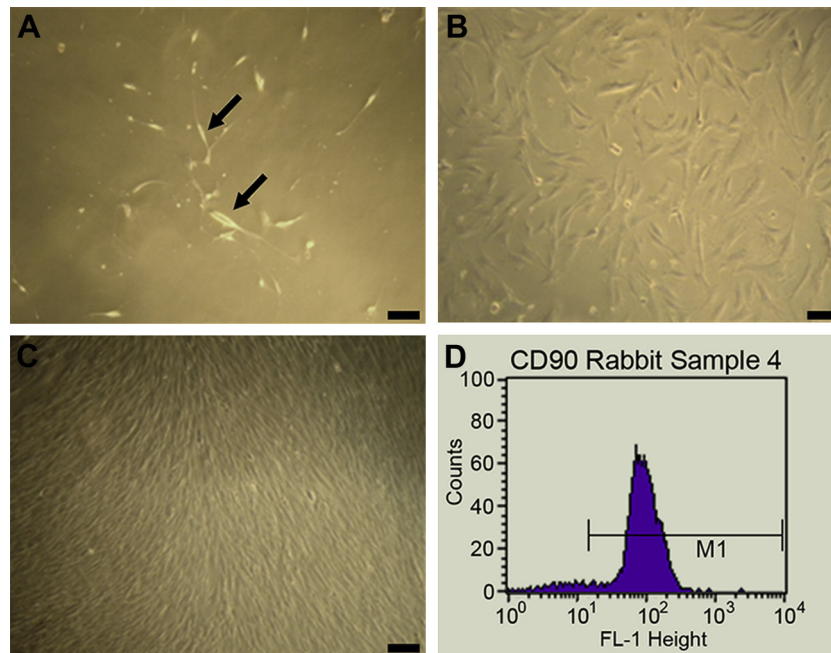
#### Histologic and immunohistochemical analysis.

After 3 weeks, cell-seeded scaffolds were prepared for histological evaluation as described.<sup>28</sup> The histologic analysis was conducted, aiming to confirm the presence of cells adhered to the internal side of the scaffolds, such as observed at the immunofluorescence analysis. All eight fragments of the scaffolds were screened at the microscope (at  $\times 200$  magnification), and the images were digitalized. Extra slides were prepared for immunohistochemical analysis conducted as previously described.<sup>32</sup> These used the anti-fascin monoclonal antibody (clone 55K-2; Dako, Glostrup, Denmark), previously described as a high expressed protein in endothelial cells.<sup>33,34</sup> Additionally, the decellularized scaffolds were processed for general morphology assessment with Masson's trichrome staining to confirm the success of cell removal. Native veins were also processed as normal controls.

## RESULTS

#### Adipose-derived MSC expansion and characterization.

After 2 days of the initial seeding of the cells, two different cell populations could be observed at the inverted microscope: nonadherent cells in suspension and adherent cells on the bottom of the culture flasks. The cells in suspension, typically white blood cells derived from the stromal-vascular portion of the processed adipose tissue, were flushed away during the culture medium exchanges. The adherent cells remained attached to the flasks (Fig 1, A) and started to proliferate. After the following 2 days, these cells started to assemble into colonies (Fig 1, B) and presented classical morphologic characteristics of MSCs: fibroblastic-shaped, small cell body, defined nucleus, presence of cytoplasmic projections, and presence of two



**Fig 1.** Mesenchymal stem cells (MSCs) morphology observed at phase-contrast inverted microscopy and characterization by flow cytometry. **A**, Initial cell adhesion to the culture flasks (*arrows*), low confluence. MSCs features fibroblastic-like morphology, well-defined nucleus, and two nucleoli (*scale bar* = 50  $\mu$ m). **B**, Cells proliferated and colonies are obtained, featuring the same morphologic characteristics (*scale bar* = 100  $\mu$ m). **C**, After the third passage, cells are cultivated until 100% confluence for CD90 cell characterization (*scale bar* = 200  $\mu$ m). **D**, CD90 positive cells, recognized as MSCs, constitute all cells in the gated area (M1) of the flow cytometry analysis.

nucleoli per nucleus (Fig 1, B). These characteristics were systematically observed during the entire cultivation period and still could be seen at the third passage (Fig 1, C). At this point, the cells were characterized as MSCs by CD90 expression, with values ranging from 95% to 98% and median of 96.5% (Fig 1, D). The negative markers CD34 and CD45 had expected results, with positive counts ranging from 0.05% to 1.32% (data not shown). Negative control (MSCs  $\times$  secondary antibody) resulted in 0.04% positive counts (data not shown). The obtained cells differentiated into osteogenic, chondrogenic, and adipogenic cells, using the respective StemPro differentiation kits, further characterizing them as MSCs (data not shown).

