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MORPHOLOGICAL AND BIOMECHANICAL MODIFICATIONS IN BLOOD VESSELS DECELLULARIZATION

341.01 TISSUE ENGINEERING AND CELL CULTURES

Summary of PhD thesis in medical sciences

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The thesis was developed within the Laboratory of Tissue Engineering and Cell Cultures, Department of Anatomy and Clinical Anatomy, *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova.

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Doctoral thesis defense will take place on 20th of December 2023, at 14:00, *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova, 165, Stefan cel Mare si Sfant Ave, room 204, in the meeting of the Commission for public defense of the doctoral thesis, approved by the decision of the Scientific Council of the Consortium from 05th of October 2023 (minutes of the meeting no. 20).

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CONTENT

CONCEPTUAL PROBLEMS IN THE RESEARCH

Actuality and importance of the studied topic

Cardiovascular diseases (CVDs) remain one of the most prevalent healthcare problems worldwide. The latest statistics in the field predict that due to the ageing of the population the annual incidence of cardiovascular disease-related mortalities will rise from 16.7 million in 2002 to 23.3 million in 2030 [1, 2, 3, 4, 5].

Available treatment options for these pathologies are variable and can be divided into the following groups: lifestyle modification, medication, and revascularization techniques, open or endovascular ones [3, 6]. Despite the benefits of both behavioral (dietary and lifestyle modifications, rehabilitation, as active counseling, and tailored exercise) and drug therapies (medications ensure regulation of the cholesterol level, amelioration of vasoconstriction, and optimal control of blood pressure), they are not always lifesaving. In some cases, the best medical treatment may fail, and alternative revascularization methods may be necessary to achieve positive outcomes. The established guidelines for the field recommend open bypass surgery in patients with persistent symptoms. The procedure supposes replacement of diseased blood vessels with a suitable biomaterial, the process called vascular grafting. In this way a new pathway for blood flow is created, and the functional blood supply is restored [7, 8].

Currently, the following types of conduits for vascular replacement are available: autografts, allografts, xenografts, and artificial prostheses. Autologous vascular tissue remains the standard for small-diameter arterial bypass. For patients who lack this tissue, the currently available prosthetic alternatives are most frequently used in clinical practice; however, the outcomes are less than satisfactory [9].

Limitations in utilizing autogenous vessels, such as the internal mammary artery, the greater saphenous vein or radial artery are related to low availability, most vessels being affected by diffuse atherosclerotic abnormalities, previous phlebitis, vessel removal, varicosities, hypoplasia, or are anatomically unsuitable; only few vessels remain indeed suitable for this therapeutic purpose. Synthetic conduits such as Dacron or Polytetrafluoroethylene (PTFE) are prone to thrombosis and neointimal hyperplasia, especially when applied in low flow high pressure position. Unsatisfactory clinical outcomes due to aneurismal degradation, infection or early thrombosis are also recorded when using xenogeneic or allogeneic tissues [7, 10, 11, 12].

Although a variety of materials used in vascular reconstruction are available, an “ideal” graft remains an unmet practical need and is not accessible “off-the-shelf”. In addition, there is an acute need for new approaches to small-diameter blood vessel substitution (<6,0 mm) [10]. Because of lack of adequate substitute, the development of alternative biomaterials for vascular grafting was initiated [7, 10, 11, 12].

Tissue engineering can overcome the limitations of the currently available vessel substitutes through the generation of biologically based functional vessels which could more closely replicate the physiological tissue, optimize tissue-biomaterial interaction and matching the properties of native vasculature, promote cell growth, facilitate extracellular matrix (ECM) production, and inhibit thrombogenicity. Convenient strategies to create vascular grafts involve seeding biocompatible, compliant scaffolds with live vascular cells. One of the approaches developed in this field refers to utilization of decellularization (DC) technique which allows to generate functional platforms [10, 13, 14, 15, 16].

Decellularization assumes removing cells and associated antigens from the scaffold while preserving the entire ECM, including the vascular architecture. Important advantage of

this technique consists in keeping the integrity of the vascular channel that makes it suitable for cellular repopulation (recellularization) [14]. So, the obtained 3D-natural platform contains the necessary cues for providing cell migration and proliferation. The main advantage of the use of decellularized scaffolds consists in avoiding any adverse immunological reactions due to loss of the major histocompatibility complex (MHC) [17, 18].

The DC process can be performed through physical, chemical, and enzymatic agents. While choosing the appropriate protocol, it is necessary to take into consideration it can alter the properties of the ECM, and thus can have a negative impact for *in vivo* stability. On the other hand, high amounts of remaining DNA may cause inflammatory reactions and hence poor graft functionality. To ensure the successful outcome of tissue engineering based therapies it is essential to strike the balance between complete removal of cells and preserving integrity of the ECM [7, 19, 20, 21].

Till now successful decellularization protocols for different tissue types/organs have already been described. Despite the enormous effort, complete DC has not yet been achieved for all tissues. For instance, decellularization of thick tissue, as blood vessel walls, is still challenging and varying [22, 23]. In addition, most published procedures are based on incubating tissues with chemical or enzymatic solutions with further evaluation of the effects of varying treatment times and intermittent washing steps on the DC efficiency [18]. There are a few data on the feasibility and efficacy of physical methods, such as osmotic treatment, agitating, perfusion, pressure, sonication, electroporation, or freeze-thaw in vascular tissue decellularization [18, 24].

The way of chemicals' administration (static conditions, agitation, shaking or perfusion) has a major impact on the characteristics of various materials and surfaces. Modifying the method may have a significant impact on the efficacy of cell removal as well as the preservationsof the functional properties of the obtained decellularized tissue (ECM integrity, stiffness, and compliance). Understanding these effects is crucial to accurately assess and measure the influence of different DC agents on DC outcomes [2, 18, 25].

The aim of the study

To develop new methods for decellularization of large-diameter and small-diameter blood vessels.

Research objectives

1. To evaluate the efficiency of sonication-assisted methods for decellularization of porcine carotid artery;
2. To test the effect of acoustic amplitude on the vascular matrix;
3. To evaluate the effectiveness of chemical (SDS, SDC, Triton X-100, hypotonic solution) and enzymatic (DNase-I) treatment in vascular tissue decellularization;
4. To evaluate whether the decellularization protocol efficiency depends on the vessel diameter;
5. To check the informativeness of qualitative methods (histological stains – H&E and DAPI) for confirmation of the decellularization process;
6. To do morphological, biochemical, and biomechanical characterization of treated blood vessels;
7. To assess the biocompatibility of acellular scaffold by performing *in vitro* contact test;
8. To determine the efficiency of perfusion decellularization for uniform cells' elimination from long segments of blood vessels.

Methodology of the scientific research

By reviewing the publications of the international researchers in the field, the

methodological support of this study was elaborated. For realization of the proposed goal and objectives an experimental study was conducted. Porcine arteries were chosen as base material for our research. Available arterial fragments (porcine aorta and porcine carotid artery) were obtained and divided into experimental groups, including control (non-treated sample). Based on successful results in utilization of SDS-SDC solution to perform decellularization of different tissues, this chemical cocktail was selected for our experiment. So, the samples were processed with detergents with/without enzymatic treatment to remove cells and cellular debris (it is supposed insufficient removal of the antigens most likely can initiate sensitization reaction). The efficiency of another detergent, namely Triton X-100, was also tested. In addition, the effects of different forms of dynamic exposure of tissue to chemicala were studied (rotation, perfusion, and ultrasound) to determine their impact on uniformity of cells elimination and improvement of cellular membranes breaking down.

The decellularized grafts were analyzed with basic histological stains (Hematoxylin-Eosin, H&E), fluorescent DAPI nuclear staining (4',6-diamidino-2-phenylindole), immunostaining against collagen type IV, and mechanical probe (suture retention strength). Also, quantitative evaluation of the remnant DNA, GAGs, Hydroxyproline, wall thickness measurement, cytotoxicity assay and SEM characterization were performed.

Statistical analysis was performed using SPSS version 16.0 software program. Where applicable, the data was reported as mean±SD. As a non-DC control, untreated blood vessels (frozen directly after harvesting and fresh not treated samples) were used. Gaussian normal distribution was tested by normality plots (Shapirko-Wilk test); and homogeneity of variance was checked by Levene's test. Differences between the groups were detected by performing independent t-test for normally distributed homogeneous values and Welch's test for normally distributed non-homogeneous values. Mann-Whitney U test was applied for non-parametric dataor parametric data which do not meet normal distribution. Differences were considered significant at p-value lower than 0.05.

The ethical committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova approved the research design (Decision no. 31 from 26.12.2017). The tests were realized at Laboratory of Tissue Engineering and Cell Cultures, Chisinau, Republic of Moldova, and Leibniz Forschungslaboratorien für Biotechnologie und künstliche Organe (LEBAO), Medizinische Hochschule Hannover (MHH), Hanover, Germany.

Novelty and scientific originality of the obtained results

The solved scientific problem consists in identifying the factors associated with efficient cells' removal and establishing a novel procedure for blood vessels decellularization with optimal characterization of acellular scaffold's structure, a fact that will allow the modification of the experimental paradigm through the scientifically reasoned selection of the optimal experimental conditions.

Conducting the experimental study with comparison and multilateral characterization of DC efficiency of different decellularization approaches in term of cells' elimination and matrix strength preservation contributed to the completion of some gaps in the current scientific literature. The results of the conducted research demonstrated there is important variability of the techniques used for blood vessels decellularization and the quality of final matrix. Analysis of interrelationships between blood vessels diameter and successful cells' removal demonstrated the necessity to have differential experimental approaches depending on the vessel's size.

Theoretic importance and main scientific results

1. Currently, the researchers' decisions on the decellularization approach for vascular tissue, as well as the selection of the testing model and examining methods for acellular scaffold characterization are not standardized and are influenced by the personal experience and preferences;
2. Wall thickness is an important parameter influencing the decellularization efficiency. In this way, large-diameter blood vessels *vs* small-diameter blood vessel have to be treated differently;
3. Even strong ionic detergents, as SDS or SDC, are not completely efficient for production the cell-free tissue. The elimination of sticky fragments of DNA from the matrix requires additional enzymatic treatment (DNase processing);
4. Multiple washing steps seem to be optimal practical method for removing SDS detergent from decellularized matrix;
5. The necessity to perform multiple tests, both qualitative (histological stains) and quantitative (quantification the DNA remnants), to confirm complete and proper elimination of cellular elements from the tissue is demonstrated. In addition, the dual approach is mandatory for evaluation of the matrix preservation;
6. Safety assessment by performing *in vitro* biocompatibility tests is indispensable before *in vivo* evaluation (animal experiments);
7. Sonication does not directly improve the cells' elimination. It can even significantly affect the matrix integrity when high amplitude waves are applied.

The applicative value of the research

Based on the study's results the decellularization efficiency of different chemicals was specified; in addition, the indispensability of the enzymatic treatment in combination with strong detergents for accelular vascular scaffolds production was demonstrated. The data obtained during the research scientifically argue for the modification of the current research strategy through the preferential use of carotid artery *vs* aorta as testing model for development of small-diameter tissue-engineered vascular grafts taking into consideration the existing differences in experimental conditions for successful efficient cells elimination depending on the wall thickness. The identification of favourable outcomes associated with the use of non-ionic detergents and repeated washing cycles for efficient ionic detergents remnants removal is important in development on biocompatible matrices. The failed attempt to use the ultrasound for vascular tissue DC defines the necessity to perform additional studies regarding the mechanism of ultrasound-induced cellular destruction.

