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Evaluation of supercritical CO2 extraction protocols in a model of ovine aortic root decellularization

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Abstract

One of the leading trends in the modern tissue engineering is the development of new effective methods of decellularization aimed at the removal of cellular components from donor tissue reducing its immunogenicity and the risk of rejection. Supercritical CO2 (scCO2) extraction can significantly improve the outcome of decellularization, reduce contamination and time costs. The resulting products can serve as personalized tools for tissue-engineering therapy of various somatic pathologies. However, decellularization of complex 3D structures, such as the aortic root, requires optimization of the parameters, including preconditioning medium composition, type of co-solvent, values of pressure and temperature insight the scCO2 reactor, etc. In our work, using an ovine aortic root model, we performed a comparative analysis of the efficiency of decellularization using approaches based on various combinations of these parameters. The protocols were based on combinations of treatment in alkaline, ethanol or detergent solutions with scCO2 extraction at different modes. Based on a histological analysis, we have selected an optimal protocol for the decellularization of ovine aortic root employing preconditioning in a detergent solution. The positive effects of scCO2 on the decellularization extent, cytotoxicity and histoarchitecture of the tissue were demonstrated.

1. Introduction

The aortic valve disorder is a widespread condition often requiring surgical replacement of the valve [1]. However, existing prostheses possess certain drawbacks, which limit their distribution in the clinical practice [2,3]. For example, the implantation of a mechanical prosthesis requires a life-long anticoagulant therapy associated with the risks of anticoagulant-related hemorrhage [4,5]. Alternative approach is based on the application of biological prostheses taking favor of superior hemodynamic properties and avoidance of a chronic anticoagulant therapy. Traditionally such valves are made from animal tissues treated with crosslinking agents in order to reduce the antigenicity. As a result of this treatment, the implanted valve undergoes continuous calcification requiring repeated surgical intervention [6]. In recent decades, numerous methods for valve decellularization have been developed [7,8]. Decellularization is a method of processing biomaterial aimed at removing cells from tissue while preserving the extracellular matrix (ECM) composition and architectonics [9, 10]. The use of this method for fabrication of aortic valve prostheses is meant to to reduce the immunogenicity of the grafts while preserving biomechanical properties and improving the long-term prognosis for patients undergoing valve replacement surgery [11,12].

Existing technologies for decellularization include treatment with detergents (sodium dodecyl sulfate, SDS

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[13], sodium deoxycholate, SD [14], Triton X-100 [15], etc.), enzymes (trypsin) [16,17] and alkali [18], as well as the methods of cyclic freezing-thawing and high pressure exposure (up to 1 GPa) [19]. However, the use of detergents often increases the cytotoxicity of the graft and requires time-consuming washing with a risk of bacterial contamination [20]. Enzymatic hydrolysis and the method of cyclic freezing-thawing are less effective and disrupt the structural organization of ECM [17,21]. The method of decellularization under the exposure to a high pressure requires complex and expensive hardware.

Since 2008, research has begun in the field of decellularization of mammalian tissues with $scCO_2$ [22]. Relative chemical inertness, favorable economic characteristics, such as cheapness and facile accessibility, as well as relatively low critical values of pressure (7.38 MPa) and temperature (31.1 0C), make $scCO_2$ a commercially viable solvent [23,24]. Due to the minimal environmental impact, the $scCO_2$ extraction is referred to as a method of the so-called "green chemistry" and is widely used in the food, textile, perfume and cosmetic industries [25,26].

$scCO_2$ can act either as an independent solvent, or in combination with traditional decellularizing agents [27-29] or co-solvents such as ethanol [22,30,31]. The use of $scCO_2$ significantly improves the decellularization outcome, reduces the risk of contamination and processing time.

Along with the decellularization, $scCO_2$ is widely used for sterilization of medical devices [32-35]. The combination of such stages as decellularization and sterilization in one environmentally friendly technological process can significantly reduce energy costs and increase economic efficiency in the production of transplants. All these advantages make $scCO_2$ a powerful tool for tissue decellularization.

However, the decellularization of complex 3D structures, such as the aortic root, in $scCO_2$medium requires optimization of the parameters, including preconditioning medium composition, type of co-solvent, values of pressure and temperature insight the $scCO_2$ reactor, etc. in order to achieve complete cell removal while preserving the ECM structure. In our work, using an ovine aortic root model, we performed a comparative analysis of the efficiency of decellularization using approaches based on various combinations of these parameters. The protocols were based on combinations of treatment in alkaline, ethanol or detergent solutions with $scCO_2$ extraction at different modes.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (Merck, USA), unless otherwise specified.

2.1. Isolation of the aortic root

The experiments were carried out after the approval of the local ethics committee (Sechenov University, Moscow). Aortic root decellularization was performed on 70 valve-containing fragments of the ovine aortas. Sheep organocomplexes were obtained at a local slaughterhouse and transported to the laboratory in a thermocontainer filled with sterile phosphate-buffered saline (PBS, EcoService, Russia) at +4 0C. The isolation of the heart from the organocomplex was carried out under sterile conditions.

