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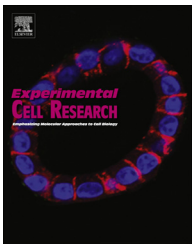

Research Article

Morphofunctional characterization of decellularized vena cava as tissue engineering scaffolds

Matheus Bertanha[a,b,*], *Andrei Moroz*[c], *Rodrigo G. Jaldin*[a], *Regina A.M. Silva*[b], *Jaqueline C. Rinaldi*[d], *Márjorie A. Golim*[e], *Sérgio L. Felisbino*[d], *Maria A.C. Domingues*[f], *Marcone L. Sobreira*[a], *Patricia P. Reis*[a], *Elenice Deffune*[b,g]

[a]*Department of Surgery and Orthopedics, Botucatu Medical School, São Paulo State University (UNESP), Vascular Laboratory, Avenue Prof. Montenegro, S/N. Rubião Júnior, Botucatu 18618-970, SP, Brazil*
[b]*Blood Transfusion Center, Cell Engineering Laboratory, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil*
[c]*Department of Bioprocess and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, SP, Brazil*
[d]*Department of Morphology, Extracellular Matrix Laboratory, Botucatu Biosciences Institute, São Paulo State University (UNESP), Botucatu, SP, Brazil*
[e]*Blood Transfusion Center, Flow Cytometry Laboratory, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil*
[f]*Department of Pathology, Immunohistochemistry Laboratory, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil*
[g]*Department of Urology, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil*

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ABSTRACT

Clinical experience for peripheral arterial disease treatment shows poor results when synthetic grafts are used to approach infrapopliteal arterial segments. However, tissue engineering may be an option to yield surrogate biocompatible neovessels. Thus, biological decellularized scaffolds could provide natural tissue architecture to use in tissue engineering, when the absence of ideal autologous veins reduces surgical options. The goal of this study was to evaluate different chemical induced decellularization protocols of the inferior vena cava of rabbits. They were decellularized with Triton X100 (TX100), sodium dodecyl sulfate (SDS) or sodium deoxycholate (DS). Afterwards, we assessed the remaining extracellular matrix (ECM) integrity, residual toxicity and the biomechanical resistance of the scaffolds. Our results showed that TX100 was not effective to remove the cells, while protocols using SDS 1% for 2 h and DS 2% for 1 h, efficiently removed the cells and were better characterized. These scaffolds preserved the original organization of ECM. In addition, the residual toxicity assessment did not reveal statistically significant changes while decellularized scaffolds retained the equivalent biomechanical properties when compared with the control. Our results concluded

*Corresponding author at: São Paulo State University—UNESP, Botucatu Medical School, Department of Surgery and Orthopedics, Vascular Laboratory, Avenue Prof. Montenegro, S/N. Rubião Júnior, 18618-970 Botucatu, SP, Brazil. Fax: +55 1438801444.
E-mail address: matheus.fameca@ig.com.br (M. Bertanha).

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that protocols using SDS and DS were effective at obtaining decellularized scaffolds, which may be useful for blood vessel tissue engineering.

Introduction

Cardiovascular disease is the leading cause of death in Western countries [1], associated with an increased incidence of peripheral arterial disease (PAD) [2]. Clinical experience in PAD treatments shows poor results when using synthetic grafts with a diameter less than 6 mm. This is especially true when it is intended to treat small caliber arteries such as those found in the lower limbs [3] and that have thus benefited from tissue engineering for the development of blood vessels using both synthetic and biological materials. The challenge of tissue engineering is to produce three dimensional (3D) biocompatible scaffolds: structures that provide seeded cells with a microenvironment mimicking natural in vivo tissue architecture [4]. Given that the development of tissues and organs using in vitro cellular engineering often suffers from a shortage of donor tissue, (which limits the potential of obtaining tissue engineered constructs), scaffolding technology is a promising approach to overcome these limitations [5,6].

Synthetic materials such as the commercially available polyglycolic acid (PLG) and polylactic acid (PLA) may be used to obtain 3D tubular structures, given that it presents some advantages; e.g., they do not induce immunogenic reactions [7]. These PLG/PLA based scaffolds were tested and proven efficient in providing mechanical support, good interaction with cells, and to have relative ease in handling. However, scaffolds from these synthetic materials merely provide three-dimensionality for cultured cells. They are artificially produced, and do not possess a natural 3D architecture [7]. An alternative is to use organs or tissues decellularized by different methods which does not promote the immunogenic reaction of allogeneic tissue transplants. Such methods have many advantages, such as the presence of extracellular matrix (ECM) proteins, bioactivity, and natural 3D tissue architecture [8].

A major concern around these bioengineered conduits for clinical application is whether these 3D tubular structures support the various mechanical forces resulting from blood flow, until the implanted cells reach a natural level of ECM deposition that allow for the development of natural tissue properties and, therefore, mechanical resistance [9].

Herein, we focus on the use of three different detergents in 12 decellularization protocols in order to obtain a decellularized blood vessel scaffold. Furthermore, the characterization of remaining ECM composition, its potential toxic residual effects, and its biomechanical properties were performed and analyzed.

Methods

Animal housing conditions and tissue harvesting

Fifty nonpregnant female adult rabbits (New Zealand) were used in all experiments. All procedures were conducted respecting the Ethical Guidelines for Animal Experimentation, after the approval by the Brazilian College for Animal Experimentation (COBEA Process no. 711). All experiments followed the US National Institutes of Health or European Commission guidelines. Rabbits were housed under controlled conditions and fed a standard pellet diet and water ad libitum, with the median age of animals being 6 months with weight ranging between 2.5 and 3 kg. Animals were anesthetized with tiletamine hydrochloride/zolazepam hydrochloride (20 mg/kg, i.m.) in association with 2% xylazine chloridate (4 mg/kg, i.m.). 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, and the animals were euthanized using high doses of pentobarbital.

The infrarenal inferior vena cava was surgically removed and their lumens washed off with sterile saline solution supplemented with unfractionated heparin (1000 UI/100 mL). These were then stored at a sterile RPMI-1640 cell culture medium (Invitrogen™, Carlsbad, USA) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 mg/mL amphotericin B (Invitrogen™) at 4 °C. Adipose tissue samples were surgically removed from interscapular region, and immediately stored in RPMI cell culture medium supplemented as described above.