Implementation of the research results

The practical impact of the present study consists in implementation of a novel technique of blood vessels decellularization in the Laboratory of Tissue Engineering and Cell Cultures, *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova. In addition, the obtained results (available decellularization procedures for vascular tissue, methods for evaluation of morphological, biomechanical, and biochemical integrity of the decellularized tissue, biocompatibility assay) have been presented to medical students within the classes at the Department of Anatomy and Clinical Anatomy and the optional learning course in Regenerative Medicine.

Approval of scientific results

The obtained results were discussed and presented within the following scientific forums: MedEspera 2018, 7th International Medical Congress for Students and Young Doctors (Chisinau, 2018), International molecular medicine symposium (Istanbul, Turkey, 2019 –

Best Poster Presentation AWARD in Third Place), the 4th International Conference on Nanotechnologies and Biomedical Engineering (Chisinau, 2019), Timisoara Anatomical Days (Timisoara, Romania, 2019), MedEspera 2020: 8th International Medical Congress for Students and Young Doctors (online edition, 2020 – DIPLOMA Ist Place Award Certification), *Congresul Consacrat aniversării a 75-a de la fondarea USMF „Nicolae Testemițanu”* (online edition, 2020), *Conferința Națională de Chirurgie* (online edition, Romania, 2021), *Conferința Științifică Anuală. Cercetarea în biomedicină și sănătate: calitate, excelență și performanță* (online edition, 2021), the 5th International Conference on Nanotechnologies and Biomedical Engineering (online edition, 2021), MedEspera 2022: 9th International Medical Congress for Students and Young Doctors, MedEspera (Chisinau, 2022 – DIPLOMA Ist Place Award Certification), *Conferința Științifică Anuală. Cercetarea în biomedicină și sănătate: calitate, excelență și performanță* (Chisinau, 2022), *Conferința Națională de Chirurgie* (Eforie Nord, Romania, 2023), 6th International Conference on Nanotechnologies and Biomedical Engineering (Chisinau, 2023 – Certificate of achievement 1st place in YOUNG INVESTIGATORS COMPETITION); salons of research, innovation and inventiveness: EUROINVENT 15th European Exhibition Of Creativity And Innovation (Iasi, Romania, 2023 – golden medal and two trophies: Innovation Award for promoting Science, Education and technology at EuroInvent 2023 and Special Award for the Invention from Titu Maiorescu University of Bucharest), 2nd edition of the International Exhibition of Innovation and Technology Transfer EXCELLENT IDEA-2023 (Chisinau, 2023 – golden medal), and public lectures: *„Ingineria tisulară și medicina regenerativă: provocări și realizări”* (*Ziua Internaţională a Ştiinţei*, 09 November 2019, Chisinau), *„Grefele vasculare decelularizate obţinute prin inginerie tisulară vor fi un standard de tratament în viitor?”* (*Expoziția MoldMedizin & MoldDent,* 11-13 September 2019, Chisinau).

Publications on the research topic

20 scientific papers were published on the research topic, including: articles in Conference Proceedings indexed in SCOPUS – 3, articles in journals from abroad – 1, articles in journals from the National Register of specialized journals – 5, materials / theses at international conferences – 4, materials / theses at international conferences organized in the Republic of Moldova – 3, materials / theses at conferences (national conferences) – 4. Number of publications without co-authors – 2. During the research period 1 innovator's certificate, 1 implementation act, 2 gold medals, 2 special trophies in the Invention Salons, and 4 awarded places at international conferences were obtained.

The thesis structure

The thesis includes annotations in Romanian, Russian and English, list of abbreviations, introduction part (reflects the actuality and scientific-practical importance of the problem addressed in the thesis, the purpose, the objectives, the scientific novelty, the theoretical importance, and the applied value of the research, the approval of the study results), 4 chapters (Literature review, Materials and Methods, Results, Discussions) with general conclusions, practical recommendations, and study limitations. The paper is followed by the list of bibliographic references with 287 sources, author’s disclaimer, and author's CV.

Keywords: cardiovascular diseases, peripheral arterial disease, bypass surgery, vascular graft, tissue engineering, tissue engineered vascular graft, decellularization, detergent, enzymatic treatment, sonication.

THESIS CONTENT

1. TISSUE ENGINEERING IS THE FUTURE OF VASCULAR REPLACEMENT

represents a brief review of the various therapeutic option that have been reported in the literature till now for cardiovascular diseases and actual methods used for the development of optimal vascular grafts. The current state-of-the-art practiced to create vascular substitutes by method of decellularization was identified.

CVDs remain one of the most prevalent healthcare problems worldwide; in addition, the number of patients who are suffering from CVD is growing; particularly, the pathologies affecting small and medium sized blood vessels are the primary cause of death [1]. In 2008, 17,3 million people died from cardiovascular related reasons; specifically, 7,3 million were due to coronary heart disease [4, 9]. The latest statistics in the field predict that due to the ageing of the population the annual incidence of cardiovascular disease-related mortalities will rise from 16.7 million in 2002 to 23.3 million in 2030 [2].

Conventional treatments for CVDs commonly involve dietary and lifestyle modifications, rehabilitation, pharmaceutical administration, and surgical options [11, 25, 26-27]. The "gold standard" for the replacement/repair of diseased blood vessels in patients with persistent symptoms is vascular substitution (bypass surgery) [7, 8]. Currently, the following types of conduits for vascular replacement are available: autografts, allografts, xenografts, and artificial prostheses [9]. Autologous vessels, such as the internal mammary artery, the greater saphenous vein or radial artery, are the best choice in case of small-caliber vascular reconstructions, where the synthetic grafts have unacceptable high failure rates [28]. However, multiple surgical procedures, diffuse atherosclerotic abnormalities, previous phlebitis, varicosities, hypoplasia, or anatomical unsuitability limit their availability [7, 10, 11, 12].

Taking into consideration a considerable number of cardiac patients and lack of appropriate vascular grafts, tissue engineering has become an alternative approach for creating new functional conduits, true blood vessels, which may promote vascular cell adhesion, proliferation, and differentiation, facilitate ECM production, respond to endogenous vasoactive compounds, and inhibit thrombogenicity [27-29]. The innovative material could potentially revolutionize vascular grafts and reduce the risk of vascular graft failure [28, 29].

Decellularization of native vascular or non-vascular tissues for vascular grafts development has gained significant attention in the past 20 years [30]. Decellularization is a complex process that requires careful consideration to create an ECM-rich, non-immunogenic vascular scaffold capable of initiating vascular regeneration [27, 31]. To ensure the successful outcome of tissue engineering based therapies it is essential to strike the balance between complete removal of cells and preserving integrity of the ECM [7, 19, 20, 21].

DC can be realized by different agents (chemical, biological, physical) allowing to induce the rupture of cellular membranes leading to production of vascular analogs comparable to native structures [21, 24, 27, 28]. There is no universal method for successful DC, as each tissue type must be considered individually to ensure optimal results. Depending on the type of target tissue, and its intended application, protocols must be carefully established to ensure safety and efficacy [22].

2. MATERIALS AND METHODS OF THE RESEARCH

This chapter describes all the materials and methods which were used during experimental phase, inclusive the methods used for morphological, biochemical, and biomechanical characterization of untreated and decellularized blood vessels. All the tests were conducted at Laboratory of Tissue Engineering and Cell Cultures, *Nicolae Testemitanu*

State University of Medicine and Pharmacy of the Republic of Moldova, Chisinau, and Leibniz Forschungslaboratorien für Biotechnologie und künstliche Organe (LEBAO), Medizinische Hochschule Hannover (MHH), Hanover, Germany under the local experts' evaluation and monitoring.

Porcine vessels were collected from Landrace Pigs (3-4 months old) in a local slaughterhouse and the animal facility of the Medizinische Hochschule Hannover (MHH). The animal care complied with the Guide for the Care and Use of Laboratory Animals. The vessels were harvested in max 2 hours after killing of the pigs and immediately transported to the laboratory in PBS at 4°C. To avoid bias based on individual variability, only specimens (three biological replicates) long enough to be cut into controls and experimental groups were used. Vessels were cleaned from fat and adjacent tissue using forceps and scissors and carefully washed to remove blood clots.

Vessels were stored at -80°C; for adequately preservation of dimensional and mechanical properties aorta were stored in DMSO. Freshly harvested carotid arteries were used as native controls for SEM analyses, freeze-thawed arteries were used as controls for all other experiments.

Chemical-Based *vs* Combined Approach in Aorta Decellularization

A chemical decellularization method based on the use of two ionic detergents, as SDS and SDC, and non-ionic one, Triton X-100, and combined approach based on additional treatment of the samples with DNase-I were adapted to decellularize porcine vessels, namely, porcine aorta.

Chemical-Based Decellularization Technique

Decellularization was performed following an initial freeze-thaw step (as mentioned above). The vessels were flushed with deionized water for 24 h at room temperature under continuous rotation to induce cell lysis via osmotic shock. Next, samples were treated with decellularization solution containing 0.5% SDS and 0.5% SDC (w/v) in hypotonic buffer (10mMTris-HCl pH 7.5, 10mM NaCl, and 10 mM EDTA) for 24 hours. At the end of the process, the vessels were washed with PBS for 24 hours to remove cell debris and residual detergent and additionally treated with 1% Triton X-100 (v/v) in hypotonic buffer for 24 hours. Finally, vessels were flushed with PBS for 48 hours.

Each decellularization step was carried out under rotation using rotating rollers (TRM50) with 35 revolutions per minute.

Combined (Detergent-Enzymatic) Decellularization Approach

At the end of decellularization process, several vessel segments (1 cm long) were additionally incubated with DNase solution of 300 U/mL DNase I (Activity 5279 U/mg) in 1 mM $MgCl_2$ in PBS at 37°C for 48 hours. The treated vessel segments were washed 3 times with PBS.

Evaluation the Decellularization Protocol Efficiency Depending on the Vessel Diameter

The combined detergent-enzymatic decellularization approach was adapted to decellularize both porcine aorta and porcine carotid artery (porcine arteries were chosen because of their similarities to the human tissues, and their availability). Four different time durations of exposure to ionic detergents were applied: 6 hours, 12 hours, 18 hours, 24 hours. After each respective decellularization time, vessels were flushed with phosphate-buffered saline (PBS) and washed additionally with 1% Triton X-100 (v/v). Finally, segments were incubated for 48 hours in DNase I solution (300 U/ml).

After each respective decellularization time, 0.5-1 cm long pieces of each vessel segment were cut off and fixed 4% paraformaldehyde (PFA) for the tissue stabilization or

directly frozen in tissue tek O.C.T. compound for fluorescent staining. Remaining vessel segments were stored at 4°C in PBS.

Decellularization of fresh *vs* frozen porcine carotid arteries

The freezing-thawing the tissue may influence the decellularization process. The study examines the efficiency of the described combined protocol in fresh porcine carotid arteries DC, the resulting data being compared to the homologous data obtained from frozen samples by performing H&E and DAPI staining.