**Scaffold decellularization.** Native veins processed as controls displayed regular morphology with intact endothelium and presence of many nuclei at the subjacent tissue (Fig 2, A). The SDS decellularization protocol was successfully employed, and all cells were removed from the tissue. No nuclei were left either at the endothelium, smooth muscle layer, and the perivascular connective tissue (Fig 2, B). The extracellular matrix (ECM) was slightly disorganized due to SDS exposure; however, large collagen content was preserved and stained in blue, at the Masson's trichrome staining (Fig 2, B). A total of 30 decellularized vein fragments (2 cm) were obtained.

**Cell-to-scaffold adhesion characterization.** It was possible to observe that the scaffolds were able to sustain

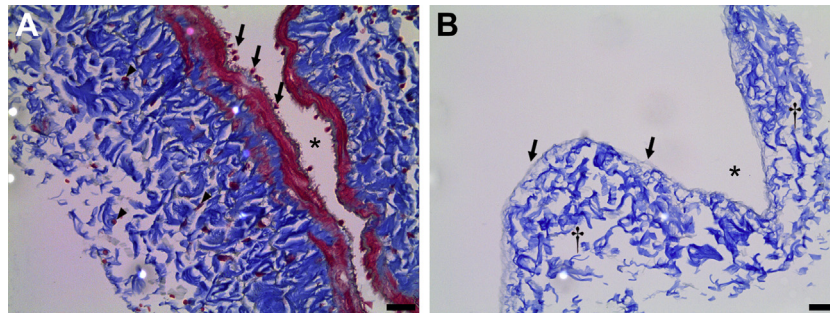
cell adherence at all samples processed (Fig 3, A-D). Although the cells were concentrated at the lumen wall (Fig 3, A), some cells migrated through the scaffolds to the inner collagenous matrix (Fig 3, B and D). Some areas in which the cells remained inside the PuraMatrix (BD Biosciences) applied to the vessel lumen could also be observed (Fig 3, C).

**Production of EIGFs.** The mean platelet count of the concentrate units was  $1.88 \times 10^{11}$ . The final growth factor concentrate was colorless and did not induce any coagulation when supplemented in the culture medium, as seen previously in our laboratory (data not published).

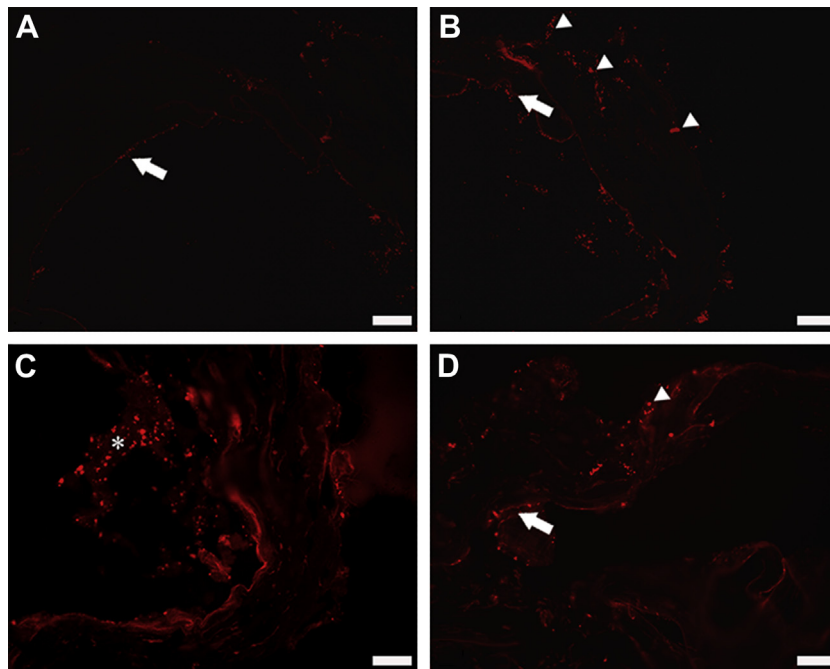
**Endothelial differentiation cell culture.** During the microscopic examination of the cell cultures, under the inverted microscope, it was possible to observe cells adhered to the vessel wall (Fig 4). These cells were morphologically rounded (Fig 4, A and B), a typical characteristic of initial cell adhesion. Within 2 weeks, the adhered cells proliferated, could be seen at the borders of the scaffolds, and cell morphology became fibroblastic-shaped (Fig 4, C and D), which is a preliminary characteristic of endothelial cells.