Adipose derived mesenchymal stem cell culture

Adipose derived mesenchymal stem cells (ADMSCs) were obtained as previously described through digestion with type I collagenase (Invitrogen™) [10]. Cell culture procedures were performed with an initial cell count of 2×10^4 cells/cm^2, obtained from five adipose tissue fragments (different donors). These cells were seeded and expanded in six-well culture plates (Techno Plastic Products™, Trasadingen, Switzerland) using 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 (Invitrogen™), and 0.5% (v/v) of 10 mM MEM nonessential amino acids solution (Invitrogen™). Upon reaching 80% confluence, the monolayers were detached from culture wells with 0.25% trypsin/ethylenediaminetetraacetic acid (Invitrogen™) and seeded at 75 cm^2 culture flasks (Nunc™, Roskilde, Denmark). After two additional trypsin exposures, cells were cryopreserved with ice-cold FBS supplemented with 10% dimethyl sulfoxide and stored in liquid nitrogen [11].

Scaffold decellularization

Sodium dodecyl sulfate (SDS), sodium deoxycholate (DS), and Triton X100 (TX100) were chosen as the decellularizing agents, based on previous literature reports [12–17]. Working concentrations were 1% and 2%, both during 1 h and 2 h of incubation at 160 rpm at 37 °C in a shaker (News Brunswick Scientific™, Nijmegen, The Netherlands). Following the decellularization of

vein fragments, scaffolds were washed 5 times with sterile PBS, and stored in fresh PBS at 4 °C until further analysis.

Histological processing and analysis of decellularization efficiency

Decellularized vein fragments were fixed in 10% buffered formalin during 48 h. After washing five times in PBS, specimens were dehydrated in a series of ethanol solutions and embedded in paraffin. From these, 5 μM sections were obtained in a microtome, and placed at microscopy slides. These slides were then stained with hematoxilin and eosin (H&E) and mounted in Permout® [18].

General morphology and absence of cells were examined by H&E staining, with tests performed in triplicate. For morphological assessment, 20 random fields at 400× magnification were captured with a mounted-microscope camera and objectively analyzed by investigating the total remaining cell nuclei/whole cells and the ECM structure. To quantify the effectiveness in removing cellular components, a scale was generated classifying as optimal result the complete absence of or up to 3 nuclei. Unsatisfactory results were classified as the presence of 4–50 nuclei, with unresponsive results being the presence of more than 50 nuclei. For the same 20 random fields, the ECM was visually analyzed for its integrity. The absence of ECM disruption areas was considered an optimum result; however the presence of one or more areas of ECM disruption was considered an unsatisfactory result. Further analyses were performed only for the scaffolds in which the decellularizing agents have proven efficient for removal of cells and maintenance of ECM network integrity.

Morphological analysis of decellularized scaffolds

Sequential slides were prepared for special histological protocols by staining them with Picrosirius red, Masson's trichrome and Calejja. In addition, slides were prepared for immunohistochemical analysis of the collagen composition of decellularized scaffolds using monoclonal antibodies against naturally occurring collagen III and IV (Dako™, Glostrup, Denmark). This was done in order to establish the integrity of collagen content. All morphological analyses allowed for comparison of the histological pattern after each treatment with the control veins [19,20]. Picrossirius stained slides were analyzed under a polarized light microscope to assess compaction and maturity of collagen fibers [21]. For each analysis, 3 blood vessels were employed.

Biomechanical characterization

Decellularized vein fragments were stretched longitudinally until failure for evaluation of mechanical properties. The extremities of each segment were fixed to the smooth plates of non-cutting metal clamps of a universal machine for mechanical studies using EMIC®, model DL 10.000 (Equipments and Testing Systems™, Ltd. Curitiba, PR, Brazil). This machine offers a precision of around