Evaluation of Ultrasound Application for the Blood Vessels Decellularization

DC of the vascular tissue has been performed by two distinct combined approaches; in both cases ultrasound was used. For sample processing a direct sonication method (direct sonicator UP200S Hielscher, Germany and Sonotrode S1 for samples from 0.1 to 5 mL) was used. Taking into consideration that collapsing bubbles may produce significant thermal loads emitted in the surrounding liquid [13], the experiments were performed in a cold room (+4°C). In addition, the samples were placed in an ice bath to prevent tissue over-heating.

Protocol 1: For decellularization, the vessels (1 cm long segment, internal diameter 4 mm) were flushed with PBS and submerged in 2.0 mL Eppendorf tubes containing 1.5 mL hypotonic lysis buffer (0.3% NaCl in distilled water). The samples were exposed to sonication with a frequency of 24kHz, 200-watt, control mode "1" (permanent acoustic irradiation). Two different amplitude values and two different exposure times for DC were applied: 20% *vs* 100% and 3 hours *vs* 12 hours, respectively.

Protocol 2: The samples from this group were placed in a 2.0 mL Eppendorf tubes containing 1.5 mL 1% Triton X-100 and exposed to sonication with a frequency of 24kHz, 200 watts, amplitude 20%, control mode "1" for 48 hours. Distance of the sample to the tip of the ultrasound probe was set at 1 cm. As control, samples were treated with the same solution under continuous rotation (50 rpm speed, Biometra WT 17).

After decellularization, samples were washed with phosphate-buffer saline (PBS). Vascular segments from each group were fixed in 4% PFA or directly frozen in tissue tek O.C.T. compound for histological and fluorescent staining. Remaining vessel segments were stored at 4°C in PBS.

Characterization of Decellularized Porcine Matrices

The decellularized scaffolds were characterized by several different tests, namely:

- ✓ Qualitative evaluation of the remaining DNA or cytoplasmic and nuclear components through H&E and DAPI staining;
- ✓ Morphometric analysis of decellularized tissue by vessel wall thickness measurement;
- ✓ Qualitative assessment of the remaining luminal surface through SEM analysis and collagen IV preservation's evaluation through immunohistochemistry;
- ✓ Quantitative analysis of the remaining DNA through spectrophotometric assays;
- ✓ Quantitative analysis of the remaining ECM components (hydroxyproline and GAGs) through spectrophotometric assays;
- ✓ Estimation of the mechanical integrity of the remaining matrix through performing the suturability test;
- ✓ Biocompatibility testing through quantification of SDS removal, cell culture method and fluorescence cell viability assay;
- ✓ Evaluation of the efficiency of perfusion decellularization for long vascular segments;

3. QUALITATIVE AND QUANTITATIVE CHARACTERIZATION OF DECELLULARIZED VASCULAR TISSUE

Chemical-Based *vs* Combined Approach in Aorta Decellularization

H&E and DAPI staining evidently showed the presence of cells nuclei in the wall layers of native porcine ascending aorta (control group). H&E staining of decellularized samples revealed no persisting cells in all groups including the samples treated exclusively with detergents, and gross preservation of ECM. The DAPI stain of the same specimens, however, uncovered substantial amounts of residual DNA. Just a 48-hour nuclease treatment led to a complete DC of aorta specimens all the layers being devoid of nuclei (figure 1).

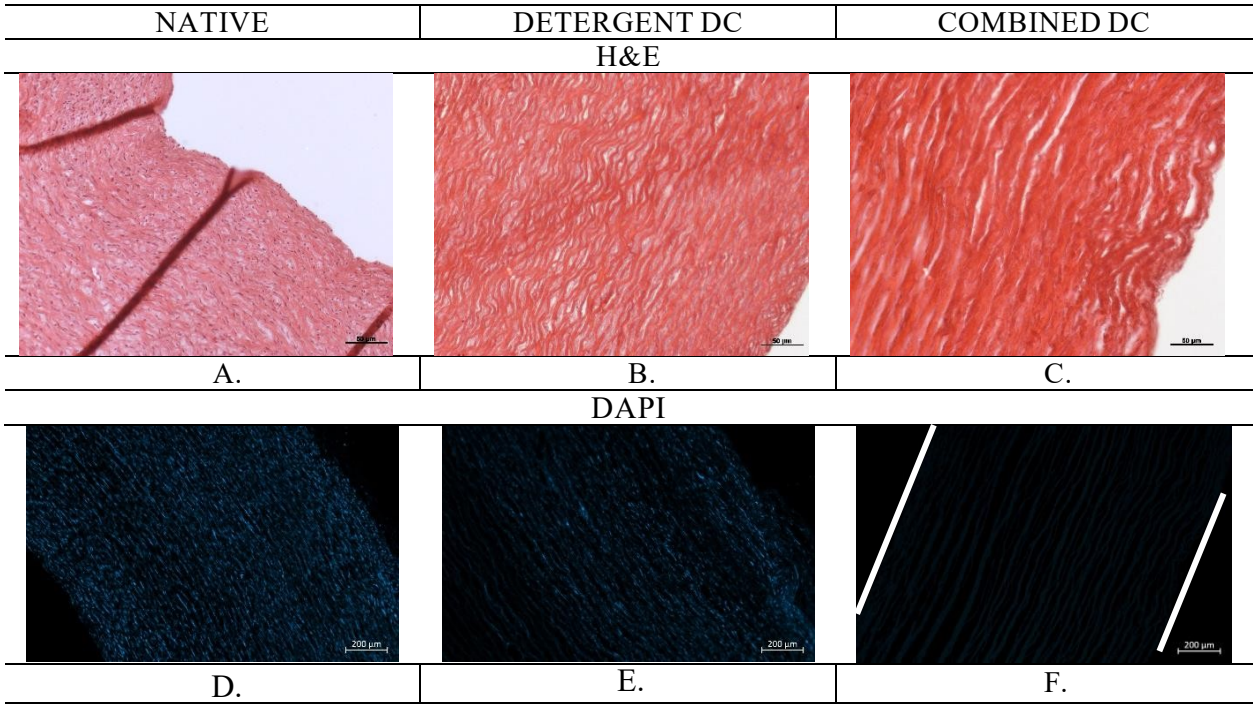

Figure 1. **Combined decellularization approach guarantee efficient cells' wash and preservation of the structure of the extracellular matrix.** H&E (A, B, C) and DAPI (D, E, F) showing cross-sections of native (A, D) and decellularized (B, C, E, F) porcine ascending aorta. **H&E**: The cytoplasm appears pinky, nucleus is blue-purple, extracellular matrix have varying degrees of pink. **DAPI**: Nuclei are blue, white lines represent the outer borders of the matrix. Scale bar. A, B: 100 µm; C 50 µm; D, E, F: 200 µm

Evaluation the Decellularization Protocol Efficiency Depending on the Vessel Diameter

Porcine aorta and carotid arteries were treated with detergents and DNase I under rotation. Even H&E staining revealed no persisting cells in all groups, the DAPI seemed to be more specific for identification substantial amounts of residual DNA. Thus, a complete DC of carotid artery resulting in the elimination of nuclei and genome required a 12-hour exposure to detergents, whereas aorta required a 24-hour treatment (figure 2).

This finding suggests large diameter blood vessels require a more extensive processing compared to small-diameter blood vessels. In such a way, no common decellularization protocol can be recommended for both types of blood vessels [33].

Decellularization of Fresh *vs* Frozen Porcine Carotid Arteries

The cross-sections of the scaffolds, fresh tissue, in this experimental group were examined by H&E staining and DAPI staining. These qualitative methods provided a visual representation of the integrity of the remaining matrix and absence of any remaining cellular

materials.

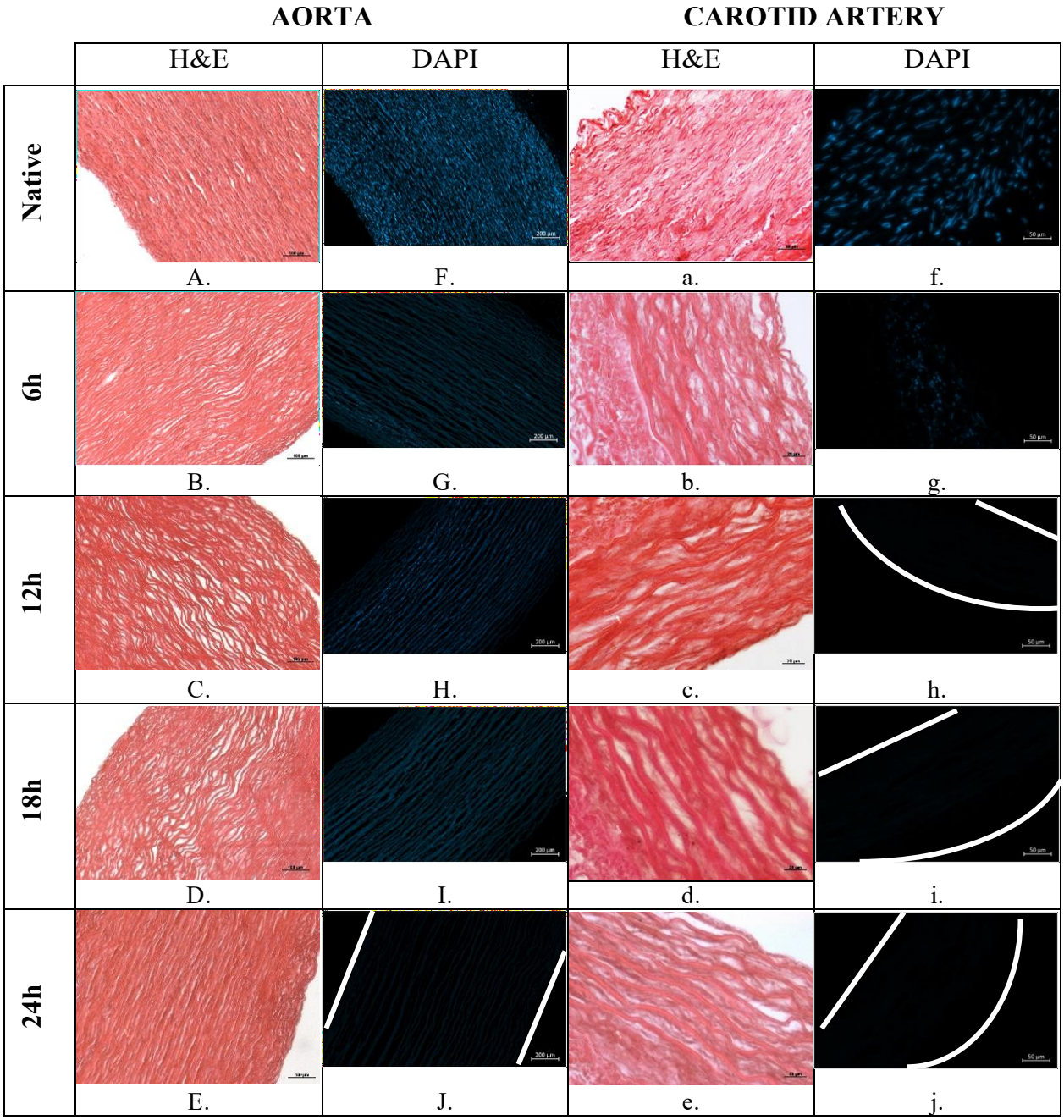

Figure 2. **Differences in decellularization efficiency of porcine vessels depending on vessels diameter [33].** Porcine aorta and porcine carotid artery decellularization: the blood vessels of different diameter require different approach in terms of effective cells' elimination. H&E staining (A, B, C, D, E, a, b, c, d, e) and DAPI staining (F, G, H, I, J, f, g, h, i, j) of native (A, F, a, f) and decellularized (B-E, G-J, b-e, g-j) vessels. Whitelines in (J, h, i, j) represent the outer of the matrix.

