DECELLULARIZATION AND CHARACTERIZATION OF RABBIT HEART

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May 2023

Declaration

I hereby declare that this manuscript, entitled "Decellularization and Characterization of rabbit heart" is the result of my work except for quotations and citations which have been duly acknowledged.

I also declare that, to the best of my knowledge and belief, it has not been previously or concurrently submitted, in whole or in part, for any other degree or diploma at Nazarbayev University or any other national or international institution.

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Date: 25.04.2023

Acknowledgments

I would like to express my deepest gratitude to my master's thesis supervisor, Dr. Cevat Erisken, for his invaluable guidance, unwavering support, and insightful feedback throughout the entire research process. His expertise and encouragement have been instrumental in the completion of this thesis.

I would also like to thank my co-supervisor Dr. Ivan Vorobyev for his assistance and support. I would like to acknowledge the BIREL team: Leya Timur, Aida Zhakypbekova, Baisunkar Mirasbek, Sanazar Kadyr, Bakhytbol Khumyrzakh, Victoria Effiong Effanga, Kamila Yeralimova, Francisco Elieso Jaramillo, Dulat Minas, Sabina Askarbekova whose contributions made this research possible. Their willingness to share their experiences and insights was critical in advancing the understanding of the research.

Furthermore, I am grateful to my parents, sister, my nearest and dearests, friends & colleagues for their constant love and encouragement, and for providing me with the motivation and inspiration to achieve my academic goals. Thank you all for your support and encouragement throughout this journey.

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List of abbreviations

CVD	Cardiovascular disease
ECM	Extracellular matrix
dECM	Decellularized extracellular matrix
LA	Left atrium
LV	Left ventricle
RA	Right atrium
RV	Right ventricle
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate
EDTA	Ethylenediaminetetraacetic acid
DAMP	Damage to the molecular pattern
DNA	Deoxyribonucleic Acid
GAG	Glycosaminoglycan
PG	Proteoglycan
DAMP	Damage-associated molecular pattern

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Abstract

In conformity with the "Bureau of National Statistics of the Agency for Strategic Planning and Reforms of the Republic of Kazakhstan", from 2010 to 2020, the incidence of cardiovascular diseases (CVD) increased by 44%. According to the Global Burden of Disease (GBD) Study 2019, the global trend of CVD cases doubled (51,8%) between 1990 and 2019. Despite new approaches in the treatment of CVD such as percutaneous coronary intervention (PCI), coronary artery bypass graft (CABG), and left ventricular assist devices (LVAD), which cannot fully compensate for the effectiveness of the original heart, heart transplantation remains as the most effective solution.

A growing body of literature recognizes the importance of developing a whole heart constructed from living tissues to provide an alternative option for patients suffering from diseases of the cardiovascular system. A potential solution that shows promise is to generate cell-free scaffolds using heart tissue to be later populated with induced pluripotent or embryonic stem cells and implanted. This study reports the initial phase, i.e., decellularization, in the creation of the whole heart scaffold. The hearts harvested from rabbits were decellularized using a perfusion method. The final bioartificial scaffolds were characterized for the efficiency of decellularization in terms of DNA content, collagen, and glycosaminoglycan (GAG). Findings revealed that the DNA content of the decellularized hearts was significantly reduced while keeping collagen and GAG content unchanged. Decellularized hearts have significant importance in treating CVD as they serve as bioartificial hearts, providing a more clinically relevant model for potential human use.

Future work will focus on the recellularization of the heart using induced pluripotent or embryonic stem cells, which allows for a deeper analysis of the effectiveness and prospects of this

treatment method.

Keywords: tissue engineering, whole organ scaffold, extracellular matrix, cardiovascular system, heart decellularization, perfusion, DNA, collagen, GAG.

CHAPTER 1- (INTRODUCTION)

Currently, cardiovascular disease (CVD) is the leading cause of mortality in most countries, including the Republic of Kazakhstan. According to the World Health Organization, over 17 million people die annually from cardiovascular diseases worldwide [1]. Signs of CVDs are not always obvious and may have similar features to the symptoms of other pathologies unrelated to the heart. The main danger of cardiovascular diseases is the inability of the heart to function or sudden death. Hence, there is a need to explore new options over the existing traditional approaches for the prevention and treatment of CVD.

1.1. Structure and function of native cardiac decellularized extracellular matrix

Since the decellularized heart retains the spatial array of matrix components, it has various benefits compared to alternative scaffolds. The decellularized extracellular matrix (dECM) plays an important role in the vital activity of the tissue itself and participates in cell communications, division, differentiation, and function. The dECM captures a complex set of proteins such as collagens, GAGs, PGs, and many other ECM compounds that are fixed in native tissues [2]. According to literature sources, dECM provides signals for the regeneration and restoration of the heart muscles [3]. One of the advantages of using a dECM is its mutability, where solid frames can be used for the native structure of the matrix, and in a soluble form, hydrogels can be formed for cell injection to restore heart function [3]. Heart ECM consists of structural and non-structural proteins, which can perform different functions. The main structural proteins are glycoproteins including elastin, fibronectin, laminin, and collagens of types I and III [4]. The amino acid

sequence of the polypeptide chains of these proteins makes it possible to form a structure with unique mechanical properties, which have enormous strength and elasticity [4]. Nonstructural proteins (proteoglycans) ensure the functioning of dECM as an information center that accumulates and transports signal data for all organ cells [4]. Amongst other things, proteoglycans create a kind of reservoir for cellular factor growth, providing adaptive regenerative capabilities for the heart [4].

1.2. Choice of decellularization approach and chemicals

Decellularization is a process aimed at removing cells from the tissues while preserving the extracellular matrix and the three-dimensionality of the organ structure [5]. One of the main goals of successful decellularization is to prevent an immune response by removing nuclear components, damage-associated molecular patterns (DAMPs) and epitopes of the cell membrane, allogeneic or xenogenic DNA [6].

