Waste and Biomass Valorization (2024) 15:4405–4417
https://doi.org/10.1007/s12649-024-02465-9

ORIGINAL PAPER

Physicochemical Decellularization of Bovine Pericardium: Effects on DNA Elimination, Extracellular Matrix Preservation, and Biocompatibility

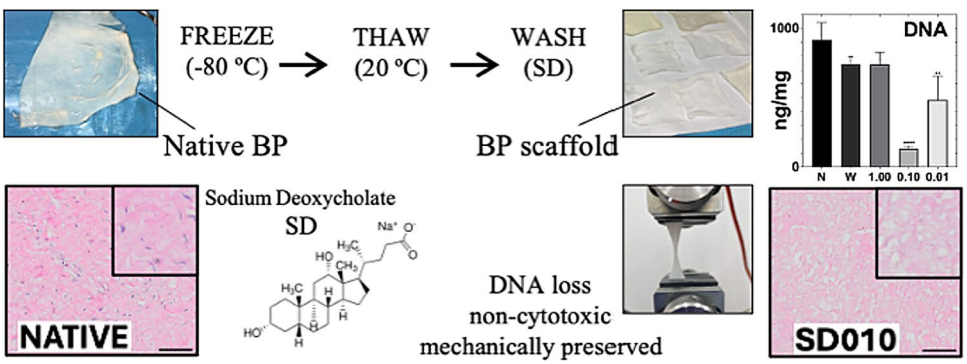
Rui C. Giorgi Filho[1] · André Miguel Martinez Junior[2] · Marilia F. Calmon[3] · Marcio José Tiera[2] · Dayane S. Alvares[4] · Guilherme Agreli[4] · José G. Nery[1]

Received: 14 September 2023 / Accepted: 10 February 2024 / Published online: 23 March 2024

Abstract

Bovine pericardium (BP) is an abundant waste biomass with applicability in tissue engineering, and decellularization is a requisite in enabling biocompatibility and suitability for medical applications. This study aimed to optimize bovine pericardium decellularization by integrating physical and chemical parameters while evaluating their effects on DNA elimination, extracellular matrix (ECM) preservation, and biocompatibility. Tissue-specific adaptations of known decellularization protocols were applied to enhance their efficacy and broaden their applicability in regenerative medicine and tissue engineering. The SD010 protocol, comprising freeze-thawing, agitation in a low-concentration (0.1% w/v) sodium deoxycholate (SD) solution, and thorough saline cleansing, emerged as a promising candidate for effective decellularization. Histological analysis revealed successful removal of cellular components, with SD010 exhibiting complete decellularization. DNA quantification indicated a significant reduction of DNA content in SD010-treated samples (85.7%). Scanning electron microscopy affirmed the preservation of ECM architecture posttreatment. Mechanical tests demonstrated minimal impact on the mechanical properties of treated tissues. The SD010 protocol's potential for xenograft applications was supported by biocompatibility tests, as evidenced by increased cell proliferation in vitro. The results indicate a potentially efficient method for bovine pericardium decellularization, highlighting its utility for repurposing biomass waste materials produced by the food and health industry.

Graphical Abstract



Keywords Decellularization · Bovine Pericardium · Extracellular matrix preservation · Biocompatibility

Extended author information available on the last page of the article

Statement of Novelty

In addition to a well-preserved extracellular matrix, decellularization of bovine pericardium yielded immune-compatible results through an innovative non-enzymatic approach.

Introduction

Pericardial membranes are an optimal choice in tissue engineering applications compared to synthetic biomaterials, considering the higher ability to simulate human tissues. One tissue that has shown potential for various applications, including biological heart valves, dental membranes, and hernia repair, is the bovine pericardium [1–4]. However, xenografts need constant development towards responses to immunological and degradation stimuli over time [5–8]. The decellularization of tissues is a leading strategy in tissue engineering, as it results in low immune response and early degradation [9]. Therefore, decellularization methods are developed synergistically towards the prevention of degradation and calcification factors [10–13].

The natural extracellular matrix (ECM) comprises collagen type I protein chains arranged in a hierarchical structure, ranging from triple-helix collagen fibrils to bundles of fibers [14]. These chains help to provide the ECM mechanical properties and offer structural integrity [15–17]. The ECM relies on various components that facilitate crucial functions of cell growth and differentiation [18]. Elastin, proteoglycans, and glycoproteins are adjacent to the ECM, and their retention significantly influences tissue engineering applications [19]. Elastin provides elasticity and flexibility, proteoglycans maintain hydration levels and structural support, and glycoproteins play a crucial role in cell adhesion and communication, collectively supporting structural functions [20, 21]. Collagen type I fiber's resistance to tensile strength is primarily attributed to the distinct helical alpha chain structure, with a minor contribution of the covalent intra- and inter-molecular cross-links between the protein [22]. The interaction between collagen and water contributes to the structural stability of collagen's triple helix and fibrils. In collagenous membranes, the mechanism of collagen-water interaction and collagen cross-linking will likely play a significant role in elasticity. After implantation, a biomimetic material must support, enhance, or replace the native ECM to form new tissue, facilitating vascularity and new cell attachment [19, 23, 24].

Trade margins over scaffolds are significantly higher than the cost of natural raw materials [25]. In Brazil, fresh bovine pericardium is obtained for less than US$ 1.00 per kilogram when the biomass can be close to 300 cm^2 per kilogram. The derived xenografts are generally costly for end-users, ranging from US$ 10.00 to 30.00 per cm^2. Various decellularization approaches often include enzymatic actions to improve cell breakdown, which may compromise the engineered tissue's mechanical properties and structural integrity [26, 27]. New techniques are needed to isolate and decontaminate biomaterials for tissue regeneration in order to meet the growing demand in health science applications.

A plethora of studies have explored the advantages of decellularization methods in the bovine pericardium and how they could influence specific parameters to target a final application, including efficacy in cell removal and preservation of mechanical function [26, 27], structural integrity, and biocompatibility [28, 29], and the regenerative potential of the decellularized bovine pericardial scaffold in the abdominal wall of rats [30] and human gingival recession [31]. Yang et al. [32]. performed an in vitro and in vivo study of human vascular cell seeding in decellularized bovine pericardium, and Nguyen et al. [33]. investigated the chondrogenic differentiation of human adipose-derived stem cells on treated bovine pericardium. The freeze-thaw technique has recently been used in decellularization processes [29, 34, 35], showing positive results for cell withdrawal. This approach involves freezing the cell contents while preserving the structure of the ECM protein. Subsequent cleansing steps are then employed to remove undesirable nucleic acids [36]. Previous studies explored variations of the freeze-thaw-wash combining method with varying outcomes [37–39]. Sodium dodecyl sulfate (SDS) is an ionic detergent with frequent use to decellularize tissues and organs. Despite its effectiveness in removing cellular components, it has been found to potentially cause permanent damage to the tissue matrix [40]. Similarly, more research is needed, especially with the bovine pericardium, to determine how sodium deoxycholate (SD) affects the ECM of membranes and how decellularization can be approached to induce less damage than enzyme-assisted methods.

Hence, the study proposes a non-enzymatic method of decellularization to reduce production costs. To decellularize bovine pericardium, established physical and chemical methodologies were currently employed. Our strategy to select the optimal approach for manufacturing a xenograft resulted in a method to decellularize bovine pericardium, removing cellular components from the pericardial tissue and consequently favoring its biocompatibility as an implant material. The efficacy of using the freeze-thaw technique to remove cells from bovine pericardium was evaluated. This

method innovates in its lack of enzymatic action associated with using lower quantities of detergents compared to the literature.

