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Pericardium decellularization in a one-day, two-step protocol

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Abstract

Scaffolds used in tissue engineering can be obtained from synthetic or natural materials, always focusing the effort on mimicking the extracellular matrix of human native tissue. In this study, a decellularization process is used to obtain an acellular, biocompatible non-cytotoxic human pericardium graft as a bio-substitute. An enzymatic and hypertonic method was used to decellularize the pericardium. Histological analyses were performed to determine the absence of cells and ensure the integrity of the extracellular matrix (ECM). In order to measure the effect of the decellularization process on the tissue's biological and mechanical properties, residual genetic content and ECM biomolecules (collagen, elastin, and glycosaminoglycan) were quantified and the tissue's tensile strength was tested. Preservation of the biomolecules, a residual genetic content below 50 ng/mg dry tissue, and maintenance of the histological structure provided evidence for the efficacy of the decellularization process, while preserving the ECM. Moreover, the acellular tissue retains its mechanical properties, as shown by the biomechanical tests. Our group has shown that the acellular pericardial matrix obtained through the super-fast decellularization protocol developed recently retains the desired biomechanical and structural properties, suggesting that it is suitable for a broad range of clinical indications.

Keywords Decellularization · Pericardial allograft · Human acellular matrix · Tissue establishment · Tissue engineering

Abbreviations

3D	Three-dimensional
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
GAGs	Glycosaminoglycans
GMP	Good Manufacturing Practices
HE	Haematoxylin–eosin
MT	Masson trichrome
RT	Room temperature
TE	Tissue Engineering
DPM	Decellularized pericardial matrix

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Introduction

Tissue Engineering (TE) is a discipline that combines cells, different material methodologies and suitable biochemical and physicochemical factors to create functional three-dimensional tissues [1]. The main aim of this field is to restore, maintain, improve or replace the function of a whole organ or tissue [2]. The scaffolds used in TE can be obtained from synthetic or natural materials, always focusing the effort on mimicking the extracellular matrix (ECM) that cells can find in vivo to support and stimulate their proliferation, differentiation, maintenance, organization and function [3]. Compared with synthetic materials,

biological materials have better biocompatibility, less cytotoxicity and induce less inflammatory reactions. Moreover, they are readily accessible and provide a broad range of cues [1]. Inner body membranes are biological materials composed by cells and ECM, which cover the surface of the internal organs [4]. These membranes can be used as versatile tools in TE applications and are classified into two main categories: epithelial membranes (amniotic membrane, mesentery, omentum, pericardium, peritoneum and pleura) and connective tissue membranes (fascia, periosteum and synovial membrane).

The heart and the roots of the great blood vessels are enclosed by conical-shaped membrane named pericardium, which protects, lubricates and maintains the heart in place. This cardiac tissue is made up of an inner serous and outer fibrous layer rich in collagen, glycoproteins and glycosaminoglycans [5]. An effective microenvironment can be created by using the pericardium as a scaffold, providing a 3D structure and promoting tissue regeneration. Clinically, pericardial tissue from both human and xenogeneic origins has widely been used, for example, to correct intracardiac and diaphragm defects [6–8], or ischaemic ventricular septal defects [9], with unquestionable benefits [2, 10]. On the other hand, there have also been reported non-cardiac pericardium-based applications, such as replacing the dura mater in the brain [11], ophthalmological surgery [12–15], odontology [16] and eardrum reconstruction [17, 18]. Furthermore, pericardium has been used to create a range of bioprostheses, including heart valves, patches for reconstruction of the abdomen or vaginal wall, and vascular grafts. [19].

Decellularization approach has been evidenced to provide a highly biocompatible pericardial ECM for TE purposes with negligible immune response and without compromising their ECM. At Barcelona Tissue Bank (BTB), several decellularization approaches have been used to eliminate the antigenic components of various tissues [20, 21], including physical, chemical or biological procedures [22]. Together with the decellularization methodology, the tissue's final preservation directly affects the composition and characteristics of the ECM [23]. We postulate that it is feasible to develop a procedure to quickly and easily decellularize pericardial tissue, decreasing the DNA content below 50ng/mg dry tissue and keeping the pericardial matrix, therefore, accomplishing standards for decellularized tissues [23]. In the context of tissue banking, avoiding repeated manipulation is crucial to reduce the risk of graft contamination and obtain a product that can be easily accessible for its intended clinical use [4]. In the same vein, terminal sterilization techniques achieved by exposure to physical or chemical sterilizing agent are employed in order to prevent potential residual microbiological contamination [24–27]. Gamma radiation is one of the most used, successful, and effective procedure for sterilizing allografts since it is based on ionizing radiation to eliminate likely microbes in the tissue [28].

The aim of this study is to define an efficient, fast protocol for obtaining decellularized pericardial matrices (DPM), gamma irradiated at low dose (8-13 KGy) and preserved at room temperature in glycerol. This protocol is tested to ensure cell removal while maintaining the pericardial ECM's biological, biochemical, and biomechanical properties. The tissue obtained may be used for clinical indications such as maxillofacial surgery, dentistry, reconstruction of the tympanic membrane and valve reconstruction, among others. Furthermore, since it does not contain cellular material, it can also be used as a scaffold in advanced therapies, as has been previously published [29].