Existing research recognizes the critical role played by the decellularization approach and solution types on the effectiveness of decellularization. Prior reports published on the decellularization of the heart suggest different techniques: chemical, enzymatic, and physical. As described in Table 1.1, each method has its pros and cons regarding nuclear content removal efficiency; impact on ECM structure integrity, and collagen content [7].

According to Table 1.1, it is important to note that in most cases, regardless of the method of decellularization, the chemicals used in the process are similar. For example, the non-ionic detergent Triton X-100 is effective in removing cells and DNA, as it reduces the concentration of glycosaminoglycan (GAGs) and cleaves tissues. Sodium dodecyl sulfate (SDS) is an anionic detergent and is often used in the decellularization of organs to remove cellular components and

debris from the tissue. Serine protease-trypsin is used in decellularization to digest cellular proteins hydrolytically and cleaves proteins [8]. Deoxycholic acid decellularizes tissue while preserving structural proteins in an intact position [9]. EDTA is important to prevent blood clotting, and biofilm formation during the decellularization procedure [8]. Peracetic acid is used for disinfection of the tissue while maintaining ECM integrity [10]. EDTA is important to prevent blood clotting and, biofilm formation during the decellularization procedure [8]. Sodium azide is used for bacteriostatic effects to prevent contamination [11].

Table 1.1: List of decellularization approaches.

#	Decellularization type	Solutions	Mechanism of action	References
1	Acidic; Alkaline solutions	Acetic acid; Peracetic acid; Sulfuric acid; Ammonium hydroxide	cell membranes and intracellular organelles are destroyed	[12]
2	Detergents	Triton X-100; Sodium dodecyl sulfate; SDC; Zwitterionic	Lipids and cell membranes are solubilized	[13]
3	Antibiotics	Penicillin; Streptomycin; Amphotericin B	Preventing contamination	[14]
4	Lipases	Gastric lipase; Pancreatic lipase	Solubilization and hydrolysis of lipids	[15]
5	Proteases	Trypsin; Pepsin; Protease K	Degradation of proteins	[16]
6	Freeze-thawing	n.d.	Ice crystals formation by freezing cause cell lysing	[17]

*n.d.- not determined

According to Table 1.1, each type of decellularization has its own mechanism of action, therefore it is necessary to combine some of them in order to achieve maximum results with minimal damage to dECM. Several studies have compared methods of decellularization, studying the elimination of cells and genetic components, and then ensuring the preservation of structural proteins [13], [18]. In this study, we utilized chemical and enzymatic methods to remove DNA content and cell, while keeping intact structural and regulatory proteins. Application of ionic and non-ionic surfactants such as SDS and Triton–X bring solubilization of lipids and cell membranes. Acids and bases with charged properties help with cell membrane solubilization, so peracetic acid and EDTA sterilize and increase the stiffness of ECM. Enzymes such as trypsin help with cell-matrix adhesion damage [18]. Each solution has an impact on ECM in decellularization, nevertheless, it is important to ensure a minimal efficient amount of solution which will not bring side effects for dECM.

1.3. Selection of animal models

From a clinical perspective, using larger animal models in heart decellularization is more relevant. Table 1.2 lists studies published on heart decellularization of different species. According to Table 1.2, the time interval varies greatly depending on the size of the heart.

One of the reasons for choosing rabbits as an animal model was that we have previously decellularized rat hearts and would like to scale up the process using a larger animal model. The other reason is the generally recognized similarity between human and rabbit hearts. Marian (2005) compared physiological changes in humans, rabbits, and mice during myocardial tissue damage. Similar heart rates were identified in humans and rabbits [19]. Additionally, from the point of view

of the practicality of the study, compared with the human and pig heart, the rabbit heart requires fewer resources and less quantity of reagents for experiments, making it more cost-effective.

Table 1.2: Review of animal heart decellularization studies.

#	Animal model	DNA removal efficiency	Chemicals/Biologicals Used	Decellularization duration	References
1	Human heart	n.d.	PBS; NaCl (hypertonic and hypotonic), NaH2PO4 and KH2PO4 in distilled water; SDS; Peracetic acid; NaOH;	8 days	[20]
2	The cadaveric rat	97%	Deionized water; Phosphate-buffered saline; Trypsin; EDTA; Sodium azide; Sodium dodecyl sulfate; Triton-X100; Deoxycholic acid; Peracetic acid; ethanol;	10 hours	[21]
3	The cadaveric rat	96%	Polyethylene glycol; SDS; Triton-X100;	12 hours	[22]
4	The cadaveric rat	Protocol I: remove 43% Protocol II: remove 80% Protocol III: remove 99% Protocol IV: remove 99%	Protocol I: PBS with adenosine; SDS; deionized water; Triton-X100; Protocol II: Trypsin; EDTA; deionized water; DCA Protocol III: Glycerol; NaN3; EDTA; DCA; Triton-X100; PBS Protocol IV: PBS; SDS; NaN3; deionized water; Glycerol; EDTA; DNase I	Protocol I: 6 days Protocol II: 5 hours Protocol III: 12 days and 12 hours Protocol IV: 16 hours	[23]
5	The porcine heart	n.d.	PBS; deionized water; SDS; Triton-X100;	7 days	[24]
6	The porcine heart	72%	Deionized water; SDS; Triton-X100;	15 hours	[24]
7	The cadaveric rat	99%	PBS with Adenosine; SDS; Triton-X100; Penicillin, Streptomycin and Amphotericin with PBS;	13 hours 55 min	[25]