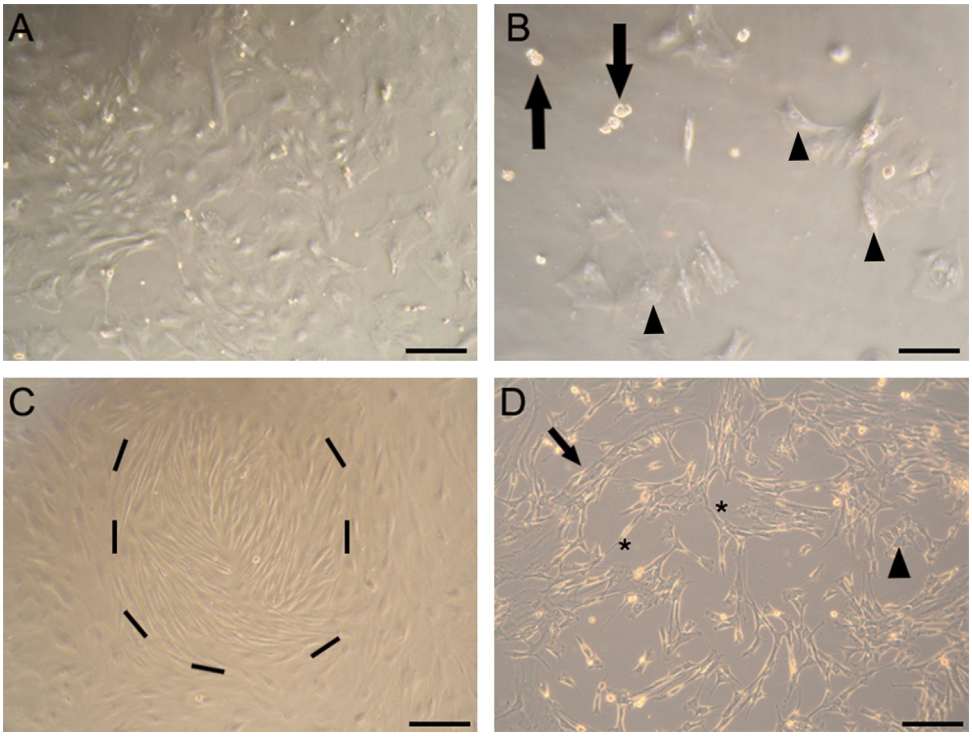

Fig. 1 – Representative images of adipose derived mesenchymal stem cells (ADMSCs) under cell culture ($n=5$). A) Following 48 h of cell seeding, ADMSCs adhere strongly to the substrate and initiate mitosis, leading to partial confluence of the culture vessel. B) Alongside the adhered ADMSCs (arrowheads) some rounded-shaped cells (arrows), mainly lymphocytes, are also present on the culture vessel. These non-adherent cells are gradually removed from the cultures, through culture medium exchange. C) After one week of cell culture, ADMSCs start to assemble into colonies (highlighted in black). These colonies will reach out to other colonies through the culture vessel, resulting in high confluence. D) Morphological features of ADMSCs, such as well defined nucleus and nucleoli (arrowhead), fibroblastic-shape (arrows) and many cytoplasmatic projections (asterisk). Scale bars = 50 μm.

0.018+F/3700 KN and operates in conjunction with a microcomputer using Mtest® 1.00 software. The velocity chosen for tension application was 30 mm/min [22,23]. At the end of the test, the software provided a load-elongation diagram and the results of yield point (N), stiffness coefficient (N/mm) and failure load (N). The yield point was obtained by Johnson's method and indicates the maximum load value at which a material still retains the capacity to return to its original length when the load is removed. On the graph it corresponds to the maximum tension value of a linear function within the load–deformation curve (elastic phase) [24]. The stiffness coefficient is obtained by dividing the force applied (N) by the deformation produced (mm) and therefore measures the stiffness of the structure [25]. Thus, stiffness is the linear and constant numeric relation between load and elongation at the yield point [26]. The failure load, or maximum load, is the greatest load withstood by either material before breaking, i.e., the tensile strength. For all measurements, 13 fragments of each decellularized scaffolds were compared to 13 control veins.

Toxic residual effects of decellularized scaffolds

To verify whether decellularized scaffolds are suitable for in vitro applications, their potential toxic residual effects were investigated. Therefore, we conducted an additional experiment in which 1×10^6 expanded ADMSCs were thawed, seeded in ultra-low attachment 6-well culture plates, cultured for 24 h, and exposed to decellularized vein scaffolds. Cells were maintained in culture for 48 h. Following exposure, the scaffolds were scrapped off to release any adhering cells. Cell viability was analyzed using a Neubauer chamber after trypan blue staining.

Moreover, remaining cells were used to observe the presence of apoptosis by flow cytometry using antibody anti-caspase 3 [27]. The BD Pharmingen™ PE Active Caspase-3 Apoptosis Kit® was used and protocols were performed according to the manufacturers' recommendations. Briefly, the cells were removed from the wells, centrifugated, and incubated with BD Cytofix/Cytoperm™ fixation/permeabilization solution for 15 min at room temperature. Cells were then washed with Perm/Wash™ Buffer and a further incubation step was performed with 20 μL of monoclonal antibody against the active form of caspase-3 in the presence of 100 μL of permeabilization solution for 45 min in dark. Subsequently, cells were analyzed by flow cytometry using the BD FACSCalibur™ equipment and the software Cell Quest™. This experiment was performed in triplicate with the controls having the same amount of cells in the absence of the scaffolds.

Results

Adipose derived mesenchymal stem cell culture

Following the initial seeding, cells actively adhered to the bottom of the cell culture flasks (Fig. 1A). A few cells were observed in suspension, typically leukocytes derived from the vascular portion of the processed adipose tissue (Fig. 1B); these cells were gradually removed during the replacement of the culture medium. In 2 days, adherent cells started to assemble into colonies (Fig. 1C), showing previously described morphologic characteristics of MSCs, such as fibroblastic-shape, well defined nucleus, and presence of cytoplasmic projections (Fig. 1D) [11].

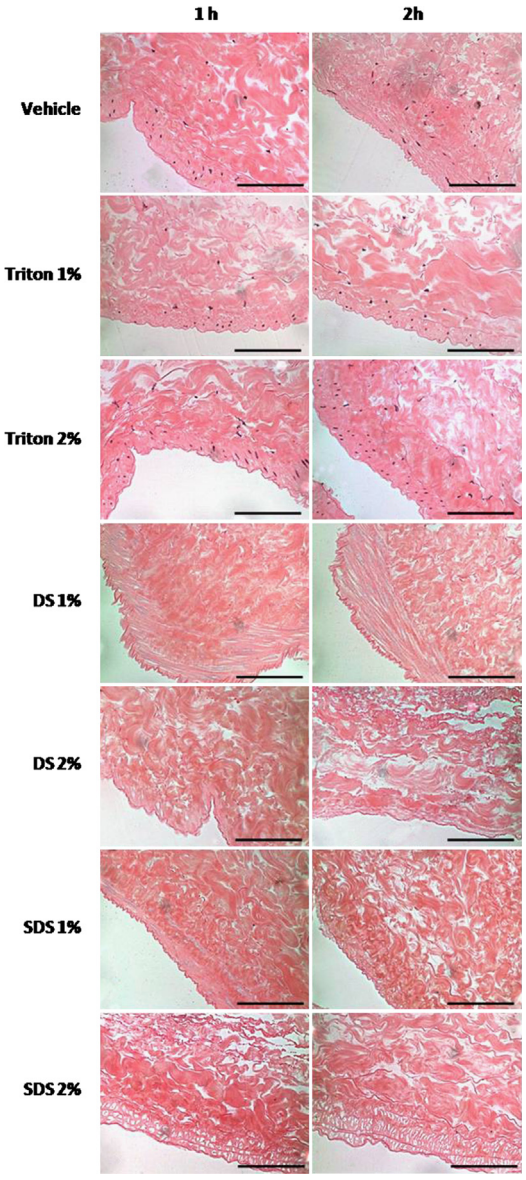

Fig. 2 – Representative sections of all rabbit vein fragments (n=42), either control (vehicle) blood vessels or blood vessels submitted to different detergent decellularization agents, stained by hematoxylin and eosin (n=3 for each treatment). The image shows that the vessels' histoarchitecture (mainly the extracellular matrix structure) was apparently preserved at the end of the decellularization process in all treatments. However, the complete absence of nuclei or cellular residues was obtained only when sodium deoxycholate (DS) and sodium dodecyl sulfate (SDS) were employed. Triton does not efficiently remove cell nuclei under the current experimental conditions. Scale bars=50 μm.

