Doctoral School in Medical Sciences

MALCOVA Tatiana

MORPHOLOGICAL AND BIOMECHANICAL MODIFICATIONS IN BLOOD VESSELS DECELLULARIZATION

341.01 TISSUE ENGINEERING AND CELL CULTURES

PhD thesis in medical sciences

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MALCOVA Tatiana

MODIFICĂRILE MORFOLOGICE ȘI BIOMECANICE ÎN DECELULARIZAREA VASELOR SANGUINE

341.01 INGINERIE TISULARĂ ȘI CULTURI CELULARE

Teză de doctor în științe medicale

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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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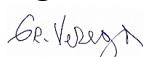
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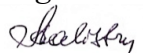Topor Boris,

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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 5th of October 2023 (minutes of the meeting no. 20).

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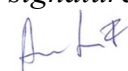Calistru Anatol,

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CONTENT

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 novel procedures 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 acelular 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 to use 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 novel techniques 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.

ADNOTARE

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

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

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

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

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

Originalitatea şi noutatea ştiinţifică. Realizarea studiului experimental cu compararea şi caracterizarea multilaterală a eficienţei diferitor tehnici de decelularizare în ceea ce priveşte eliminarea celulelor şi păstrarea rezistenţei matricei pentru completarea unor lacune din literatura ştiinţifică actuală.

Problema ştiinţifică rezolvată în teză constă în: identificarea factorilor asociaţi cu îndepărtarea eficientă a celulelor din matrice şi stabilirea unei proceduri noi de decelularizare a vaselor sanguine de calibru mic, şi caracterizarea optimă a structurii acelulare, fapt care va permite modificarea paradigmei experimentale prin selectarea raţională din punct de vedere ştiinţific a condiţiilor experimentale optime.

Semnificaţia teoretică şi valoarea aplicativă. A fost specificată eficienţa de decelularizare a diferitor substanţe chimice; în plus, a fost demonstrat caracterul indispensabil al tratamentului enzimatic în combinaţie cu detergenţi puternici pentru producerea matricei vasculare acelulare. Datele obţinute argumentează ştiinţific modificarea strategiei actuale de cercetare prin utilizarea preferenţială a arterei carotide *faţă de* aortă ca model de testare pentru dezvoltarea grefelor vasculare de diametru mic prin inginerie tisulară. Încercarea eşuată de a utiliza ultrasunetul pentru decelularizarea ţesutului vascular defineşte 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 şi culturi celulare, Universitatea de Stat de Medicină şi Farmacie „Nicolae Testemiţanu", Chişinău, 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 287 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. Описать 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.

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special thanks to Dr. SLADIĆ Sanja and DELIBALTOVA Sofia for being great friends at work and in between work.

Finally, I would like to thank my friends and best colleagues, Dr. GURGHIS Radu, Dr. BZOVII Florin, URSU Alexandr, and KÄFER Martin for never ending moral support and always having an open ear for all my problems.

LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
bp	Base pairs
C/A buffer	Citric acid buffer
CVD	Cardiovascular disease
DAPI	4', 6-diamidino-2-phenylindole
DC	Decellularization
DMMB	Dimethyl-methylene blue
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
GAG	Glycosaminoglycans
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin
HUVECs	Human umbilical vein endothelial cells
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PTFE	Polytetrafluoroethylene
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TRIS	Trisaminomethane

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INTRODUCTION

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). In addition, the effects of different forms of dynamic exposure of tissue to chemicals 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 b a s i c histological s t a i n s (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 I st Place Award Certification), *Congresul Consacrat aniversării a 75-a de la fondarea USMF „Nicolae Testemiţanu"* (online edition, 2020), *Conferinţa Naţională de Chirurgie* (online edition, Romania, 2021), *Conferinţa Ştiinţifică Anuală. Cercetarea în biomedicină şi sănătate: calitate, excelenţă şi performanţă* (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 I st Place Award Certification), *Conferinţa Ştiinţifică Anuală. Cercetarea în biomedicină şi sănătate: calitate, excelenţă şi performanţă* (Chisinau, 2022), *Conferinţa Naţională 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 şi medicina regenerativă: provocări şi realizări"* (*Ziua Internaţională a Ştiinţei*, 09 November 2019, Chisinau), *„Grefele vasculare decelularizate obţinute prin inginerie tisulară vor fi un standard de tratament în viitor?"* (*Expoziţia 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.

1. TISSUE ENGINEERING IS THE FUTURE OF VASCULAR REPLACEMENT

1.1 The Clinical Problem: Cardiovascular Disease

Cardiovascular diseases 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, and pharmaceutical administration [11, 26, 27]. Rehabilitation strategy incorporates active counseling and tailored exercise, while recommended medications ensure regulation of the cholesterol level, amelioration of vasoconstriction, and optimal control of blood pressure [27, 28]. Despite the benefits, increased popularity, and good clinical results of these procedures, because their longevity has been called into question [6, 29], in the patients with advanced CVDs and extremely severe symptoms for the revascularization of occluded vessels surgical options, which are more invasive (as endovascular technologies, namely angioplasty and stenting, atherectomy, endarterectomy, thrombectomy or vascular bypass), may be needed [25, 27, 30-32]. Some forms of replacement therapy mentioned above may also help patients to overcome life-threatening conditions associated with traumatic vascular injuries [27].

1.2 Currently Available Vascular Grafts: Safety and Performance

Vascular conduits are essential for treating a range of vascular conditions, such as aneurysms, carotid artery stenosis, and atherosclerosis. These conditions can lead to severe complications if left untreated, and the use of vascular conduits has been proven to provide effective treatment. This technology ensures improvement the patients' life quality [8].

According to the latest statistics in the field, over 600,000 vascular grafts are implanted annually to replace damaged blood vessels; this number is steadily growing [5, 20]. Choosing the appropriate conduit depends on the vessels size range. According to this criterion blood vessels are classifying into several groups (table 1), such as microvessels (<1 mm in diameter), small vessels (1–6 mm), medium vessels (6–8 mm), and large vessels (>8 mm) [15, 33]. During the bypass graft surgery using substitute vessels a new pathway for blood flow is created and the functional blood supply is restored [8, 34].

Another valorous practical application of the vascular conduits refers to the creation of a surgical connection between an artery and a vein necessary in patients requiring hemodialysis or treating life-threatening conditions associated with traumatic vascular

injuries [35].