The procedure for isolating the aortic root was carried out in a laminar flow biological safety box, class II, Safe 2020 1.2 (Thermo FS, USA) in several stages. At the first stage, the access to the aortic root was provided. Then the pericardium was opened and dissected, the main vessels were inspected. Surrounding tissues located distal to the great vessels, as well as around them, were removed. Aortopulmonary curtain was dissected, separating the aorta from the main pulmonary artery. Then, using scissors, connective tissue cords were sequentially separated, gradually approaching the aortic root. The pulmonary artery was removed. Coronary arteries were isolated. At the second stage, the excessive myocardium tissue was removed. To this purpose, the myocardium of the right ventricle and atrium was dissected. Next, the left atrial appendage was excised, the posterior leaflet was identified and the myocardium of the left ventricle was dissected along it. Then the chords of the anterior mitral valve leaflet were removed and most of the myocardium was cut off, leaving only the basal part. Finally, with high precision steps, the muscle cuff of the aortic root was gradually formed. During the isolation process, the material was periodically irrigated with non-buffered isotonic saline

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(0.9% NaCl) to prevent tissue drying. Aortic valves without apparent macroscopic pathology were included in the study.

2.2. Aortic root decellularization

Decellularization was carried out within 4-6 hours after the isolation of the aortic roots as summarized in Table 1. 250 ml sealing glass containers (Simax, Czech Republic) were used for preconditioning in order to eliminate the risk of unwanted contamination. The aortic valves were rinsed with povidone-iodine solution (10% Betadine, Egis, Hungary) and dipped into the containers with sterile preconditioning medium at 25 0C under constant stirring at a speed of 200 rpm on OS-20 orbital shaker (Biosan, Latvia) followed by a washing cycles with PBS (12 h). The $scCO_2$ extraction was performed using an Applied Separations unit (USA). The experimental setup is shown in Supplementary Figure S5. The extraction was carried out at a temperature of 37 ± 0.2 0C, pressure of 10-25 MPa and flow rate of 3 ± 0.5 ml/min. An ovine aortic root was placed in a high-pressure reactor (working volume 100 ml), the CO_2 chamber was filled up at a pressure of 6-7 MPa, then the thermostat was turned on and the temperature was set at 37 0C and the pressure was adjusted to 10-25 MPa (achievement of the operating parameters was about 20 minutes). Next, the fine adjustment valve set the flow rate to 3 ml/min. Under such conditions, the samples were kept for 3 hours, then the thermostat was turned off and slowly, over 30 minutes, the pressure was reduced.

Table 1. The protocols for decellularization of ovine aortic root.

Treatment method	**Ref.**	**Preconditioning**	**Extraction parameters**	**Co-solvent**
$scCO_2$-Ethanol	[22]	None	T=37 0C, P=15 MPa, t=3 h	95% Ethanol
			T=37 0C, P=25 MPa, t=3 h	95% Ethanol
Alkali-salt + $scCO_2$	[27]	1 M NaOH+0.8 M Na_2SO_4 (1 h)	T=37 0C, P=10 MPa, t=1 h	None
			T=37 0C, P=15 MPa, t=1 h	None
Detergent	[8]	0.5% SDS/0.5% SD (12 h)	-	None
Detergent + $scCO_2$	[27,29]	0.5% SDS/0.5% SD (12 h)	T=37 0C, P=15 MPa, t=3 h	None
			T=37 0C, P=25 MPa, t=3 h	None

The control groups included untreated native samples (negative control) and decellularized in detergent solution according to a state-of-the-art protocol (positive control).

2.3. Histological analysis

Aortic walls and valve leaflets of 5 samples in each experimental group were harvested for the histological study. The tissue samples were fixed in a solution of 10% neutral formalin, dehydrated, embedded in paraffin and sectioned into 4 μm transverse sections (3 sections per block). The transverse sections were then stained with hematoxylin and eosin to evaluate the general histoarchitecture of the tissues. Toluidine blue staining was used to visualize glycosaminoglycans (GAGs), picrosirius red – for collagen fibers, Weigert's resorcin fuchsin staining – for elastic fibers and lamellae. After embedding into the ≪Shandon mount TM≫ mounting medium (USA), and covering with coverslips, the samples were studied with a Leica DM4000 B LED universal microscope, equipped with a Leica DFC7000 T camera running under LAS V4.8 software (Leica Microsystems, Switzerland). The morphological analysis was performed using bright-field and polarized light microscopy techniques.

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The morphometric study of the tissue structure was performed using microscopic images acquired in 10 representative and randomly selected fields of view (FOV, 400×). We analyzed the mean numbers of intact fibroblasts and endothelial and smooth muscle cells, the density of collagen fibers, elastic fibers and lamellae in FOVs in each layer of aortic walls and valve leaflets. The density of collagen fibers and elastic structures was calculated as a ratio of their area to the area of each layer of aortic walls and valve leaflets and carried out by digital analysis with Adobe Photoshop CS6 software. The degree of porosity (histoarchitecture preservation) and metachromatism (GAGs content) of the scaffolds in the samples were also assessed by a semi-quantitative evaluation (Supplementary Table S1).