Materials and methods

Ethical considerations

Human samples were obtained, processed and analysed in accordance with current European guidelines on the collection and preservation of human tissues for clinical use (EEC regulations 2004/23/EC and 2006/17/EC) and in accordance with the protocol and legal requirements for the use of biological samples and biomedical research in Spain (Law 14/2007 and RD 1716/2011). In addition, the acquisition, processing and preservation of the tissues used in this study were carried out in accordance with Spanish law on the development and application of organ transplants (RD 9/2014). All the information provided before donation, together with informed consent, guaranteed that the samples obtained were to be used for clinical applications and/or research purposes. The use, protection, communication and transfer of personal data complied with local regulations (Law 15/1999).

Pericardium procurement

The anterior part of pericardium was procured from thirteen human cadaveric donors between 40 and 70 years old (3 women and 10 men), with consent for research purposes. Donor screening included but may not be limited to review of the complete social and medical history, physical examination of the donor, complete serological and microbiological testing during retrieval, histopathological analysis, as well as any other information pertaining to risk factors for relevant communicable diseases. After retrieval, the pericardium was wrapped in a double-layer surgical drape and directly frozen at − 80 °C until processed in a clean room environment.

Reagents

The following reagents were used in the study: 0.9% NaCl (Braun, Kronberg, Germany, cat #3570470), 5 M NaCl stock solution (Sigma Aldrich, cat #S5150), DNAse (Roche, Basel, Switzerland cat #6922859), PBS (Gibco, MA, USA, cat #14190-094), amikacin (Normon Laboratories—Spain; cat #791301), metronidazole (B. Braun Medical SA- Spain; cat #600496), ciprofloxacin (Altan Farmaceuticals, S.A.; cat #643494), vancomycin (Lab. Reig Jofre, S.A; cat #606390) and amphotericin B (XalabarderFarma, Barcelona, Spain, cat #820152268), RPMI (Roswell Park Memorial Institute medium, Corning, cat #15040) glycerol (Sigma Aldrich, cat #G7757-1L), DMEM (Gibco, MT, United States, cat # 61965-026), FBS (Biowest, Nuaillé, France, cat #S1860-500), and a penicillin/streptomycin/amphotericin cocktail (Antibiotic Antimycotic Solution, Merck, Darmstadt, Germany, cat #A5955-20ML), WST-1 (Abcam, Cambridge, UK cat #ab65475), thioglycolate broth media (Biomerieux, France, cat #28410), BD BACTEC™ Plus Aerobic medium (BD Bioscience, New Jersey, United States, cat #442192), BD BACTEC™ Lytic Anaerobic (BD Bioscience, New Jersey, United States, cat # 442021), paraformaldehyde (VWR, Leuven, Belgium, cat #9713.50000), ethanol (Sigma Aldrich, Darmstadt, Germany, cat #1009831000), PicoGreen (Thermo Fisher, Massachusetts, United States, cat #p11496), agarose (Merck, Darmstadt, Germany cat #1012360500), 10xTAE buffer (Sigma Aldrich, cat #T9650-4L), SYBR Safe (ThermoFisher, Waltham, MA, USA cat #S33102), loading buffer 6× (ThermoFisher, Waltham, MA, USA ca t#J62157-AC) and DNA size marker ladder (TrackIt™ 100 bp ThermoFisher, Waltham, MA, USA cat #10488058).

Decellularization protocols

The pericardium was thawed overnight at 4ºC and incubated at room temperature in 500 mL antibiotic cocktail containing amikacin 0.6 mg/mL, metronidazole 0.6 mg/mL, ciprofloxacin 0.15 mg/mL, vancomycin 0.6 mg/mL and amphotericin B 0.01 mg/mL in RPMI medium (Roswell Park Memorial Institute Medium). The pericardium decellularization protocol was defined to remove the cellular content while maintaining its structure and mechanical properties. The super-fast protocol consisted of the following steps: (i) mechanical cleaning, (ii) cell osmosis and (iii) cell remnants and DNA content removal. First, the pericardium was cleaned mechanically, using scissors and forceps, to eliminate other tissues such as fat that could be attached to it. Subsequently, the tissue was inspected in order to select regular fragments while discarding holes, areas with heterogeneous thickness or fibre separation. The obtained tissue sizes were between 25 and 60cm^2 with a thickness of between 0.4 and 1 mm. Following macroscopic evaluation, the pericardium was incubated in 100 mL of 2 M NaCl during 2.5 h, which causes cell lysis by osmosis. Next, it was incubated in 50 mL of 0.2 mg/mL DNAse during 1 h, which causes the lysis of amino acid sequences, resulting in the elimination of the genetic material. All incubations were performed under gentle stirring at 37 ± 2 °C, and three rinsing steps with sterile water were performed after each incubation. The obtained pericardial matrix was preserved in a glycerol solution, and then a final decontamination step by gamma radiation (8–13 KGy) was performed. The complete process, starting from pericardium thawing and ending with completion of the decellularization protocol and final packaging, lasts less than one working day.

Microbiological assessment

Pericardium tissue samples were taken during retrieval and included in thioglycolate broth media. Before starting the process, a second biopsy was taken and during processing, microbiological samples were obtained in the final step of the decellularization protocol. Biopsies of the tissue were included in thioglycolate broth media for anaerobic growth and liquid samples were included in BD Bactec FX bottles for aerobic/anaerobic growth. The samples were analysed by the Biomedical Diagnostic Center (CDB) of the "*Hospital Clínic de Barcelona*".