Materials and Methods

Tissue Harvesting and Decellularization

Fresh bovine pericardium (BP) was obtained from a certified slaughterhouse facility (JBS Frigorifico, Promissao, Sao Paulo, Brazil) within 12 h of slaughter. Phosphate buffered saline (PBS) solution was prepared using Sodium Chloride, Potassium Chloride, Sodium Phosphate Dibasic, and Potassium Phosphate Monobasic (Sigma-Aldrich) in Milli-Q® water, achieving a concentration of 151.6 ± 0.1 mM with pH adjusted to 7.42 ± 0.05. The pericardial membranes were rinsed with PBS to eliminate visible traces of blood and body fluids. However, it is important to note that this method may not remove all microscopic traces of blood, and further testing may be required to confirm the complete absence of blood contamination. Subsequently, they were stored in fresh PBS at 0 °C. Upon arrival at the laboratory, the pericardial sacs were dissected to remove the external fat. The selected parts of the pericardia had a thickness below 500 µm, and the samples were cut into 10 mm x 10 mm squares and 5 mm x 40 mm rectangles. The BP samples selected for the treatments were immediately subjected to thermal shock by placing them individually inside 5 mL cryogenic tubes in an ultra-low temperature freezer for 12 h at − 80 °C without the addition of any cryoprotectant. Subsequently, the samples were transferred to a conventional freezer for 2 h at − 20 °C and then left until completely thawed at room temperature of 20 °C. BP tissue samples were divided into different groups for the decellularization process: Pure Water (WATER), 1.00% (w/v) sodium deoxycholate (SD100), 0.10% (w/v) sodium deoxycholate (SD010), and 0.01% (w/v) sodium deoxycholate (SD001). The control group (NATIVE) corresponded to bovine pericardium without any treatment. Each sample was randomly immersed in the corresponding solution of the predefined group and subjected to agitation at 120 rpm on the bench for 24 h at 20 °C. After decellularization treatment, the samples underwent repeated rinsing by replacing the detergent solution with a sterile saline solution (NaCl 0.9%, JP Farma, Sao Paulo, Brazil) for two days under the same conditions as the decellularization phase. The saline solution was replenished with a fresh solution at half-time. Finally, a third wash with sterile saline solution at 80 rpm was performed for 24 h at 20ºC. These washing steps were executed to remove residual detergent substances and to stabilize the tissue's pH. All procedures adhered to the regulations and guidelines outlined in Regulatory Ordinance n° 2600, published on October 21, 2009, and ANVISA Resolution - RDC n° 625, published on September 17, 2021, according to the Brazilian National Regulations.

Histological Analysis

Samples of control ($n = 3$) and treated ($n = 3$) groups were fixed for 24 h at 20 °C in 10% buffered formaldehyde (Alkimia, Sao Paulo, Brazil) before processing. Histological slides containing 5 µm segments of deparaffinized samples were prepared for analysis. Cuts were subjected to hematoxylin and eosin (HE) staining, and subsequent cuts were subjected to alcian blue (AB) staining. Samples were observed under an Olympus BX61 VS slide scanner microscope (Olympus, Tokyo, Japan). The average number of intact nuclei was calculated at approximately 3.0 mm^2 in each group with ImageJ software (NIH, Bethesda, USA) – the retention of sulfated glycosaminoglycan (sGAG) after the treatments, was qualitatively compared to the control group.

DNA Extraction and Quantification

DNA of native ($n = 3$) and decellularized ($n = 3$) BP was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To determine the DNA quantity, absorbance scans were carried out using 1 µL of the eluted DNA on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Each DNA reading was performed in triplicate, with the elution buffer as the blank. The DNA amount was calculated based on the dry weight of the sample and expressed as ng/mg.

Scanning Electron Microscopy

Samples comprising native and treated specimens were fixed using 10% buffered formaldehyde. The samples underwent gradual dehydration by subjecting them to a series of ethanol immersions. BP samples were then dried in the presence of silica pellets at room temperature of 20 °C. Before analysis, samples were mounted on stubs with a double-sided conductive carbon adhesive and gold-sputtered (coating ~22.5 nm) for 90 s in a Balzers Sputter Coater SCD 04 (Bal-Tec AG, Balzers, Liechtenstein). Then, high-resolution scanning electron microscopy (SEM) analysis was performed using a FEI Inspect S50 instrument (FEI Company, Hillsboro, USA) at a voltage of 2.00 KV under high vacuum conditions.

Biomechanical Assay

Rectangular specimens ($n = 10$) of each group were uniaxially pulled until failure at a rate of 10 mm/min in both the longitudinal (0°) and transverse (90°) directions. Biomechanical tests were conducted at a room temperature of 20 °C, with 10 mm (l_o) between the clamps. The mechanical reaction to stress on decellularized tissue was evaluated by comparing it to native tissue. Meticulous tests were performed using an AME-2kN tensile testing machine (Oswaldo Filizola, Sao Paulo, Brazil), following the methodology described by Oswal [41]. The test termination criterion was predefined in DynaView software when tissue disruption was identified. The elastic modulus was calculated as the slope of the linear region of the stress-strain curve. Close to 60% of data from the deformation curve was used to calculate Young's elastic modulus (E), ignoring the membrane's elastic, transition, and rupture phases [42]. The mean thickness of the specimens was determined by measuring three different points in the center portion using a Mitutoyo 7301 thickness gauge (Mitutoyo, Tokyo, Japan). Based on these measurements, the area (A_o) was calculated. To minimize the influence of natural degradation, newly obtained native tissue and freshly treated scaffolds were used for the tests. The data obtained from the stress-strain curves were exported to Microsoft Excel. The elastic modulus, ultimate tensile strength (UTS), and tensile strain (TS%) were quantified using the following equations:

$$\epsilon_x = \frac{\Delta l_x}{l_0} \tag{1}$$

$$\sigma_x = \frac{F_x}{A_0} \tag{2}$$

$$E_x = \frac{\sigma_x}{\epsilon_x} \tag{3}$$

Denaturation and Tissue Shrinkage

A shrinkage test was performed to obtain the denaturation temperature (Td). Square specimens (n = 3) of each group were suspended individually on clamps in deionized water at room temperature. By gradually increasing the temperature by 1 °C per minute, the temperature was monitored using a thermometer and a heated magnetic stirrer until the temperature reached 70 °C or until specimen shrinkage was observed (Refer to Figure SM-3 in Supplementary Material for setup details). The transformation caused the specimens to assume a distinct 'C' shape, indicating a phase change in the collagen protein. This test was performed to determine if the collagen protein underwent denaturation during the treatment.

Cell Culture

Mouse fibroblasts (NIH/3T3) obtained from ATCC® (ATCC, Virginia, USA) were cultured according to the manufacturer's instructions. Briefly, the culture medium consisted of high-glucose DMEM (Dulbecco's Modified Eagle Medium) from Sigma-Aldrich, supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 units.mL^{-1} penicillin, 100 µg·mL^{-1} streptomycin, and 0.25 µg·mL^{-1} amphotericin B), from Sigma-Aldrich. The cells were grown at 37 °C in a humidified chamber with a 5% CO_2 enriched atmosphere (in air) and were applied to the cytotoxicity studies.

In vitro Cytotoxicity Assay

Cytotoxicity was evaluated following a previously described method [43] with minor modifications. NIH/3T3 fibroblasts (ATCC, Virginia, USA) were seeded in 96-well plates at a density of 2×10^4 cells/well one day prior to the experiment. To assess cell viability, the CellTiter96® AQueous One Solution commercial kit (Promega Corporation, Fitchburg, USA) was utilized. This kit contains a mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) and phenazine ethosulphate (PES). To obtain tissue extracts, 5 mg of newly decellularized tissues were incubated in 1 mL of DMEM supplemented only with antibiotics for 72 h at 37 °C under orbital stirring (approx. 100 rpm). For control, DMEM-antibiotic without tissue (i.e., only the solvent) was kept at the same condition applied to the obtainment of the extracts. Next, the fibroblast growth medium was replaced by different concentrations of tissue extracts (10 to 80%, v/v), which were obtained by the extract dilution in fresh DMEM-antibiotics medium. After 24 h of incubation at 37 °C, the medium was removed, and 100 µL of DMEM along with 20 µL of commercial MTS/PES solution were added to the cells. Following a 3-h incubation period, the absorbance of the wells was measured at 490 nm using the ELx808 microplate reader (BioTek Instruments, Winooski, USA). The cytotoxicity of the treated samples was expressed in terms of cell viability relative to cells treated only with DMEM-antibiotics (solvent; 100% cell viability). The extracts were prepared in triplicate, and each one was applied in two different wells, resulting in a total of six measurements ($n = 6$) for each group at different concentrations.

Statistical Analysis

Statistical studies were performed with one-way analysis of variance (ANOVA), and differences were analyzed by Dunnett's multiple comparison test to report statistical differences between the control and treated groups. Results are expressed as the mean ± standard deviation. Differences were considered statistically significant at $p < 0.05$. Statistical analysis by GraphPad Prism 10 (GraphPad Software, Inc., USA).