Evaluation of Ultrasound Application for the Blood Vessels Decellularization

Application of US in blood vessels decellularization did not appear to be an efficient strategy. Both H&E and DAPI staining revealed the presence of huge amounts of intact cells. In addition, the tissue structure was significantly affected when high amplitude waves (100%) were utilized [34] (figure 3).

Ultrasonic waves used in combination with a non-ionic detergent offered the same result

– lack of DNA reduction and cellular elements elimination. The presence of intact cellular elements suggested that reagents were not able to solubilize/destroy the membranes and to induce cell removal (figure 4).

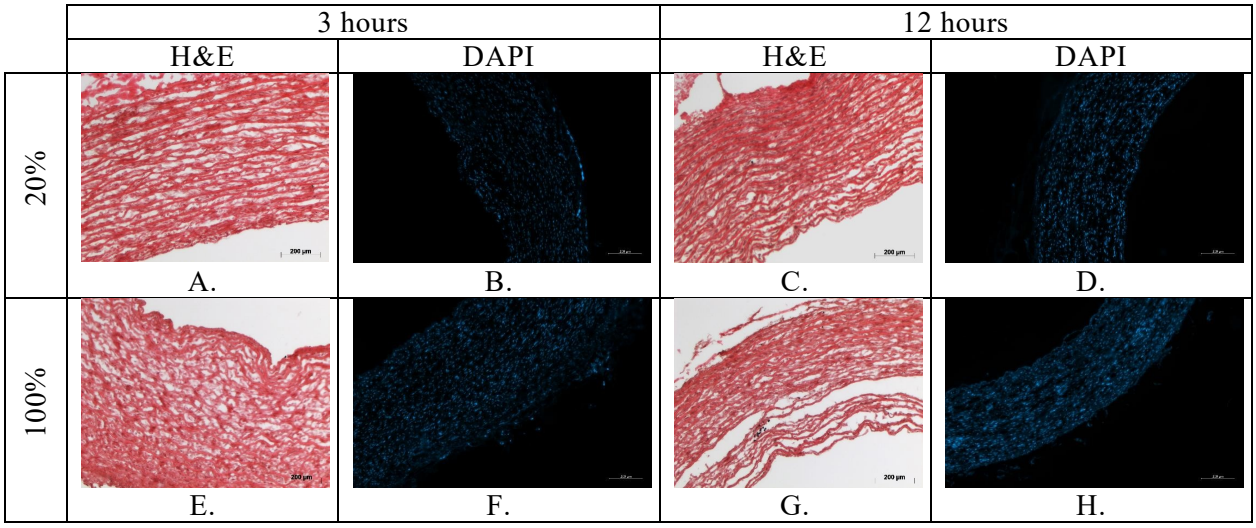

Figure 3. **Ultrasound application in blood vessels decellularization.** H&E staining (A, C, E, G) and DAPI staining (B, D, F, H). Scale bar. A, B, C, D: 200 µm

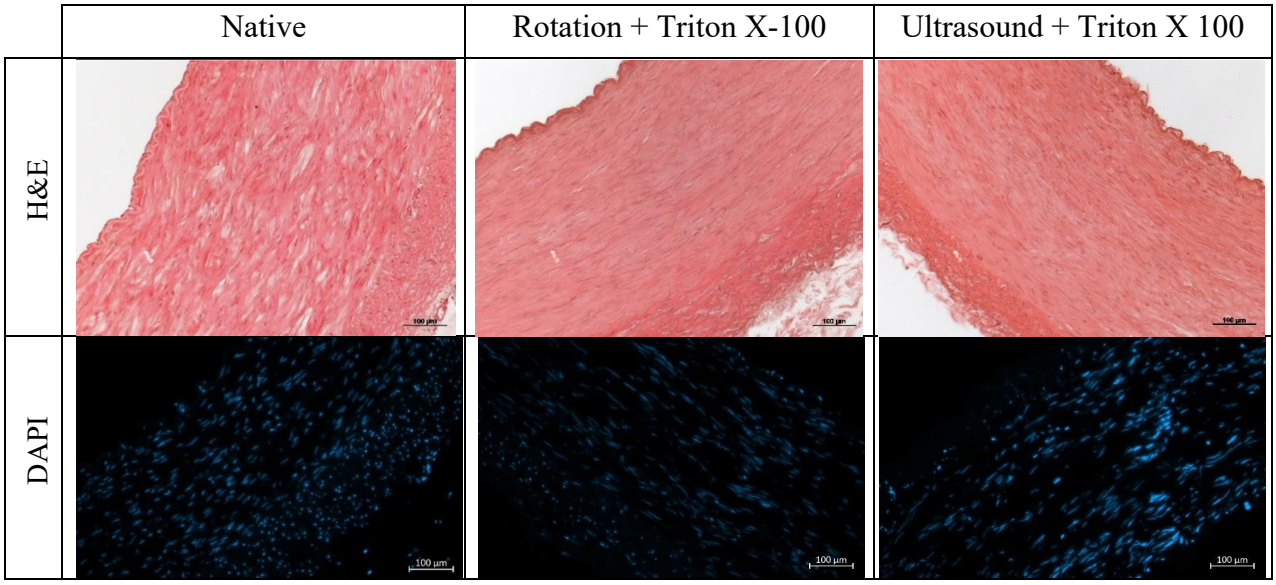

Figure 4. **Combined sonication-based approach in carotid artery decellularization.** H&E staining (A, B, C) and DAPI staining (D, E, F). Scale bar: 100 µm

Characterization of Decellularized Porcine Matrices

Decellularized porcine carotid arteries were characterized by different methods to analyze remaining components after decellularization and their quality, to evaluate mechanical integrity, and ability to host GFP-HUVECs. Each test has its own limitations and is focused on different aspects of the vessel structure or composition. Therefore, applying a variety of evaluation techniques results in a better description of the derived matrix and understanding of the decellularization process.

Qualitative methods as H&E, DAPI, and immunostaining provide a visual representation of the integrity of the remaining matrix and any remaining acellular materials. DNA, GAG, and collagen content were quantified additionally to test for remaining cellular material and retention of matrix components. Assessment the thickness of the decellularized tissue allowed to perform the morphometric analysis of the scaffold. Suture retention test was

conducted to have an impression about the mechanical strength of the remaining vessel. SEM photos of the luminal surface of decellularized vessels were taken to have an impression about any damage to the basal lamina. Biocompatibility was tested by seeding GFP-HUVECs onto opened, decellularized porcine matrices.

Qualitative Methods

Histological Evaluation

Three biological replicates were conducted and analyzed by H&E and DAPI staining (figure 5) for visualization of decellularization efficiency. The histological inspection confirmed successful cell removal – no nuclear material was detectable without harming the architecture of the ECM. As measured on H&E-stained sections statistic significant difference in arterial wall thickness before (555.94±59.22 μm) and after (427.06±37.47 μm) decellularization were detected ($p = 0.0001$).

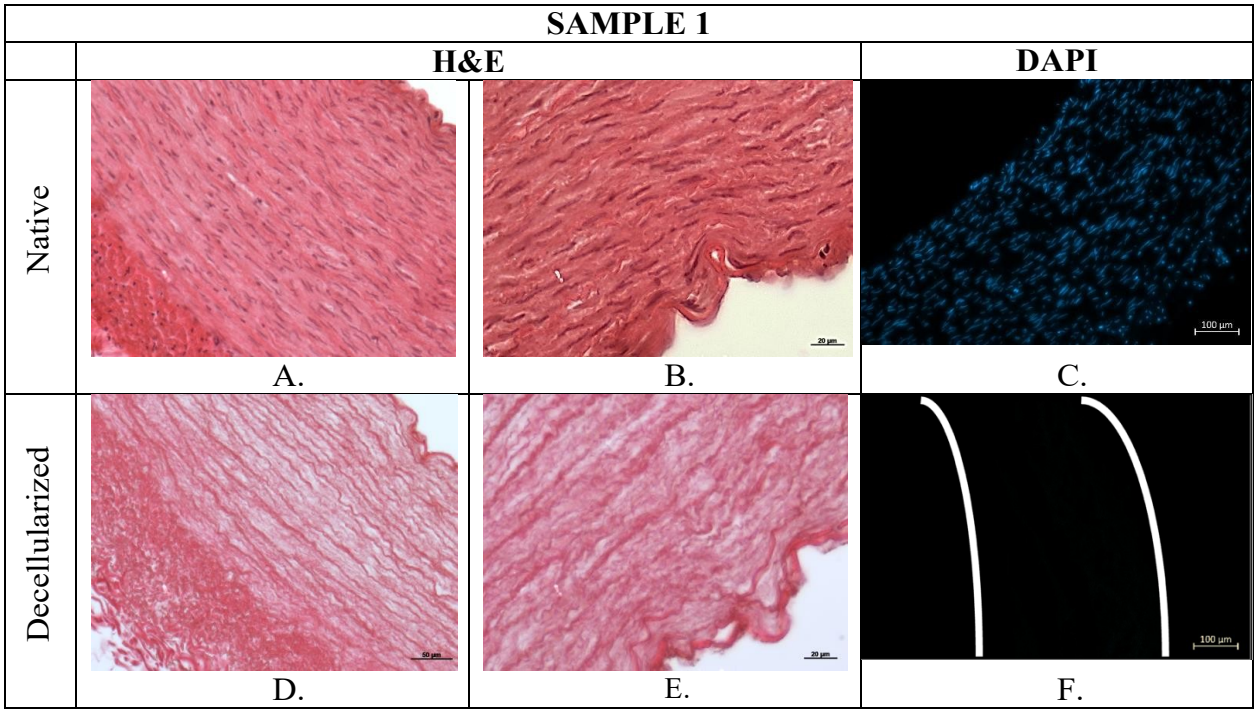

Figure 5. **Efficient decellularization of a porcine carotid artery visualized by H&E and DAPI staining (SAMPLE 1).** Native vessel (A, B, C). Decellularized vessel (D, E, F). Scale bar. A, D: 50 µm; B, E: 20 µm; C, F: 100 µm

Basement Membrane's Evaluation by Using Immunohistochemistry

Immunohistochemical approach was relevant to analyze the presence of common scaffolds' components. The major element of vascular basement membrane is collagen Type IV; staining against it using monoclonal antibody was performed to make a qualitative characterization of remaining membrane architecture and determination of its integrity. A layer of collagen IV stained in brown-yellow was observed along the luminal surface and within the vessel wall of both the control and treated arteries (alterations being detected just in some areas), revealing a relatively good preservation of basement membrane (Figure 6).