Analysis of the extracellular matrix

Qualitative assay: histology and structure

The structure of the pericardial extracellular matrix was assessed by means of histology. Biopsies around 0.25mm^2 from each pericardium were obtained before and after the decellularization process and fixed overnight with 4% paraformaldehyde solution at 4 °C. The samples were subsequently washed with PBS and preserved with 30% ethanol solution until a paraffin embedding was achieved. Serial sections of 3 µm thickness were stained using the haematoxylin–eosin (HE) and Masson trichrome (MT) protocols. Images were taken from each slide using an Axio Scope A1 (Zeiss) bright-field microscope and an AxioCam MRc5 camera.

Quantitative assays: ECM biomolecule analysis

The main ECM biomolecules contained in pericardium samples were quantified using commercially available kits according to manufacturer's instructions: Soluble Collagen Assay Sircol™ (Biocolor life science assays, cat #S1000) for total collagen; Fastin Elastin Assay™ (Biocolor life science assays, cat #F2000) for elastin; and Glycosaminoglycan Assay Blyscan™ (Biocolor life science assays, cat #B1000)

for total glycosaminoglycan (GAGs). Briefly, after freeze drying of the samples, 5 mg of each donor was weighed (in duplicate) to quantify each molecule. Samples used for collagen quantification were lysed with 1 mL of HCL 6 M during 18 h at 95 °C; samples for elastin quantification were lysed with three serial lysis of 500 µL oxalic acid 0.25 M, lasting 1 h each at 100 °C; and samples for GAGs quantification were lysed with 10 µL of papain in 1 mL papain buffer during 18 h at 65 °C. The resulted lysates were analysed using dye-binding methods, and the absorbance was read with an Epoch microplate spectrophotometer (Biotech) at 570 nm (collagen), 513 nm (elastin), and 656 nm (GAGs). The results were presented as µg biomolecule/mg dry weight tissue.

DNA evaluation

A commercially available kit QIAamp DNA Mini Kit™ (Qiagen, cat #51304) was used to extract the DNA from the pericardium before and after decellularization. Tissue biopsies were freeze-dried, and 5 mg of each sample was used for DNA isolation through affinity columns. Briefly, the tissue was incubated in 200 µl ATL buffer with proteinase K and maintained at 56 °C during 24 h before its inclusion in affinity columns. Two serial 200 µL elutions were used to elute the DNA through the columns. The amount of DNA was quantified by spectrophotometry using the PicoGreen commercial kit using a Triad Multi-Mode Microplate Reader (Dynex Technologies). The amount of DNA remaining in decellularized pericardium was compared with native samples, and both DNA percentages were calculated. The results were presented as ng DNA/mg dry weight tissue.

The DNA electrophoresis was performed as follows: agarose was added to 1 × TAE buffer to a final concentration of 0.8%. The mixture was heated until complete homogenization and 5 µl of SYBR Safe were added to the warm solution, which was gently mixed and poured on the gel tray until solidified. Samples were prepared with loading buffer 6 × concentrated, and 20 µL of each sample were loaded on gel. The gel was run in 1 × TAE buffer at 120 V for 30–40 min. A molecular size marker was always used, and DNA fragments were visualized under UV light.

Cell viability

An in vitro cytotoxicity assay of DPM extracts was performed following ISO 10993 recommendations [30]. To assay the pericardium extracts, the DPM was washed four times and 100 mg/ml of DPM were immersed in culture media (DMEM medium complemented with 10% FBS and 1% of penicillin, streptomycin, and amphotericin B) for 24 h at 37 °C under agitation. First, 3T3-J2 fibroblasts were seeded on a 96-well plate and incubated in a water-jacketed incubator at 37 °C and 5% CO_2. Having obtained a subconfluent layer of 3T3-J2 fibroblasts, the culture medium was removed and the filtered pericardium extract was added to the cells, which were incubated in a water-jacketed incubator at 37 °C and 5% CO_2 for 24 h. Afterwards, a WST-1 assay was performed, incubating the cells in the reagent for 3 h in accordance with the manufacturer's recommendations. Plates were read at 450 nm with a reference wavelength of 680 nm in an absorbance plate reader (Biotek). Cells with conditioned DMEM medium and TRITON™ X-100 surfactant were used as a negative and positive cytotoxicity controls, respectively.

Biomechanical testing by uniaxial mechanical assay

Pericardium samples (native and decellularized) were tested using a uniaxial tensile test, increasing stress until rupture is achieved by means of a universal tensile testing machine (Instron 3366), which measures the material's resistance to an applied force. Control samples stored at 4 °C were brought to RT and then measured. Decellularized samples stored in 50% glycerol at RT were washed 5 times with 0.9% NaCl before measuring. Bone-shaped samples of 4 × 1 cm were prepared and gripped to measure the mechanical properties (Fig. 4A–C). The specific thickness of each sample was measured using a micrometer. Samples were pre-conditioned using a speed of 12 mm/min until a load of 0.5 N was reached, which was defined as an unstretched length (L_o). The samples were stretched at a speed of 12 mm/min until rupture and the mechanical properties of each sample were obtained from the stress–strain (σ–ε) curve. The following mechanical properties were evaluated: maximum load (N), Young's modulus (N/mm), and elongation at maximum load (%).

Residual glycerol quantification

To define the washing steps (deep soaking with manual agitation) that would need to be performed before implantation by the clinical team, the residual glycerol in the pericardial matrix after each of the four rinsing steps (with 500 ml of saline solution for 5 min) was quantified using a commercially available Glycerol Assay Kit™ (Sigma Life Science, cat #MAK117-1KT) following the manufacturer's instructions. The absorbance was read at 570 nm using the Epoch microplate spectrophotometer (Biotech). The results were presented as ppm glycerol/mg tissue.