2.4. DNA quantification test

To determine the DNA concentration, the samples of decellularized and native aortic tissues were freeze-dried and cut into fragments weighing ˜ 5 mg. Fragments obtained from the same sites were digested in Collagenase A (from *Clostridium histolyticum*) solution (2.5 mg/ml) in a buffer (50 mM Tris-HCl, pH 7.4) containing 10 mM calcium chloride and 0.02 mg/mL sodium azide (Paneco, Russia). The samples were then incubated at 37 °C with periodic stirring on an IKA MS 3 BASIC SHAKER vortex mixer (Sigma Aldrich, USA). For the following isolation of DNA, a standard set of reagents (Evrogen, Russia) was used in accordance with the recommendations of the manufacturer. The amount of DNA was measured using the QuantiFluor dsDNA kit and a Quantus fluorimeter (Promega, Madison, WI, USA).

2.5. MTT-test

MTT-test was adopted from ISO 10993 in order to evaluate the effect of soluble components of decellularized ovine aortic roots on the viability of 3T3 murine fibroblast cells. Tissue specimens were freeze-dried, sterilized by γ-radiation and incubated in 1 ml (180 mg dry weight) of DMEM/F12 culture medium supplemented with 100 U/mL streptomycin, 100 g/mL penicillin, 1% (v/v) GlutaMAX (Gibco), 5% (v/v) fetal bovine serum (HyClone) for 24 h at 37 °C. Serial dilutions of the obtained extracts in the culture medium were added in triplicates (100 µl) to a subconfluent monolayer of 3T3 murine fibroblasts in 96-well plates. Culture medium alone was used as a control. The plates were further incubated for 24 h at 37 °C in 5% (v/v) CO_2 in air. For the MTT-test, the extract and control media were replaced with 100μ L of the MTT solution (0.5 mg/mL in the culture medium without supplements) followed by the incubation in a CO_2-incubator at 37 °C for 3 h. After discarding the MTT solution, 100 μ L aliquots of dimethyl sulfoxide were added to all the wells and shaken. The color developed was quantified by measuring absorbance at 567 nm using the *Multiscan FC* microplate photometer (reference, 650 nm).

2.6. Scanning electron microscopy

A standard sample preparation protocol was used as follows. After washing a cut block in PBS, it was incubated in buffered 2.5% glutaraldehyde for 24 h. After the fixation, the samples were washed again in PBS and dehydrated through an ascending ethanol gradient (30%, 40%, 50%, 60%, 70% and "absolute"). Final dehydration was achieved by exposure to acetone and critical-point-drying. After the dehydration, a metallic conductive film (Au-Pd, 50-100 nm) was applied onto the surface of the samples by the plasma spraying. The surface structure of the samples was visualized using a Zeiss EVO LS10 scanning electron microscope (Zeiss, Germany) operating under high vacuum.

2.7. Atomic-force microscopy

The mechanical characteristics of the aortic wall were evaluated with atomic force microscopy (AFM) using a Bruker Bioscope Resolve microscope in the Force Volume mode in a PBS buffer solution. Scanning parameters: all the pictures were taken on the same SQUBE cantilever with a spherical tip (tip radius = 2500 Nm), stiffness coefficient k = 0.0551 N/m. Force curve measurement speed 5 Hz, maps with a resolution of 32x32 points, scan size of map 5x5 µm. Mean value of the Young's modulus was calculated from 6-8 maps for each sample with use on the Hertz model.

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2.8. Statistical analysis

Statistical data analysis was performed using JASP software v0.11.1, KNIME v4.1.0, GraphPad Prism v8.4. Descriptive statistics minimum, maximum, mean ±SD, median (from 25 to 75 percentile) were calculated and Shapiro-Wilk test was performed. Spearman correlation coefficient was calculated for groups. For normally distributed data the one-way Brown-Forsythe ANOVA followed by post-hoc Dunnet T3 test was performed, in other cases the Kruskal-Wallis test was used, followed by post-hoc Dunn test. Graphs are presented as the medina ± 95% CI for non-parametric data and the mean ± 95% CI for parametric. Significance of differences was considered at p value <0.05.

3. Results

The histological analysis of the ovine aortic roots indicated three layers in aortic valve leaflets of all studied groups: ventricularis, fibrosa and spongiosa (Figure 1). The aortic walls in all groups were also composed of three layers: intima, media and adventitia.