Table 1. **Choosing vascular substitutes according to vessel size and use goals [15, 33]**

Vascular substitute choice	Large vessels (>8 mm)	Medium vessels (6–8 mm)	Small vessels (≤6 mm)	Hemodialysis arterio-venous access
	Aorta, arch vessels, iliac and common femoral artery	Carotid, subclavian, common femoral, visceral and above-the-knee arteries	Coronary, below the knee, tibial and peroneal arteries	Upper > lower extremity
1st choice	Prosthesis (Dacron, ePTFE)	Prosthesis or Autograft (equal)	Arterial or Venous autograft	Native material
2nd choice	Allograft or deep venous autograft	Prosthesis or Autograft	Composite graft, vein interposition, prosthesis (ePTFE, Dacron), allograft, biosynthetic	ePTFE, PU, xenografts, biografts, TEBV (clinical trial)

Currently, four types of conduits for vascular grafting are available, such as autografts, allografts, xenografts, and artificial prostheses [9, 15, 36-38]. Detailed characteristic of each group is presented below.

The gold standard for vascular replacement remains the autologous native vessel, which possesses the most physiological properties and exhibit excellent patency, being used in clinical practice more than 50 years [15, 21]. Patients' own vasculature is considered the unique acceptable option in situations where small-diameter (<6,0 mm) vessels, such as below the knee and or coronary artery bypass grafting (CABG), are required [35]. Internal mammary artery or radial artery are the superior choice in CABG [39]; the greater saphenous vein is an optimal substitute in both coronary artery and lower limb bypass surgery [40]. Valuable alternatives to traditional autologous substitutes are presented by right gastroepiploic artery [41] and lesser saphenous vein for coronary application [42, 43]; arm veins for coronary and peripheral bypasses [44]; and deep leg veins for infected graft replacement, visceral revascularization, or even primary lower limb bypass [45].

However, this approach is not always feasible. Despite good clinical performance of autologous native vessels are undoubtedly, in up to 40% of patients needing bypass surgery the tissue's sources may be inadequate or unavailable, most vessels being affected by diffuse atherosclerotic abnormalities, previous phlebitis, vessel removal, varicosities, hypoplasia or are anatomically unsuitable, and only few vessels remain indeed "good" for this purpose [10, 46, 47]. Moreover, the extraction of these grafts conveys some drawbacks: adds time, cost, and the potential for additional donor site morbidity (substantial pain, infections, decreased function, and additional reconstructive or cosmetic surgeries) [27, 48-50]. In addition, autologous native vessels are also liable to atherosclerosis [51] and intimal hyperplasia [12, 52] occurring throughout the length. As the result, a reduction of the patency rate during the

time of implantation may happen [53, 54]. For patients who lack this tissue vital for successful surgery, the currently available alternatives are prosthetic, xenogeneic, and allogeneic graft.

Vascular allografts have been used in variety of medical procedures for decades. In the 1950s, Gross et al. pioneered their use in coarctation repair, marking a pivotal milestone in the medical community. Subsequently, vascular allografts had become increasingly popular for their efficacy and safety when performing surgeries [55]. However, in the early 1960s they were abandoned because of difficulties with procurement and preserving them, late graft's deterioration, aneurysm formation and no availability of suitable prosthetic grafts. The specific scenario in the field have changed in the 1990s. Vogt PR et al. (1998) successfully used cryopreserved allograft valves in the treatment of infective endocarditis [56], while Bahnini A et al. (1991) used aortic allograft in aortobifemoral reconstruction in a case of aortic prosthetic infection with favorable practical experiences. These good clinical outcomes led to a new era in the use of cryopreserved vascular allografts in vascular surgery [57].

Since then, fresh (cold-stored) or cryopreserved arterial homografts (i.e., human allografts from cadaver donors) have been broadly reintroduced in practice for revascularization. The renewed interest to this material is based on the need for managing aortic prosthetic graft infection [58], lower extremity primary revascularization [59] or in redo surgery [15]. They are considered superior to artificial prostheses due to relatively resistance to infection, minimal thromboembolic complications, and avoidance of anticoagulation, but inferior to autologous vascular substitutes [60].

The restrictions to their use include graft rejection related to immunogenicity, late degenerative change due to calcification, aneurysmal dilatation, and pseudoaneurysm formation, thrombotic occlusion of the graft's limited availability, durability, and high risk of rupture [1, 61-66]. Another key issue of allograft tissues is the risk of disease transmission, which requires additional disinfection and sterilization techniques [67].

Xenografts are another biological material which are increasingly applied in arterial reconstruction procedures in vascular surgical routine [68, 69]. Following the favorable experiences of Norman Rosenberg et al. in 1966 with bovine arterial grafts (just two immediate and three long-term failures were identified), further research had been undertaken in this field [70]. The advantages of these grafts are the adequate biomechanical properties, unlimited availability, and high resistance against infections [71]. However, the future published reports in the field indicate a high incidence of complications and disadvantages of this material due to relatively shorten life span [9], poor control over physical and mechanical properties, aneurysmal degradation (3-29%), infections (3-6%) and unknown transfer of animal related infectious diseases, early thrombosis, considerable cost, no availabilityof the

graft in different dimensions, poor control over inflammation and calcification, and last but not the least xenogeneic rejection patterns [69, 72].

A bovine or porcine graft is a medical procedure that has been used to help pediatric patients with a variety of medical issues. While the graft may last for up to fifteen years, this poses a major problem for those pediatric patients who will need to replace the implant every ten to fifteen years. In addition, various xenogeneic tissue components are to be potentially immunogenic (e.g., lipids, DNA, glycosylation products) [16, 71, 73]. In particular, the α-Gal epitope presented in non-primate tissues is presumably involved in the rejection of xenografts [1, 69, 74, 75]. Thereby, use of xenografts cannot be recommended for vascular reconstruction due to not encouraging long-term results.

Synthetic vascular conduits are only suggested as a standard choice and optimal clinical alternative to autologous grafts when the patient's own artery or vein is not available [76, 77]. The history regarding the use of synthetic conduits in reconstructive vascular surgery is long and starts with the first reports published during the early 1950s [78]. Currently, available materials used to fabricate synthetic vascular grafts are non-biodegradable and include Polytetrafluoroethylene (PTFE), Teflon or Gore-Tex©; expanded polytetrafluoroethylene (ePTFE); Polyethyleneterephthalate (PET), Dacron©; Heparin-bonded ePTFE (HePTFE), Propaten©; and Polyurethanes (PUs) [15, 47, 79-81]. These grafts can be successful used exceptionally in high flow, low-resistance conditions. Thus, the conduits had great success in replacing large diameter vessels (>8mm), such as in aortoiliac or aortobifemoral substitutes where the patency is around 90% [82], and in medium-diameter arteries (6-8mm), such as carotid or common femoral artery replacements [15, 33].