Statistical analysis

PRISM software version 5.00 (GraphPad Software, San Diego CA, USA; www.graphpad.com, accessed on 18 October 2022) was used for the statistical analysis. All results

are presented as the mean ± standard deviation (M ± SD) obtained from minimum five samples corresponding to independent donors. Each sample was analysed in triplicate. The non-parametric two-tailed Mann–Whitney test was used and p values less than 0.05 ($p < 0.05$) were considered statistically significant. Differences between results are considered statistically significant at a value of $p < 0.05$: $*p < 0.05$, $**p < 0.01$. Non-statistically significant results are indicated as ns.

Results

Histological and macroscopic evaluation of DPM

No evidence of residual cell material was observed in the DPM after the decellularization protocol since Hematoxylin–Eosin, Masson Trichrome and Collagen/DAPI staining show the elimination of the nucleus in the tissue (Fig. 1F–J). The structural architecture of the matrix was maintained in after decellularization (Fig. 1F-I) when compared to the native pericardium (Fig. 1A–D). Macroscopically, Fig. 1K shows the pericardium immediately after extraction. Following antibiotic incubation and mechanical cleaning, the pericardium is fat free, as depicted in Fig. 1L. The decellularized pericardium is presented in Fig. 1M.

Microbiological assessment

There was no evidence of bacterial or fungal growth on either the thioglycolate media or the blood culture for any of the samples taken at the end point of the protocol. Moreover, after processing the decellularized pericardial tissue was irradiated at low dose. Thus, obtained tissue is microbiologically safe since all quality controls that monitor the microbiology of the process were negative.

DNA quantification

After pericardium decellularization, DNA quantity was below 1,7 ng/mg dry tissue (Fig. 2A). Therefore, the proposed protocol eliminates 99.4% of the genetic material, giving a statistically significant DNA reduction. Moreover, the electrophoresis analysis showed the absence of genetic fragments larger than 200 bp (Fig. 2B).

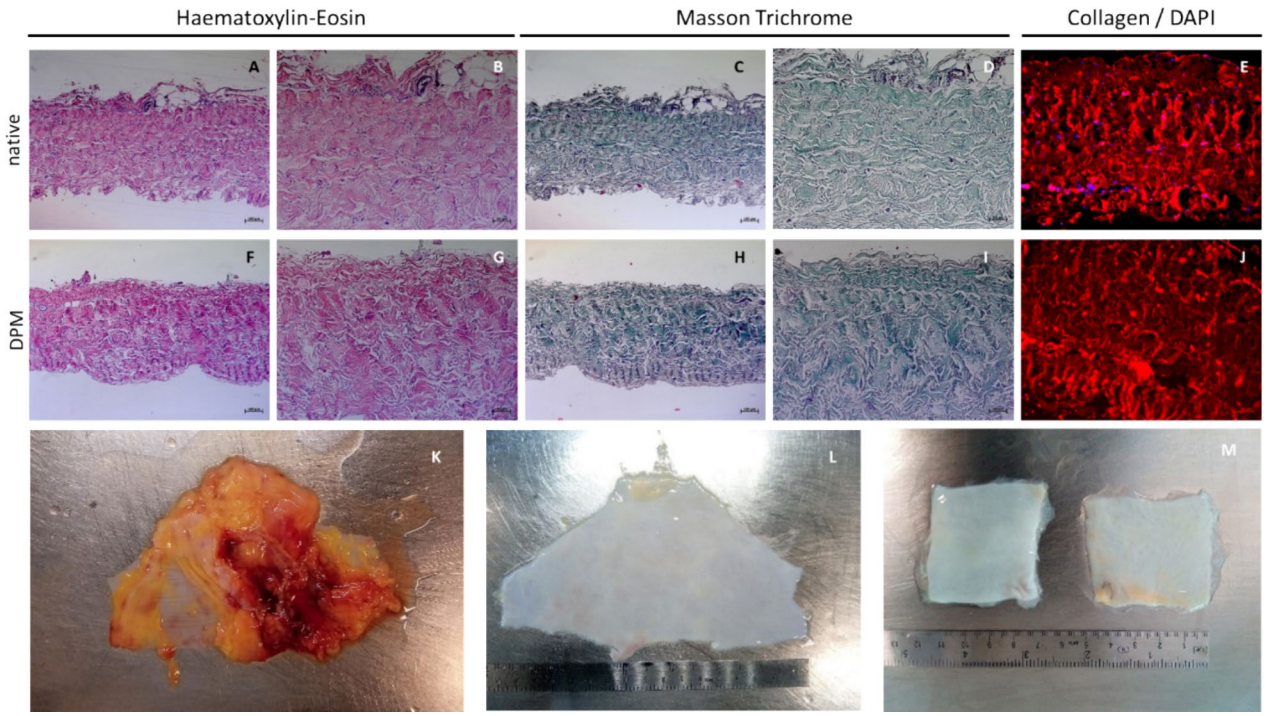


Fig. 1 Microscopic and macroscopic evaluation of native and decellularized pericardium. Histological sections of native (**A** to **E**) and decellularized (**F** to **J**) pericardium are shown. **A** and **F** Haematoxylin–Eosin staining with a scale bar of 100 µm; **B** and **G** Haematoxylin–Eosin staining with a scale bar of 50 µm. **C** and **H** Masson Trichrome staining with a scale bar of 100 µm; **D** and **I** Masson Trichrome staining with a scale bar of 50 µm. **E** and **J** Immunofluorescence staining for collagen/DAPI with a scale bar of 50 µm. Macroscopic images of the pericardium are shown before cleaning and dissection (**K**), after processing to eliminate fat and connective tissue (**L**), and after decellularization and preparation of 4×4 samples (**M**)