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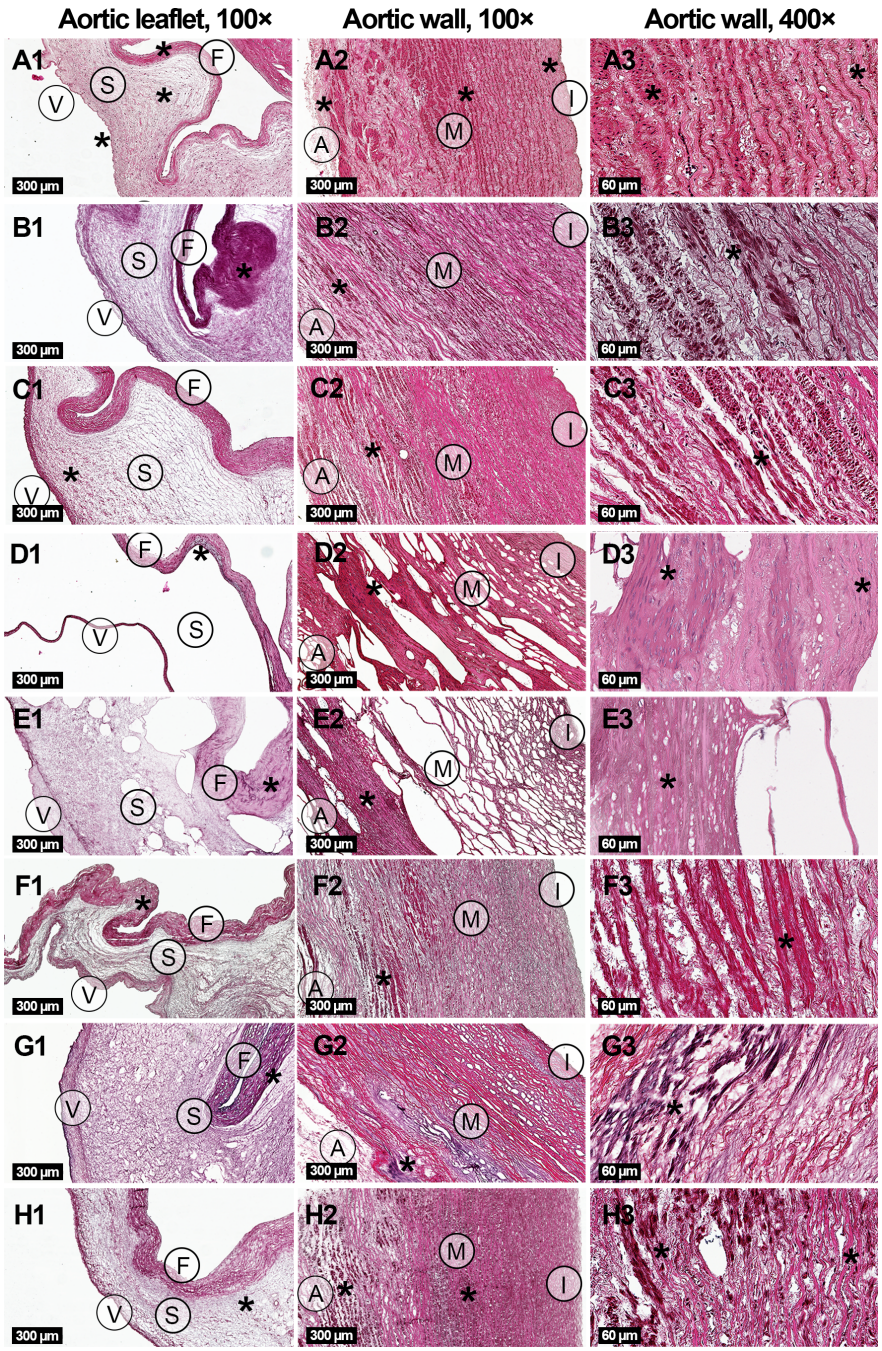

Figure 1. Histological study of native (A1-3) and decellularized ovine aortic roots under hematoxylin-eosin staining. Decellularization protocols included $scCO_2$ extraction at the pressure of 15 MPa **(B1-3)** and 25 MPa **(C1-3)** with the use of ethanol as a co-solvent; combination of alkaline treatment with $scCO_2$ extraction at the pressure of 10 MPa**(D1-3)** and 15 MPa **(E1-3)** ; detergents without $scCO_2$ extraction **(F1-3)** or with following $scCO_2$ extraction at the pressure of 15 MPa**(G1-3)** and 25 MPa **(H1-3)** . Three layers are indicated in aortic valve leaflets: ventricularis (V), fibrosa (F) and spongiosa (S); as well as three layers are indicated in aortic walls: intima (I), media (M) and adventitia (A). Accumulations of cells are shown with asterisks (*).

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Picrosirius red staining applied with bright-field and polarized light microscopy techniques confirmed the preservation of the native structure of collagen fibers in ECM in all sample groups (Supplementary Figure S1-S2). However, significant structural differences were found between the groups including the degree of changes in the sample histoarchitecture, decellularization and ECM organization (Figure S1-S4, Table S1). Hematoxylin-eosin staining revealed retained focal accumulations of intact cells (without karyopyknosis, karyolysis, plasmolysis) in some experimental groups (Figure 1).