Results

Histological Analysis

The effectiveness of the combined decellularization method was evaluated with the HE staining of native and treated bovine pericardium. The histological results indicate a decrease in cell count for all decellularization protocols. Two distinct levels of decellularization efficacy, concerning the absence of cell nuclei, were observed in bovine pericardium samples. A remarkable reduction of approximately 99% was documented in samples subjected to SD100 and SD010 protocols compared to NATIVE samples. Conversely, samples treated with the WATER and SD001 protocols exhibited an approximate 50% reduction compared to NATIVE samples (Table 1). Figure 1 illustrates both the native material (Fig. 1a) and the results for decellularized bovine pericardium samples treated with the SD010 decellularization protocol (Fig. 1b) (Refer to Fig. SM-2 in Supplementary Material for photomicrograph of WATER, SD100, and SD001). The photomicrograph in Fig. 1a reveals visible nuclei in the NATIVE pericardium, marked in blue. Conversely, the complete absence of nuclei in samples treated with the SD010 decellularization protocol (Fig. 1b) was identified. While the sGAG content decreased following all decellularization processes (seen in Fig. 2), it was notably more noticeable in the SD100 group compared to the other groups.

DNA Quantification

The DNA quantification of control and treated samples is shown in Fig. 3. The decellularization method significantly reduced DNA content in the SD010 group, with a decrease of 85.7% ($p < 0.0001$) in detectable DNA compared to the NATIVE group (917.8 ± 122.2 ng/mg to 131.0 ± 20.0 ng/mg of dry tissue) and 34.4% reduction ($p = 0.0016$) for SD001 group (484.5 ± 168.7 ng/mg). The SD100 group experienced a slight decrease in the DNA content (19.4%), indicating no significant change compared to the NATIVE group.

Scanning Electron Microscopy

Native and treated samples were analyzed with SEM. Figure 4 depicts surface photomicrographs of the serous (internal) and fibrous (external) sides of the native and decellularized pericardium treated with freeze-thawing, followed by either water or sodium deoxycholate solution. A consistent

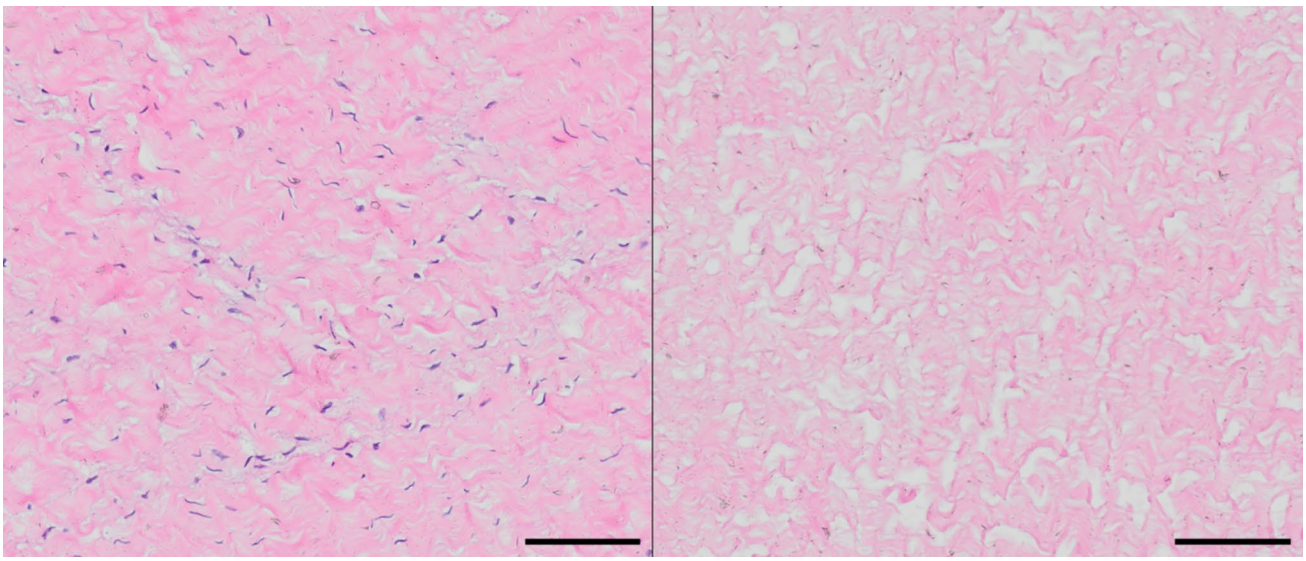


Fig. 1 Histopathology of bovine pericardium. Representative images of fragments of bovine pericardium samples by HE staining magnified at 400X of **a** NATIVE and **b** SD010. The SD010 group experienced remarkable preservation of the collagen fibrous pattern, and the absence of cell nuclei suggested a successful decellularization protocol. Bar: 100 µm

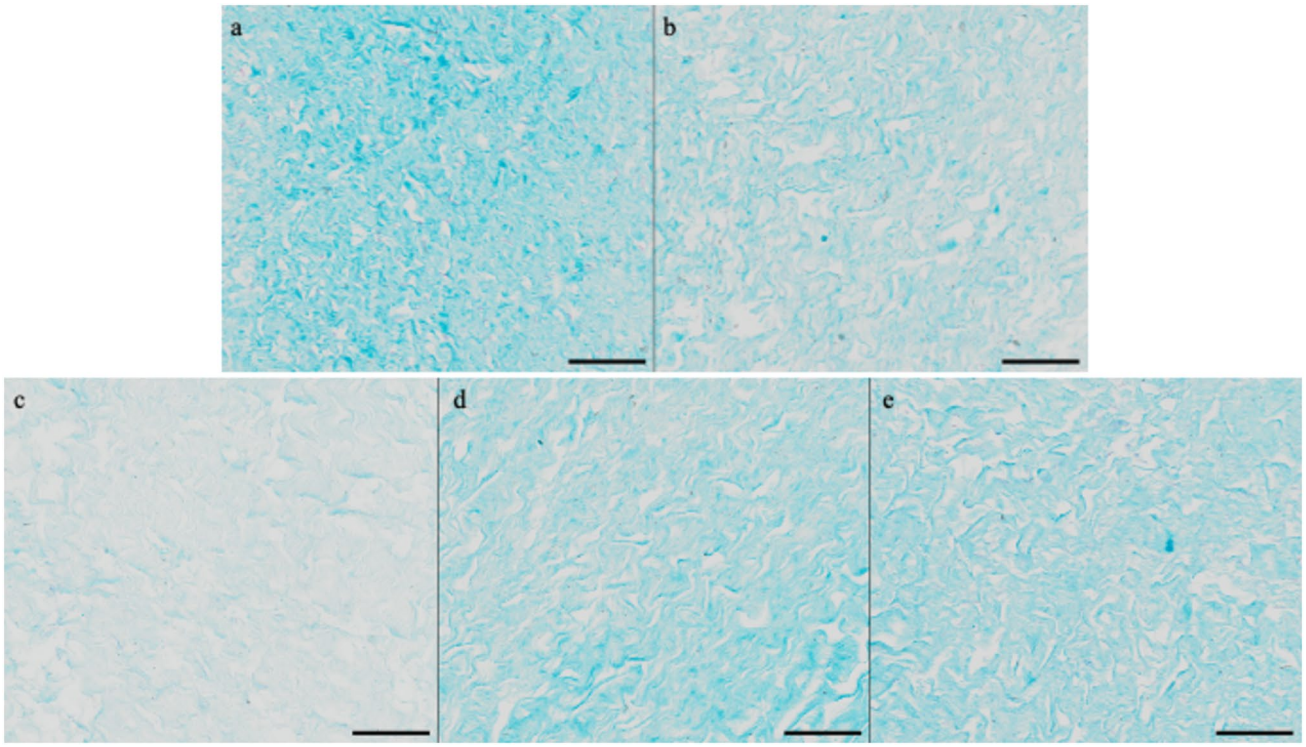


Fig. 2 Histological examination of ECM sulfated glycosaminoglycans by AB staining of bovine pericardium samples. Representative images of fragments of bovine pericardium samples magnified at 400X. The SD100 treatment significantly reduced sGAG in ECM related to the interaction with the highest concentrated solution. **a** NATIVE **b** WATER **c** SD100 **d** SD010 **e** SD001. Bar: 100 µm

Table 1 Average cell nuclei quantification from histopathology of control and decellularized pericardial samples

Group	Cells/mm^2
NATIVE	536.1 ± 65.2
WATER	235.2 ± 46.7
SD100	9.4 ± 11.8
SD010	2.6 ± 4.3
SD001	252.0 ± 41.9

structure with densely arranged fiber bundles is observed on the serous side of the bovine tissue surface. Similarly, the fibrous side exhibits relatively looser bundles while maintaining a dense fiber network. Notably, the freeze-thawing treatment effectively preserved the integrity of the collagen network without signs of denaturation, as evidenced by the orientation of collagen fibers within the layered structures of the treated samples compared to the control.