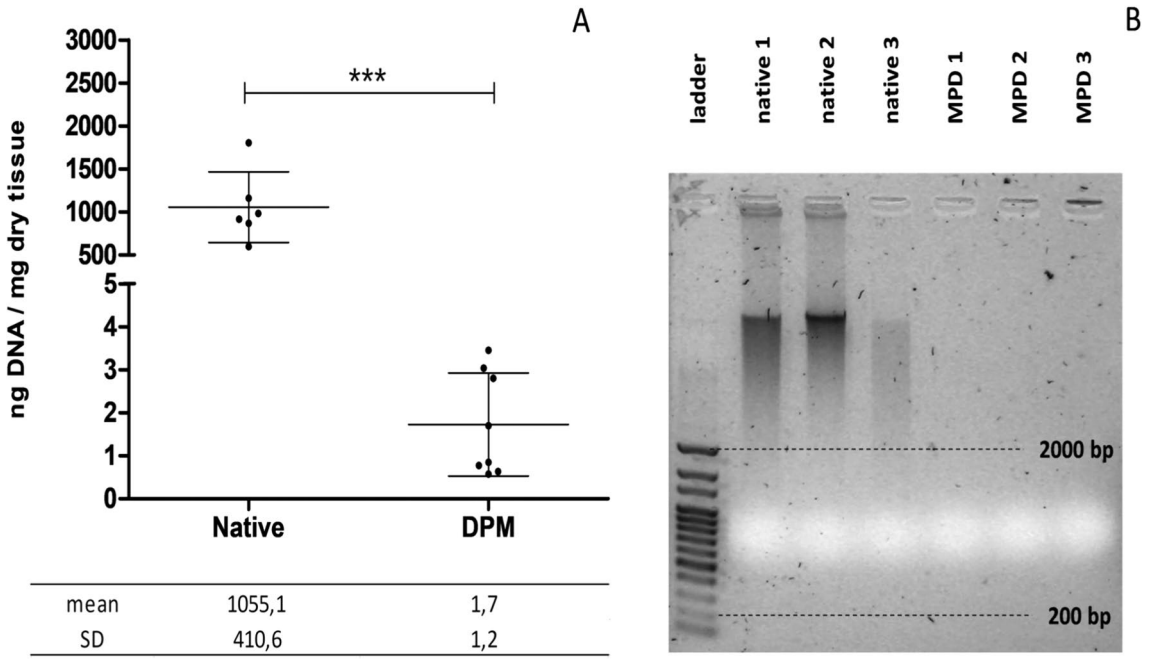


Fig. 2 DNA content in the native and decellularized samples (DPM). **A** DNA content and **B** Electrophoresis Gel of DNA extracted from the pericardial matrix was compared with DNA extracted from the native pericardium. The results are presented as mean ± SD, and statistical differences were determined using the non-parametric two-tailed Mann–Whitney test (minimum $N = 6$, in triplicate). Differences are significant with p-value < 0.05

Structural ECM biomolecule content

The decellularization protocol did not affect the quantity of total collagen (Figure 3A) and elastin (Figure 3B), despite observing a significant reduction in GAGs content in the DPM compared to native pericardium (Figure 3C).

Biomechanical properties

The mechanical properties of both native pericardium and DPM, preserved in glycerol and sterilized by low-dose gamma irradiation, were assessed using stress–strain tensile strength. The stress–strain curve (Fig. 4D) illustrates the relationship between the mean stress of all analysed samples and strain. Although the mean stress values for DPM are generally higher than those for native pericardium at the same strain percentage, there is significant variability in the stress–strain curves among donors within the same group (native or decellularized), resulting in no significant differences between the groups. When comparing DPM with native pericardium, no significant differences were observed in maximum load (Fig. 4E), Young's modulus (Fig. 4F), elongation at maximum load (Fig. 4G) or Ultimate Tensile Strength (Fig. 4H). The decellularized pericardium maintained its intrinsic elastic properties compared to native pericardium, and the results indicate that DPM preservation in glycerol and terminal sterilization by low-dose gamma irradiation do not affect its mechanical properties (Figs. 4E–H).

Residual glycerol quantification

Residual glycerol decreased progressively and significantly after four serial washes of 5 minutes each (Figure 5). After the first 5-min wash, a decrease of 83.6% was observed, followed by a total decrease of 96.8% after the second wash. 99.7% of the glycerol had been removed (maintaining a residue of 0.91ppm/mg dry tissue) by the fourth wash.

Cell viability

Decellularized pericardium matrix preserved in glycerol proved to be non-cytotoxic, as shown by the viability test performed following ISO 10993-5 [30]. As can be seen in Fig. 6, cell viability is above 70% for all washing steps. Therefore, the DPM produced is biocompatible and safe for transplanting.