3.1. Ethanol-assisted decellularization

Decellularization of ovine aortic roots by means of $scCO_2$ extraction with the use of ethanol as a co-solvent under the pressure of either 15 MPa or 25 MPa did not lead to significant changes in the histoarchitecture of the tissue samples and to their remarkable decellularization. However, a marked redistribution of fibers in the ECM appeared (Supplementary Figures S1-S4, Tables S2-S10). In particular, this protocol promoted condensation of collagen fibers in ventricularis. The density of ventricularis collagen fibers was significantly higher than that of the rest of the protocols, except for the alkali-assisted treatment (see below).

Condensation of the elastic fibers in aortic wall occurred in the intima and was the most pronounced at the pressure of 25 MPa when compared to 15 MPa. The density of elastic fibers in this experimental group was the greatest among all the protocols we studied. Elastic lamellae also condensed in the media and fibers in spongiosa of valve leaflets at 25 MPa and were denser than in most other experimental groups. However, the differences were not statistically significant compared to that achieved at 15 MPa. The density of the elastic lamellae in the aortic wall was higher compared to all other protocols, except for the combination of detergent treatment with $scCO_2$ extraction at the pressure of 15 MPa. Interestingly, after $scCO_2$extraction at 15 MPa the elastic fibers of fibrosa loosened, while at 25 MPa – condensed.

As the result of the decellularization procedure, a decrease in the number of valve fibroblasts and aortic endotheliocytes was achieved independent of the pressure value. Additionally, at the pressure of 25 MPa removal of smooth muscle cells occurred in the aortic wall.

The content of GAGs decreased in both the aortic wall and valve leaflets with not significant difference at 15 and 25 MPa and with native tissue.

3.2. Alkali-assisted decellularization

In contrast to the previous protocol, decellularization in $scCO_2$ after preconditioning in an alkali-salt solution caused conspicuous structural changes in both the aortic wall and valve leaflet ECM (Figure S1-S4, Tables S2-S10). In particular, all samples, regardless of the pressure value (10 or 15 MPa) acquired high porosity and were almost deprived of spongiosa, which significantly distinguished this protocol from all others.

Unlike with the previous alcohol-assisted protocol, the alkali-assisted decellularization provided removal of substantial number of smooth muscle cells in the aortic wall. As per endotheliocytes and fibroblasts, the significance varied when compared with the ethanol-assisted decellularization at either 15 MPa (greater in both the aortic wall and valve leaflets) or 25 MPa (greater endothelial decellularization in valve leaflets and fibroblast decellularization in the aortic wall).

ECM reorganization following alkali-assisted decellularization included condensation of collagen fibers in ventricularis of the valve leaflets, which was similar to the ethanol-assisted protocol with the maximum at the pressure of 10 MPa. In the aortic wall, a loosening of the fibers of the media was detected comparable with other protocols.

The collagen fiber packing in the aortic wall and valve leaflets was affected differently depending on the pressure value. For example, at 10 MPa, the collagen fibers in spongiosa were more loosely arranged compared to that in the native tissue and after ethanol-assisted decellularizaion, while 15 MPa, collagen fiber packing in fibrosa was denser.

The elastic fibers in fibrosa and elastic lamellae in media appeared loosened after alkali-assisted decellularization at both 10 and 15 KPa. The loosening of the elastic fibers in fibrosa at the pressure of 15 MPa was

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less pronounced than after the ethanol-assisted decellularization at 15 MPa, but higher than in all other groups. Loosening of the elastic lamellae in media at both 10 and 15 MPa also significantly differed from other protocols except for the detergent treatment followed by $scCO_2$ extraction at the pressure of 25 MPa. The arrangement of elastic fibers in the intima was similar to that after alcohol-assisted decellularization. Loosening of the elastic fiber in ventricularis tool place only at 15 MPa.

As opposes to the ethanol-assisted decellularization, the use of alkali led to a significant decrease in the content of GAGs in aortic wall and valve leaflets independent of the pressure value.

3.3. Detergent-assisted decellularization

The state-of-the-art protocol based on treatment in a detergent solution (SDS/SD) provided relatively high level of decellularization including removal of endothelial cells and fibroblasts from the aortic wall and valve leaflets, as well as smooth muscle cells from the aortic wall, with slight alterations in the ECM and histoarchitecture (Figure S1-S4, Tables S2-S10). The extent of the decellularization of the aortic valve leaflets, as well as removal of fibroblasts and smooth muscle cells from aortic wall was higher than that of ethanol-assisted protocol and comparable with the alkaline treatment. However, the endothelial decellularization in the aortic wall did differ when compared to all previously described methods. The inclusions of residual cellular material (fragments of cell membranes and nuclei) were identified in dense or deep structures, mostly between collagen fiber bundles in the pars fibrosa or spongiosa of the valve tissues and aortic media.

After the decellularization, the collagen fiber packing was only affected in the aortic valve leaflet fibrosa and spongiosa leading to their more loose arrangement. For the fibrosa, this effect was even more significant than after the alcohol- and alkali-assisted treatments.