Shrinkage Tests

Shrinkage temperature testing was employed to assess whether the decellularization protocols led to collagen protein denaturation. The results indicated negligible disparities between the control sample (NATIVE) and the treated samples (WATER, SD100, SD010, SD001). This suggests

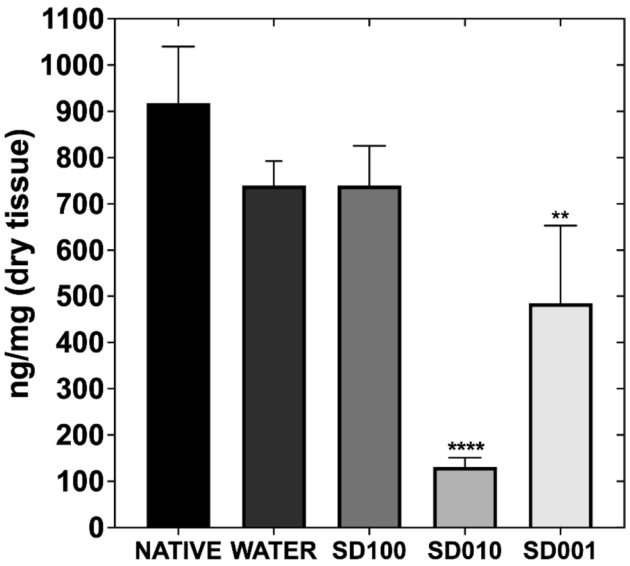


Fig. 3 Residual DNA quantification of decellularized BP. DNA quantification of native bovine pericardium samples (dark bar) and decellularized samples (grayscale bars). The DNA quantification indicated a significant reduction in DNA for the decellularization protocols SD010 and SD001 compared to the control. **($p < 0.01$) ****($p < 0.0001$)

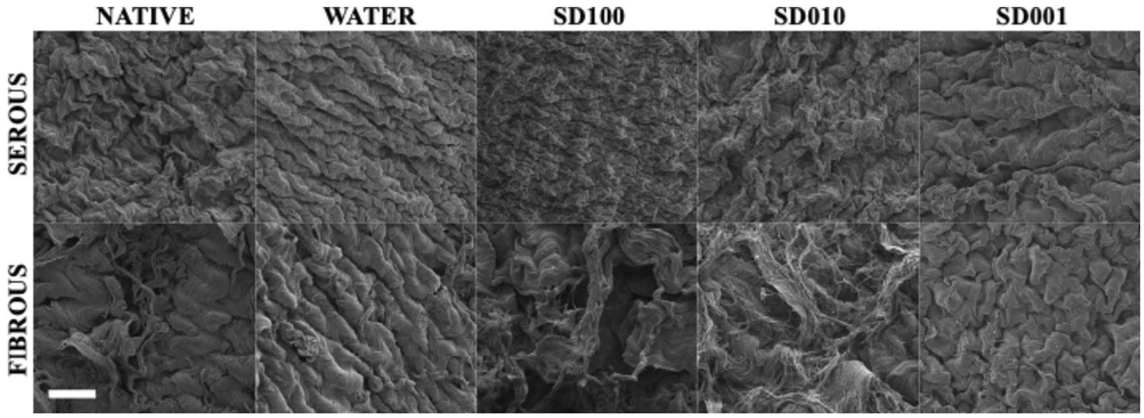


Fig. 4 SEM examination of bovine pericardium decellularized by freeze-thaw and detergent protocol. Representative surface photomicrographs of bovine pericardium samples at a magnification of 1000X. The analysis encompassed both sides of the sample surface – a comparative physical examination of the ECM structure and conformation before and after the decellularization treatment. The micrographs revealed minimal damage to the collagen fibers and no fractures within the ECM. Bar: 50 µm

Table 2 Tensile test results of longitudinal (0°) orientation of collagen fibers on bovine pericardium samples for control and treated groups

Group	Elastic Modulus (MPa)	UTS (MPa)	Elongation (%)
NATIVE	60.2 ± 10.1	14.5 ± 4.8	39 ± 4
WATER	72.6 ± 12.3	20.0 ± 3.9	45 ± 15
SD100	59.8 ± 21.9	14.2 ± 9.2	55 ± 10
SD010	61.3 ± 18.1	18.8 ± 6.7	54 ± 15
SD001	60.8 ± 11.5	17.9 ± 5.4	49 ± 16

Table 3 Tensile test results of transverse (90°) orientation of collagen fibers on bovine pericardium samples for control and treated groups

Group	Elastic Modulus (MPa)	UTS (MPa)	Elongation (%)
NATIVE	21.8 ± 6.8	5.2 ± 1.9	32 ± 8
WATER	19.9 ± 10.6	6.1 ± 2.9	42 ± 8
SD100	12.0 ± 3.2	3.9 ± 1.0	44 ± 9
SD010	22.3 ± 9.1	6.4 ± 2.3	46 ± 11
SD001	27.8 ± 9.8	6.4 ± 2.9	38 ± 5

that the decellularization process did not induce any collagen denaturation. The control sample exhibited a shrinkage temperature of 66.3 ± 0.5 °C, while the treated samples had no statistical difference, ranging from 65.9 ± 0.2 to 67.0 ± 0.4 °C.

3.5 Biomechanical Tests

Tables 2 and 3 display the data related to tensile testing according to fiber orientation. This data highlights the influence of the orientation (longitudinal or transverse) on the elastic modulus and ultimate tensile strength (UTS) values. The elongation parameter was higher for all groups compared to the native pericardium. Group WATER experienced a more distinct resistance to stress longitudinally, reflecting higher values for the elastic modulus and UTS if compared to NATIVE. In the case of the SD100 group, the elastic modulus in the transverse direction was lower than that of NATIVE. Remarkably, the partially and fully decellularized groups (SD100, SD010, and SD001) exhibited consistent Young's modulus and UTS values longitudinally, with no statistically significant deviation from the NATIVE group.

In vitro Cytotoxicity Assay

To determine the cell viability of the decellularized specimens, a series of different extract ratios to cultured cells were tested in this study. Specifically, NIH/3T3 fibroblasts were exposed to various extract ratios obtained from decellularized specimens, and cell viability (%) was assessed using the MTS/PES assay. The results depicted in Fig. 5 indicate no statistically significant differences in cell viability for groups WATER, SD100, and SD001 compared to the 100% cell viability considered for the control medium (DMEM-antibiotic) to create the extracts. Extracts of group SD010 indicated a higher fibroblast proliferation and a p-value of 0.0002 compared to control. This finding suggests that the biomaterial produced is safe for cell growth and proliferation.