This examination of the ECM demonstrated that the applied combined decellularization protocol did not significantly alter vascular wall morphology; however, additional quantitative tests are mandatory for better understanding in matrix modifications induced by decellularization agents.

SEM Analysis Reveals a Preserved Basal Lamina

SEM analysis allowed to perform a more comprehensive analysis of the inner structure

of obtained matrix, its cellularity, and morphology of the blood vessels. In fact, it was used to visualize the impact of the decellularization agents on the luminal surface of vascular tissue and to evaluate the efficiency of debris elimination.

The control displayed a groovy structure, namely, a densely packed surface with elongated, smooth endothelial cells lining up in the direction of the blood flow. The luminal surface of decellularized porcine carotid arteries appeared flattened and free of cell-likes structures after decellularization. The basal lamina of treated samples appeared intact, no tearing was identified, exposed collagen fibers to various degrees being visible accidentally in a few areas (figure 7) [35].

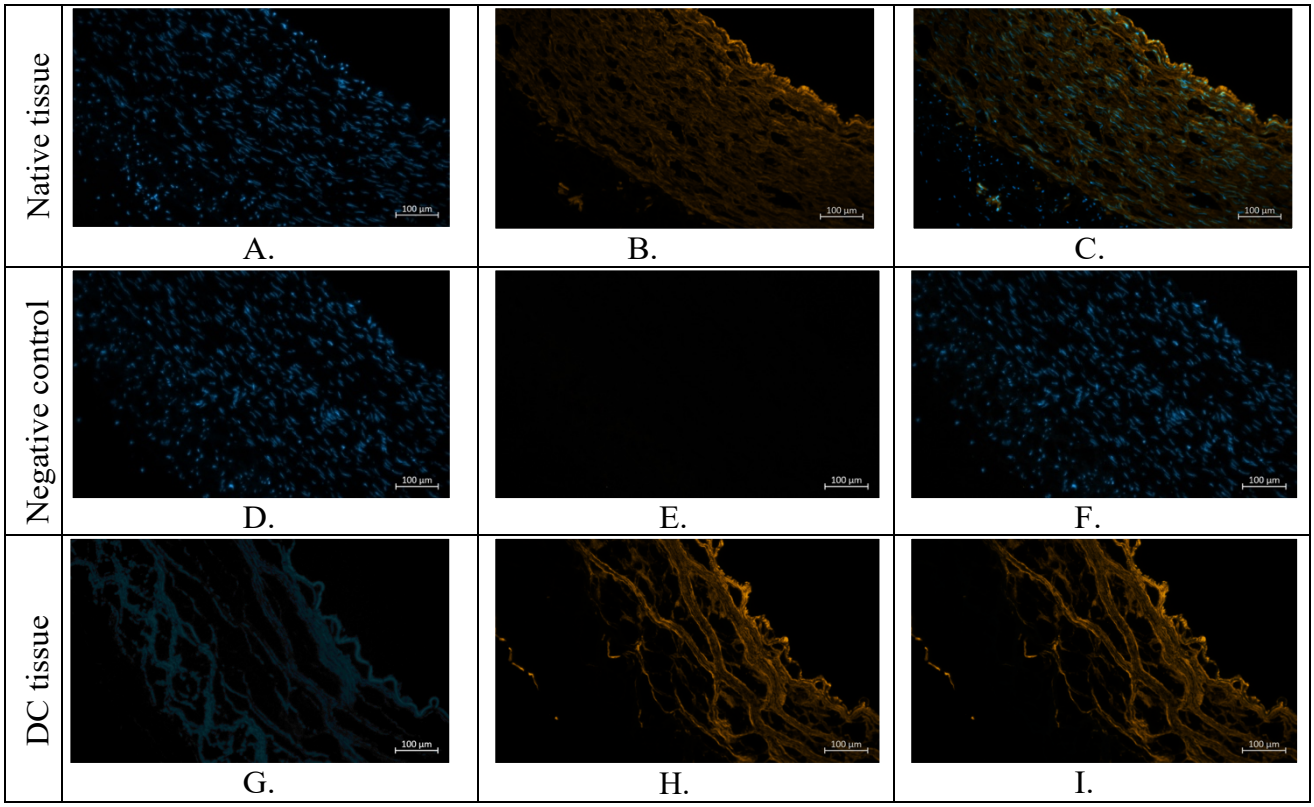

Figure 6. **Basement membrane's evaluation by using immunohistochemistry confirmed preservation the Collagen IV.** Native tissue (A, B, C). Negative control (D, E, F). Decellularized tissue (G, H, I). Scale bar: 100 µm

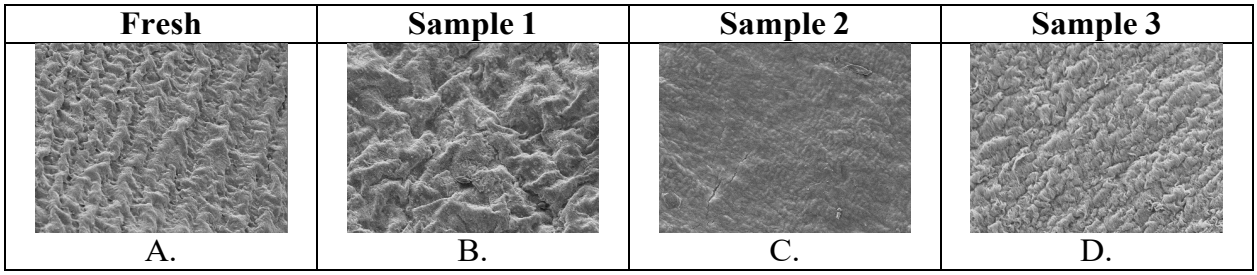

Figure 7. **SEM analysis of the luminal surface of carotid vessels.** Fresh native carotid artery (A). Decellularized carotid artery: SAMPLE 1 (D). SAMPLE 2 (G). SAMPLE 3 (J). The luminal surface of arteries appeared free of cellular remnants after DC (B-D). Scale bar. A, B, C, D: 50 µm

Perfusion decellularization

Perfusion decellularization method was evaluated also for long vascular segments (6-cm piece of porcine carotid artery being used as testing model) by application of qualitative

tests. To analyze if the vessels were decellularized evenly by perfusion, middle and end segments of the treated specimens were stained with H&E and DAPI. Additionally, the SEM analysis at decellularized matrices was performed (figure 8).

A consistent and even elimination of intact nuclei over the length of the vessel in the decellularized samples compared to native vessels was observed. On SEM analysis the luminal surface appeared free of cellular remnants after decellularization with satisfactory keeping of basal membrane.

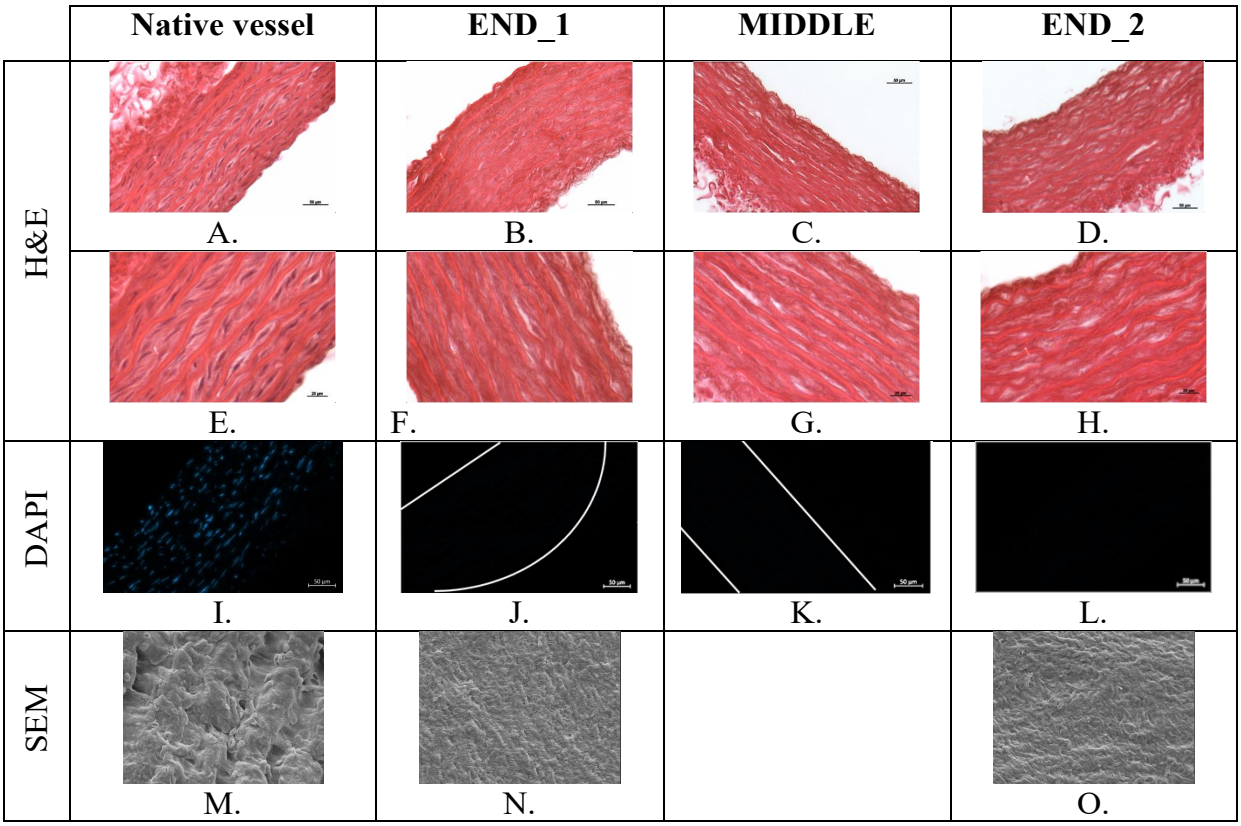

Figure 8. **Perfusion decellularization.** The efficiency of decellularization is evaluated by H&E staining (A-H) and DAPIstaining (I-L) and SEM analysis (M-O). Native vessels (A, B, I, M). END_1 (B, F, J, N). Middle section (C, G, K). END_2 (D, H, L, O).

Quantitative Methods

DNA quantification confirmed the results from the staining with DAPI and showed DNA content in decellularized vessels was reduced when combined decellularization protocol is applied. DNA quantification showed that native vessels contained 23.56±4.73 μg/mg DNA on average. Decellularization decreased the DNA content to 1.03±0.49 μg/mg on average (table 1). Decellularization reduced the DNA content to 95.6% compared to native vessel samples proving the efficiency of the in-house protocol in terms of cell nuclei removal. A Welch's test showed that the treatment groups were significantly different compared to native vessels (p=0.0001) [36].

Quantification of GAG revealed a decrease in GAG content in all decellularized groups compared to controls. GAG quantification showed that native vessels contained 13.01±3.56 μg/mg GAG on average. Decellularization decreased the GAG content to 1.30±0.72 μg/mg on average (table 1). Decellularization decreased the GAG content to 90% compared to native vessel samples (p=0.00001) [36]. In contrast, higher levels of hydroxyproline were observed in decellularized groups compared with native samples. Hyp quantification showed that native vessels contained 54.26±10.68 μg/mg on average. Decellularized vessels had 69.70±7.60

μg/mg Hyp content on average (table 1). There was 28.5% increase in Hydroxyproline content compared to native vessel samples (p=0.0744) [36].