**Histologic, immunohistochemical analysis, and vWF dosage.** After the third week of differentiation culture, cell-seeded scaffolds were processed for general histology. The lined cells observed at the phase contrast inverted microscopy and immunofluorescence were confirmed at the histologic sections and were well organized and with





**Fig 2.** Native and sodium dodecyl sulfate (SDS)-decellularized vein fragments after histologic processing and Masson's trichrome staining. **A**, Naturally occurring vein morphology with lumen (\*), well preserved endothelial cells (*arrows*), smooth muscle cells, and multiple nuclei at the inner collagenous tissue (*arrowheads*) (*scale bar* = 120  $\mu$ m). **B**, Decellularized vein morphology displaying its lumen (\*), cell-free endothelium (*arrows*), and cell-free inner collagenous tissue (*crosses*). Note that the *thick red layer* on **A**, comprised by endothelial cells and smooth muscle cells, is totally absent on the decellularized vein fragment. All cells (rejection inductor) were successfully removed (*scale bar* = 120  $\mu$ m).

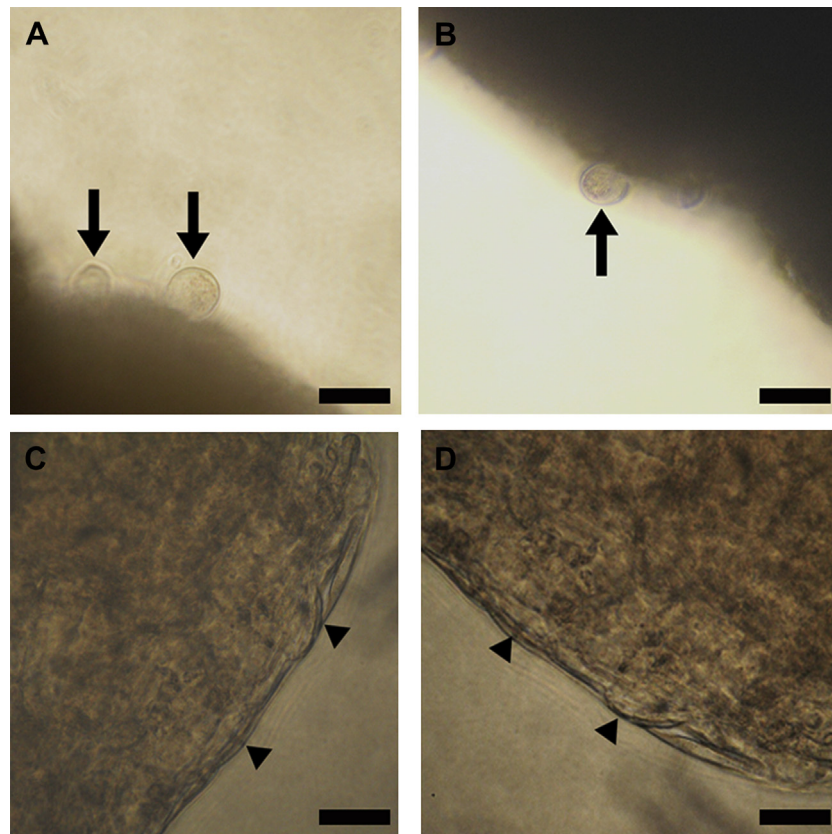


**Fig 3.** Immunofluorescence analysis of the bioscaffold seeded with mesenchymal stem cells (MSCs) after 60 minutes of initial seeding. MSCs were stained with Qtracker 605 Cell Labeling Kits (Molecular Probes; Life Technologies) and diluted at the PuraMatrix peptide hydrogel (BD Biosciences). The stained cells/PuraMatrix solution was applied at the lumens of the decellularized vein scaffolds, with repetitive pipetting. **A**, Note the accumulation of cells lined in the vessel lumen wall (*arrow*) (*scale bar* = 300  $\mu$ m). **B**, Again, cells lined on the lumen (*arrow*) can be seen alongside with cells in the inner portions