In contract, the distribution of elastic fibers in the spongiosa was the most widespread of the all the experimental groups.

With this treatment protocol, the content of GAGs in the valve leaflets and aortic wall was lower than that after the ethanol-assisted decellularization. However, in aortic wall it was higher when compared to that after the alkaline treatment.

When the stage of $scCO_2$ extraction at the pressure of 15 MPa was introduced to the protocol, not significant improvement in the decelluarization outcome was achieved. The porosity of the valve leaflets increased, yet significantly less than after the alkaline treatment. The elastic fiber packing in spongiosa and ventricularis was less dense than before $scCO_2$ extraction.

A satisfactory decellularization avoid of the accumulations of residual nuclear and membrane fragments of fibroblasts and smooth myocytes was achieved after the $scCO_2$ extraction at the pressure of 25 MPa. In contract to the previous protocol employing the pressure of 15 MPa, at 25 MPa the increased porosity appeared in the aortic wall, but not in the valve leaflet.

Even though, no significant differences in the distribution of collagen fibers in the aortic wall and valve leaflets were found at different pressure values; when higher pressure was applied, the elastic fibers of fibrosa became more lose, while in spongiosa and ventricularis they remained intact. Elastic membranes were also looser in the media.

The content of GAGs after $scCO_2$ extraction did not differ from the state-of the-art protocol.

In order to confirm the extraction of residual nuclear material in $scCO_2$ ($P = 25$ MPa) medium following treatment with SDS/SD, we evaluated the level of residual DNA in the samples which was reduced from $5.14 \pm 0.01\%$ to $2.70 \pm 0.22\%$ of initial value.

Additionally, the cytotoxicity inherent to detergents was examined before and after $scCO_2$ extraction ($P = 25$ MPa). Figure 2A shows that $scCO_2$ improved cytocompatibility of the tissues by means of washing out residual detergents.

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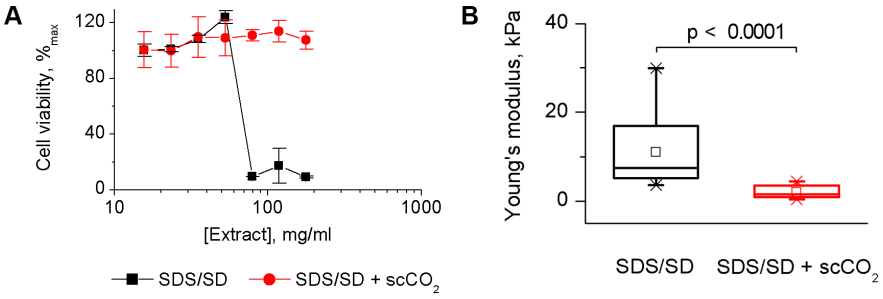

Figure 2. Cytotoxicity and mechanical characteristics of ovine aortic walls decellularized in a 0.5% SDS/0.5% SD detergent solution (SDS/SD) and after the subsequent extraction in $scCO_2$medium (T = 37 0C, P = 25 MPa, t = 3 h). **A.** MTT assay for the cytotoxicity estimation. **B.** Mechanical characteristics established by atomic force microscopy.

Measurement of the micromechanical characteristics of the aortic wall using AFM in a liquid environment revealed slight decrease in Young's modulus following $scCO_2$ extraction at 25 MPa. It is known that the mechanical properties of collagenous tissues are predetermined by the arrangement of the collagen fibrils [36]. The results of this study correlates with the increased porosity in the aortic wall revealed in histological study. Nonetheless, the preservation of the structural integrity of the material was confirmed by scanning electron microscopy in both aortic wall and valve leaflets following $scCO_2$ extraction at 25 MPa (Figure 3).

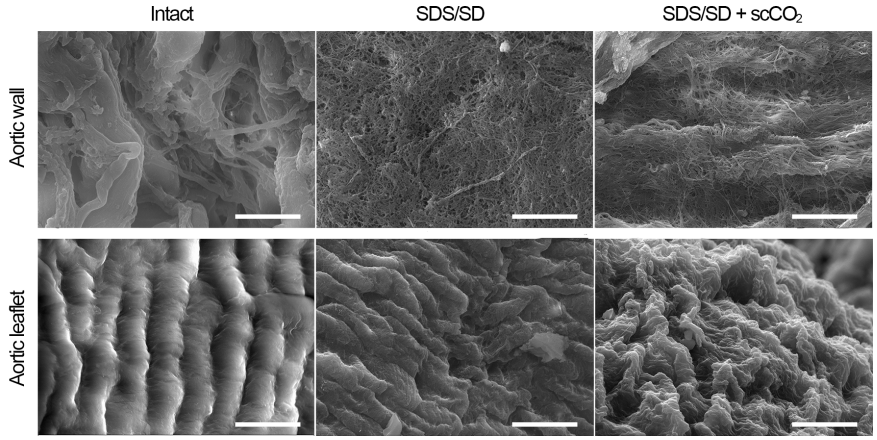

Figure 3. Scanning electron microscopy of ovine aortic rootsdecellularized in a 0.5% SDS/0.5% SD detergent solution (SDS/SD) and after the subsequent extraction in $scCO_2$ medium (T = 37 0C, P = 25 MPa, t = 3 h). Scale bar = 10 μm.