8	The rabbit heart	87%	EDTA; PBS; SDS; deionized water; ethanol; Sodium azide;	10 days	[26]
9	The cadaveric rat	98.9%	PBS; phenylmethylsulfonyl fluoride; N- ethylmaleimide; benzamidine; iodoacetamide; sodium ascorbate; EDTA; dimethyl sulfoxide; SDS; deionized water; Triton-X100; 2,3-butanedione monoxime; penicillin-streptomycin; amphotericin;	2 days 21 hours 15 min	[27]
10	The ovine heart	88%	SDS; Deoxycholic acid; deionized water; PBC;	24 hours	[28]
11	The cadaveric rat	n.d.	PBS; SDS; deionized water; Triton-X100; PBS with penicillin, streptomycin;	-	[29]
12	The porcine heart	92%	Trypsin; EDTA; NaN3; Triton-X100; deoxycholic acid; peracetic acid; ethanol;	6 hours 25 min	[30]
13	The porcine heart	82%	Trypsin; EDTA; NaN3; Triton-X100; deoxycholic acid; peracetic acid; ethanol;	6 hours 25 min	[31]
14	The cadaveric rat	n.d.	PBS; SDS; deionized water; Triton-X100; PBS with penicillin, streptomycin, amphotericin;	13 hours 45 min	[5]

*n.d.- not determined

The main goal of decellularization of the heart is to eliminate immune response by removing allo- and xenoantigens from the tissues. There are many protocols for effective decellularization of the heart, however, it is necessary to choose a procedure that strikes an ideal balance between eliminating cells and maintaining the extracellular matrix (ECM) proteins that make up its diverse structures, and reducing any negative changes that interfere with the biological activity of the ECM [27]. For example, it should be considered that the selected decellularization method should not leave the residues of the solutions used. This study, therefore, attempts to

decellularize rabbit hearts using perfusion and evaluate the efficiency of the process in terms of its capacity to remove the nuclear component and preserve the structural and non-structural proteins.

1.4. Problem statement, study hypothesis, and aims

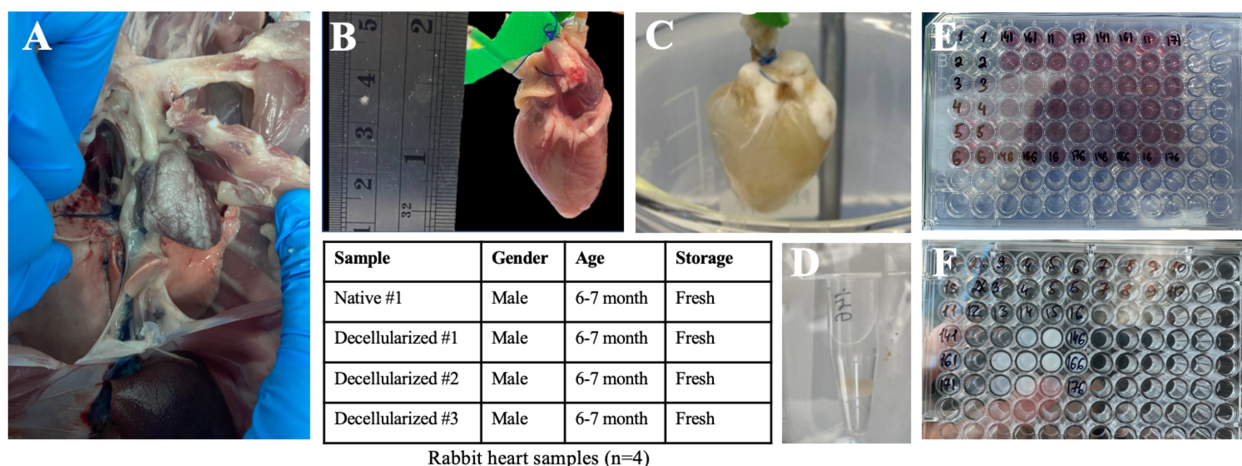
The inadequate functioning of heart tissue is irreversible and may lead to complete dysfunction. In the history of development medicine, CVD has been considered a central worldwide health-related problem since it can be caused by various factors that may not seem dangerous at first glance, making it difficult to find a compromise in the treatment. Even though there are methods of treatment of CVD including allogeneic heart transplantation, angioplasty, and valve reconstruction/replacement, they cannot be considered permanent clinical solutions due to some side effects such as lifelong immunosuppressive therapy, renal failure, and diabetes.

We hypothesized that the utilization of the perfusion decellularization method on rabbit hearts would result in the production of decellularized whole hearts while keeping their structural properties and non-cellular composition unchanged.

This research aims to create a technique that can decellularize rabbit heart tissue, assess the efficiency of decellularization in terms of residual nuclear content and structural characteristics, and determine its potential use in cardiovascular tissue engineering.

CHAPTER 2 - (MATERIALS AND METHODS)

This research study involves two main experimental parts: i) harvesting and decellularization of rabbit hearts, and ii) characterization of decellularized rabbit hearts in terms of DNA content as well as collagen and GAGs. Figure 2.1 presents the processes for harvesting (A), decellularization (B, C), and characterization (D-F) of rabbit hearts.



Sample	Gender	Age	Storage
Native #1	Male	6-7 month	Fresh
Decellularized #1	Male	6-7 month	Fresh
Decellularized #2	Male	6-7 month	Fresh
Decellularized #3	Male	6-7 month	Fresh

Figure 2.1: Experimental design and representation of harvesting and characterization of native and decellularized rabbit hearts.

2.1. Materials

All chemicals were acquired from Sigma Aldrich and Fisher Scientific, and catalog numbers are listed below. Trypsin (Sigma Aldrich, #T4049), Sodium dodecyl sulfate (Sigma Aldrich, #L3771), Sodium azide (Sigma Aldrich, #S2002), Triton-X (Sigma Aldrich, #T9284), Ethylenediamine tetraacetic acid (Sigma Aldrich, #E5134), Peracetic acid (Sigma Aldrich, #433241), ethanol (Sigma Aldrich, #E7023), Phosphate buffered saline (Sigma Aldrich, # P4417),

SIRCOL Collagen Standard Type 1 (Sigma Aldrich, #NC9541732), Sodium Formate (Sigma Aldrich, #247596), 1,9-dimethyl methylene blue (Sigma Aldrich, #341088), Sodium acetate trihydrate (Sigma Aldrich, #S7670), L-Cysteine Hydrochloric acid (Sigma Aldrich, #C7477), Papain (Sigma Aldrich, #P3125), PicoGreen double-strand DNA assay (Invitrogen #P7589), chondroitin-6-sulfate (Sigma Aldrich, #C4384).