Scaffold decellularization

Aproximately 42 vein fragments were obtained, decellularized and employed for all characterization assays, as described above. Regardless of the agent used for decellularization, scaffolds acquired some common macroscopic characteristics, such as pronounced opacity and slight elongation. The general structure of all 42 vein fragments was preserved (Fig. 2).

Table 1 – For the morphological assessment, 20 random fields (combined from 3 different sections of each vehicle or decellularized vein) at 400× magnification were captured at a mounted-microscope camera and objectively analyzed by investigating the total remaining cell nuclei/whole cells and the ECM structure. To quantify the effectiveness in removing cellular components, a scale was created, adopting as optimal result: the complete absence or up to 3 cells, an unsatisfactory result: the presence of 4–50 cells, and an unresponsive result: the presence of more than 50 cells.

Analysis of decellularizing efficiency

Treatment	≤3 cells	4–50 cells	≥51 cells
Vehicle	0	6/20 (30%)	14/20 (70%)
Triton 1%—1 h	0	6/20 (30%)	14/20 (70%)
Triton 1%—2 h	0	16/20 (80%)	4/20 (20%)
Triton 2%—1 h	0	14/20 (70%)	6/20 (30%)
Triton 2%—2 h	0	18/20 (90%)	2/20 (10%)
DS 1%—1 h	16/20 (80%)	4/20 (20%)	0
DS 1%—2 h	20/20 (100%)	0	0
DS 2%—1 h	20/20 (100%)	0	0
DS 2%—2 h	20/20 (100%)	0	0
SDS 1%—1 h	16/20 (80%)	4/20 (20%)	0
SDS 1%—2 h	20/20 (100%)	0	0
SDS 2%—1 h	20/20 (100%)	0	0
SDS 2%—2 h	20/20 (100%)	0	0

Histological processing and analysis of decellularizing efficiency

The decellularization with Triton X-100 protocol was not efficient for cell removal. Multiple nuclei were consistently observed under microscopy analysis (Fig. 2). Triton X-100 was thus considered a poor decelularizing agent (presence of many cells, and some areas of ECM disruption were observed).

Both decellularization protocol using SDS and DS, at a concentration of 1% for 1 h, showed that there was not a complete decellularization of the scaffolds (20% containing between 4 and 50 cells), as evidenced by incomplete absence of nuclei, while maintaining the ECM structure intact (absence of areas of ECM disruption) (Fig. 2). However, when these detergents were used at a concentration of 1% for 2 h, a total removal of cell nuclei was observed, while still maintaining the structure of the ECM (100% containing between 0 and 3 cells), and absence of areas of ECM disruption (Fig. 2). SDS and DS at 2% during 1 h of exposure completely removed all cells (100% containing between 0 and 3 cells) while maintaining an intact ECM structure (absence of areas of ECM disruption). Higher concentrations of SDS or DS at 2% and with a higher exposure time of 2 h resulted in efficient removal of all cells, but led to a high degree of ECM disruption (100% containing between 0 and 3 cells) (Fig. 2). However, while many areas of ECM disruption were observed, they were considered unsatisfactory decellularized agents (data not shown).

Among all tested protocols, DS at 2% for 1 h and SDS at 1% for 2 h exposure led to more efficient decellularization results, with complete cell removal while preserving ECM network architecture. Table 1 summarizes these findings. Further characterization assays were thus performed for these protocols only (DS at 2% for 1 h and SDS at 1% for 2 h).

Morphological analysis of decellularized scaffolds

For a more complete investigation of the post-decellularization ECM architecture, a complementary analysis with Sirius red, Masson's trichrome and Calejja staining was performed. The results strengthened our findings, highlighting that the ECM of the scaffolds that have been decellularized with DS and SDS have good maintenance of integrity, with emphasis in the apparent maintenance of elastic and collagen fibers (Fig. 3). Immunohistochemical analyses showed that type III collagen content was slightly reduced (Fig. 3), and type IV collagen content was further decreased compared to type III collagen (Fig. 3).

Polarized light assessment of collagen fibers showed that almost 100% of fibers were stained in red, which indicates that these fibers are compact and mature (Fig. 4). Moreover, neither detergent significantly influenced collagen organization on the scaffolds, when compared to controls.

Biomechanical characterization

Decellularized scaffolds produced using SDS, DS, and control veins ($n=13$) were longitudinally stretched to failure for the evaluation of mechanical properties. Test results were interpreted as the load elongation diagram, results of the yield point (N), the coefficient of stiffness (N/mm), and failure load (N) values. Results showed a large variability (standard deviation) amongst decellularized scaffolds as well control scaffolds (when inside groups were compared), which can be explained by fissures on the vessels as well as fragility caused by the sectioning of lumbar tributary vein. There were no statistically significant differences between the three groups for all tested parameters. However, slightly reduced (non-significant) elasticity and rigidity of the decellularized veins, (compared to controls), were observed (Table 2).

Toxic residual effects of decellularized scaffolds

Both employed assays (Trypan blue and caspase-3) did not result in statistically significant differences among control veins and decellularized veins for their viability or apoptosis of cells exposed to scaffolds (Table 3). The exception was the DS which showed viability significantly superior to the other experiments (Table 3).

Discussion

Autologous transplantation (AT) still remains the main form of treatment for PAD; however AT is not always possible and the remaining treatment alternative is the use of synthetic materials whose longevity is less known [28]. Tissue engineering is a promising alternative for the production of tissues and organs, especially when there is a lack of appropriate donor tissues, aiming at providing biocompatible grafts to replace damaged, unfunctional organs or tissues [29–31].