of the scaffold (*arrowheads*) (*scale bar* = 300  $\mu$ m). **C**, A transversal section of the scaffold showing cells inside the PuraMatrix peptide solution inside the vessel lumen can be seen (\*; *scale bar* = 100  $\mu$ m). **D**, Another section showing cells lined in the lumen (*arrow*) and inside the inner collagenous portion of the scaffold (*arrowhead*) (*scale bar* = 100  $\mu$ m).

endothelial morphology (Fig 5, A). Moreover, the cells migrated inside the scaffold and reorganized into small capillaries of three to four cells (Fig 5, B). Cells were also positive for fascin antibody staining, which is a highly expressed protein in endothelial cells<sup>33</sup> (Fig 5, C).

The CM of cells cultivated with EIGF supplement presented statistically significant higher concentrations of

secreted vWF ( $P = .0289$ ) compared with the CM of cells cultivated under regular culture medium (Fig 5, D). Fresh culture medium with the same EIGF supplement concentration presented no detectable levels of vWF; therefore, all the detectable vWF in the CM of the cultivated cells was secreted by the cells. Normal human serum (positive reaction control) presented 10  $\mu$ g/mL of vWF.



**Fig 4.** Mesenchymal stem cells (MSCs) seeded in decellularized vein scaffolds, cultivated with endothelial inductor growth factor (EIGF) supplementation, observed at phase-contrast inverted microscopy. **A** and **B**, Note the initial cells adhering in the lumen of the decellularized scaffold (arrows) (scale bar = 20  $\mu$ m). **C** and **D**, After 1 week of cell cultivation, the initial adhering cells seen at the previous figures presents a morphology shift, from rounded-shaped cells to scamous/flattened epithelial-shaped cells (arrowheads) (scale bar = 40  $\mu$ m).