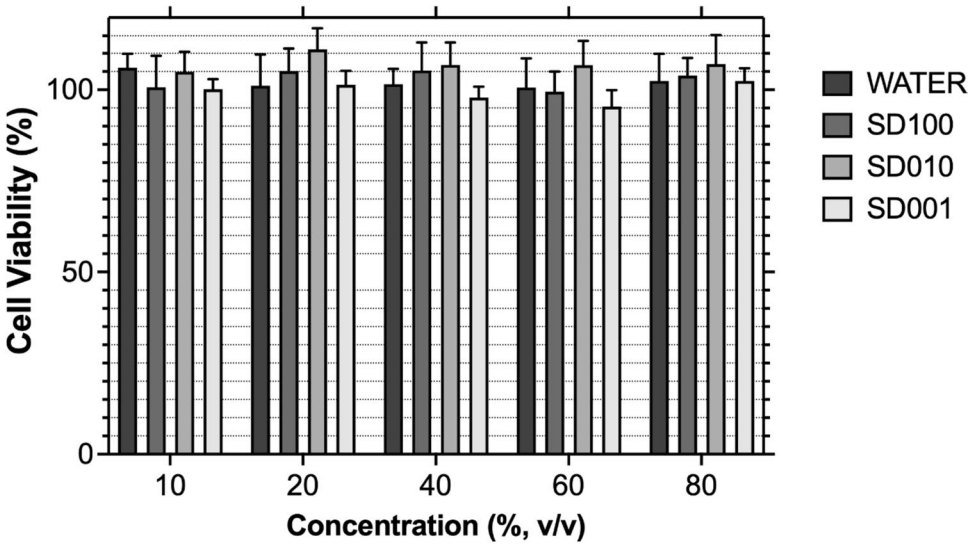


Fig. 5 MTS/PES extract cytotoxicity assay. The graphic shows five dilution ratios of the extract to the cultured cells after 3 h incubation, and the Cell Viability (%) of treated samples is expressed as percentages of control. The cytotoxicity was related to cell viability in different concentrations of the extract. The extracts of cultured samples (grayscale bars) were spectrophotometrically compared at 490 nm

Discussion

The successful utilization of bovine pericardium xenografts hinges on minimizing the presence of endogenous DNA and preserving the organic structure and biochemical composition. The use of enzymes in decellularization is known to facilitate DNA breakdown and improve decellularization to minimal levels. The present study shows an entirely non-enzymatic method to decellularize bovine pericardium, from which we obtained results comparable to those with the use of enzymes [4, 26, 44]. The SD010 protocol, involving a sequence of steps including a single freeze-thaw cycle, 24 h agitation in a 0.1% sodium deoxycholate solution, and intensive saline cleansing, yielded promising outcomes, achieving substantial DNA elimination of around 85.7% (131.0 ± 20.0 ng/mg, dry tissue) and improved ECM preservation. Decellularization protocols yield a wide range of outcomes in terms of DNA/RNA elimination when applied to diverse biological matrices. For instance, Gardin et al. [4] employed an enzymatic protocol for bovine pericardium, resulting in a DNA reading of 142 ng/mg (dry tissue), while Mendoza-Novelo et al. [26] conducted enzymatic protocols on bovine pericardium, leading to DNA readings ranging from 75 to 125 ng/mg (dry tissue). Ozeki et al. [44], in their work on decellularized esophagus scaffolds, reported values of 140 ng/mg (dry tissue). Furthermore, Heuschkel et al. [28] utilized a detergent-based protocol for bovine pericardium, which yielded a DNA reading of 198 ng/mg (dry tissue).

It is important to note the present results do not entirely align with the consensus proposed by Crapo et al. [45], which suggests that decellularized materials should contain less than 50 ng of DNA per mg of dry ECM to minimize immune reactions. However, it's worth acknowledging that this threshold remains a subject of debate, particularly in relation to the specific use of xenografts and the type of tissue being isolated. Additionally, other variables, such as the presence of alpha-gal epitopes, can also contribute to adverse immune reactions in xenotransplantation [46]. Furthermore, all medical devices undergo meticulous biocompatibility testing for clinical approval [7, 47].

In a study by Suss et al. [48], their decellularization protocols resulted in DNA remnants above 275 ng/mg, consisting of DNA fragments below 100 bp in size, and yet, no cytotoxicity was observed. Cramer and Badylak [49] emphasize that there are no universal criteria for producing ECM scaffolds, and a comparison of various commercially available materials revealed a reduction of DNA remnants, however still above the proposed threshold [50].

The DNA quantification analyses demonstrated the importance of combining physical and chemical parameters, i.e., freeze-thaw and detergent solutions, in treating the bovine pericardium for DNA elimination and utter preservation of the ECM components. Additionally, SD010-treated samples were non-cytotoxic to cultured fibroblasts, with higher cell proliferation ($p = 0.0002$) compared to the different protocols (Fig. 5).

The scientific literature extensively discusses the combination of various treatments for optimal decellularization. Freeze-thaw cycles are commonly used to ensure cell devitalization through the repetitive formation of ice crystals around cells [29]. When applying thermal shocks, it is critical to consider the specific tissue being targeted because different tissue types respond differently to physical and chemical treatments. In this study, the use of a single freeze-thaw cycle in the tissue was appropriate to break and detach cellular remnants from the scaffold, as evidenced by the pyknotic appearance of the nucleus in partially decellularized samples (Refer to Fig. SM-2 in Supplementary Material). Therefore, the appropriate level of thermal stimulation and detergent concentration must be determined for each

case. Proper consideration of the tissue type can help prevent damage or other adverse effects [51, 52]. Freeze-thaw cycles have been applied in the decellularization of equine tendons [37], porcine menisci [53], arteries [39], and bovine pericardium [4]. Enzymatic processes have also been employed as benchmarks for removing cell debris and catalyzing RNA and DNA hydrolysis for easier ECM removal [45, 54]. However, nuclease solutions' prolonged exposure negatively affects the pericardial ECM [43, 55], with ECM preservation being attributed to SD in the same studies. Literature is limited about non-enzymatic procedures combined with freeze-thaw treatment. Cheng et al. [39]. used three cycles and 1.0% Triton X-100 to decellularize carotid arteries. Gao et al. [56]. used three freeze-thaw cycles and ten days of detergent agitation (1.0% SDS) to decellularize articular cartilage. Li et al. [29]. used five freeze-thaw cycles and detergent agitation (1.0% Triton and 0.5% SD combined) to decellularize bovine pericardium membranes. Moreover, to our knowledge, there is no reported evidence of a low-cost approach involving a single freeze-thaw cycle and subsequent interaction with a low-concentrated detergent solution to completely eliminate cells from the bovine pericardium. It was evidenced in this work that one freeze-thaw cycle was sufficient to devitalize cells from the bovine pericardium and help their eradication throughout the washing phase.

Histological examination confirmed a collagen pattern (Fig. 1) and the natural aspect of sGAG (Fig. 2) that are consistent with the literature. Notably, the SD100 and SD010 groups lacked nuclei material, while the results were less significant for the WATER and SD001 groups (see Table 1). Laker et al. [57]. demonstrated substantial ECM and collagen structure damage due to detergents in bovine pericardium, whereas the present study showed minor damage. Gaps observed in SD010-treated samples (Fig. 1b) were likely related to freeze-thaw treatment rather than detergent interaction with ECM [39]. Analysis of the data reveals that SD010 outperforms in minimizing residual cellular and nucleic acid concentrations. Contrary to expectations, elevated SD concentration did not correlate with a reduction in cellular and nucleic acid quantities. This observation is consistent with Meezan et al. [58], who reported a concentration-specific influence of SD on DNA agglutination in tissue samples. Immunohistochemical imaging of scaffolds further corroborated DNA agglutination [59, 60]. At a 1% SD concentration, there is an increased tendency for DNA to cluster and adhere to the collagen matrix, which may hinder acid removal. Conversely, a 0.1% concentration of SD effectively disrupts cell structures without causing substantial DNA agglutination, thereby enhancing decellularization efficiency. These results highlight the critical importance of fine-tuning detergent concentrations to balance decellularization efficiency with the preservation of tissue integrity and composition.

It was reported that Trypsin, associated with SD and Triton X-100 treatment, can preserve sGAG content; however, it noticeably modifies the ECM structure [61]. In this study, alcian blue staining revealed a higher sGAG depletion in tissues treated with SD100 (Fig. 3c) and great preservation of sGAG in samples treated with lower or zero detergent concentration.

Decellularization protocols can vary widely based on tissue type and applications. For whole organs, SD solutions with concentrations up to 4.0% have been used [40, 62] for chemical interactions, while tissue and membrane decellularization often utilize 1.0% SD reported [9, 55, 63]. The present study's distinguishing factor among the four decellularization protocols was the detergent concentration, derived from a 1.0% (w/v) SD stock, and group WATER used no detergent. Interestingly, after the agitation with detergent, a cloudier solution was observed in the reservoir containing the SD010 group (Refer to Fig. SM-1 in Supplementary Material). In contrast, the pool containing samples of other groups remained clear. This observation could be related to the higher cellular and DNA depletion experienced by group SD010 because of the higher concentration of bioparticles in the solution, corresponding to cellular debris or dissolved substances, that could influence different degrees of cloudiness between the solutions containing samples [64, 65]. The cleansing phase was performed in a sterile saline solution. Based on the study of Cebotari et al. [9]. , three washings in saline solution were established, changing the solution every 24 h.