Over the years, biotechnology-derived techniques have boosted the development of numerous scaffolds which may be artificially constructed or derived from natural compounds. Most synthetic scaffolds are produced with polyglycolic acid (PGA) and its variants, such as polyglycolic acid-poly-L-lactic acid (PGAPLLA), among others [7,25,32]. Scaffolds derived from these materials can be produced in a controlled way, in different shapes, and with different porosity coefficients. However, synthetic

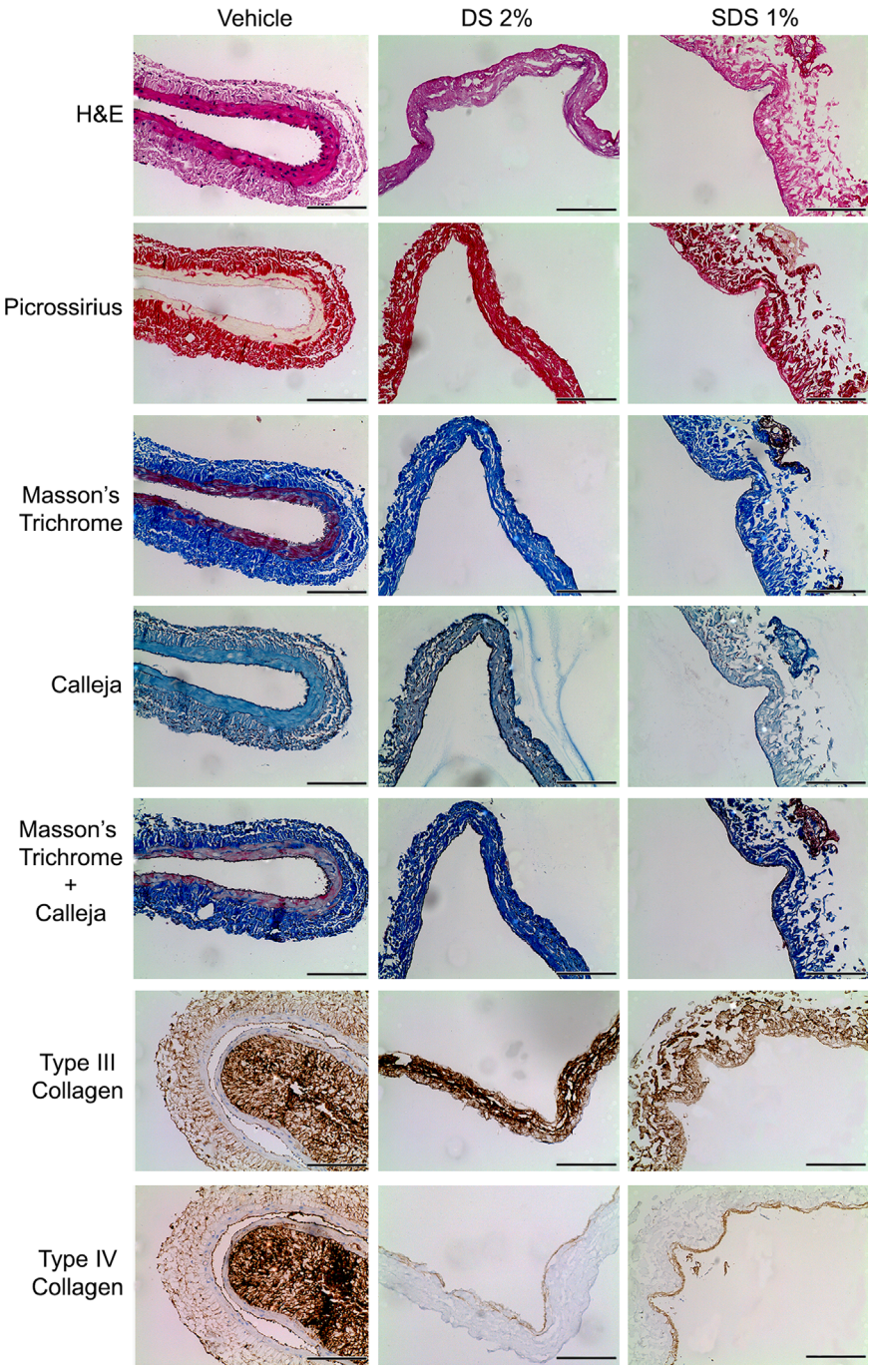

Fig. 3 – Representative sections of rabbit control (vehicle) blood vessels and blood vessels submitted to different detergent decellularization agents, stained with H&E (for general histology), picrossirius (collagen stains in red), Masson's trichrome (collagen stains in blue), calleja (elastic fibers stains in dark blue/black), combined Masson's and calleja (collagen stains in blue, elastic fibers in dark blue/black), and sections probed with anti-collagen III and IV after immunohistochemistry ($n=3$ for each analysis). Generally, all decellularization agents preserved the extracellular matrix structure of the blood vessels in comparison to vehicle (control) vessels. This can be verified as the red, blue, and dark blue colors are present at decellularized vessels (in sections stained with picrossirius, Masson's trichrome, and calleja, respectively). Type III collagen content was slightly reduced by the treatments, and type IV collagen reduced after the decellularization processes. Scale bars=500 μm.

polymer scaffolds are more thrombogenic, more rigid, potentially inflammatory, display higher immunogenic reactions, possess toxic degradation, and may induce fibrosis, leaflet retraction, and early stenosis [8]. In addition, previous reports show that biodegradable polyglactin–PGA used to produce vascular scaffolds is unstable and causes aneurysm formation [33].