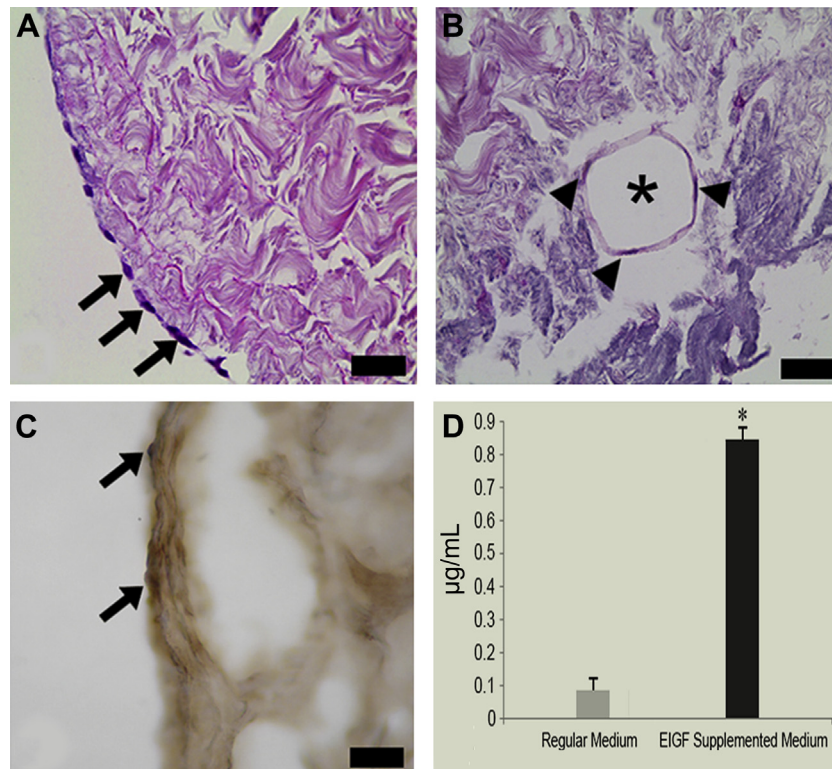
## DISCUSSION

In this study, we evaluated the feasibility of a bioscaffold obtained from decellularized veins, together with adipose-derived MSCs, which were cultivated with EIGF supplementation, aiming at the reconstruction of a functionally active endothelium on the decellularized veins. Initially, we demonstrated that when the MSCs were cultivated during three passages, with constant culture medium exchange, all the cells from the five different samples (animal donors) presented morphologic features highly characteristic of MSCs, and high expression of CD90, considered as a MSC marker, in accordance to previous reports.<sup>27,28</sup> It is important to notice that these obtained cells may have some degree of residual endothelial progenitor cells, which would contribute for the final constructed vessel. In this sense, future investigators should be aware of this and if possible, employ further phenotypic profiling using endothelial markers such as CD34, vascular endothelial growth factor R2, and CD133.

After the characterization of the cell source, the next step to obtain the desirable blood vessel substitute was the seeding of these cells in a suitable 3D scaffold. Given

that two-dimensional cell culture models does not allow complex cell-to-ECM interactions<sup>35</sup> and represent only a fraction of the in vivo scenario, previous researchers have reported that MSCs require a 3D structure to allow ECM deposition and to successfully differentiate into endothelial cells.<sup>36</sup> In this sense, the following experiments were performed to obtain the 3D scaffold capable of housing the MSCs, thus giving them conditions to differentiate into endothelial cells.

Three major characteristics are important for the successful implementation of a scaffold in tissue engineering. First, it needs to present a well-designed porous network<sup>37</sup> (eg, the pore size and pore number needs to be well balanced given that higher porosity makes cell migration/proliferation more favorable). However, this same higher porosity could also compromise the mechanical structure. Second, it needs to degrade in a speed that is compatible to the ECM production speed by the seeded cells so that the scaffold material is gradually replaced by ECM proteins.<sup>38</sup> Third, and easily understandable, it needs to be biocompatible.<sup>28,37,38</sup> Aiming to address all three required characteristics, we proposed to employ decellularized vein fragments



**Fig 5.** Histologic sections of mesenchymal stem cell (MSC)-seeded vein fragments after the third week of endothelial differentiation using endothelial inductor growth factor (EIGF) supplementation. **A**, Cells with evident endothelial cell morphology lined on the lumen of the decellularized scaffolds (arrows) (low magnification; hematoxylin-eosin staining; scale bar = 20  $\mu$ m). **B**, Transversal section showing a capillary-like structure (\*) composed of three endothelial cells (arrowheads) (hematoxylin-eosin staining; scale bar = 20  $\mu$ m). **C**, Anti-fascin monoclonal antibody staining of cells lined in the scaffold's lumen (arrows) (scale bar = 10  $\mu$ m). **D**, Quantification of the von Willebrand factor (vWF) on the conditioned medium (CM) of cells cultivated in the decellularized scaffolds, with regular cell culture medium and EIGF supplemented medium. While the CM of cells cultivated under regular medium presents low levels of vWF, the CM of cells cultivated with EIGF supplementation presents high detectable levels of this endothelial function marker. Data are expressed as the mean  $\pm$  standard deviation of seven individual enzyme-linked immunosorbent assay readings. \* Statistically significant values with  $P < .05$ .

as the housing scaffold, since it removes the donor cells (rejection component), displays a naturally occurring porous network, possesses a nonimmunogenic 3D ECM structure, and is a biocompatible structure. Moreover, this scaffold can be considered bioactive, since it is accepted that the ECM gives differentiation cues for the seeded cells.<sup>39,40</sup>