2.2. Collection of hearts for decellularization

The rabbit hearts were collected from the National Biotechnology Center (Astana, Kazakhstan), and kept at -4°C in PBS. The hearts were harvested along with the aorta and pulmonary artery by the surgical blade (Figure 2.2). Since rabbit hearts are relatively small, all valves, such as the aortic valve, were left in place. The perfusion system employed 24G butterfly needles "bioflyject". To cannulate each heart, the aorta was secured to the end of a cannula using 5.0 Vicryl Plus suture thread. The harvested hearts were used for decellularization and further characterization of cardiac dECM.

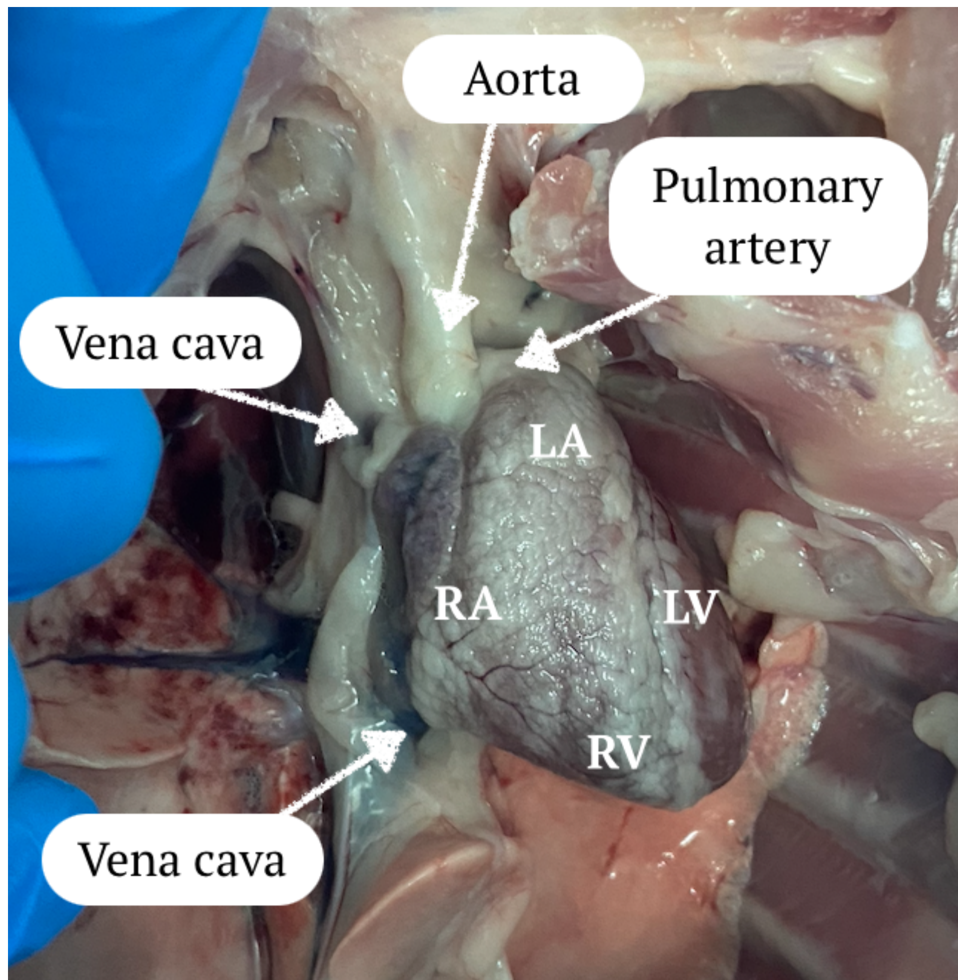


Figure 2.2: Rabbit heart harvesting process.

2.3. Rabbit heart decellularization setup

For the perfusion system, a syringe pump (KDS Legato 210) was used with a configured fluid flow rate parameter of 2.5 ml/min through 60 ml syringes (inner diameter: 26.72 mm). Considering the individual characteristics created by the heart tissue of each rabbit, the actual pressure and blood flow rate can vary and affect the efficiency of decellularization. In perfusion of the heart, butterfly systems with a diameter of 24G were used for syringing. For static location

and hydrostatic pressure, the heart was attached to a custom-made apparatus (Figure 2.3).