Prosthetic grafts are becoming increasingly interesting approach for use in peripheral vascular bypass surgeries. However, synthetic prosthetic grafts have unacceptable high failure rates and are rejected within a few months in the body if the diameter of the vessel is smaller than 6 mm (coronary arteries, infrainguinal arteries, infrageniculate arteries) [33, 83]. ePTFE prostheses have been shown to provide 40–50% patency at 5 years when used to bypass the proximal popliteal artery and 20% patency at 3 years when used for infrapopliteal bypass, unacceptable low values [84]. These results are incomparable to those of autologous blood vessels, which typically have a patency rate of over 70% [70]. In addition, when applied in arteriovenous access for hemodialysis, the synthetic conduits, usually PTFE grafts, remain open to flow for only 10 months [35].

Thus, these materials are relative different in mechanical properties compared to the native vasculature [75], such as vascular elasticity, cannot provide growth environment for the adherence of endothelial cells and fail to "bio-integrate" after implantation *in vivo*, are predisposed to infection, aneurysm formation, thrombosis, and intimal hyperplasia, that lead

to graft stenosis or occlusion [20, 83, 85]. These drawbacks are mainly correlated to the regeneration of a nonfunctional endothelium and mismatch of compliance between the graft and native blood vessels [84, 86-89]. In addition, in the pediatric population, synthetic grafts are further limited by their lack of growth potential [90]. Seeding autologous endothelial cells (ECs) onto the luminal surface of synthetic grafts is known to improve patency; however, these grafts have been unable to surpass the capabilities of autologous vessels [78, 91].

Overall, currently available grafts do not satisfy completely the clinical requirements of modern medicine. Although the refinements in techniques and suture materials continue to occur, significant morbidity, mortality, and expenditure incurs from graft failure [91].

Vascular graft failure is a major problem that threatens the lives of thousands of people every year. The most common failure modes of existing vascular grafts may be classified into early, midterm, and late. Early post-implantation failure of a medical device is a serious problem often caused by technical complications, flow disturbances or acute thrombosis. Understanding the cause of these early failures (within 30 days after the implantation) has become increasingly important to improve the design and function of future medical devices. Intimal hyperplasia is the major cause of midterm failure (3 months to 2 years after implantation). Late failures (>2 years) are often associated with atherosclerosis. Careful follow-up and monitoring after an implantation are essential to ensure long-term success [91, 92].

1.3   Type of cells in the blood vessels

The predominant cells in the structure of the blood vessels' wall are endothelial cells, smooth muscle cells (SMCs), and fibroblasts with specific location within the layers (tunica intima, media, and adventitia). ECs are the cells of the tunica intima; they are responsible for the maintenance in the vascular tone by secretion the substances that regulate vascular relaxation and contraction, thrombosis prevention by managing blood clotting and platelet adhesion, and immune function [94, 97, 99].

SMCs are present in the medial layers, circumferentially, along with collagen and elastin fibers, which provide mechanical support and control the vascular diameter, wall movement, and wall stiffness. SMCs are a vital component of the cardiovascular system, playing an important role in the keeping of blood vessel integrity. SCMs possess contractile properties that allow them to expand and contract as blood flows through them, while also creating the ECM to replenish degraded fibers. As such, SMCs are integral for maintaining tissue homeostasis, and their presence is essential for proper functioning of our cardiovascular system.

Fibroblasts are essential component of the vascular wall, located primarily in the

adventitia. They play a critical role in providing mechanical support along with large bundles of collagen fibers and other structural components. Fibroblasts help maintain tissue integrity and aid in wound healing, making them an invaluable part of our bodies [94, 97, 99].

The ECM is a microenvironment which regulate the behavior and functions of the cells, namely proliferation, adhesion, migration, differentiation, and apoptosis [286]. It contains an abundance of glycoproteins, proteoglycans, glycosaminoglycans (GAGs), as well as collagen, elastin, fibronectin, and laminin. Among them, two fibrous proteins, namely collagen, the most abundant one, and elastin, determine the dominant mechanical responses of the vascular tissue [27, 100].

1.4 A Potential Solution for Vascular Surgery: Tissue Engineered Vascular Grafts

To address the issue of graft failure researchers and manufacturers have been working to develop a novel biomaterial that can optimize tissue-biomaterial interaction and matching the mechanical properties of native vasculature, promote cell growth, facilitate ECM production, and inhibit thrombogenicity. The innovative material could potentially revolutionize vascular grafts and reduce the risk of vascular graft failure [83, 92, 93].

The multiple demands placed on choosing an appropriate cardiovascular graft and great disadvantages of existing conduits have meant the necessity of elaboration of criteria of an "ideal" cardiovascular bypass graft. The "ideal" cardiovascular bypass graft is a state of evolution and requires a broad range of characteristic to be fit for this purpose, including biocompatibility, non-immunogenicity (no need for immunosuppressing drugs), easy processing and handling, compliance, ability to grow, remodel, and self-repair *in vivo* (table 2) [15, 16, 38, 83, 84, 93-96]. There have been developed for decades synthetic materials to address these requirements; however, till now it seems challenging to obtain vessel substitutes that meet all the issues simultaneously [27].

Taking into consideration a significant 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, differentiation, and respond to endogenous vasoactive compounds. Advances in tissue engineering have enabled the manufacturing of vascular grafts which are designed to mimic natural tissue. These tissue-engineered vascular grafts (TEVGs) offer a viable alternative for patients who lack suitable autologous tissue or those who are ineligible for synthetic grafts, providing an immediately available option [27, 35, 73, 83, 93].

The first model of a blood vessel created *in vitro* was developed by Weinberg and Bell in 1986 [97]. Their approach included the usage of scaffolds made of collagen gel and Dacron onto which bovine endothelial cells, fibroblasts, and smooth muscle cells were added. Since then, several different procedures have been developed to produce a clinically available

TEVGs that differ according to the scaffold type applied.

In conformity with this criterion (scaffold type), available TEVGs can be broadly divided into the following groups: (1) methods based on natural or synthetic biodegradable polymers, such as polyglycolic acid, polyhydroxyalkanoates, polycaprolactone and polyethylene glycol, (2) decellularized natural matrix techniques, (3) hybrid scaffolds, and (4) TEVGs without scaffolds, or completely biological grafts [16, 79, 83]. Decellularization is considered a more efficient option *vs* those of a synthetic nature allowing to obtain accurate replicas of the vascular matrix with superior immunogenicity due to elimination of immunogen cell materials, biodegradability, and bioactivity by release bioactive substances for cell maintenance upon recellularization [98].