Discussion

Nowadays, human or animal grafts and synthetic materials are used as bio-substitutes in a broad range of clinical indications [6–9, 11–19, 31]. Most commercial pericardium

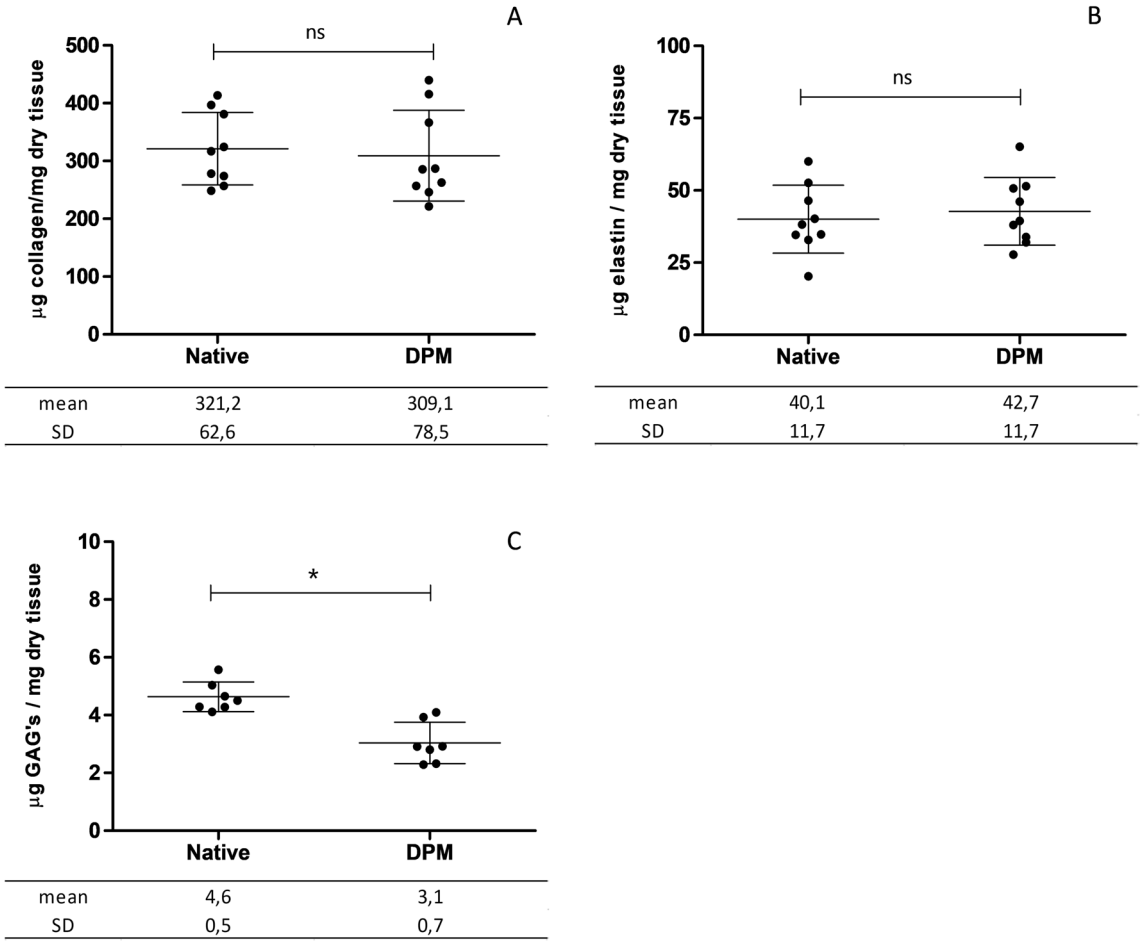
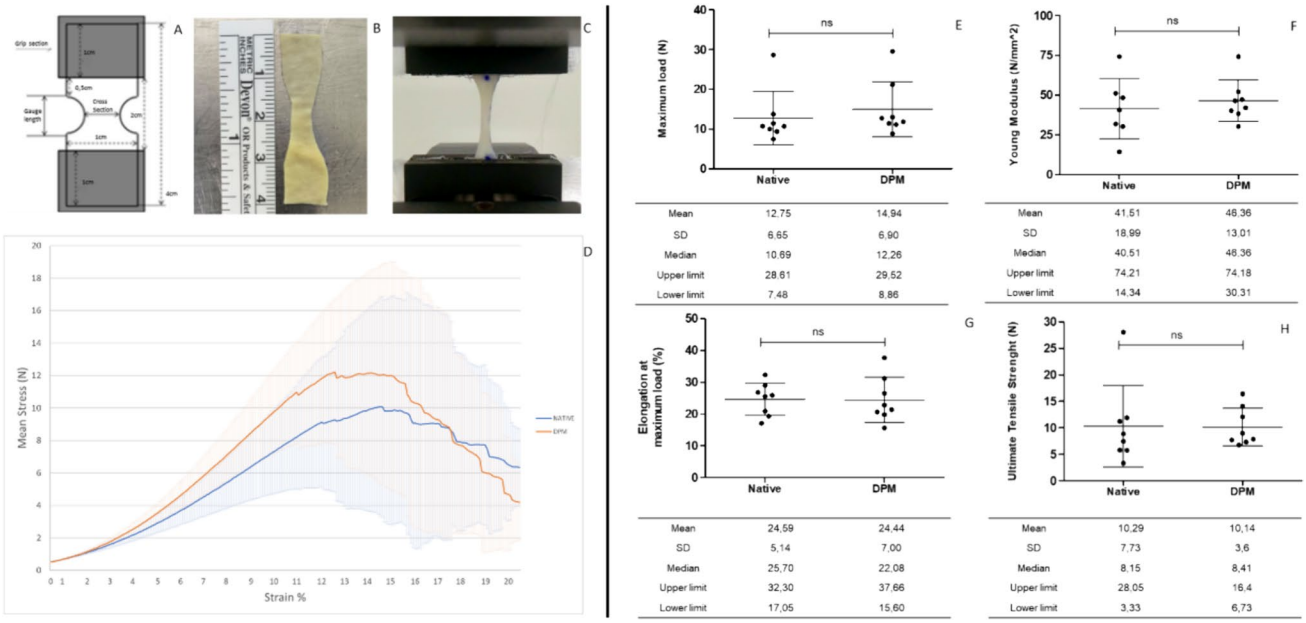