Mechanical testing

Mechanical strength of the remaining matrix after decellularization was determined by measuring the suturability of vessels compared to untreated tissue. Nine vessels from native group and eight samples (technical replicates) from decellularized were tested. The average suture retention strength of native porcine vessels (n=9) was 1.08±0.39 N. The average suture retention strenght of decellularized vessels (n=8) was 1.14±0.38 N (table 1). So, there was no statistically significant difference between untreated and decellularized samples (p=0.0731). These fundings suggested the graft scaffold had sufficient suture retention strength to withstand anastomotic forces [37].

Table 1. **Quantitative approach in characterization the decellularized vascular tissue**

	DNA		**GAG**		**Hyp**		**Suturability**	
	concentration (µg/mg dry tissue)						***Maximum load (N)***	
	Native tissue	**DC tissue**	**Native tissue**	**DC tissue**	**Native tissue**	**DC tissue**	**Native tissue**	**DC tissue**
SAMPLE 1	25.93	1.05	14.48	2.69	45.13	65.75	0.7	1.44
	24.58	0.84	13.35	1.56	51.62	70.24	1.27	1.32
	24.81	0.84	13.01	0.44	53.37	71.60	0.94	
	31.15	2.30	14.83	8.21	45.87	52.09		
	30.54	1.40	14.64	0.26		60.67		
	30.42	1.70	14.39			61.62		
SAMPLE 2	26.94	0.80	12.47	0.87	63.29	65.27	0.79	0.92
	24.41	1.52	10.41	1.39	71.89	69.76	0.93	0.62
	24.87	1.08	10.55	1.23	72.68	71.40	0.66	0.80
	25.71	0.53	13.01	1.53	58.39	76.09		
	23.33	1.02	13.36		65.17	82.94		
	23.22	1.11	13.72		67.49	80.36		
SAMPLE 3	17.37	0.60	10.22	1.78	41.81	70.35	1.74	1.16
	15.75	1.01	10.48	1.03	46.67	69.14	1.61	1.82
	16.50	0.98	10.50	0.62	48.05	77.20	1.04	1.05
	20.28	0.38	11.99	2.19	41.20	65.81		
	19.39	0.42	5.19		46.65	106.93		
	18.86	3.50	10.40		48.97	74.63		

Evaluating the Washing Method to Remove Residual SDS

Removal of detergents, especially anionic detergents as SDS, from large acellular scaffoldsis known to be critical problems in decellularization method until it may cause undesirable host response *in vivo* towards an implanted material. The study finding (table 2) revealed that the combined decellularization method allowed to remove most of the SDS.

Table 2. **SDS quantification**

Type of tissue	**SDS/tissue [µg/mg]**										
NATIVE vessel	0.261	0.059	0.172	0.219	0.109	0.022	0.155	0.146	0.539	0.454	0.63
DC vessel	0.017	0.021	0.008	0.070	0.149	0.106	0.113	0.099	0.363	0.076	0.205	0.20

Biocompatibility Assay: *in vitro* cytocompatibility by contact test with cells

The biocompatibility of decellularized porcine matrices was tested be seeding GFP-labeled HUVECs onto the luminal surface of decellularized matrices. All matrices retrieved

after decellularization supported the attachment of HUVECs, a confluent layer was observed after 5 days of cultivation (figure 9); no apparent differences were observed between the experimental groups and the control group. After 6 days of culture live/dead assay showed live cells evenly distributed with a few dead cells within the sample. The results indicated that the treated tissues do not contain residual detergents or other harmful components to affect the cells' survival. As conclusion, the current protocol with specific components may be considered eligible for further *in vivo* evaluation of the biocompatibility [38].

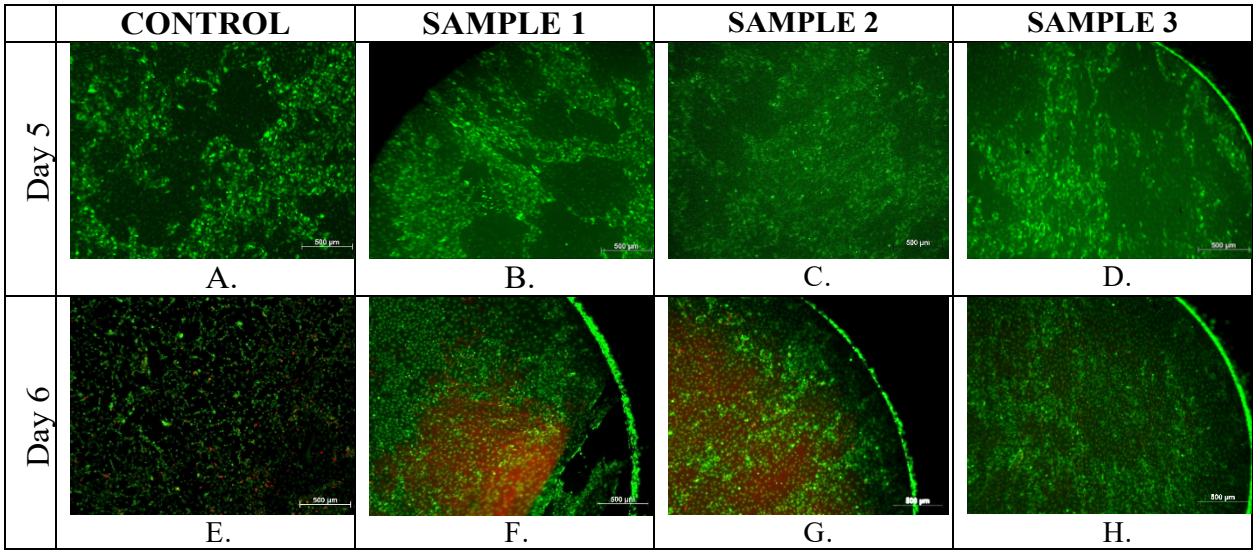

Figure 9. **Successful re-seeding of GFP-HUVECs onto decellularized porcine matrices.** 200000 cells/well seeded onto the luminal surface of matrix or plastic culture dish as control. Images were taken with a Stereomicroscope at 5th and 6th day post-seeding. All matrices retrieved after decellularization supported the attachment of HUVECs, as a confluent layer was observed after 5 days of cultivation (B-D). Live/dead staining showed live cells evenly distributed in decellularized samples while dead cells were red (F-G). Plastic culture dish, as control (A, E). Decellularized carotid artery (SAMPLE 1 – B, F. SAMPLE 2 – C, G. SAMPLE 3 – D, H).

GENERAL CONCLUSIONS

1. Sonication-assisted methods do not appear to be efficient strategy to remove cells from vascular tissue;
2. High-amplitude ultrasound is inappropriate for vascular tissue decellularization because of its harmful effects on the matrix;
3. Large-diameter blood vessels require an extended processing time than small-diameter blood vessels; therefore no common decellularization protocol can be recommended;
4. The described chemical-enzymatic approach is an efficient tool in development the acellular vascular scaffolds for both large-diameter and small-diameter blood vessels; only chemical treatment cannot allow to obtain complete cells elimination;
5. The represented chemical-enzymatic approach can be applied for both fresh and freeze-thawed vascular tissue;
6. H&E in not efficient qualitative tool for confirmation of cellular residues elimination;
7. Complex characterization (morphological, biochemical, and biomechanical tests) of decellularized scaffold is mandatory in order to be able to predict the *in vivo* performanceof tissue-engineered vascular grafts;

8. Perfusion decellularization is an efficient tool for uniform decellularization of long segments of blood vessels.

PRACTICAL RECOMMENDATIONS

1. A large-scale characterization of sonication assisted techniques of decellularization require experimental testing of other existing indirect and direct sonication methods;
2. The lack of DNA reduction when applying Triton X-100 or hypotonic solution in combination with ultrasound suggests the necessity of using sonication with stronger chemicals, such as sodium dodecyl sulfate or sodium deoxycholate, for blood vessels' decellularization;
3. Porcine carotid artery is an optimal testing model for evaluation of decellularization protocols' efficiency and development of small-diameter tissue-engineered blood vessels;
4. Freezing without cryoprotectant as a method of prolonged storage of biological tissues can be safely used in practice; no negative impact on the mechanical properties were recorded;
5. Detergents do not allow cellular components elimination from the scaffold, for successful DC the working protocol should be supplemented with enzymatic treatment for DNA removal;
6. H&E staining cannot be used as a sole proof of DC and should be completed, at least, with a DNA stain like DAPI;
7. Multiple washing steps and treatment with Triton X-100 are an optimal instrument to wash SDS remnants from the scaffold.

STUDY LIMITATIONS

1. More detailed histological analysis is necessary; Masson's trichrome staining and picrosirius red staining may reveal the collagen and elastin morphology;
2. Suture retention test in not enough for mechanical evaluation of the scaffold, uniaxial and biaxial mechanical testing would be more informative about the tissue behavior. Simultaneously the behavior after re-cellularization would be an interesting study;
3. If *in vitro* biocompatibility tests had good results, animal testing could be the last important test;
4. Testing for different matrix and cellular proteins levels, and for known and unknown antigens, would give a better understanding about the immunological potential of these grafts.

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38. **Malcova T,** Nacu V, Ciubotaru A, Rojnoveanu Gh. Biocompatibilitatea țesutului vascular decelularizat: model *in vitro* pentru testarea grefelor obținute prin metode inginerești. Jurnalul de Chirurgie. 2023;19(2):143-149.

LIST OF SCIENTIFIC PUBLICATIONS AND EVENTS
at which the results of the research for the doctoral thesis in Medical Science with the topic "Morphological and biomechanical modifications in blood vessels decellularization" were presented

I. Articles in scientific journals

- **Articles in international scientific journals:**

1. **Malcova T.,** Nacu V., Ciubotaru A., Rojnoveanu Gh. Biocompatibilitatea țesutului vascular decelularizat: model *in vitro* pentru testarea grefelor obținute prin metode inginerești. In: *Jurnalul de Chirurgie*. 2023; 19(2): pp. 143-149. ISSN: 1584-9341 (Online).