Table 2. **Requirements of an ideal vascular graft [15, 16, 38, 83, 84, 93-96]**

Biocompatibility	■ Nonthrombogenicity ■ No healing disturbances ■ Noncytotoxicity and no allergic reactions ■ No induction of malignancies ■ Minimally trauma to blood compound ■ Nonsusceptibility to infection ■ Nonimmunogenicity, or the graft should not trigger chronic inflammation, complement cascade initiation, or activation of the adaptive immune system ■ Nonirritating to adjacent tissues ■ Complete incorporation into the host tissue with satisfactory graft healing ■ Ability to grow, remodel, and self-repair *in vivo* (especially necessary, when the graft is placed in children)
Mechanical properties	■ Suitable compliance necessary to prevent the formation of high stresses around the anastomosis ■ Flexibility, and elasticity like native vessels ■ Burst pressure and tensile strength similar to native vessels ■ Kink and compression resistance ■ Resistance to intimal hyperplasia and deformation through aneurysm ■ High patency rate
Processability	■ Availability in a variety of sizes and lengths for emergency care (off-the shelf) ■ Sterilizability ■ No need for special storage conditions ■ Easy suturing and ability to be handled
Optional	■ Capability of local drug delivery ■ Reasonable, economically viable manufacturing costs

1.5 Decellularization as a New Strategy in the Development of Vascular Grafts

With the help of tissue engineering, a variety of treatments can be developed for a wide range of diseases and conditions. The use of tissue-engineering approaches in medical and therapeutic treatments require precise protocols to ensure successful outcomes [22].

Decellularization is a complex process that requires careful consideration to create an ECM-rich, non-immunogenic vascular scaffold capable of initiating vascular regeneration [27, 101]. Successful decellularization requisites a delicate balance between removing cells effectively while preserving the ECM structure. 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, 102].

The efficiency of DC is dependent on a range of factors. These include the type of chemicals used, the intensity and duration of the applied treatment, but also the complexity, structure, components, thickness, size, and density of the tissue itself. By understanding these variables and their impact on DC efficiency, better experimental results can be obtained bridging the gap between basic science and clinical applications [2, 4, 6, 10, 25, 103, 104].

By application of any decellularization technology, all the vascular cell elements are removed with preservation of the complex ECM, abundant in the cell signaling components, its main components, such as collagen, elastin, and GAGs, and original mechanical properties necessary to withstand changes in blood pressure [2, 21, 38, 40, 83, 105-109]. The collagen and elastin fibers in the medial layer provide elasticity necessary to withstand pulsatile blood flow within a physiological range. Besides that, collagen is responsible for the retention of tensile strength, elastin fibers maintain the elastic properties of the scaffold, and GAGs provide viscoelasticity [110]. Preservation of adventitia layer is crucial within ECM provide signals to cells in adhesion, proliferation, migration, differentiation and ultimately gain high regeneration capacity within these natural delivered scaffolds [40, 111]. After implantation, this acellular immunological inert matrix completely integrates into the organism and, thus, gradually transforms from an allograft to an implant of primarily autogenous, self-renewing and living tissue due to *in vivo* invasion and ingrowth of SMCs and fibroblasts into it from the adjacent tissue that is fit for vasculogenesis [40, 112-114].

The main advantage of the use of decellularized scaffolds consists in avoiding any adverse immunological reactions due to loss of the major histocompatibility complex. Concerning tissues and organ transplantation's concept, MHC molecules act as antigens themselves and can evoke an immune response in the host, directed against the transplanted organ, consequently causing transplant rejection. It was demonstrated that by DC both MHC I and II cell membrane antigens can be removed [31]. Thus, limiting the immune response by

the host and avoiding transplant hyperacute or chronic rejection allows the usage of allogenic or xenogeneic material. The assay in this field demonstrated that the use of decellularized tissue stimulated minimal to no panel reactive antibody response, confirming minimal immune allosensitization to transplanted decellularized vessels, while fresh or cryopreserved tissue evoked a strong to moderate antibody response [115, 116].

The first reports regarding development of biological vascular grafts by DC appeared in the 1960s [68]. Since then, a range of commercial off-the shelf products have become available for clinical use. These include Artegraft®, Solcograft®, ProCol®, MatraCELL®, and SynerGraft®, derived from decellularized xenogeneic materials, such bovine blood vessels (carotid artery or mesenteric vein) or bovine ureter [117-121]. Clinical applications for tissue engineered vascular grafts using decellularized matrices include arterial bypass and hemodialysis vascular access [113, 207, 208]. The main advantages of these materials include reduced thrombosis, long-term durability, naturally biocompatibility, non-immunogenicity, and flexibility [83].

Preliminary randomized studies of DC concept in animal models have not provided encouraging clinical results yet, graft infection, thrombosis, and pseudoaneurysm formation being the most frequent postprocedural complications compared with other alternatives such as synthetic conduits [27]. Probably, the high failure rate is determined by the presence of residual immunogenic contaminants, such as alpha-gal epitope. Besides that, the implantation of decellularized xenografts demonstrates the potential risk of viral transmission from animal tissue. In addition, their production cost is still high if compared to synthetic grafts [83]. As a result, their large application in practice is still limited and further investigations in the field are necessary. Moreover, because the disadvantages of xenografts transplantation are well studied, the use of homologous acellular matrices seems to be a more suitable approach for vascular replacement [31].

While choosing the appropriate donor material it is necessary to take into consideration that ECM matrices may be mainly affected by the age and health status of the animal at harvest and by the manufacturing process. Eventually, these factors influence quality, mechanical and biochemical properties, biocompatibility, and clinical performance of the vascular substitute [122].

However, there are several limitations associated with using decellularized natural matrices. The limited performance of commercially available grafts has been suggested to be due to their lack of cellularity upon implantation [209]. So, to reduce the risk of thrombosis, recellularization with endothelial cells, either *ex vivo* before implantation or *in situ* after implantation, is regarded necessary for a long-term functional vascular graft. The endothelium prevents blood clotting and protects against the formation of intimal hyperplasia and graft

atherosclerosis [24, 123].

Substantial gaps in knowledge limit the extensive commercialization of decellularization-based vascular tissue engineered products. As a result, further studies are required to support optimization techniques and ways to engineer small- to large-caliber vascular substitutes that truly mimic their native counterparts [27].