Fig. 3 Structural ECM biomolecule content. **A** Collagen, **B** Elastin and **C** GAGs concentrations. The results are presented as mean ± standard deviation and statistical differences were determined using the non-parametric two-tailed Mann–Whitney test (minimum $N = 7$, in triplicate). Differences are significant with p-value < 0.05

patches are made from bovine material, such as BioIntegral Surgical No-React® [32], Dura-Guard® [33], Duravess® [34], XenoSure® [35], SJM® [36] or TutoMesh® [37], and few are also marketed that come from humans (Tutoplast®) [38]. A biocompatible, non-cytotoxic and non-immunogenic human epithelial membrane such as a decellularized pericardium graft provides a natural microenvironment for use in TE applications [4].

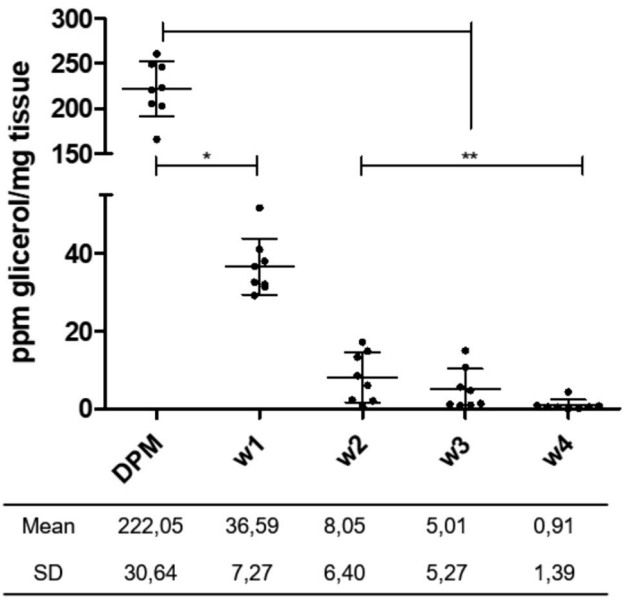
In this study, we have developed a novel, extremely fast protocol to obtain decellularized human pericardium for clinical applications. This protocol succeeds in reducing the decellularization processing time in comparison with other already published protocols. For instance, Wollman et al. describe a method using non-ionic detergents, which needs ten days of washing steps to obtain 511.23 ng DNA/mg dry tissue after pericardium decellularization [39]; Musilkova et al. present a protocol without non-ionic detergents which lasts six days [40], whereas Montagner et al. uses a non-denaturated detergent consisting in three full days to obtain 2 ng DNA/mg dry tissue after pericardium decellularization [41]. Our protocol can achieve decellularized pericardium in one working day without any sort of detergents that could be potentially cytotoxic [42] and avoiding undesired adverse side effects. This new protocol is able to remove 99.5% of the DNA (remaining 1.7 ng DNA / mg dry tissue), while maintaining the pericardium's native-like structure and preserving its biomechanical properties as well as its main ECM biomolecules. Consequently, the decellularized pericardium obtained is expected to be a suitable graft for several clinical indications and TE applications.

A number of protocols for pericardium decellularization of different origins have been described and DPM products have been available on the market for several decades, for instance, CardioCel® [43] or Supple Peri-Guard®. Efficiency of the decellularization is highly dependent on the type and quantity of cells, ECM density, lipid content and thickness of the tissue [22]. Other protocols have been published and shown to be effective for decellularizing pericardium, using a combination



	E Native	E DPM	F Native	F DPM	G Native	G DPM	H Native	H DPM
Mean	12,75	14,94	41,51	48,36	24,59	24,44	10,29	10,14
SD	6,65	6,90	18,99	13,01	5,14	7,00	7,73	3,6
Median	10,69	12,26	40,51	48,36	25,70	22,08	8,15	8,41
Upper limit	28,61	29,52	74,21	74,18	32,30	37,66	28,05	16,4
Lower limit	7,48	8,86	14,34	30,31	17,05	15,60	3,33	6,73

Fig. 4 Mechanical uniaxial assay. **A** Representative scheme of the samples used for mechanical testing; **B** Representative image of the samples used for the mechanical testing. **C** Sample gripped in the tensile testing machine. **D** Stress–Strain curve of native and decellularized pericardium represented as the mean ± SD of all evaluated samples per group. The SD is represented as density areas. The DPM's mechanical properties were compared with native pericardium. **E** Maximum load (N), **F** Young's modulus (N/mm^2), **G** Elongation at maximum load (%), and **H** Ultimate Tensile Strength (N/mm^2). The results are presented graphically as mean ± standard deviation, and table contains present results of mean ± standard deviation and median with range. Statistical differences were determined using the non-parametric two-tailed Mann–Whitney test (minimum $N = 8$, in triplicate). Differences are significant with p-value < 0.05