4. Discussion

The fabrication of aortic valve bioprostheses requires a precise control of structural-functional properties of the resulting product. Therefore, the decellularization procedure aiming at the elimination of the donor cell material must preserve the structural integrity of ECM with its inherent functions and cytocompatibility [37]. The approaches to decellularization based on the extraction in $scCO_2$medium have been recently established for various tissues [38]. In our work, we aimed to evaluate the feasibility of existing protocols and their modifications for aortic root decellularization. The object of our study was ovine aortic root decellularized in various combinations of $scCO_2$ medium with traditional decellularizing agents. The choice of the donor animal was due to the need for the most accurate modeling of immunological characteristics and hemodynamic parameters close to the human.

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Decellularized materials should not contain cellular components, including the cytoplasm and nuclei. Their presence in ECM can contribute to the violation of biocompatibility and cause adverse reactions after implantation. To assess the effectiveness of decellularization and maintaining the structural integrity of the ECM, we employed histological analysis.

In [22], Sawada et al. on the model of porcine aorta demonstrated the effectiveness of decellularization in $scCO_2$ (15 = MPa, T = 37 0C, t = 20 min) with ethanol employed as a co-solvent. In our work, we adopted this protocol to decellularization of a complex 3D structure, ovine aortic root, with a modification concerning the processing time (t = 3 h). However, no satisfactory decellularization was achieved, even at higher pressure (25 MPa), since numerous nuclei remained in the tissue. In addition, this protocol promoted condensation of the collagen fibers in ventricularis. The density of elastic fibers in aortic wall intima and elastic lamellae in the media and spongiosa of the valve leaflets also increased and was the greatest among all the protocols we used. This result may be related to tissue dehydration due to the hygroscopic nature of ethanol. Surprisingly, a dramatic difference was found between the elastic fiber organization in fibrosa after $scCO_2$ extraction at different pressure values. At 15 MPa, the fibers loosened, while at 25 MPa – condensed. These disadvantages limit the application of this protocols to the aortic root decellularization purposes.

In contrast to the previous protocol, alkali-assisted decellularization provided satisfactory removal of cells and GAGs from aortic wall and valve leaflets independent of the pressure value. However, structural derangements (porosity) in both the aortic wall and valve leaflet ECM and deprivation of spongiosa that was the hallmarks of this protocol, regardless of the pressure value (10 or 15 MPa). Even though in [27] this protocol enabled decellulariztion of porcine and bovine pericardim without significant effect on its mechanical and structural properties, it appeared unsuitable for the decellularization of ovine aortic root.

Finally, the detergent-based protocols were assessed using SDS/SD solution as a preconditioning medium followed by $scCO_2$extraction at the pressure of either 15 or 25 MPa. To begin with, we reproduced a state-of the-art protocol of decellularization based on detergent treatment. The extend of decellularization and GAG removal was comparable with that after the alkaline treatment. However, the inclusions of residual cellular material (fragments of cell membranes and nuclei) were identified in dense or deep structures, mostly between collagen fiber bundles in the pars fibrosa or spongiosa of the valve tissues and aortic media. The decellularization process appeared to result in the most widespread distribution of elastic fibers in the spongiosa of the all the experimental groups. The collagen fiber packing was only affected in the valve leaflet fibrosa and spongiosa leading to the more loose arrangement.

Not significant improvement in the decelluarization outcome was achieved after $scCO_2$ extraction at the pressure of 15 MPa, however, the aortic valve leaflet porosity increased. The opposite effects were found at 25 MPa. A satisfactory decellularization avoid of the accumulations of residual nuclear and membrane fragments of fibroblasts and smooth myocytes was achieved after the $scCO_2$ extraction that is in accordance with the data obtained earlier for porcine aorta in [29]. The increased porosity appeared in the aortic wall, but not in the valve leaflet.

Since the use of detergents is known to increase the cytotoxicity of the graft [20] we carried out a comparative *in vitro* cytotoxicity study of aortic valves decellularized in a detergent solution (SDS/SD) and after the subsequent extraction in $scCO_2$ medium (T = 37 0C, P = 25 MPa, t = 3 h). The dramatic decrease in cytotoxicity level was revealed after $scCO_2$ extraction (P = 25 MPa) which is favorable for possible clinical application of this protocol.

A decrease in the Young's modulus when measured by atomic force microscopy correlated with the increase in the porosity in the aortic wall revealed in histological study. It is known that an increase in transplant porosity contributes to cellularization [39] and angiogenesis [40]. Yet, the histoarchitecture of valve leaflets was preserved.

To conclude, we have selected an optimal protocol for the decellularization of ovine aortic root using the technology of extraction in $scCO_2$ medium and preconditioning in a detergent solution. The positive effect of $scCO_2$ on the decellularization, cytotoxicity, and porosity of the ovine aortic root decellularized by the

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detergent treatment method was shown.