1.6 An Overview of Decellularization Agents

The decellularized vascular replacements offer better results than alternative synthetic conduits [21]. DC can be realized by different agents (table 3), as chemical, biological, and physical ones, allowing to induce the rupture of cellular membranes and cellular components wash out, and thus leading to production of vascular analogs comparable to native structures [21, 24, 27, 83, 113, 123-126].

Most of the existing studies refer to application of chemical and biological DC agents; physical and procedural DC factors have been assessed inadequately so far [18]. Currently, a combination of several agents is popular in DC process to avoid single side effects for only one agent [83, 111]. Also, the most appropriate technique varies depending on the type of tissue and the species of origin [24].

So, there is not a systematic, standardized report regarding the effectiveness, advantages, and disadvantages of all the indicated procedures and their combinations in the specialized literature [127]. In addition, the presented results are frequently conflicting due to varying experimental conditions, which makes direct comparison of different protocols difficult [128]. Difficulties to compare the DC protocols between centers are determined by differences in initial treatment chemicals' concentration, exposure time, the ratio of tissue weight to washing solution volume and the cells type. Therefore, systematically analysis and evaluation are needed to provide "ideal" DC techniques that are effective in different tissue types [129].

Considerable progress and improvements in recent years in the field of TEVG development are obvious, however, identifying an optimal protocol to create a decellularized natural scaffold appropriate for small-diameter (<6 mm) blood vessels replacement is still difficult to find and elusive [127]. In addition, an important drawbacks of existing DC procedures are trials and tribulations of the inter-centers' comparison, technical complexity, and multi-step execution that requires numerous solutions' changes, largely carried out manually increasing the rate of human errors. It means it is time-consuming demanding process, which holds no assurance of reliability and reproducibility of the experiment. These aspects indicate the necessity of automatic systems establishment and implementation, which may become a valuable tool in development of functional and practically applicable decellularized scaffolds [131].

Table 3. **Characterization of different decellularization techniques**

Chemical agents	▪ Acids (acetic acid) and Bases (calcium hydroxide, sodium sulphide, and sodium hydroxide) ▪ Hypotonic and Hypertonic solutions (osmotic gradients) ▪ Detergents: ionic (sodium dodecyl sulfate –SDS, sodium deoxycholate –SDC), non-ionic (Triton X-10), and zwitterionic (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate – CHAPS) ▪ Solvents: alcohols (isopropanol, ethanol, methanol, and chloroform), acetone, tributyl phosphate – TBP
Biological agents*	▪ Enzymes: endo- and exonucleases, trypsin, collagenase, lipase, dispase, thermolysin, and α-galactosidase (for xenogeneic tissues) ▪ Non-enzymatic agents: chelating agents (ethylenediaminetetraacetic acid, or EDTA, ethylene glycol tetraacetic acid, or EGTA), toxins (latrunculin), xenogeneic serum associated with nucleic acid fragments
Physical and miscellaneous methods	▪ Temperature: freeze-thaw processing ▪ Force and pressure: mechanical abrasion, high hydrostatic pressure (HHP) ▪ Non-thermal irreversible electroporation

*NB: *Serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), aprotonin, and leupeptin prevent undesirable damage to ECM [130], while antibiotics and antimycotics such as penicillin, streptomycin, amphotericin B, and sodium are used to minimize microbial contamination during decellularization*

Describing chemical decellularization methods

Various chemicals, such as acids, bases, detergents, and hyper-/hypotonic solutions have been investigated for their ability to decellularize tissues while maintaining the integrity of ECM, and consequently, the mechanical properties of the scaffold [27, 125]. These chemicals are generally selected to solubilize the cell membranes and cytoplasmic components as well as to remove nucleic acids such as RNA and DNA [123].

Osmotic shock produced by application of high and low molarity ionic solutions is used for cells removal within tissues and organs [131]. Hypotonic solutions as distilled water can cause cell lysis by simple osmotic effects with minimal changes to matrix molecules and architecture [132], while hypertonic solutions in addition may determine DNA dissociation from proteins [133]. However, there are controversial data on the effects of hypertonic solutions to the basal membrane and GAGs preservation. So, Uzarski et al. (2013) described disruptions in the basal membrane 5-10 μm wide when hypertonic solution is used [134], in contrast to Goktas et al. (2014) who found no alterations in it structure but did describe a significant decrease in GAGs [135].

Osmotic gradient cannot be recommended as the sole decellularization technique, because they do not generally remove the resultant cellular remnants from the matrix [131],

for a better result being recommended alternatively immersion in hyper- and hypotonic solutions through several cycles [136]. In addition, they can be used in combination with the detergents or enzyme-based methods as an initial pre-treatment step [27, 137]. In this way the required enzyme/detergent concentration and/or exposure time can be reduced, and the duration of exposure can be kept as short as possible. Such strategies have been already applied in experimental practice for creation the acellular templates of blood vessels [27].

Acids, such as acetic acid, peracetic acid CH_3CO_3H (PAA), hydrochloric acid HCl, and sulfuric acid H_2SO_4, and *bases*, as sodium hydroxide NaOH and ammonium hydroxide NH_4OH, can disrupt the cell membrane and solubilize the intracellular organelles; in addition, they are useful agents in removing residual nucleic acids (RNA and DNA) and plasma membrane lipids [138]. Some of the acid treatments, for example PAA, simultaneously disinfect the material by entering microorganisms and oxidizing microbial enzymes [139] and are useful in preservation the many growths factors resident in the ECM [140]. However, while removing the cellular parts, the acid agents also tend to denaturate structural proteins and damage collagens. Thus, these chemicals may damage the ECM structure, reduce the scaffolds' strength, and significantly increase yield stress and elastic modulus [105, 141]. So, these chemicals are not suitable in tissues in which compliance and mechanical stability are desired properties.

Application of *bases* may even more significantly decrease the mechanical properties of ECM by cleavage and swelling of collagen fibrils and the disruption of collagen crosslinks [142]. Also, a reduction in the tissue's GAG content and viscoelasticity in found with no evident benefits in terms of reducing immunogenicity [105].

Ionic, anionic, or cationic (SDS, SDC, NLS), *non-ionic* (Triton X-100), and *zwitterionic* (CHAPS) *detergents* are amphipathic molecules composed of a hydrophilic polar head-group and a hydrophobic non-polar part (tail); there molecular structure is very similar to the amphiphilic phospholipids that make up cellular membranes (figure 1A) [143]. Detergents are used for the disruption of cell membranes and the release of intracellular materials and are probably the most studied decellularization agents nowadays [143, 144].