While these artificial products are widely employed and characterized, little effort has been put on the use of natural

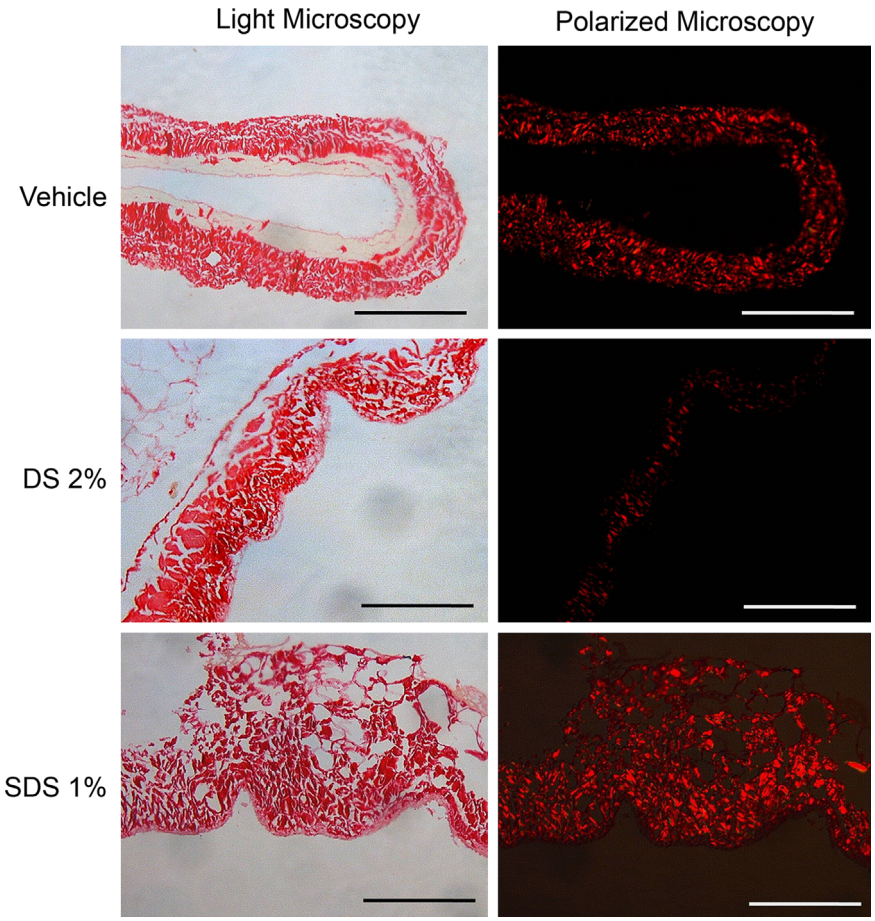

Fig. 4 – Representative sections of rabbit control (vehicle) blood vessels and blood vessels submitted to DS 2% and SDS 1% decellularization agents, stained with picrossirius, under light (left column) and polarized (right column) microscopy ($n=3$ for each analysis). The image shows that the vessels' collagen content, stained in red with picrossirius, (on the left column), was characterized mainly as compact old collagen fibers, stained in dark red, as seen in the right column, under the polarized light. Apparently, sodium deoxycholate (DS) and sodium dodecyl sulfate (SDS), at these concentrations, are able to preserve the collagen architecture. This resembles the collagen architecture of the vehicle (control) vessels. Scale bars = 500 μm.

biological compounds as the main source of scaffold. Biological scaffolds may circumvent the hurdles of synthetic scaffolds, as they are much more cell-compatible, such as the components of the ECM: collagen, elastin, fibronectin, hyaluronic acid, glycosaminoglycans (GAGs), and others, which benefits from their bioactive, biocompatible, and similar mechanical properties compared to in vivo tissues [8,33,34]. One approach to obtain biological 3D scaffolds is to employ the original tissue where the cell cultures shall be seeded in the moment of transplantation. To this purpose, however, in a heterologous approach, these scaffolds need to be decellularized to prevent immune rejections. Previous reports suggest that scaffolds derived from decellularized tissues have a facilitating role in the differentiation and obtainment of the expected tissue, as they contain the bioactive molecules and ligands necessary for stem cell maturation and diffusion of nutrients, as well as the naturally occurring 3D tissue architecture [11,33].

Given that the most commonly used tissue graft substitute for cardiovascular surgery in humans is the great saphenous vein, it seemed logical to study, initially in animal models, the behavior of decellularized veins, given that it is feasible to set up banks of decellularized veins from cadaveric donors, aiming at, in the future, the customization of blood vessel production. In this report, we have chosen the infrarenal inferior vena cava, due to similarities such as diameter, thickness, and histological structure to the great saphenous vein in humans [35].

We first demonstrated that the production of decellularized veins was feasible, depending on the detergent selected for this purpose. While Triton X100 does not completely remove cell nuclei, SDS and DS are more efficient for decellularization, being also able to preserve ECM matrix architecture. Interestingly, other authors have reported successful decellularization using Triton X100; however, not only were they decellularizing other tissues, but they also employed different concentrations and time of exposure that could account for the discrepancies observed in the efficacy of cell removal by Triton X100 [4,35,36]. Given that for heterologous usage these veins must be cell-free, and Triton X100 did not completely remove cell nuclei, this detergent cannot be used to produce blood vessel scaffolds for therapeutic purposes and was removed early from our study.

Even though cell removal is a key point in the process of decelularization for scaffold production, it also is important that the remaining ECM structure is maintained. Previous reports have shown that ECM components such as collagen, elastin, fibronectin,

Table 2 – Biomechanical characterization of vehicle (control) and decellularized blood vessels. Biomechanical analysis of vehicle treated and decellularized blood vessels. Data are expressed as mean ± standard deviation (SD) ($n=13$). After statistical analysis (described in "Materials and Methods") no significant values were observed when all treatments were compared to control vessels, meaning that DS and SDS are able to preserve the scaffold's biomechanical properties.

Biomechanical parameters	Treatments		
	Vehicle	DS	SDS
Yield point (N)	2.275 ± 0.709	1.890 ± 0.562	2.073 ± 0.894
Stiffness coefficient (N/mm)	0.683 ± 0.237	0.493 ± 0.190	0.613 ± 0.270
Failure load (N)	2.540 ± 0.735	2.164 ± 0.612	2.255 ± 0.872

Mean ± SD. All obtained results were non-significant.

Table 3 – Toxic residual effects of decellularized scaffolds. Data is expressed as mean ± standard deviation (SD). After statistical analysis (described in "Materials and Methods") only a significant value was observed, when DS treatment was compared to control vessels, however, with higher viability. In this sense, the data shows that the scaffolds do not have significant toxic residual effects, as observed by the high viability and low positive counts for caspase 3 cells. Experiments were carried out in triplicate.