The concentration of sodium deoxycholate (SD) in the solution significantly influences the interaction between decellularization and the removal of sulfated glycosaminoglycans (sGAG). In the SD010 protocol, SD exhibited a gentler interaction with collagen's protein structure compared to the SD100 protocol, leading to the effective preservation of glycosaminoglycan content. SD is an anionic detergent [66] with the capacity to dissolve nuclear membranes, potentially affecting collagen stability and mechanical properties in pericardial membranes. Additionally, sGAG contributes to the hydrophilic domains of the structure [67, 68], potentially explaining the regulation of sGAG removal by sodium deoxycholate solution concentration [65].

The histological results highlight the critical role of SD concentration in controlling the interaction between decellularization and sGAG removal. The SD010 protocol successfully preserved glycosaminoglycan content and facilitated efficient DNA extraction with a milder interaction as evidenced in Fig. 2. In contrast, the SD100 protocol, with a higher concentration, SD may induce agglutination of scaffold DNA, which could interfere with the scaffold's structural integrity and its interaction with GAG molecules.

The response to mechanical stress determined Young's elastic modulus, ultimate tensile strength, and elongation to

understand the physical conditions of collagen fibers after interaction with water and detergent solutions. All groups treated with SD responded similarly to stress compared to the native material (see Tables 2 and 3). The curves obtained in this analysis show the different slopes that relate to the fiber alignment with the elongation axis (Refer to Fig. SM-4 in Supplementary Material). Electron microscopy revealed that our decellularization method preserved the pericardial structure effectively, in line with prior studies [28, 69]. The freeze-thaw process maintained the collagen network's integrity, with no cell debris visible and only minimal morphological changes observed, contrasting with the damage often caused by repetitive freezing [8, 36], or enzymes [61, 70]. By ensuring the preservation of tissue architecture, our method holds promise for developing scaffolds that could support tissue regeneration and repair, in the field of regenerative medicine.

This result shows that the protocol had no significant impact on the material and did not cause protein denaturation. Shrinkage temperature tests (Refer to Fig. SM-3 in Supplementary Material) confirmed this, consistent with previous pericardial decellularization studies [71, 72]. Beyond the laboratory, our findings hold potential implications for advancements in regenerative medicine, tissue engineering, and the utilization of xenografts. As we navigate the intersection of science and practicality, this research has yielded an efficient approach to eliminating native DNA from cow pericardium waste – a product of the food and health industry. This endeavor speaks to the broader pursuit of sustainable solutions and innovative applications in waste management.

Conclusion

In summary, achieving effective decellularization of bovine pericardium necessitates a meticulous blend of physical and chemical parameters. The SD010 protocol effectively decellularized bovine pericardium, utilizing a detergent concentration that outperformed others following freeze-thaw treatment, preserved the extracellular matrix (ECM) integrity, and maintained biocompatibility. This establishes it as a promising option for xenograft applications. Additionally, our findings highlight that a single freeze-thaw cycle has minimal impact on ECM integrity, while a low-concentration SD solution effectively decellularized bovine pericardium, preserving native ECM attributes and bolstering in vitro cell proliferation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12649-024-02465-9.

Acknowledgements The authors thank the Ministry of Science, Technology, and Innovation for the Academic Doctorate for Innovation (DAI) grant and financial support. Special thanks go to P+F Products and Features Brasil for their valuable insights throughout the research process. We also thank the Laboratory of Structural Characterization (LCE/DEMa/UFSCar) for the general facilities. R. C. Giorgi Filho acknowledges the National Council for Scientific and Technological Development (CNPq) for the doctorate fellowship (# 159566/2019-3). J G. Nery acknowledges FAPEMIG (APQ-03610-17 / CNPq: 465594/2014-0).

Author contributions RCGF: Decellularization method development, histological analysis, DNA extraction and quantification, tensile tests, and related figures. AMMJ: Cytotoxicity tests. DSA: Writing and formal analysis on the decellularization method. MFC: Formal analysis of DNA quantification and statistical analysis. MJT: Laboratory facilities. GA: Project design and Company coordinator. JGN: Writing, review & editing; data analysis; project supervision and administration.

Funding This work was supported by CNPq (Grant N.: 159566/2019-3, 465594/2014-0) and FAPEMIG (Grant N.: APQ-03610-17).

Data Availability Data will be made available on request.

Declarations

Competing Interest The authors have no competing interests to declare that are relevant to the content of this article.

References

1. James, N.L., et al.: Comparative evaluation of treated bovine pericardium as a xenograft for hernia repair. Biomaterials. **12**(9), 801–809 (1991). https://doi.org/10.1016/0142-9612(91)90065-I
2. Coburn, J., Pandit, A.: Development of naturally-derived biomaterials and optimization of their biomechanical properties. Top. Tissue Eng. **3**, 1–33 (2007)
3. Gauvin, R., et al.: A comparative study of bovine and porcine pericardium to highlight their potential advantages to manufacture percutaneous cardiovascular implants. J. Biomater. Appl. (2013). https://doi.org/10.1177/0885328212465482
4. Gardin, C., et al.: Decellularization and delipidation protocols of bovine bone and pericardium for bone grafting and guided bone regeneration procedures. PLoS One (2015). https://doi.org/10.1371/journal.pone.0132344
5. Specke, V., et al.: Virus safety in xenotransplantation: First exploratory in vivo studies in small laboratory animals and non-human primates. Transpl. Immunol. **9**, 2–4 (2002). https://doi.org/10.1016/S0966-3274(02)00039-4
6. Schoen, F.J., Levy, R.J.: Calcification of tissue heart valve substitutes: Progress toward understanding and prevention, *Annals of Thoracic Surgery*, vol. 79, no. 3. Elsevier USA, pp. 1072–1080, (2005). https://doi.org/10.1016/j.athoracsur.2004.06.033
7. Wang, S., Zinderman, C., Wise, R., Braun, M.: Infections and human tissue transplants: Review of FDA MedWatch reports 2001–2004. Cell Tis. Bank. (2007). https://doi.org/10.1007/s10561-007-9034-3
8. Hussein, K.H., Park, K.M., Kang, K.S., Woo, H.M.: Biocompatibility evaluation of tissue-engineered decellularized scaffolds for biomedical application. Mater. Sci. Eng. C (2016). https://doi.org/10.1016/j.msec.2016.05.068
9. Cebotari, S., et al.: Detergent decellularization of heart valves for tissue engineering: Toxicological effects of residual detergents on