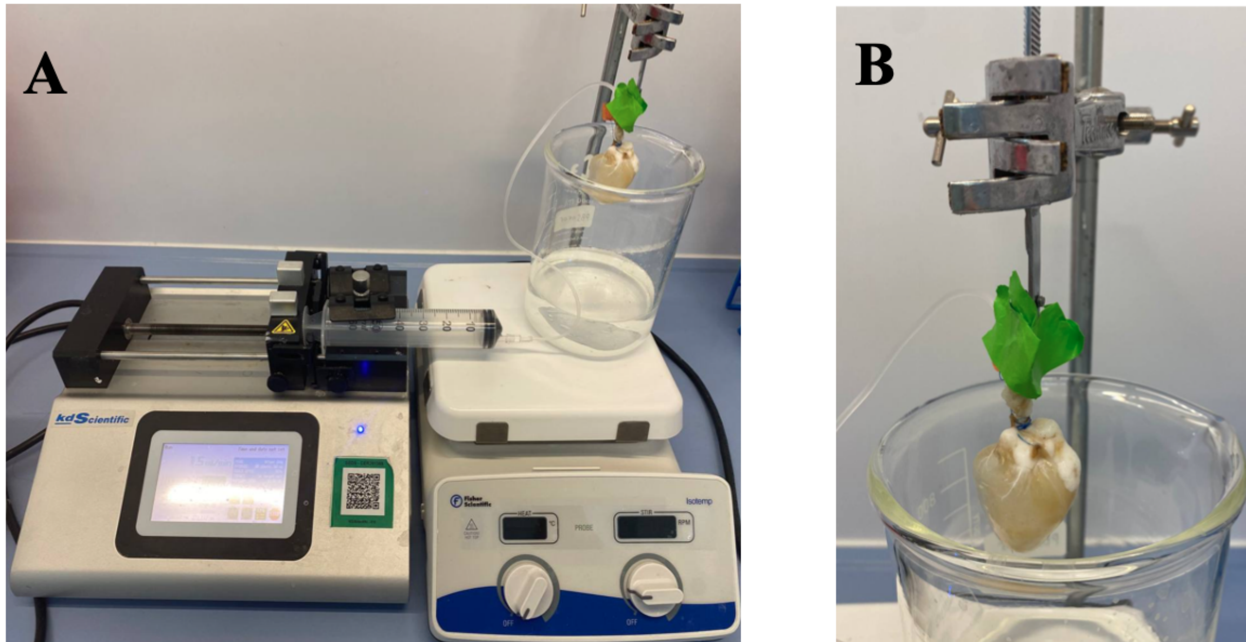


Figure 2.3: The experimental setup for the decellularization procedure includes (A) an overall illustration of the setup and (B) the magnified portion of the cannulated heart area.

2.4. Decellularization procedure

Decellularization of the rabbit hearts was performed according to the modified protocol to remove cellular content properly. We followed our previously published protocol [21], with longer durations proportionate to the size of the rabbit heart compared to the rat heart. Heart decellularization involves 11 solutions for 1 cycle, and 2 cycles were applied to fully remove cellular content.

To compare and ensure effective cell removal, we applied two perfusion methods, namely perfusion through the aorta only and bi-ventricular perfusion (Table 2.3) through the aorta and pulmonary artery.

Table 2.3: Bi-ventricular perfusion procedure.

#	Solution	The aorta (1.5 ml/min)	The pulmonary artery (1 ml/min)
1.	Deionized water	103 ml	77 ml
2.	Phosphate-buffered saline (PBS)	206 ml	154 ml
3.	A mixture of 0.02% trypsin, 0.05% EDTA, and 0.05% Sodium azide, distilled water	206 ml	154 ml
4.	A mixture of 1% sodium dodecyl sulfate, and 0.05% Sodium azide, distilled water	103 ml	77 ml
5.	A mixture of 3% Triton X-100, 0.05% EDTA, and 0.05% sodium azide, distilled water	103 ml	77 ml
6.	A deoxycholic acid, distilled water	103 ml	77 ml
7.	A mixture of 0.1% peracetic acid, and 90% ethanol, distilled water	103 ml	77 ml
8.	PBS	154 ml	116 ml
9.	Deionized water	154 ml	116 ml
10.	PBS	154 ml	116 ml
11.	Deionized water	154 ml	116 ml

During this study, for only aorta perfusion for both cycles, the flow rate of perfusion was 2.5 mL/min for a 60 mL syringe. Firstly, deionized water 180 ml was perfused through the aorta to prevent clotting and wash out excess blood. Next, PBS (360 mL) was perfused. After that, the mixture (360 mL) of 0.02% trypsin, 0.05% ethylenediaminetetraacetic acid, and 0.05% sodium azide in deionized water was perfused. This solution was replaced with a solution (180 ml) of 1% sodium dodecyl sulfate, and 0.05% sodium azide. Next, the mixture (180 ml) of 3% Triton X-100,

0.05% ethylenediaminetetraacetic acid, and 0.05% sodium azide was perfused. The solution (180ml) of deoxycholic acid and 75% ethanol with deionized water was run through the system. Next, 270 ml of PBS followed by 270 ml of deionized water was perfused twice. The perfusion of rabbit hearts was repeated in the same order of solutions. The decellularized hearts were stored in PBS at 4°C.

During decellularization, cells are completely separated from the extracellular matrix, this helps to fully analyze the mechanics of the heart wall and study the corresponding properties of myocardial tissue to cellular/extracellular components [32]. Figure 2.2 represents the setup for the rabbit heart decellularization. Several reports have shown that owing to the decellularization of the heart, the possibility of a biocompatible strategy is growing since it relies on a specific extracellular matrix (ECM) in which all biomolecules are dispersed and arranged in the same way as in a naturally healthy organ [21], [24]. When the ECM is completely cleared of cells, the ECM and all its elements retain the proper organization of the ability to direct the repopulation of the decellularized framework into a functional tissue or organ [27].

2.5. Samples preparation

Samples of intact and decellularized hearts were prepared for further examinations (DNA, collagen, GAG assays). 12 pieces of tissue were cut from both sides of the heart for analysis. In total, 6 pieces of tissue from different parts of the heart (atria, ventricles, apex) were used for each analysis (Figure 2.4).