Conclusion.

In the course of our experiments, we evaluated the effects of different protocols for decellularization of ovine aortic roots based on various combinations of treatment in alkaline, ethanol or detergent solutions with $scCO_2$ extraction. The ethanol-assisted protocols did not suffice in decellularization, while alkaline treatment deranged the ECM organization that can lead to the valve dysfunction, as a result of hemodynamic shifts and thromboembolism after implantation. These features make these protocols unsafe. However, favorable effects were demonstrated for the ovine aortic root decellularization by means of preconditioning in a detergent solution followed by $scCO_2$ extraction at the pressure of 25 MPa. They included satisfactory decellularization avoid of the accumulations of residual nuclear and membrane fragments of fibroblasts and smooth myocytes; low cytotoycity; preservation of the histoarchitecture of ECM of aortic valve leaflets and increase of porosity of aortic wall.

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Conflict of interest

None

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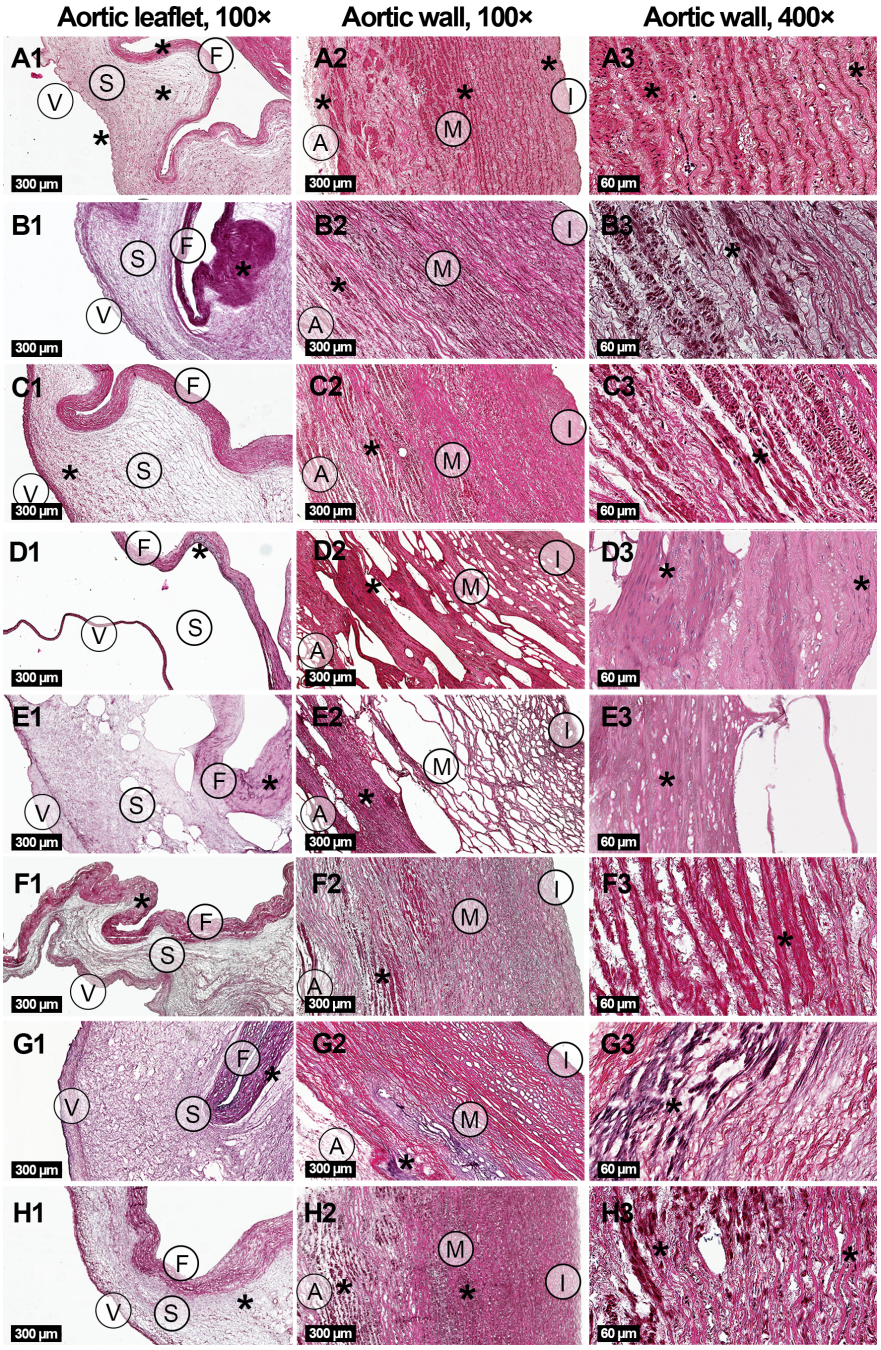

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