Parameters	Treatments		
	Vehicle	DS	SDS
Apoptosis—trypan blue (% live cells)	74.933 ± 12.219	88.133 ± 4.502*	83.767 ± 1.563
Apoptosis—caspase 3 (% positive cells)	0.883 ± 0.329	1.250 ± 0.581	1.567 ± 1.266

Mean ± SD.
* Statistically significant difference with $p<0.05$ in comparison with vehicle treated vessels.

vitronectin, and laminin are responsible for cell adhesion, proliferation, differentiation, and thus maintaining the structure of the vessel [37]. Herein, scaffolds obtained by the two best protocols for cell removal were further analyzed for ECM composition.

In the second step, we demonstrated that the adjacent connective tissue content was fairly preserved after decellularization with SDS and DS, with special emphasis for elastin and type-I collagen (results also obtained and reported for other tissues) [8,35]. For a better understanding of the remaining collagen content on these decelularized scaffolds, we performed immunohistochemistry for type-III collagen, which was well preserved after treatment. However, we also performed the same technique for type-IV collagen, which was slightly decreased in both treatments compared with the controls. This finding raised a concern about the mechanical stability of the decellularized scaffolds and prompted us to move to the next phase of investigation [35].

In a third step, the biomechanical tensile experiments showed no significant statistical differences with the control blood vessels ($p>0.05$), demonstrating that the strength of the scaffolds, produced with SDS and DS, is very similar to the veins that did not undergo decelularization. This leads to the conclusion that the scaffolds maintain their natural mechanical tensile resistance after the decellularization process, mainly because the elastin and collagen (type I and III) content are preserved. Other mechanical properties were investigated through yield point, stiffness, and failure load testing, which also were similar to non-decellularized blood vessels ($p>0.05$). Other authors performed similar experiments with aorta or aortic segment of other animals with similar results [35,38].

Having demonstrated that SDS and DS are efficient at removing cells, preserving the ECM proteins, and preserving the desired biomechanical properties, we finally investigated whether these agents had residual toxicity in the produced scaffolds that could subsequently impair their use in cell culture. We showed that toxicity was not observed when stem cell cultures were exposed to these scaffolds, as there was no decrease in viability of living cells, as observed by Tripan blue dye. To confirm this finding, new cultures were exposed to these scaffolds, probed with anti-caspase 3 antibodies, and analyzed using flow cytometry. The results confirmed the previous findings that SDS and DS do not induce significant toxicity; therefore it is likely safe to use these detergents, at the optimal concentration and time of exposure demonstrated herein, as a base of decelularization for tissue engineering.

Conclusion

The methods of decellularization of blood vessels, in rabbits, can be performed efficiently while maintaining ECM architecture with two different protocols tested: 1% SDS for 2 h or 2% DS for 1 h of exposure. Decelularized scaffolds were mechanically tested to evaluate their strength and stiffness and proved surprisingly similar with control veins, indicating that they are resistant enough to be tested in vivo. Moreover, there was no residual toxicity of these scaffolds as observed in stem cell cultures. In this sense, these techniques may be suitable for tissue engineering of blood vessels for future clinical use.

Conflict of interest statement

The authors declare no conflicts of interest.