human endothelial cells. Artif. Organs. **34**(3), 206–210 (2010). https://doi.org/10.1111/j.1525-1594.2009.00796.x
10. Goissis, G., Marcolino Braile, D., Carnevalli, C., Aparecido Ramirez, V.: Mechanical, thermal and morphological properties of Glutaraldehyde crosslinked bovine pericardium followed by glutamic acid treatment. Mater. Res. **12**(1), 113–119 (2009). https://doi.org/10.1590/S1516-14392009000100015
11. Jorge-Herrero, E., Fernhndez, P., Escudero, C., Castillo-Olivares, J.L.: Calcification of pericardial tissue pretreated with different amino acids. Biomaterials (1996). https://doi.org/10.1016/0142-9612(96)88707-2
12. García Páez, J.M., et al.: The influence of chemical treatment and suture on the elastic behavior of calf pericardium utilized in the construction of cardiac bioprostheses. J. Mater. Sci. Mater. Med. **11**(5), 273–277 (2000). https://doi.org/10.1023/A:1008901128613
13. Collatusso, C., et al.: Decellularization as a method to reduce calcification in bovine pericardium bioprosthetic valves. Interact. Cardiovasc. Thorac. Surg. (2019). https://doi.org/10.1093/icvts/ivz041
14. Parenteau-Bareil, R., Gauvin, R., Berthod, F.: Collagen-based biomaterials for tissue engineering applications. Materials. **3**(3), 1863–1887 (2010). https://doi.org/10.3390/ma3031863
15. Wilkinson, S.J., Hukins, D.W.L.: Determination of collagen fibril structure and orientation in connective tissues by X-ray diffraction. Radiat. Phys. Chem. (1999). https://doi.org/10.1016/S0969-806X(99)00282-0
16. Wells, H.C., Edmonds, R.L., Kirby, N., Hawley, A., Mudie, S.T., Haverkamp, R.G.: Collagen fibril diameter and leather strength. J. Agric. Food Chem. (2013). https://doi.org/10.1021/jf4041854
17. Wells, H.C., Sizeland, K.H., Kirby, N., Hawley, A., Mudie, S., Haverkamp, R.G.: Acellular dermal matrix collagen responds to strain by intermolecular spacing contraction with fibril extension and rearrangement. J. Mech. Behav. Biomed. Mater. **79**, 1–8 (2018). https://doi.org/10.1016/j.jmbbm.2017.12.009
18. Badylak, S.F.: The extracellular matrix as a biologic scaffold material. Biomaterials (2007). https://doi.org/10.1016/j.biomaterials.2007.04.043
19. Soiderer, E.E., Lantz, G.C., Kazacos, E.A., Hodde, J.P., Wiegand, R.E.: Morphologic study of three collagen materials for body wall repair. J. Surg. Res. **118**(2), 161–175 (2004). https://doi.org/10.1016/S0022-4804(03)00352-4
20. Yeung, B.K.S., Chong, P.Y.C., Petillo, P.A.: Synthesis of glycosaminoglycans. J. Carbohydr. Chem. (2002). https://doi.org/10.1081/car-120016490
21. Mavrilas, D., Sinouris, E.A., Vynios, D.H., Papageorgakopoulou, N.: Dynamic mechanical characteristics of intact and structurally modified bovine pericardial tissues. J. Biomech. (2005). https://doi.org/10.1016/j.jbiomech.2004.05.019
22. Lin, J., Shi, Y., Men, Y., Wang, X., Ye, J., Zhang, C.: Mechanical roles in formation of oriented collagen fibers. Tiss. Eng. - Part B: Rev. (2020). https://doi.org/10.1089/ten.teb.2019.0243
23. Stosich, M.S., et al.: Bioengineering strategies to generate vascularized soft tissue grafts with sustained shape. Methods (2009). https://doi.org/10.1016/j.ymeth.2008.10.013
24. Stosich, M.S., Mao, J.: Stem cell-based soft tissue grafts for plastic and reconstructive surgeries. Semin Plast. Surg. **19**(3), 251–260 (2005). https://doi.org/10.1055/s-2005-919720
25. Santos, M.H., Silva, R.M., Dumont, V.C., Neves, J.S., Mansur, H.S., Heneine, L.G.D.: Extraction and characterization of highly purified collagen from bovine pericardium for potential bioengineering applications. Mater. Sci. Eng. C (2013). https://doi.org/10.1016/j.msec.2012.11.003
26. Mendoza-Novelo, B., et al.: Decellularization of pericardial tissue and its impact on tensile viscoelasticity and glycosaminoglycan content. Acta Biomater. **7**(3), 1241–1248 (2011). https://doi.org/10.1016/j.actbio.2010.11.017
27. Yang, M., Chen, C.Z., Wang, X.N., Bin Zhu, Y., Gu, Y.J.: Favorable effects of the detergent and enzyme extraction method for preparing decellularized bovine pericardium scaffold for tissue engineered heart valves. J. Biomed. Mater. Res. B Appl. Biomater. (2009). https://doi.org/10.1002/jbm.b.31409
28. Heuschkel, M.A., et al.: In vitro evaluation of bovine pericardium after a soft decellularization approach for use in tissue engineering. Xenotransplantation (2019). https://doi.org/10.1111/xen.12464
29. Li, N., Li, Y., Gong, D., Xia, C., Liu, X., Xu, Z.: Efficient decellularization for bovine pericardium with extracellular matrix preservation and good biocompatibility. Interact. Cardiovasc. Thorac. Surg. **26**(5), 768–776 (2018). https://doi.org/10.1093/icvts/ivx416
30. Thampi, P., Nair, D., Venugopal, L.R.V.N.S., Ramachandra, U.: Pathological effects of processed bovine pericardial scaffolds-a comparative in vivo evaluation. Artif. Organs. **37**(7), 600–605 (2013). https://doi.org/10.1111/aor.12050
31. Schlee, M., Ghanaati, S., Willershausen, I., Stimmlmayr, M., Sculean, A., Sader, R.A.: Bovine pericardium based non-cross linked collagen matrix for successful root coverage, a clinical study in human, *Head Face Med*, vol. 8, no. 6, pp. 1–7, [Online]. Available: (2012). http://www.head-face-med.com/content/8/1/6
32. Yang, M., Chen, C.Z., Shu, Y.S., Shi, W.P., Cheng, S.F., John Gu, Y.: Preseeding of human vascular cells in decellularized bovine pericardium scaffold for tissue-engineered heart valve: An in vitro and in vivo feasibility study. J. Biomed. Mater. Res. B Appl. Biomater. **100B**, 1654–1661 (2012). https://doi.org/10.1002/jbm.b.32734
33. Nguyen, M.T.N., Doan, V.N., Tran, H.L.B.: In vitro study on chondrogenic differentiation of human adipose-derived stem cells on treated bovine pericardium. Turkish J. Biology. **43**(6), 360–370 (2019). https://doi.org/10.3906/biy-1908-10
34. Xing, Q., Yates, K., Tahtinen, M., Shearier, E., Qian, Z., Zhao, F.: Decellularization of fibroblast cell sheets for natural extracellular matrix scaffold preparation. Tiss. Eng. Part. C Methods **21**(1), 77–87 (2015). https://doi.org/10.1089/ten.tec.2013.0666
35. Wu, H., Yin, G., Pu, X., Wang, J., Liao, X., Huang, Z.: Preliminary Study on the Antigen-Removal from Extracellular Matrix via Different Decellularization. Tiss. Eng. Part. C Methods **28**(6), 250–263 (2022). https://doi.org/10.1089/ten.tec.2022.0025
36. Rabbani, M., Zakian, N., Alimoradi, N.: Contribution of physical methods in decellularization of animal tissues. J. Med. Signals Sens. (2021). https://doi.org/10.4103/jmss.jmss_2_20
37. Burk, J., et al.: Freeze-Thaw cycles enhance decellularization of large tendons. Tiss. Eng. Part C Methods (2014). https://doi.org/10.1089/ten.tec.2012.0760
38. Hung, S.H., Su, C.H., Lee, F.P., Tseng, H.: Larynx Decellularization: Combining Freeze-Drying and Sonication as an Effective Method. J. Voice **27**(3), 289–294 (2013). https://doi.org/10.1016/j.jvoice.2013.01.018
39. Cheng, J., Wang, C., Gu, Y.: Combination of freeze-thaw with detergents: A promising approach to the decellularization of porcine carotid arteries. Biomed. Mater. Eng. **30**(2), 191–205 (2019). https://doi.org/10.3233/BME-191044
40. White, L.J., et al.: The impact of detergents on the tissue decellularization process: A ToF-SIMS study. Acta Biomater. (2017). https://doi.org/10.1016/j.actbio.2016.12.033
41. Oswal, D., et al.: Biomechanical characterization of decellularized and cross-linked bovine pericardium. J. Heart Valve Dis. **16**(2), 165–174 (2007)
42. Joyce, K., Rochev, Y., Rahmani, S.: Assessment of the uniaxial experimental parameters utilised for the mechanical testing of bovine pericardium. J. Mech. Behav. Biomed. Mater. **96**, 27–37 (2019). https://doi.org/10.1016/j.jmbbm.2019.04.025