When dissolved in water at appropriate concentrations and temperatures amphiphilic molecules self-assemble into structures that keep their hydrophilic headgroups on the exterior and the hydrophobic tails on the interior away from the water. Due to their molecular differences, detergent molecules form spherical micelles (figure 1C) [143]. Decellularization with detergents is based on this molecular property of soap to form micelles (spontaneously formed and soap-specific aggregates). These properties allow it to imitate, destruct, and insert into cell membranes [145]. Furthermore, the hydrophobic core of the micelle can bind to hydrophobic regions of proteins (figure 1B) [143].

So, the detergents may lyse cells by penetrating the extracellular matrix and cell membranes due to reducing the surface tension of the local environment and dissociate DNA from proteins [146]. Detergents may reduce the scaffold immunogenicity by removing greater than 90% of remnant DNA. Obviously, by addition the detergents to a decellularizing protocol are possible to make difference between complete and incomplete removal of cellular matrix [147], however it may cause ultrastructure disruption with reducing the biomechanical strength of obtained cell-free scaffold [148] and elimination of growth factors [149].
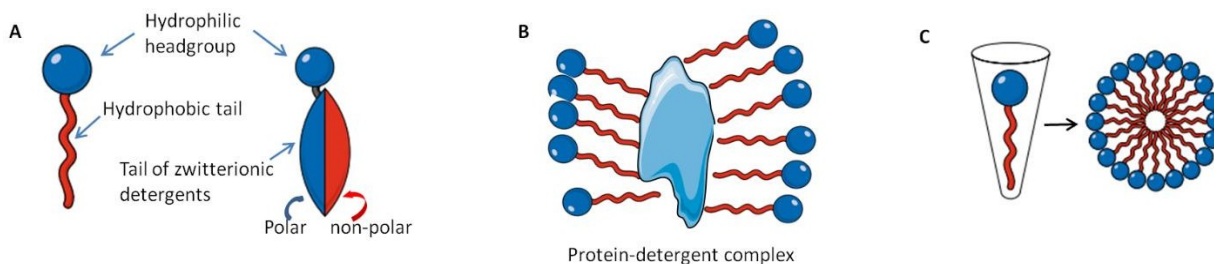
Figure 1. **The general structure of detergent monomers** [143]

A. Schematic structure of detergent molecules. B. Hydrophobic proteins (light blue) can be solubilized in aqueous solutions using detergents. C. The molecular structure of detergents, with a large hydrophilic headgroup and long hydrophobic tail, makes them curvophilic, inducing them to form spherical micelles in aqueous solutions

Successful decellularization with detergents depend on numerous factors, such as agents' concentration, pH of the solution, ionic strength, and temperature [143, 150]. The minimal detergent concentration at which micelles are observed at a given temperature is called the Critical Micelle Concentration (CMC). The size and shape of micelles is temperature dependent. Likewise, the lowest temperature at which micelles are formed is called Critical Micelle Temperature (CMT). CMC is also affected by the degree of lipophilicity of the headgroup. Generally, a low lipophilic or lipophobic character results in high CMC [143].

Decellularization with increased temperatures of 37°C–50°C showed an improved removal of cellular residues due to the formation of more micelles at the same concentrations [151]. Daugs A et al. (2017) demonstrated that application of 50°C during decellularization permits to remove cellular components efficiently with no damage of the fiber network confirmed via histology [145]. However, temperatures higher than 50°C can lead to protein denaturation, dehydration, and shrinkage of collagen fibers [152].

Ionic detergents, such as SDS, SDC, and NLS, contain a head group with net charge that can be either negative (anionic) or positive (cationic). They can disrupt protein-protein interactions long with lipid-lipid and lipid-protein interactions; in this way, ionic detergents may solubilize cytoplasmic and nuclear cellular membranes, but also may denaturate

structural proteins [153, 154]. In contrast to non-ionic detergents, ionic detergents generally have higher CMC values and are stronger solubilizing agents. Thus, they are generally thought to be harsher to the tissue because of great disruption of protein structure and loss of matrix components, particularly, glycosaminoglycans [143, 155]. Furthermore, additional precautions should be taken when using them because some of their properties may be altered in buffers with variable ionic strength (e.g., CMC can fall dramatically when the NaCl concentration increases from 0 to 500 mM) [155].

SDS (Sodium-Dodecyl-Sulphate), a steroid acid, is a good candidate anionic detergent (figure 2) due to its known ability to solubilize both cytoplasmic and nuclear cellular membranes and to remove the cellular components from tissue in this way [105, 156]. It disrupts non-covalent bonds within and between proteins, denaturing them, and resulting in the loss of their native conformation and function [143]. Being quite effective in cell removal, it is a widely preferred surfactant in antigen shifting especially in dense and thick tissues like aorta [145, 153, 157-159].
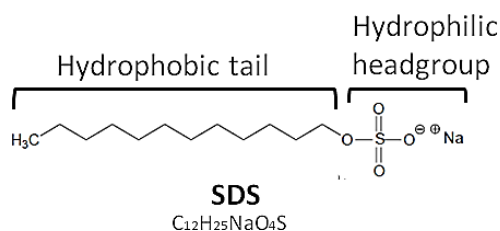
Figure 2. **Structure and formula of SDS** [137]

Despite its effectiveness, some specific drawbacks are associated with its application; they are determined by its reported disruptive effect on protein-protein interactions [24]. For instance, SDS has the potential to reduce the biomechanical strength of obtained scaffold and to affect ECM integrity. These effects result from denaturation most proteins, disruption native tissue structure, decreasing the GAG concentration, elastin, and collagen content (hyalunoric acid, heparin sulfate, chondroitin sulfate A, and dermatan sulfate), compaction of collagen with altering fibril morphology and cross-linking network [24, 105, 145, 157, 160]. So, Yang et al. (2010) reported that incubation of pig bladder at room temperature for 24 h in hypertonic Tris buffer containing 1% SDS decreased sGAG content with 25% that might be related with 70% bioactive factor loss [147]. In addition, Liu et al. (2018) demonstrated that decellularization using SDS may negatively affect structural preservation of elastin, collagen, and GAG [157]. These damaging effects prevent tissue cell repopulation and full retention of its mechanical properties. Also, because of this adverse impact to ECM composition, there appears to be a high risk of aneurysm formation once *in vivo* and increased immunogenic potential of the allograft due to denaturation of the ECM proteins. In addition, because of

inadequate permeability of SDS to decellularized tissue, for a sufficient removal of cellular components fully in the deep structure the exposure time should be increased; as a result, adversely effects to ECM components are intensified and the resulting tissue in mechanically weak. In addition, SDS is toxic, and efforts are needed to ensure its optimal removal from the scaffold at the end of the treatment; extensive wash process is recommended efficient tool for its elimination [105].