- **Articles in accredited national scientific journals:**

✓ **Articles in category B + journals**

2. **Malcova T.,** Balutel T., Ciubotaru A., Nacu V. Tissue engineering of heart valves-challenges and opportunities. In: *The Moldovan Medical Journal*. 2019; 62(4): pp. 49-55. ISSN: 2537-6373 (Print) / ISSN: 2537-6381 (Online).
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✓ **Articles in category B journals**

4. **Malcova T.,** Cirimpei D., Cirimpei O., Nacu V. Tehnologii de cultivare *in vitro* a fibroblastelor din piele pentru tratamentul pacienților cu ulcere cronice. In: *Curierul Medical*. 2016; 59(3): pp. 74-81. ISSN: 2537-6373.
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- **Articles in conference proceedings:**

✓ **Articles in conference proceedings included in databases Web of Science and SCOPUS:**

7. **Malcova T.,** Globa L., Vascan A., Tugui E., Stoian A., Nacu V. Mechanical and morphological characterization of decellularized umbilical vessels as tissue engineering scaffolds. In: Tiginyanu I., Sontea V., Railean S. (eds). *Springer, Cham, 4th International Conference on Nanotechnologies and Biomedical Engineering. ICNBME 2019. IFMBE Proceedings*. 2020; 77: pp. 589-593. ISSN: 1680-0737 / ISSN: 1433-9277 (electronic). ISBN 978-3-030-31865-9 ISBN 978-3-030-31866-6 (eBook). DOI: 10.1007/978-3-030-31866-6 **(SJR: 0.155, SCOPUS)**.
8. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Evaluation of ultrasound application for the decellularization of small caliber vessels. In: Tiginyanu I., Sontea V., Railean S. (eds). *Springer, Cham, 5th International Conference on Nanotechnologies and Biomedical Engineering. ICNBME 2021. IFMBE Proceedings*. 2022; 87: pp. 350-357. ISSN: 1680-0737 / ISSN: 1433-9277 (electronic). ISBN 978-3-030-92327-3. ISBN 978-3-030-92328-0 (eBook). DOI: 10.1007/978-3-030-92328-0_46 **(SJR: 0.155, SCOPUS)**.
9. **Malcova T.**, Rojnoveanu Gh., Ciubotaru A., Nacu V. Mechanical characterization of decellularized blood vessels: a valuable tool to provide comprehensive information about the scaffold. In: Sontea V., Tiginyanu I., Railean S. (eds). *Springer, Cham, 6th International Conference on Nanotechnologies and Biomedical Engineering. ICNBME 2023. IFMBE*

Proceedings. 2024; 91(Volume 1): pp. 386-396. ISSN: 1680-0737 / ISSN: 1433-9277 (electronic). ISBN 978-3-031-42774-9. ISBN 978-3-031-42775-6 (eBook). DOI:/10.1007/978-3-031-42775-6_42 **(SJR: 0.155, SCOPUS)**.

II. Abstracts / thesis submitted at scientific conferences:

✓ **international conferences from abroad**

10. **Malcova T.,** Balutel T., Popescu V., Nacu V. Characterization of decellularized porcine aorta as tissue engineering scaffolds for vascular application. Timișoara Anatomical Days. In: *Abstract Book. Research and Clinical Medicine*. Timisoara, Romania: 2019; 3 (1), p. 49. ISSN: 2360-1124.

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13. **Malcova T.,** Rojnoveanu Gh., Ciubotaru A., Andrée B., Hilfiker A. Cuantificarea ADN și a proteinelor matricei extracelulare: instrument util în caracterizarea vaselor sanguine decelularizate. In: *Chirurgia (Bucur*). *Conferința Națională de Chirurgie 2023.* 2023; 118(Suppl.1), pp. 163-164. ISSN: 1221-9118. ISSN (online): 1842-368X.

✓ **international conferences organized in Republic of Moldova**

14. **Malcova T.** Blood vessel decellularization – challenges and perspectives. In: *Abstract Book. MedEspera 2018: 7th Intern. Medical Congress for Students and Young Doctors*. Chisinau: 2018; p. 204-205.

15. **Malcova T.,** Balutel T., Cociug A., Popescu V. Tissue engineered vascular grafts: decellularization of porcine aorta through three different methods. In: *Abstract Book. MedEspera 2020: 8th Intern. Medical Congress for Students and Young Doctors*. Chisinau: 2020; p. 101.

16. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Qualitative evaluation of detergent-enzymatic decellularized small-caliber blood vessels. In: *Abstract Book. MedEspera 2022: 9th Intern. Medical Congress for Students and Young Doctors*. Chisinau: 2022; p. 437.

✓ **national conferences**

17. **Malcova T.,** Balutel T., Globa T., Popescu V. Eficiența comparativă a procedurilor de decelularizare cu detergenți a grefelor vasculare. In: *Abstract Book. Congresul Consacrat aniversării a 75-a de la fondarea USMF "Nicolae Testemițanu"*. Chisinau: 2020; p. 422.

18. Pavlovschi E., Stoian A., **Malcova T.,** Iordachescu R., Verega G., Nacu V. Decelularizarea combinată a alografei osoase vascularizate. Etapă de studiu experimental in vivo. În: *Abstract Book. Congresul Consacrat aniversării a 75-a de la fondarea USMF "Nicolae Testemițanu"*. Chisinau: 2020; p. 519.

19. Stoian A., Nacu V., Pavlovschi E., Macagonova O., **Malcova T.,** Mihaluta V. Perspectiva de viitor a alotransplantului osos vascularizat. In: *Abstract Book. Congresul Consacrat aniversării a 75-a de la fondarea USMF* "*Nicolae Testemițanu"*. Chisinau: 2020; p. 525.

20. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Decelularizarea de succes a aortei porcine pentru generarea scaffoldului acelular necesar în obținerea grefelor vasculare inginerești. In: *Abstract Book. Conferința Științifică Anuală. Cercetarea în biomedicină și sănătate: calitate, excelență și performanță*. Chisinau: 2021; p. 250.

III. Invention patents, patents, registration certificates, materials of invention salons:

21. **Malcova T.,** Nacu V. Procedeu de decelularizare a vaselor sanguine de calibru mic. Innovator Certificate no. 5937, 12.08.2022

22. **Malcova T.,** Nacu V. Procedeu de decelularizare a vaselor sanguine de calibru mic. Implementation Act no. 60, 20.03.2023.

23. **Malcova T.,** Rojnoveanu Gh., Ciubotaru A., Nacu V. *In vitro* model of biocompatibility evaluation: a new approach for testing the decellularized vascular scaffolds. Diploma GOLD MEDAL. EUROINVENT: 15th European Exhibition Of Creativity And Innovation. Special Award For The Invention. Titu Maiorescu University Of Bucharest. Certificate of Recognition. Innovation Award for Promoting Science and Technology at Euroinvent 2023. Iasi, Romania. 11.05.-13.05.2023.

24. **Malcova T.,** Jian M., Cobzac V., Mostovei A., Bujor M., Nacu V. New methods in tissue engineering: decellularization of small-caliber blood vessels and colagen concentration. DIPLOMA of GOLD MEDAL. 2nd edition of the International Exhibition of Innovation and Technology Transfer EXCELLENT IDEA – 2023. Chisinau. 19.09.-21.09.2023.

IV. Participation with communications at scientific forums:

✓ **international**

25. **Malcova T.,** Globa L., Vascan A., Tugui E., Stoian A., Nacu V. Evaluation of the efficacy of decellularization treatment in preparing decellularized umbilical cord artery. The 4th International Conference on Nanotechnologies and Biomedical Engineering – ICNBME-2019. Chisinau, 18-21 September 2019.

26. **Malcova T.,** Balutel T., Cociug A., Popescu V. Tissue engineered vascular grafts: decellularization of porcine aorta through three different methods. The 8th MedEspera International Congress for Students and Young Doctors. Chisinau, 24-26 septembrie 2020.
(DIPLOMA Ist Place Award Certification)

27. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Protocolul de decelularizare a vaselor sanguine este dependent de diametrul acestora. Conferința Națională de Chirurgie. Online Edition, Romania, 9-12 June 2021.

28. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Evaluation of ultrasound application for the decellularization of small caliber vessels. The 5th International Conference on Nanotechnologies and Biomedical Engineering – ICNBME-2021. Online Edition, Chisinau, 3-5 November 2021.

29. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Qualitative evaluation of detergent-enzymatic decellularized small-caliber blood vessels. The 9th International Medical Congress for Students and Young Doctors, MedEspera. Chisinau, 12-14 May 2022.
(DIPLOMA Ist Place Award Certification)

30. **Malcova T.**, Rojnoveanu Gh., Ciubotaru A., Nacu V. Mechanical characterization of decellularized blood vessels: a valuable tool to provide comprehensive information about the scaffold. 6th International Conference on Nanotechnologies and Biomedical Engineering – ICNBME 2023. Chisinau, 20-23 September 2023.
(Certificate of achievement 1st place in YOUNG INVESTIGATORS COMPETITION)

✓ **national**

31. **Malcova T.** Public lecture "Grefele vasculare decelularizate obținute prin inginerie tisulară vor fi un standard de tratament în viitor?". MoldMedizin & MoldDent. Chișinău, 11-13 September 2019.

32. **Malcova T.** Public lecture "Inginerie tisulară și Medicină regenerativă: provocări și realizări". Ziua Internațională a Științei. Chisinau, 09 November 2019.

33. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Decelularizarea de succes a aortei porcine pentru generarea scaffoldului acelular necesar în obținerea grefelor vasculare inginerești. Conferința Științifică Anuală. Cercetarea în biomedicină și sănătate: calitate, excelență și performanță. Online Edition, Chisinau, 20-22 October 2021.

34. **Malcova T.** Decelularizarea vaselor sanguine. Curs educațional "Medicina regenerativă și nanomedicina". Conferința Științifică Anuală Cercetarea în Biomedicină și Sănătate: Calitate, Excelență și Performanță. Chisinau, 19-21 October 2022.

V. Participation with posters at scientific forums:

✓ **international**

35. **Malcova T.,** Globa L., Vascan A., Țugui E., Stoian A., Nacu V. Evaluation of the efficacy of decellularization treatment in preparing decellularized umbilical cord artery. International Molecular Medicine Symposium by the Bosphorus. Istanbul, Turkey, 16-18 May 2019.
(Certificate of AWARD - Best Poster Presentation AWARD in Third Place)

36. **Malcova T.,** Balutel T., Popescu V., Nacu V. Characterization of decellularized porcine aorta as tissue engineering scaffolds for vascular application. Timișoara Anatomical Days. Simpozionul "Zilele Anatomice Timișorene", ediția I, cu participare internațională. Timisoara, Romania, 6-7 December 2019.

37. **Malcova T.,** Rojnoveanu Gh., Ciubotaru A., Andrée B., Hilfiker A. Cuantificarea ADN și a proteinelor matricei extracelulare: instrument util în caracterizarea vaselor sanguine decelularizate. Conferința Națională de Chirurgie 2023. Eforie Nord, Romania, 24-27 May 2023.

✓ **national**

38. Pavlovschi E., Stoian A., **Malcova T.,** Iordachescu R., Verega G., Nacu V. Decelularizarea combinată a alografei osoase vascularizate. Etapă de studiu experimental *in vivo*. Congresul Consacrat aniversării a 75-a de la fondarea USMF "Nicolae Testemițanu". Online Edition, Chisinau, 21-23 October 2020.

39. Stoian A., Nacu V., Pavlovschi E., Macagonova O., **Malcova T.,** Mihaluta V. Perspectiva de viitor a alotransplantului osos vascularizat. In: Abstract Book. Congresul Consacrat aniversării a 75-a de la fondarea USMF "Nicolae Testemițanu". Online Edition, Chisinau, 21-23 October 2020.

40. **Malcova T.,** Balutel T., Cociug A., Popescu V. Tissue-engineered vascular grafts: decellularization of porcine aorta through three different methods. Congresul consacrat aniversării a 75 ani de la fondarea USMF "Nicolae Testemițanu". Online Edition, Chisinau, 21-23 October 2020.
(Laureat al Concursului „Performanțe în cercetare" pentru ciclul de lucrări în domeniul Chirurgiei generale, Ingineriei tisulare și culturilor celulare)

VI. Participation in media shows / projects:

41. **Malcova T.** Ambasador-trainer of Science, Scientific field – Tissue engineering and cell cultures, Surgery. Grant Agreement nr. 101060678, project "Green Science to the Service of Healthy Society" (GreenSCI), program HORIZON EUROPE (01 April 2022 – 30 November 2023).
(Diploma de Onoare a Academiei de Științe a Moldovei; Diploma de Gratitudine)

VII. Research internship:

42. Exchange program at University of Chester, Chester, Great Britain, European Union's Erasmus+ programme International Credit Mobility (agreement number: 2016-1-UK01-KA107-024078), 15 January 2018 – 15 Juny 2018.