43. Mirsadraee, S., et al.: Development and characterization of an acellular human pericardial matrix for tissue engineering. Tissue Eng (2006). https://doi.org/10.1089/ten.2006.12.763
44. Ozeki, M., et al.: Evaluation of decellularized esophagus as a scaffold for cultured esophageal epithelial cells. J. Biomed. Mater. Res. A (2006). https://doi.org/10.1002/jbm.a.30885
45. Crapo, P.M., Gilbert, T.W., Badylak, S.F.: An overview of tissue and whole organ decellularization processes. Biomaterials (2011). https://doi.org/10.1016/j.biomaterials.2011.01.057
46. Naso, F., et al.: First quantification of alpha-Gal epitope in current glutaraldehyde-fixed heart valve bioprostheses. Xenotransplantation (2013). https://doi.org/10.1111/xen.12044
47. Prüfungsarbeit, W., Des Titels, E.: The new U.S. FDA regulations on biocompatibility and reprocessing for medical devices. [Online]. Available: https://ssrn.com/abstract=3104134
48. Suss, P.H., Ribeiro, V.S.T., Motooka, C.E., de Melo, L.C., Tuon, F.F.: Comparative study of decellularization techniques to obtain natural extracellular matrix scaffolds of human peripheral-nerve allografts. Cell Tissue Bank (2022). https://doi.org/10.1007/s10561-021-09977-x
49. Cramer, M.C., Badylak, S.F.: Extracellular matrix-based biomaterials and their influence upon cell behavior. Ann. Biomed. Eng. (2020). https://doi.org/10.1007/s10439-019-02408-9
50. Pashos, N.C., Scarritt, M.E., Eagle, Z.R., Gimble, J.M., Chaffin, A.E., Bunnell, B.A.: Characterization of an acellular scaffold for a tissue engineering approach to the Nipple-Areolar complex reconstruction. Cells Tissues Organs (2017). https://doi.org/10.1159/000455070
51. Deeken, C.R., Eliason, B.J., Pichert, M.D., Grant, S.A., Frisella, M.M., Matthews, B.D.: Differentiation of biologic scaffold materials through physicomechanical, thermal, and enzymatic degradation techniques. Ann. Surg. (2012). https://doi.org/10.1097/SLA.0b013e3182445341
52. Keane, T.J., Swinehart, I.T., Badylak, S.F.: Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance. In: Methods, vol. 84, pp. 25–34. Academic Press Inc. (2015). https://doi.org/10.1016/j.ymeth.2015.03.005
53. Stapleton, T.W., et al.: Development and characterization of an acellular porcine medial meniscus for use in tissue engineering. Tissue Eng. Part A (2008). https://doi.org/10.1089/tea.2007.0233
54. Costa, A., Naranjo, J.D., Londono, R., Badylak, S.F.: Biologic scaffolds. Cold Spring Harb Perspect. Med. (2017). https://doi.org/10.1101/cshperspect.a025676
55. Zhou, J., et al.: Impact of heart valve decellularization on 3-D ultrastructure, immunogenicity and thrombogenicity. Biomaterials. **31**(9), 2549–2554 (2010). https://doi.org/10.1016/j.biomaterials.2009.11.088
56. Gao, S., Yuan, Z., Xi, T., Wei, X., Guo, Q.: Characterization of decellularized scaffold derived from porcine meniscus for tissue engineering applications. Front. Mater. Sci. **10**(2), 101–112 (2016). https://doi.org/10.1007/s11706-016-0335-y
57. Laker, L., Dohmen, P.M., Smit, F.E.: Synergy in a detergent combination results in superior decellularized bovine pericardial extracellular matrix scaffolds. J. Biomed. Mater. Res. B Appl. Biomater. (2020). https://doi.org/10.1002/jbm.b.34588
58. Meezan, E., Hjelle, J.T., Brendel, K., Carlson, E.C.: A simple, versatile, nondisruptive method for the isolation of morphologically and chemically pure basement membranes from several tissues. Life Sci. (1975). https://doi.org/10.1016/0024-3205(75)90119-8
59. Keane, T.J., Londono, R., Turner, N.J., Badylak, S.F.: Consequences of ineffective decellularization of biologic scaffolds on the host response. Biomaterials **33**, 1771–1781 (2012). https://doi.org/10.1016/j.biomaterials.2011.10.054
60. Chen, T.A., et al.: Detergent-Based Decellularization for Anisotropic Cardiac-Specific Extracellular Matrix Scaffold Generation. Biomimetics (2023). https://doi.org/10.3390/biomimetics8070551
61. Yu, B.-T., Li, W.-T., Song, B.-Q., Wu, Y.-L.: Comparative study of the Triton X-100-sodium deoxycholate method and detergent-enzymatic digestion method for decellularization of porcine aortic valves. Eur. Rev. Med. Pharmacol. Sci. **17**(16), 2179–2184 (2013)
62. Marzaro, M., et al.: In vitro and in vivo proposal of an artificial esophagus. J. Biomed. Mater. Res. A (2006). https://doi.org/10.1002/jbm.a.30666
63. Tudorache, I., et al.: Tissue engineering of heart valves: biomechanical and morphological properties of decellularized heart valves. J. Heart Valve Dis. **16**(5), 567–573 (2007)
64. Flynn, L., Semple, J.L., Woodhouse, K.A.: Decellularized placental matrices for adipose tissue engineering. J. Biomed. Mater. Res. A (2006). https://doi.org/10.1002/jbm.a.30762
65. Kusuma, G.D., Yang, M.C., Brennecke, S.P., O'Connor, A.J., Kalionis, B., Heath, D.E.: Transferable matrixes produced from decellularized extracellular matrix promote proliferation and osteogenic differentiation of mesenchymal stem cells and facilitate scale-up. ACS Biomater. Sci. Eng. **4**(5), 1760–1769 (2018). https://doi.org/10.1021/acsbiomaterials.7b00747
66. Faulk, D.M., et al.: The effect of detergents on the basement membrane complex of a biologic scaffold material. Acta Biomater. **10**(1), 183–193 (2014). https://doi.org/10.1016/j.actbio.2013.09.006
67. Korducki, J.M., Loftus, J.: Stimulation of glycosominoglycon production in cultured human retroocular fibroblasts. Investig. Ophthalmol. Vis. Sci. **33**(6), 2037 (1992).
68. Miller, T., Goude, M.C., Mcdevitt, T.C., Temenoff, J.S.: Molecular engineering of glycosaminoglycan chemistry for biomolecule delivery. Acta Biomaterialia (2014). https://doi.org/10.1016/j.actbio.2013.09.039
69. Chang, Y., Lee, M.-H., Liang, H.-C., Hsu, C.-K., Sung, H.-W.: Acellular Bovine Pericardia with Distinct Porous Structures Fixed with Genipin as an Extracellular Matrix. Tissue Eng. **10**, 5–6 (2004). https://doi.org/10.1089/1076327041348509
70. Funamoto, S., et al.: The use of high-hydrostatic pressure treatment to decellularize blood vessels. Biomaterials **31**(13), 3590–3595 (2010). https://doi.org/10.1016/j.biomaterials.2010.01.073
71. Courtman, D.W., Pereira, C.A., Kashef, V., McComb, D., Lee, J.M., Wilson, G.J.: Development of a pericardial acellular matrix biomaterial: Biochemical and mechanical effects of cell extraction. J. Biomed. Mater. Res. **28**(6), 655–666 (1994). https://doi.org/10.1002/jbm.820280602
72. Cunanan, C.M., et al.: Tissue characterization and calcification potential of commercial Bioprosthetic Heart valves. Ann. Thorac. Surg. **71**(5), S417–S421 (2001). https://doi.org/10.1016/s0003-4975(01)02493-6

Authors and Affiliations

Rui C. Giorgi Filho[1] · André Miguel Martinez Junior[2] · Marilia F. Calmon[3] · Marcio José Tiera[2] · Dayane S. Alvares[4] · Guilherme Agreli[4] · José G. Nery[1]

✉ Dayane S. Alvares
dayalvares@gmail.com; dayane.alvares@unesp.br

✉ José G. Nery
geraldo.nery@unesp.br

[1] Department of Physics, Institute of Biosciences, Languages, and Exact Sciences, São Paulo State University (UNESP), São José do Rio Preto, Brazil

[2] Department of Chemistry and Environmental Sciences, Institute of Biosciences, Languages, and Exact Sciences, São Paulo State University (UNESP), São José do Rio Preto, Brazil

[3] Laboratory of Genomic Studies, Institute of Biosciences, Languages, and Exact Sciences, São Paulo State University (UNESP), São Jose do Rio Preto, Brazil

[4] Department of Research and Development, P+F Products and Features Brasil Indústria e Comércio, Pesquisa e Desenvolvimento LTDA, São Jose do Rio Preto, Brazil