Obviously, by improvement SDS access to tissue the reaction time can be reduced. In this way, the potential damage to crucial ECM elements may be minimized. Guler et al. (2018) proposed a modified SDS technique which was improved by adding of dimethyl sulfoxide (DMSO), as a penetration enhancer. They demonstrated this new protocol is efficient both for nearly complete removal of cellular components with preservation of crucial ECM parts and reduction of tissue's exposure time to detergents [161].

In general, it seems SDS is a practical solution for removing cellular molecules like proteins, DNA or glycans from tissue compared to other detergents, but it is also disruptive to ECM's microstructure due to a deficient access to deep structure [162]. This finding suggests that SDS decellularization protocols should be improved in future.

SDC (Sodium Deoxycholate), bile salts detergents [150], is another anionic detergent used in experimental practice (figure 3). Even it tends to act more like a non-ionic detergent, it is classified as ionic one because of its polar properties. It is usefully applied for disrupting and dissociating many types of protein interactions while preserving ECM structural proteins, such as fibronectin and elastin; the position of collagen and elastin fibers [163, 164], and even von Willebrand factor, a protein key maintaining hemostasis [98]. Another SDC effect consists in dissociation histones II from DNA [165]. Probably, due to this consequence on DNA molecule structure, SDC may cause it agglutination on the tissue's surface. To address this issue, it is recommended to apply a combination of SDC and deoxyribonucleases I (DNase I) [105]. The scaffolds produce with SDC are highly biocompatible and seems to be more suitable for cell seeding and growth [27].

Unfortunately, there are no studies of tissue decellularization using SDC alone; as a result, controversial reports about its efficacy appear. For instance, according to Gilbert et al. (2006), when compared SDS to SDC, it tends to cause greater disruption to the native tissue architecture and to reduce its mechanical properties in this way [123, 157]. In contrast, Pellegata et al. (2015) demonstrated that when extensive wash process is performed a detergent-enzymatic protocol in vessels decellularization (SDC and DNase I) is efficient for cell removal, allows preservation of ECM, and limits alteration of mechanical properties of the tissue [131]. Li et al. (2016) obtained in the same experimental data. They confirmed that

combinative enzyme-detergent procedure (SDC, Triton X-100 and EDTA) is efficient in vascular cells elimination with a lesser DNA residue and less damaging to vessels' mechanical features compared to trypsin-based enzymatic decellularization method [158]. So, by application of a combination of SDC with other detergents, the removal efficacy of the procedure can be improved [157].
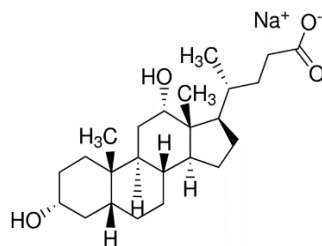
Figure 3. **Structure and formula of SDC** [166]

With respect to the early study, tissue-engineered heart valve based on SDS and SDC technique have been successfully used in clinical practice. In 2011, Cebotari et al. reported the clinical midterm results on implantation of fresh decellularized pulmonary homografts comparedwith glutaraldehyde-fixed bovine jugular vein and cryopreserved homografts in children and young adults. According to the presented data, decellularized grafts provided enough resistance and stability for suture lines and showed robust mechanical properties with no signs of cusp thickening or reduction of cusps mobility, as well as no signs of relevant conduit stenosis or dilatation during the entire follow-up. In addition, there were no cases of graft rupture or dislodgment, the freedom from operative reintervention being of 100% at 5-year follow-up in the first group. Interestingly, decellularized valves provided a tendency to adapt to somatic development of the patients. So, they remain unchanged or even decrease in annulus size in case of oversizing or dilate together with physiological growth of the patient [72].

Improvement of SDC efficacy can be made by choosing the optimal pH solution; so, SDC forms giant micelles at pH 7.8 and aggregates at pH 6.9. To prevent gelation of the decellularization solution and to improve the solubility of SDC, Daugs A et al. (2017) used 0.1 M Tris HCl to buffer the system at pH 8.3. This procedure significantly improved blood vessels decellularization [145].

So, it is evident that by addition of SDC decellularization effectiveness can be improved with maintenance of the key ECM's proteins. In this way, mechanical properties of the scaffold are preserved. At the same time, it is still unclear in which way SDC may influence the vascular framework if applied alone.

NLS (N-Lauroyl Sarcosinate) is another mild ionic detergent (figure 4) with bactericidal properties applied in laboratory experiments and the practice of tissue decellularization due to its good water solubility, high foam stability, and strong sorption capacity to proteins [167].

As a result, it may effectively solubilize the membrane proteins and thus to lyse cells [168]. According to its denaturation power, it is milder that SDS [169]; however, it was demonstrated that N-Lauroyl Sarcosinate in conjunction with a recombinant endonuclease can be successfully utilized for decellularization of ovine pulmonary valves [115, 116]. In addition, sarkosyl was included as a component of MatrACELL© decellularization technology, which is effectively applied to remove potential immunogenic material and to prepare useful ECM for tissue engineering with preservation of its biomechanical properties [170]. Moore M et al. (2015) used this procedure to obtain human acellular dermal matrix, which is widely recommended for acute and chronic wound healing, soft tissue reconstruction, and sports medicine applications [171]. It has been also applied to human pulmonary patches production which were used in clinic since 2009 with satisfactory outcomes [159].
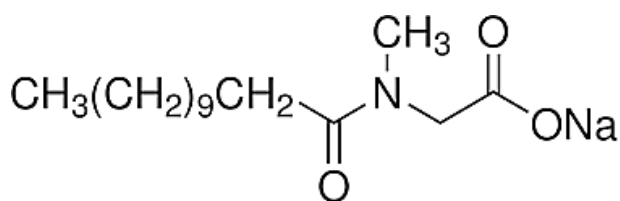
Figure 4. **Structure and formula of NLS** [172]

Even the usefulness of ionic surfactants application is undoubtedly, gentler decellularization procedures should be developed in future. When compared to ionic detergents, non-ionic detergents contain unchanged hydrophilic head groups and should not have ionic change. They are suited for breaking lipid-lipid and lipid-protein interactions and preservation intact of protein-protein interactions [154, 173]. Due to the least impact on the protein structure, minimum toxicity, and superior preserving ECM architecture due to smaller tendency to denaturate proteins, non-ionic detergents are theoretically the most desirable to use [27, 152].