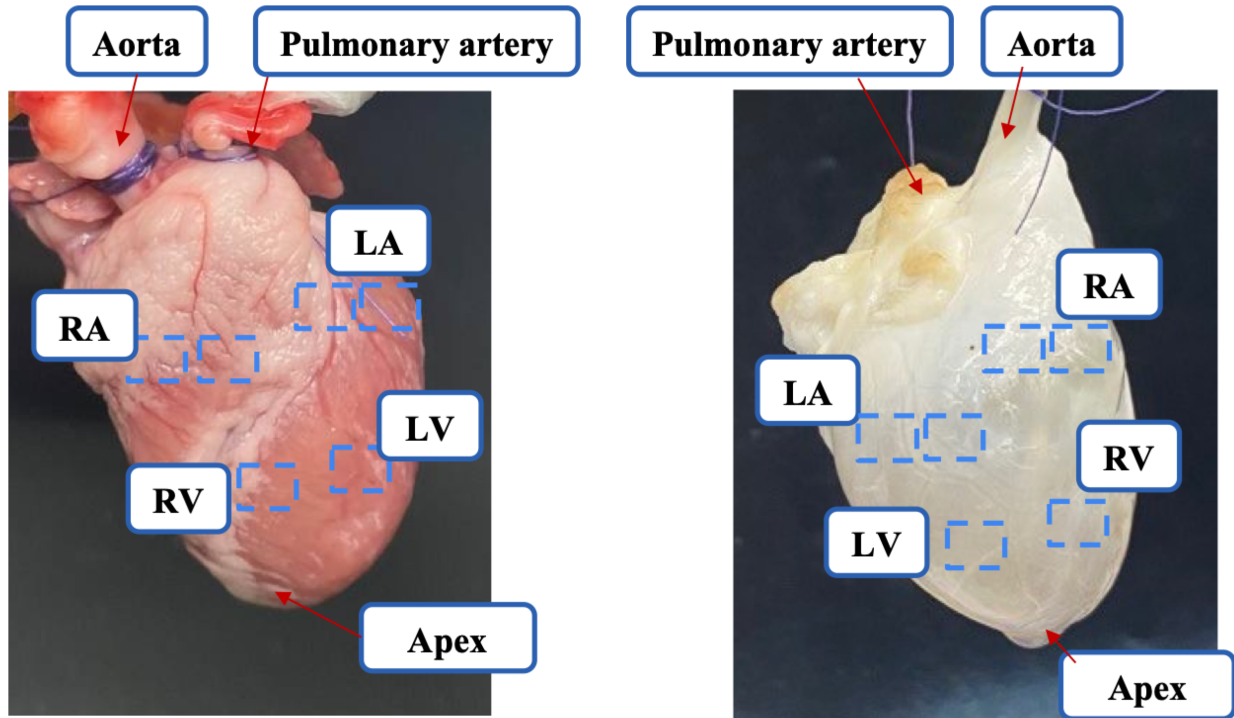


Figure 2.4. Intact heart tissue (A); Decellularized heart tissue (B).

2.6. Collagen assay

By using the Sircol Collagen assay kit (Biocolor Ltd, Belfast, N Ireland), we analyzed how much collagen remains in the ECM after decellularization. The heart samples were prepared by drying in a vacuum dryer (Buchi Glass Oven B-585) for 22 hours at 40°C. For the colorimetric assay of collagen amount, we added Sircol dye reagent to samples and standards, after that, we incubate at 60ºC for 24 hours. The next step is the addendum of the digestion buffer (pH 6.0), centrifuging the samples and standards to pellet the collagen-dye complex. Afterward, we discarded the liquid supernatant and mix the solid sediment with an alkaline solution to separate the combination of collagen and dye. Finally, the absorbance of samples was read at 540 nm by SkanIt Software 2.4.5 RE for Varioskan Flash. A native intact heart was used as a control group.

2.7. DNA assay

For a comprehensive analysis of the effectiveness of decellularization, the cellular content of the decellularized heart was analyzed by Picogreen dsDNA quantification kit, following recommendations of the manufacturer. Samples were quantified using a fluorometric spectrophotometer SkanIt Software 2.4.5 RE for Varioskan Flash with a fluorescein excitation (485 nm) and emission (535 nm) wavelength. A standard curve was created to correlate the DNA content with fluorescence, and the number of cells was determined using the DNA/cell conversion factor. A native intact heart was used as a control for DNA quantification.

2.8. GAG-DMMB assay

To determine the number of GAGs in the decellularized heart, we applied the GAG-DMMB dye-binding assay, using chondroitin-6-sulfate as a standard. For the control group during analysis, a native intact heart was used. The intact and decellularized heart samples were prepared by the same procedure as for collagen assay in a vacuum dryer. A digestion buffer (pH 6.0) with Sodium acetate trihydrate, L-Cysteine Hydrochloric Acid, EDTA was added to the sample's tubes. Prepared 1,9-dimethylmethylene blue dye (pH 1.5) was added to samples and standards. Formic acid was used to detect sulfated GAG-DMMB complexes with a spectrophotometer, which reduced the pH level to 1.5. The absorbance of samples was read in the first 5 minutes after the addendum of DMMB dye since the GAG-DMMB complexes precipitate out in 5min and the plate becomes unreadable. We used SkanIt Software 2.4.5 RE for Varioskan Flash with dual-wavelength at 540 nm (for a positive change) and 595 nm (for a negative change) to improve signal detection.

2.9. Statistical analysis

The efficiency of decellularization in terms of DNA, collagen, and GAG contents was evaluated by comparing it with an intact heart using a student t-test. A p-value of less than 0.05 was considered a significant difference.

CHAPTER 3 – (RESULTS)

3.1. DNA Content

Before examination for cellular content, ECM components, etc., we made a visual comparison of color change during decellularization (Figure 3.5).