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REFERENCES

[1] V.L. Roger, A.S. Go, D.M. Lloyd-Jones, R.J. Adams, J.D. Berry, T.M. Brown, M.R. Carnethon, S. Dai, G. de Simone, E.S. Ford, Heart disease and stroke statistics—2011 update a report from the american heart association, Circulation 123 (2011) e18–e209.
[2] B.C. Isenberg, C. Williams, R.T. Tranquillo, Small-diameter artificial arteries engineered in vitro, Circ. Res. 98 (2006) 25–35.
[3] O.E. Teebken, A. Haverich, Tissue engineering of small diameter vascular grafts, Eur. J. Vasc. Endovasc. 23 (2002) 475–485.
[4] P.M. Crapo, T.W. Gilbert, S.F. Badylak, An overview of tissue and whole organ decellularization processes, Biomaterials 32 (2011) 3233–3243.
[5] H. Nagase, R. Visse, G. Murphy, Structure and function of matrix metalloproteinases and timps, Cardiovasc. Res. 69 (2006) 562–573.
[6] P. Bornstein, E.H. Sage, Matricellular proteins: extracellular modulators of cell function, Curr. Opin. Cell Biol. 14 (2002) 608–616.
[7] M. Peck, D. Gebhart, N. Dusserre, T.N. McAllister, N. L'Heureux, The evolution of vascular tissue engineering and current state of the art, Cells Tissues Organs 195 (2011) 144–158.
[8] J.G. Nemeno-Guanzon, S. Lee, J.R. Berg, Y.H. Jo, J.E. Yeo, B.M. Nam, Y.-G. Koh, J.I. Lee, Trends in tissue engineering for blood vessels, J. Biomed. Biotechnol. 2012 (2012) 9563451.
[9] R.M. Nerem, Tissue engineering a blood vessel substitute: the role of biomechanics, Yonsei Med. J. 41 (2000) 735–739.
[10] A. de Mattos Carvalho, A.L.G. Alves, M.A. Golim, A. Moroz, C.A. Hussni, P.G.G. de Oliveira, E. Deffune, Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue, Vet. Immunol. Immunopathol. 132 (2009) 303–306.
[11] M. Bertanha, A. Moroz, R. Almeida, F.C. Alves, M.J. Acorci Valério, R. Moura, M.A.C. Domingues, M.L. Sobreira, E. Deffune, Tissue-engineered blood vessel substitute by reconstruction of endothelium using mesenchymal stem cells induced by platelet growth factors, J. Vasc. Surg. S0741–5214 (13) (2013) 00973–00977 (pii).
[12] P.J. Schaner, N.D. Martin, T.N. Tulenko, I.M. Shapiro, N.A. Tarola, R.F. Leichter, R.A. Carabasi, P.J. DiMuzio, Decellularized vein as a potential scaffold for vascular tissue engineering, J. Vasc. Surg. 40 (2004) 146–153.
[13] T.W. Gilbert, T.L. Sellaro, S.F. Badylak, Decellularization of tissues and organs, Biomaterials 27 (2006) 3675–3683.
[14] B.D. Elder, S.V. Eleswarapu, K.A. Athanasiou, Extraction techniques for the decellularization of tissue engineered articular cartilage constructs, Biomaterials 30 (2009) 3749.
[15] S. Funamoto, K. Nam, T. Kimura, A. Murakoshi, Y. Hashimoto, K. Niwaya, S. Kitamura, T. Fujisato, A. Kishida, The use of high-hydrostatic pressure treatment to decellularize blood vessels, Biomaterials 31 (2010) 3590–3595.
[16] S.L. Dahl, J. Koh, V. Prabhakar, L.E. Niklason, Decellularized native and engineered arterial scaffolds for transplantation, Cell Transplant. 12 (2003) 659–666.
[17] E. Rieder, M.-T. Kasimir, G. Silberhumer, G. Seebacher, E. Wolner, P. Simon, G. Weigel, Decellularization protocols of porcine heart valves differ importantly in efficiency of cell removal and susceptibility of the matrix to recellularization with human vascular cells, J. Thorac. Cardiovasc. Surg. 127 (2004) 399–405.
[18] A. Moroz, R.A.C. Bittencourt, R.P. Almeida, S.L. Felisbino, E. Deffune, Platelet lysate 3d scaffold supports mesenchymal stem cell chondrogenesis: an improved approach in cartilage tissue engineering, Platelets 24 (2012) 219–225.
[19] A. Patel, B. Fine, M. Sandig, K. Mequanint, Elastin biosynthesis: the missing link in tissue-engineered blood vessels, Cardiovasc. Res. 71 (2006) 40–49.
[20] C.B. Weinberg, E. Bell, A blood vessel model constructed from collagen and cultured vascular cells, Science 231 (1986) 397–400.
[21] L.C. Junqueira, G. Bignolas, R. Brentani, Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections, Histochem. J. 11 (1979) 447–455.
[22] W. Yoshida, S. Müller, I. Carvalho, V. Fabris, L. Naresse, F. Maffei, Tensile strength and histological changes of abdominal aorta of malnourished rats, Vascular 3 (1995) 437–439.
[23] N.F. Cerqueira, W.B. Yoshida, S.S. Müller, J.L. Sequeira, de Rodrigues A.C., C.R. Padovani, Morphological and biomechanical study of abdominal aorta of rats submitted to experimental chronic alcoholism, Acta Cir. Bras. 20 (2005) 213–218.
[24] S. Lee, Y. Fung, M. Matsuda, H. Xue, D. Schneider, K. Han, The development of mechanical strength of surgically anastomosed arteries sutured with dexon, J. Biomech. 18 (1985) 81–89.
[25] N. L'heureux, S. Pâquet, R. Labbé, L. Germain, F.A. Auger, A completely biological tissue-engineered human blood vessel, FASEB J. 12 (1998) 47–56.
[26] R.G. Jaldin, É. Castardelli, J.E. Perobelli, W.B. Yoshida, A. de Castro Rodrigues, J.L. Sequeira, S.A.R. Paiva, Morphologic and biomechanical changes of thoracic and abdominal aorta in a rat model of cigarette smoke exposure, Ann. Vasc. Surg. 27 (2013) 791–800.
[27] M.D. Jacobson, M. Weil, M.C. Raff, Programmed cell death in animal development, Cell 88 (1997) 347–354.
[28] L. Norgren, W.R. Hiatt, J.A. Dormandy, M.R. Nehler, K.A. Harris, F.G. Fowkes, Inter-society consensus for the management of peripheral arterial disease (tasc ii), J. Vasc. Surg. 45 (2007) S5–S67.
[29] A. Atala, Tissue engineering and regenerative medicine: concepts for clinical application, Rejuvenation Res. 7 (2004) 15–31.
[30] J.R. Fuchs, B.A. Nasseri, J.P. Vacanti, Tissue engineering: a 21st century solution to surgical reconstruction, Ann. Thorac. Surg. 72 (2001) 577–591.
[31] M. Patruno, T. Martinello, Treatments of the injured tendon in veterinary medicine: from scaffolds to adult stem cells, Histol. Histopathol. 29 (2014) 417–422.
[32] G. Matsumura, N. Nitta, S. Matsuda, Y. Sakamoto, N. Isayama, K. Yamazaki, Y. Ikada, Long-term results of cell-free biodegradable scaffolds for in situ tissue-engineering vasculature: in a canine inferior vena cava model, PLoS One 7 (2012) e35760.
[33] H.L. Prichard, R.J. Manson, L. DiBernardo, L.E. Niklason, J.H. Lawson, S.L. Dahl, An early study on the mechanisms that allow tissue-engineered vascular grafts to resist intimal hyperplasia, J. Cardiovasc. Transl. Res. 4 (2011) 674–682.
[34] H. Naderi, M.M. Matin, A.R. Bahrami, Review paper: critical issues in tissue engineering: biomaterials, cell sources, angiogenesis, and drug delivery systems, J. Biomater. Appl. 26 (2011) 383–417.
[35] A.F. Pellegata, M. Asnaghi, I. Stefani, A. Maestroni, S. Maestroni, T. Dominioni, S. Zonta, G. Zerbini, S. Mantero, Detergent-enzymatic decellularization of swine blood vessels: insight on mechanical properties for vascular tissue engineering, Biomed. Res. Int. 2014 (2014) 412838.
[36] J.S. Uzarski, A.B. Van De Walle, P.S. McFetridge, Preimplantation processing of ex vivo-derived vascular biomaterials: effects on peripheral cell adhesion, J. Biomed. Mater. Res. A 101 (1) (2013) 123–131.
[37] J. Rouwkema, et al., The use of endothelial progenitor cells for prevascularized bone tissue engineering, Tissue Eng. 15 (8) (2009) 2015–2027.
[38] J.C. Fitzpatrick, P.M. Clark, F.M. Capaldi, Effect of decellularization protocol on the mechanical behavior of porcine descending aorta, Int. J. Biomater. (2010) pii: 620503. http://dx.doi.org/10.1155/2010/620503. Epub 2010 Jul 4.