43. Exchange program at Hannover Medical School, or. Hanovra, Germania, în cadrul proiectul Orizont2020 NanoMedTwin "Promoting smart specialization at the Technical University of Moldova by developing the field of Novel Nanomaterials for BioMedical Applications through excellence in research and twinning" (Grant agreement ID: 810652), 01 September 2020 – 28 February 2021.

44. Scholarship program offered by the World Federation of Scientists for PhD students and young researchers from the Republic of Moldova, Chisinau, 01 June 2023 – 31 May 2024.

VIII. Educational courses on research topic:

45. Autumn School on Nano-Bioengineering 2019. Chisinau, 14-17 September 2019.

46. Advanced Training Course on Nanotechnologies and Biomedical Engineering organised in the framework of the Horizon2020 project "NanoMedTwin". Chisinau, 19 October 2019 – 16 May 2020.

47. Training Course on Intellectual Property Protection and Technology Transfer in the framework of the Horizon2020 project "NanoMedTwin". Chisinau, 01 October – 19 December 2020.

48. Summer school "Nanotehnologii și biomedicină în contextul provocărilor secolului XXI". Online edition, Chisinau, 5-13 June 2021.

49. Workshop of Polymerase Chain Reaction and Cell Culture, Bahçeşehir University Faculty of Medicine. Istanbul, Turkey, 16 May 2019.

ANNOTATION

Malcova Tatiana „**Morphological and biomechanical modifications in blood vessels decellularization**". The thesis for the degree of PhD in medical sciences, Chisinau, 2023.

Structure of the thesis. The thesis includes annotations in Romanian, Russian and English, list of abbreviations, 48 figures, 6 tables, introduction, 4 chapters with general conclusions, practical recommendations, and study limitations. The paper is followed by the list of bibliographic references with 287 sources, author's disclaimer, and author's CV. The principal results of the study were published in 20 scientific papers.

Keywords: Cardiovascular diseases, peripheral arterial disease, bypass surgery, vascular graft, tissue engineering, tissue engineered vascular graft, decellularization, detergent, enzymatic treatment, sonication.

The aim of study. To develop new methods for decellularization of large- and small-diameter blood vessels.

Objectives of the study. (1) To evaluate the efficiency of sonication-assisted methods for decellularization of arterial vessels; (2) To test the effect of acoustic amplitude on the vascular matrix; (3) To evaluate the effectiveness of chemical (SDS, SDC, Triton X-100, hypotonic solution) and enzymatic (DNase-I) treatment in vascular tissue decellularization; (4) To evaluate whether the decellularization protocol efficiency is depending on the vessel diameter; (5) To check the informativeness of qualitative methods (H&E and DAPI) for confirmation of the decellularization process; (6) To do morphological, biochemical, and biomechanical characterization of treated blood vessels; (7) To assess the biocompatibility of acellular scaffold by performing *in vitro* contact test; (8) To determine the efficiency of perfusion decellularization for uniform cells' elimination from long segments of blood vessels.

Scientific originality and novelty. Conducting the experimental study with comparison and multilateral characterization of decellularization efficiency of different decellularization approaches in term of cells' elimination and matrix strength preservation contributed to the completion of some gaps in the current scientific literature.

The scientific problem solved in the thesis consists in identifying the factors associated with efficient cells' removal and establishing a novel procedure for blood vessels decellularization and optimal characterization of acellular scaffold's structure, a fact that will allow the modification of the experimental paradigm through the scientifically reasoned selection of the optimal experimental conditions.

Theoretical significance and applicative value. Decellularization efficiency of different chemicals was specified; in addition, the indispensability of the enzymatic treatment in combination with strong detergents for accelular vascular scaffolds production was demonstrated. The data obtained during the research scientifically argue for the modification of the current research strategy through the preferential use of carotid artery *vs* aorta as testing model for development of small-diameter tissue-engineered vascular grafts. The failed attempt touse the ultrasound for vascular tissue DC defines the necessity to perform additional studies regarding the mechanism of ultrasound-induced cellular destruction.

The practical impact of the present study consists in implementation of a novel technique of blood vessels decellularization in the Laboratory of Tissue Engineering and Cell Culture, *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova.

ADNOTARE

Malcova Tatiana „**Modificările morfologice și biomecanice în decelularizarea vaselor sangiuine**". Teza pentru obținerea titlului de doctor în științe medicale, Chișinău, 2023.

Structura tezei. Teza include adnotările în limbile engleză, română și rusă, lista abrevierilor, 48 de figuri, 6 tabele, introducere, 4 capitole cu concluzii generale, recomandări practice și limitări ale studiului. Lucrarea este urmată de lista de referințe bibliografice cu 287 de surse, declarația autorului și CV-ul autorului. Principalele rezultate ale studiului au fost publicate în 20 de lucrări științifice.

Cuvinte-cheie: boli cardiovasculare, boala arterială periferică, chirurgie bypass, grefă vasculară, inginerie tisulară, grefă vasculară obținută prin inginerie tisulară, decelularizare, detergent, tratament enzimatic, ultrasunet.

Scopul studiului. Dezvoltarea unor tehnici noi de decelularizare a vaselor sanguine de calibru mare și mic.

Obiectivele studiului. (1) Evaluarea eficienței metodelor asistate de sonicare în decelularizarea vaselor arterelor; (2) Testarea efectului amplitudinii acustice asupra matricei vasculare; (3) Determinarea eficacității tratamentului chimic (SDS, SDC, Triton X-100, soluție hipotonică) și enzimatic (DNază-I) în decelularizarea țesutului vascular; (4) Evaluarea eficienței protocolului de decelularizare în funcția de diametrul vasului; (5) Aprecierea informativității metodelor calitative (H&E și DAPI) pentru confirmarea procesului de decelularizare; (6) Caracterizarea morfologică, biochimică și biomecanică a vaselor sanguine prelucrate; (7) Testarea biocompatibilității matricei acelulare prin efectuarea testului de contact *in vitro*; (8) Determinarea eficienței decelularizării prin perfuzie pentru eliminarea uniformă a celulelor din segmentele lungi ale vaselor sanguine.

Originalitatea și noutatea științifică. Realizarea studiului experimental cu compararea și caracterizarea multilaterală a eficienței diferitor tehnici de decelularizare în ceea ce privește eliminarea celulelor și păstrarea rezistenței matricei pentru completarea unor lacune din literatura științifică actuală.

Problema științifică rezolvată în teză constă în: identificarea factorilor asociați cu îndepărtarea eficientă a celulelor din matrice și stabilirea unei proceduri noi de decelularizare a vaselor sanguine de calibru mic, și caracterizarea optimă a structurii acelulare, fapt care va permite modificarea paradigmei experimentale prin selectarea rațională din punct de vedere

științific a condițiilor experimentale optime.

Semnificația teoretică și valoarea aplicativă. A fost specificată eficiența de decelularizare a diferitor substanțe chimice; în plus, a fost demonstrat caracterul indispensabil al tratamentului enzimatic în combinație cu detergenți puternici pentru producerea matricei vasculare acelulare. Datele obținute argumentează științific modificarea strategiei actuale de cercetare prin utilizarea preferențială a arterei carotide față de aortă ca model de testare pentru dezvoltarea grefelor vasculare de diametru mic prin inginerie tisulară. Încercarea eșuată de a utiliza ultrasunetul pentru decelularizarea țesutului vascular definește necesitatea de a efectua studii suplimentare privind mecanismul de distrugere celulară indusă de undele sonore.

Impactul practic al prezentului studiu constă în implementarea unei tehnici noi de decelularizare a vaselor sanguine în cadrul Laboratorului de inginerie tisulară și culturi celulare, Universitatea de Stat de Medicină și Farmacie „Nicolae Testemițanu” din Republica Moldova.

АННОТАЦИЯ

Малкова Татьяна "**Морфологические и биомеханические модификации при децеллюляризации кровеносных сосудов**". Диссертация на соискание степени кандидата медицинских наук, Кишинев, 2023.

Структура диссертации. Диссертация включает аннотации на румынском, русском и английском языках, список сокращений, 48 рисунков, 6 таблиц, введение, 4 главы с общими выводами, практические рекомендации и ограничения исследования, список из 286 библиографических источников, авторскую декларацию и резюме. Основные результаты исследования опубликованы в 20 научных работах.

Ключевые слова: сердечно-сосудистые заболевания, заболевания периферических артерий, шунтирование, сосудистый трансплантат, тканевая инженерия, сосудистый трансплантат полученный тканевой инженерией, децеллюляризация, детергент, ферментативная обработка, ультразвук.

Цель исследования. Развить новые техники децеллюляризации кровеносных сосудов большого и малого калибра.

Задачи исследования. (1) Оценить эффективность методов децеллюляризации сонной артерии свиньи с помощью ультразвука; (2) Проверить влияние акустической амплитуды на сосудистый матрикс; (3) Оценить эффективность химического (ДСН, ДДК, Тритон Х-100, гипотонический раствор) и ферментативного (ДНКаза-I) лечения при децеллюляризации сосудистой ткани; (4) Оценить эффективность протокола децеллюляризации в зависимости от диаметра сосуда; (5) Проверить информативность качественных методов (H&E и DAPI) для подтверждения процесса децеллюляризации; (6) Провести морфологическую, биохимическую и биомеханическую характеристику обработанных кровеносных сосудов; (7) Оценить биосовместимость бесклеточного каркаса путем проведения контактного теста *in vitro*; (8) Определить эффективность перфузионной децеллюляризации для однородного удаления клеток из длинных сегментов кровеносных сосудов.

Научная оригинальность и новизна. Проведение экспериментального исследования со сравнением и многосторонней характеристикой эффективности различных методов децеллюляризации с точки зрения удаления клеток и сохранения прочности матрикса.

Научная проблема, решаемая в диссертации, заключается в выявлении факторов, связанных с эффективным удалением клеток, и создании новой процедуры децеллюляризации кровеносных сосудов и оптимальной характеристики структуры ацеллюлярного матрикса, что позволит модифицировать экспериментальную парадигму

путем научно обоснованного выбора оптимальных условий эксперимента.

Теоретическая значимость и прикладное значение. Уточнена эффективность децеллюляризации различными химическими веществами; продемонстрирована незаменимость ферментативной обработки в сочетании с сильными детергентами для производства сосудистого ацеллюлярного матрикса. Неудачная попытка использования ультразвука определяет необходимость проведения дополнительных исследований относительно механизма разрушения клеток, индуцированного ультразвуком.

Практическая значимость настоящего исследования заключается во внедрении новой методики децеллюляризации кровеносных сосудов в Лаборатории Тканевой Инженерии и Культуры клеток Государственного Университета Медицины и Фармации им. Николае Тестемицану, Кишинев, Республика Молдова.