	DPM	w1	w2	w3	w4
Mean	222,05	36,59	8,05	5,01	0,91
SD	30,64	7,27	6,40	5,27	1,39

Fig. 5 Reagent residues. Quantification of glycerol in the DPM after serial washes of 5 min each (w1: one wash, w2: two washes, w3: three washes, w4: four washes). The results are presented as mean ± standard deviation and statistical differences were determined using the non-parametric two-tailed Mann–Whitney test ($N = 8$, in triplicate). Differences are significant with p-value < 0.05

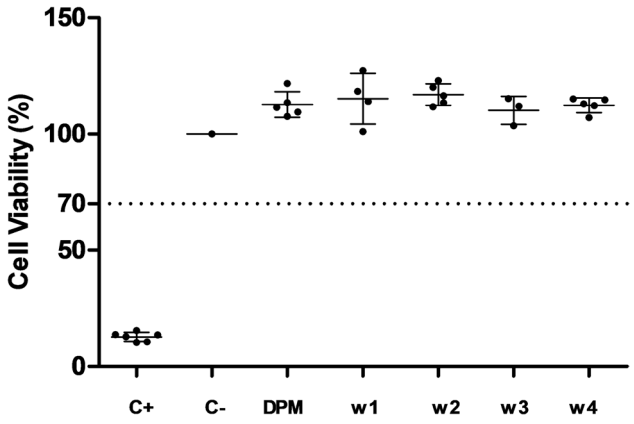


Fig. 6 Cell viability of DPM after four serial washes. The results are presented as mean ± standard deviation and statistical differences were determined using the non-parametric two-tailed Mann–Whitney test (minimum $N = 3$, in triplicate)

of different decellularization agents and incubation times and temperatures. However, with these protocols, the complete process takes at least 2–3 days [29, 39, 44–47]. In contrast, our new protocol proved to be effective in a few hours, which is a very short time. Our methodology is capable of significantly reducing the DNA content below the accepted threshold of 50 ng/mg dry tissue [22], while keeping the native

histological architecture intact. The histological conformation of the pericardium, composed of a membrane of fibrous and epithelium tissue, is rich in several biomolecules such as collagen, elastin and glycosaminoglycans [5]. Collagen is the main structural protein as well as the primary mechano-structural element, being stiff and lacking extensibility. Elastin fibers dictate the tissue's mechanical behaviour at small stresses and strains and interplay closely with the collagen, facilitating return to its wavy configuration at rest [48]. Compared with native pericardium, collagen and elastin quantity were preserved, while a significant decrease in GAGs was observed, as has been published previously by other decellularization protocols [49, 50]. GAGs reduction after decellularization could be caused by the reduction in the thickness of the epithelium layer caused by the chemical reagents involved in the process. However, no significant changes were observed in terms of mechanical behaviour. As described previously, the contribution of the glycosaminoglycans to elasticity and tensile strength is minimal [48]. Moreover, a glycerol-based preservation solution, which was chosen for its antimicrobial properties and its ease of handling, has been shown to not cause a cytotoxic response after four washes with 0.9% NaCl. As a result of the protocol described here, a safe pericardium graft without cells is obtained which has the potential for use in the treatment of cardiovascular pathologies or other applications.

According to the DNA quantification and histological results, the reagents are capable of penetrating the different pericardium layers and decellularizing the pericardium effectively. In spite of all these benefits, some limitations affect this study. As each donor's pericardium is different (intra-donor variability), it is difficult to obtain a homogeneous thickness for all pericardium samples. For this reason, homogeneous DPM fragments were carefully selected for decellularizing. Moreover, in order to assure biocompatibility of the allograft, repeating serial washes must be performed at the end of the protocol to remove the reagents used in the process and obtain a product suitable for clinical requirements. Finally, as commented above, the GAGs content in the DPM is reduced when compared to native pericardium, but this effect does not result in a decrease in mechanical properties and a DPM suitable for clinical transplantation is obtained.

European tissue establishments perform their activities in accordance with a set of guidelines that demand an ethical approach to tissue procurement, a manufacturing licence granted in accordance with the current state of the art in GMP, a procedure that ensures a minimum viral, bacterial and fungal load, and quality control measurements that continuously guarantee tissue quality. In terms of disease transmission and adverse reactions, a decellularized human pericardium graft manufactured on this basis may be considered safe. We can be sure that the DPM produced in GMP facilities under strict sterility control poses a remarkably low risk of infectious disease transmission. Our protocol reduces the processing time in clean rooms while ensuring the decellularization of the native pericardium, thereby reducing the probability of human-caused contamination. Moreover, this time reduction increases process efficiency with the corresponding cost savings and risk reduction.

Biological scaffolds derived from decellularized human tissues are promising for supporting tissue growth and regeneration in vivo for preclinical research and clinical practice [51–53]. Tissue specifications must be tailored to each clinical application, but in all cases, the decellularized pericardium should integrate with the recipient's own tissue and promote autologous tissue regeneration.

Conclusion

This study describes an easy, extremely fast step-by-step procedure, from retrieval and decellularization of native pericardium to final preservation of the DPM. The one-day decellularization protocol delivers a human cell-free pericardium matrix that meets the product specifications, with negligible levels of residual genetic material, while maintaining the major ECM biomolecule content and biomechanical elasticity properties. This time reduction increases process efficiency with the corresponding cost savings and risk reduction. The acellular pericardium matrix developed is, therefore, considered a safe allograft endowed with a series of mechanical, structural, biochemical, and storage properties, which suggests its suitability for a broad range of clinical indications or TE applications.

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Data availability No supporting data are available.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the study reported in this paper.

Consent to participate All tissue samples come from donors whose family signed an informed consent form.

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