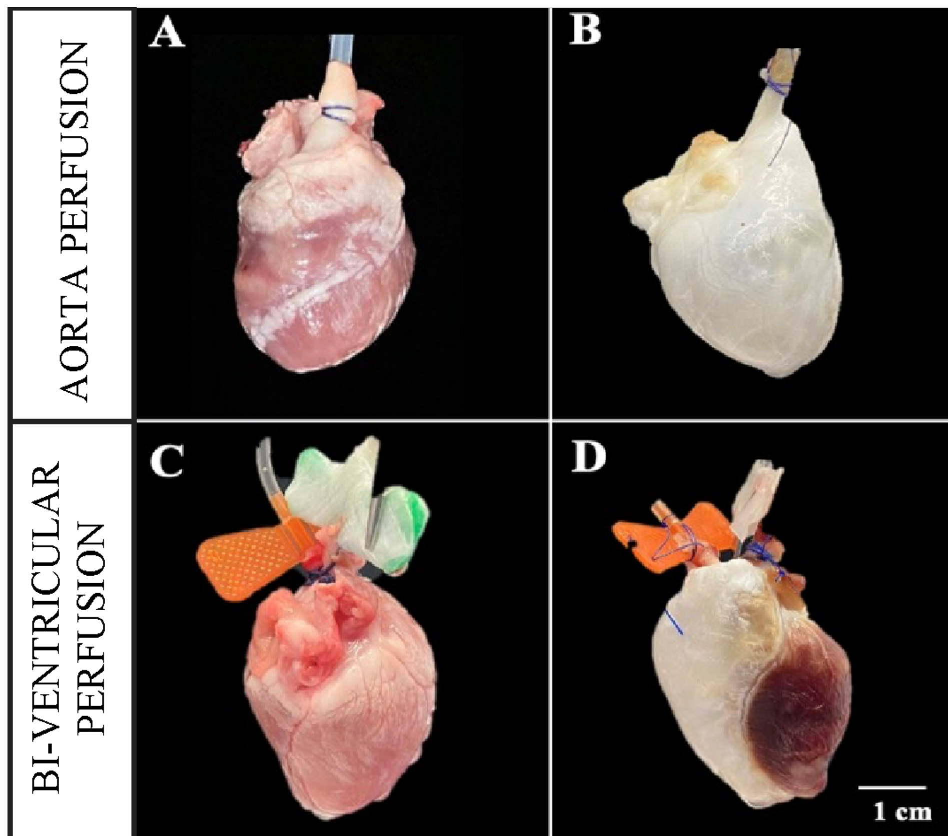


Figure 3.5: Comparison of aorta perfusion and bi-ventricular perfusion of whole rabbit heart. A- before decellularization by aorta perfusion; B- after 2 cycles perfusion in flow rate 2,5 ml/min; C-before decellularization by bi-ventricular perfusion; D - after bi-ventricular perfusion through the aorta (1,5 ml/min) and pulmonary artery (1 ml/min).

Visual comparison after each step of decellularization by aorta perfusion is evidence of the success of the procedure, namely, a color change from reddish pink in fresh native tissue to translucent white in decellularized tissue (Figure 3.6).

Figure 3.6: Images of incremental color change during decellularization of the whole rabbit heart by aorta perfusion. A - 1st cycle of whole heart decellularization; B - 2nd cycle of whole heart decellularization.

3.2. Decellularization indicated by residual nuclear material

A comparison of the results between the native heart and the decellularized one showed that the DNA content is present in both samples. The DNA content in the samples of the

decellularized heart was lower as compared to the native heart (Figure 3.7, $p<0.05$). A 66% reduction in the DNA content was achieved.

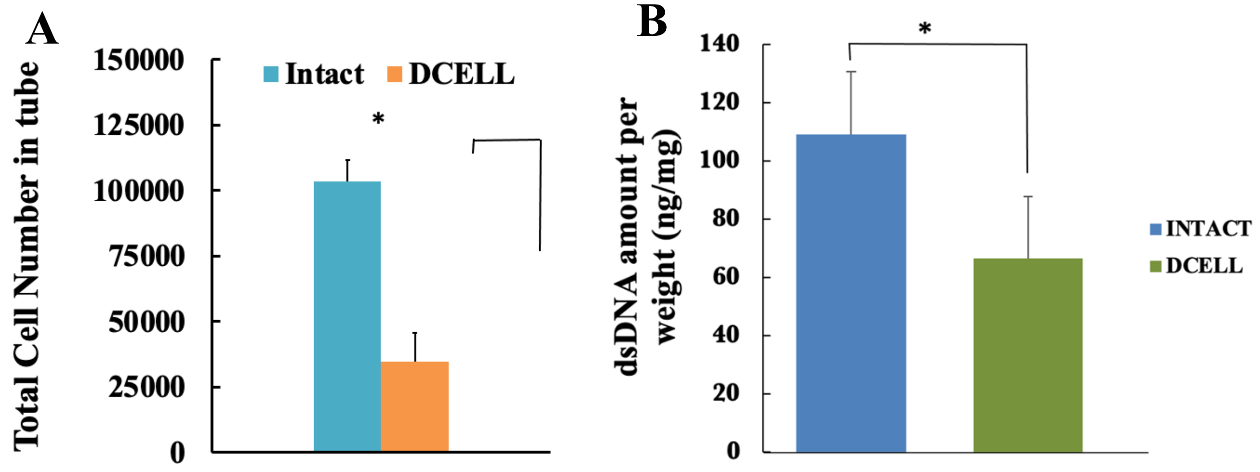


Figure 3.7: Total cell number measurements in native and decellularized heart samples (A). dsDNA amount per ECM wet weight in native and decellularized heart samples (B). The values were presented as mean and STD. * indicates difference at p<0.05.

3.3. Decellularization assessment by Collagen and GAG assay

According to Figure 3.8, the collagen content in decellularized samples remained unchanged compared to native tissue samples. Considering these data, it has been confirmed that the protocol is safe with respect to the preservation of collagen levels after decellularization.

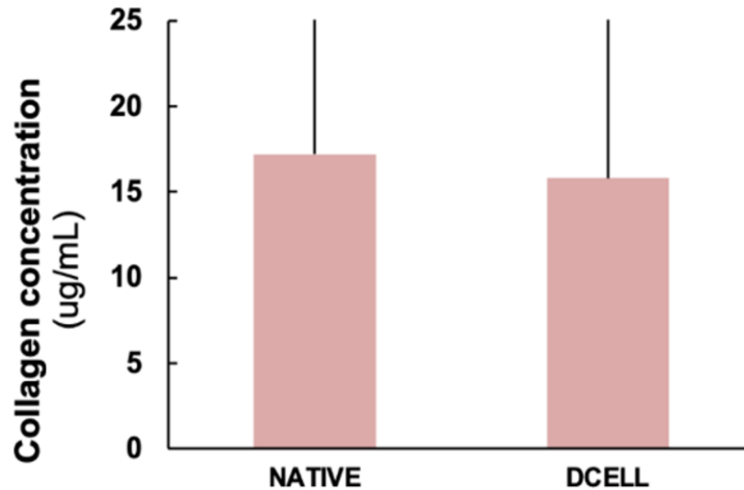


Figure 3.8: Measurements of collagen content in native and decellularized dry hearts. Total Collagen amount in native and decellularized hearts. No significant difference between the groups was detected at p<0.05.