Therefore, we tested the SDS chemical decellularization agent, which has proven to be efficacious at removing the immunogenic agents, in accordance with previous literature reports.<sup>25</sup> Interestingly, although it is alleged that SDS may interfere with the ECM structure,<sup>41</sup> it was only slightly disorganized in our experiments, indicating that this effect may be less deleterious at small tissue fragments, such as blood vessels, which requires less exposure time to the agent. Although only the Masson's staining was performed, further data in our laboratory support that SDS does not compromise the ECM structure (data not published). In fact, previous reports that SDS action on human great saphenous veins did not compromise collagen morphology, induced only a slight decrease in elastin staining, and left the

basement membrane unaffected<sup>41,42</sup> further support our findings. On the next experiments, when the characterized MSCs were seeded on the scaffolds, the immunofluorescence analysis revealed that the SDS treatment did not interfere with the ability of cells to adhere to the scaffold, since high cellularity was observed after only 60 minutes of cell adhesion time. Moreover, some cells were seen at the inner portions of the scaffold, indicating cell migration.

Having obtained the characterized cell source and the decellularized scaffold, we then moved on to the final step of the study, which was to obtain a functional endothelium. To accomplish this in the following experiments, we employed the obtained decellularized scaffolds in conjunction with characterized MSCs that were cultivated on the scaffolds and in the presence of growth factors that were obtained from platelets' granules (EIGF). MSCs that were cultivated on the scaffolds with basal medium only were also investigated.

After 3 weeks of cell culture, the scaffolds were subjected to histology, which revealed epithelial-like cells lined



in the scaffolds' lumen. Not only did these cells have a scamous/flattened cell morphology, but they were also stained with a monoclonal antibody anti-fascin, which is considered to be an endothelial differentiation marker.<sup>33,43</sup> Intriguingly, some sections revealed groups of three to four cells that reorganized into capillary-like structures, with lumens, inside the 3D scaffolds. To further characterize these cells as endothelial cells, we quantified the vWF protein concentration at the CM of cells cultivated with and without EIGF supplementation. These final analyses revealed that when the cells are cultivated on the scaffold with basal medium, low levels of vWF are detectable, indicating that some differentiation cues are due to the retained ECM proteins on the decellularized scaffolds, as described elsewhere.<sup>39,40</sup> However, when EIGF supplementation is added to the cell cultures, statistically significant high levels of vWF are obtained, indicating that the combination of the bioscaffold and EIGF are important to the endothelial cell differentiation. Moreover, these high levels of vWF further support the findings on the histology and general morphology analyses.

Although there are a number of published reports and interesting findings in the field of blood vessel tissue engineering, most of them employ synthetic scaffolds for the cells with purified recombinant growth factors. For a detailed review on these findings, readers should seek the paper by Nemen-Guanzon et al.<sup>36</sup> Only a few authors have investigated the use of decellularized bioscaffolds for blood vessel engineering.<sup>38</sup>

In this sense, future studies should explore the present reported findings, possibly expanding it with the addition of smooth muscle cells given its crucial role in the homeostasis on blood vessels physiology.<sup>36</sup>

In conclusion, this report demonstrates that adipose-derived MSCs cultivated at a decellularized vein scaffold differentiated into functional endothelial cells. These secreted vWF on the culture medium through the induction of growth factors obtained from platelets. Moreover, the decellularized bioscaffolds promoted cell adhesion, suggesting that it can be employed as a cell-housing facility. Taken together, bioscaffold, cell source, and the method of differentiation seem to be an interesting approach to obtain bioengineered blood vessel substitutes. These, then, could be upgraded with smooth muscle cells and further explored within in vivo experiments as vein grafts in future investigations.

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Conception and design: MB, FA, RM, MS  
Analysis and interpretation: MB, AM, MV, MD, ED  
Data collection: MB, AM, RA, MV, MD

Writing the article: MB, AM

Critical revision of the article: MB, AM, RM, MS, ED

Final approval of the article: MB, AM, RA, FA, MV, RM, MS, MD, ED

Statistical analysis: AM, RA

Obtained funding: MB, FA, ED

Overall responsibility: MB, ED

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