Data of GAG measurements are presented in Figure 3.9 with the comparison of intact and decellularized rabbit hearts. According to Figure 3.9, the decellularization process did not change the amount of GAG present in the tissue.

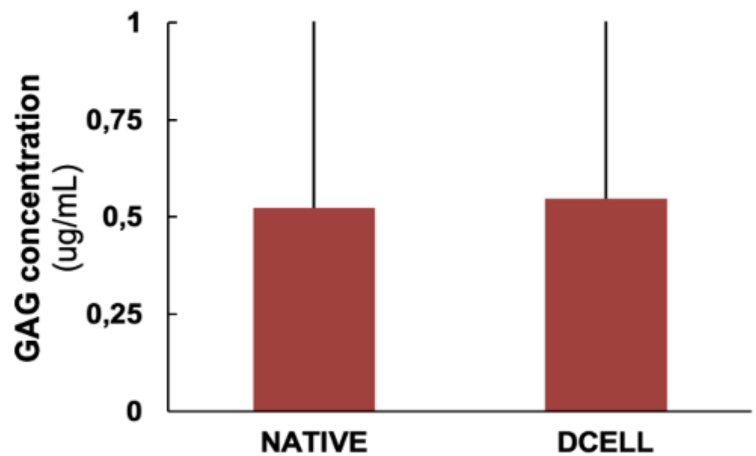


Figure 3.9: Measurements of GAG amount in native and decellularized dry heart samples. No significant difference between the groups was detected at p<0.05.

CHAPTER 4 - (DISCUSSION)

The decellularization protocol described in this study was adapted from the cadaveric rat heart decellularization system developed by Ozlu et al., (2009). The novelty of the current study is that we applied two methods of perfusion: perfusion by the aorta and bi-ventricular perfusion. Bi-ventricular and aortic decellularization are two different methods used to create acellular scaffolds for tissue engineering and regenerative medicine applications. Bi-ventricular decellularization involves the removal of cellular components from the left and right ventricles of the heart, while aortic decellularization involves the removal of cellular components from the aorta, the main artery that carries blood from the heart to the rest of the body. To save reagents, heart samples, and time, we decided to follow only aortic perfusion. In accordance with physiological characteristics blood flow rate in the aorta is much higher than in the pulmonary artery, due to this applied syringe pump could not compensate for hydrostatic pressure between two ventricles artificially [33].

Usually, the process of tissue decellularization involves the removal of cellular material and the preservation of the components of the ECM. The primary aim of decellularization is to remove all cellular components while preserving the extracellular matrix; however, complete decellularization is not possible, and residual cell debris may promote tissue rejection and calcification. Thus, decellularization procedures must be optimized for various species. Different methods can be used to assess the effectiveness of the removal of cellular contents. Some methods include quantitative measurement of bio-loading in flowing solutions over time, biochemical quantitative determination of histological/ immunofluorescence staining, and analytical assays to determine the DNA content.

In this study, a perfusion system was employed to decellularize the entire rabbit heart using various detergents. The effectiveness of detergents in removing cellular material from tissues is attributed to their ability to dissolve the cell membrane and separate proteins from DNA. To analyze the effectiveness of the applied rabbit heart decellularization procedure, many methods were used. First, a qualitative comparison after each step of decellularization can be taken as evidence of decellularization. A color change from reddish pink in fresh native tissue to translucent white in decellularized tissue was observed after the process. The second method, DNA assay using fluorescence spectrophotometry, indicated that the DNA content of the heart was significantly reduced as compared to the intact heart. According to previous research on tissue decellularization, the tissue engineering society agreed-upon measurable standards for adequate decellularization. These criteria include less than 50 ng of double-stranded DNA per milligram of extracellular matrix weight; DNA fragment length less than 200 base pairs; no visible nuclear material in tissue sections in staining. In this study, we measured decellularization efficiency in terms of the first criterion "less than 50 ng of DNA per ECM weight (mg)" [34]. The results were 66 ng/mg of DNA amount, according to this we assumed to add an additional cycle of decellularization and utilization of bioreactor in further studies. One of the main goals of tissue decellularization is to preserve the maximum integrity of the native structure and components of the extracellular matrix (ECM) to create a scaffold that most accurately mimics the native tissue. Depending on the intended use of the tissue, various quantitative and qualitative methods of analysis can be used. In this study, further recellularization and endothelization of the heart are planned, so it is important to preserve the integrity of the basal plate, which is part of the ECM and is directly dependent on the circulatory system. During the endothelization of blood vessels, endothelial cells attach to the basal plate. The main components of the basal plate are type IV

collagen, glycosaminoglycans, laminin, fibronectin, elastin, and proteoglycans. Due to the limited time of the study, the content of only collagen and GAGs was studied. The collagen content in the native and decellularized tissue was studied to confirm the safety of solutions for the ECM structure. Similarly, a GAG assay was performed to confirm the preservation of the tissue matrix components. Our findings indicate that the decellularization process did not affect the structural integrity of the extracellular matrix or the composition of the tissue.

In future studies, for a more accurate assessment of the efficiency of decellularization of the heart, it is recommended to include histological evaluation, quantitative determination of elastin content, the diameter of muscles, and analysis of biomechanical properties. Literature reports suggest that the use of pressure-controlled bioreactors affects the rate of cell leaching from blood vessels. It may also be necessary to extend the continuous decellularization procedure since similar studies on rabbits were conducted continuously for up to 10 days.

CHAPTER 5 - (CONCLUSION)

In this study, the hearts harvested from rabbits were decellularized using a perfusion method. Findings revealed that the DNA content of the decellularized hearts was significantly reduced while keeping collagen and GAG content unchanged. Given the structural similarity between decellularized hearts and native hearts, it is anticipated that the decellularized hearts will provide a more clinically relevant model for potential human use and will have a significant impact in treating CVD.

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