Triton X-100 is the most widely studied non-ionic detergent for decellularization protocols (figure 5); it derives from polyoxyethylene and contains an alkylphenyl hydrophobic group [124,143]. It is considered a relatively mild detergent and a contradictory data on its aggressivity is reported [174, 175].

Triton X-100 has proven effective at cell and DNA removal; in addition, it allows better ECM retention compared to SDS treatments [176]; thus, the mechanical properties are well preserved following the DC process [177]. However, it demonstrated mixed results about its effectiveness in decellularization of various tissues. For instance, Triton X-100 has not sufficient strength to decellularize cardiovascular tissue. Grauss R et al. (2005) demonstrated in their experience that after 24 hours treatment of heart valve with Triton X-100 cellular material was found in the adjacent myocardium and aortic wall and the content of laminin and

fibronectin (components of ECM) was reduced [178]. Also, Triton X-100 is aggressive against the basal membrane, causing its disruption [134, 175].

Besides, Dahl et al. (2003) showed that Triton X-100 in ineffective for decellularization of blood vessels (H&E histological staining revealed presence of intact nuclei and DNA quantification demonstrated there was no significant difference in DNA content before and after decellularization). In addition, they established that this treatment can negatively influence the mechanical characteristics of the blood vessels by reducing the compliance and ultimate wall stress [179]. Liu et al. (2018) presented the same unsatisfactory results. They demonstrated that the effectiveness of Triton X-100-based protocols for the removal of antigenic cellular components is low and recommended SDS-based procedures as more successful in developmentof decellularized matrices [113]. In addition, it was speculated that application of Triton X-100 detergent may result in increased cytotoxicity of the obtained decellularized matrix [113, 173].

So, Triton X-100 can be considered an appropriate chemical for decellularization when used in appropriate tissue and in good combination with other methods [167, 169, 180].

Triton X-100
$C_{34}H_{62}O_{11}$

Figure 5. **Structure and formula of Triton X-100** [143]

Examples of zwitterionic detergents include (3-[(cholamidopropyl)dimethylammonio]-1- propansulfonate) (CHAPS), sulfobataine-10 (SB-10) and sulfobetaine (SB-16) [123]. They are chemically synthesized and combines useful features of both ionic and non-ionic detergents (figure 6) [8, 24]. They have a net zero electrical charge on the hydrophilic head groups which protects the native state of proteins during decellularization (nondenaturating features of zwitterioning surfactants) [24, 105]. However, it is expected they may break protein-protein interactions like ionic detergents and thus manifest disruptive effect upon the ECM [8]. These reactions are less harsh than ionic detergents, but greater than non-ionic detergents [143, 175, 181]. In addition, zwitterionic detergents are less effective in complete elimination cytoplasmic proteins [182].

The detergent CHAPS has been studied for decellularization of thinner tissues, such as lung or blood vessels [182]. Even it demonstrated to be an effective DC method, exhibiting complete DC by the histological analysis, remaining cytoplasmic proteins and cellular debris are still present when thicker tissues are decellularized [105, 175, 183]. CHAPS may be also used in combination with other detergents. For instance, Dahl et al. (2003) used a combination of CHAPS, EDTA, and SDS for decellularization of native porcine carotid arteries. They

showed this procedure is efficient in vascular cells' removal but may also produce a significant reduction in vascular compliance and ultimate wall stress [179]. Otherwise, Gilpin et al. (2017) stated positively about ECM proteins maintenance and thus retainment the tissue compliance when CHAPS is used in DC purpose [105]. However, it is not clear if the disturbing results reported by Dahl et al. are due to CHAPS used for vessels treatment or effects of SDS, an ionic denaturing detergent, on them. Thus, further studies regarding feasibility of zwitterionic detergents in development of acellular scaffolds are necessary [175].
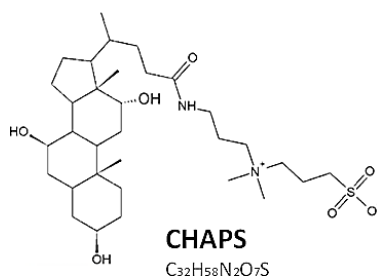
CHAPS
$C_{32}H_{58}N_2O_7S$

Figure 6. **Structure and formula CHAPS** [182]

The general conclusion on efficacy of detergents was formulated by Hudson TW et al. (2004). According to their study (a blinded categorical comparison of detergents) non-ionic and zwitterionic detergents showed better preservation of ultrastructure, while ionic detergents – better cell removal [184].

After decellularization it is mandatory to remove the chemical from the ECM. Complete removal from the tissue is difficult and residual detergents can adversely affect cell adhesion and repopulation (the cytotoxicity phenomenon) [105]; thus, this could inhibit or completely nullify the beneficial properties of a cell-free ECM scaffold. That is why for complex tissue it is advantageous to combine numerous chemicals through a series of short intensive washes in cycles to increase the efficiency of each chemical, to reduce the overall time that the tissue is exposed to any one chemical and to lower the residual contamination below a hazardous threshold and allows their successful *in vivo* or *in vitro* repopulation [185]. For example, when working with SDS it is important to know that SDS precipitates at low temperatures, and this effect is enhanced in the presence of potassium salts. This phenomenon can sometimes be exploited to remove SDS from a protein sample [143].

To summarize, there are many different detergents that can be used in decellularization protocols, but it is critical to understand how different detergents with distinct chemical properties effect ECM scaffolds in the process of decellularization. Unfortunately, the detergents' classification presented above in not helpful in practice – the literature is full of conflicting results on the effects that each of these detergents has on the tissue of interest in the study [24, 123]. It may be determined by variations in the concentration of detergent used, the combination to other physical and chemical methods used, inconsistencies of the means

of analysis, and the tissue studied. These findings have raised important question about the need of new controlled investigations to determine the effects of each detergent on each tissue of interest [124, 175].

Describing biological decellularization methods

Enzyme agents (nucleases, trypsin, collagenase, lipase, dispase, thermolysin, α-galactosidase) are used in tissue decellularization to breakdown specific biological molecules and facilitate cell removal by disrupting the interactions between the cells and the ECM; however, they are not efficient when applied alone. In addition, the proper flushing may impair recellularization or evoke adverse immune response [24].

Nucleases (DNase/RNase) successfully cleave nucleic acids sequences into shorter segments, expediting their removal from the ECM or eliminating their function [147] and thus limit the potential immunogenicity *in vivo* [105]. Therefore, by adding of nucleases to decellularization protocol, scaffolds' immunorejection may be prevented. However, including nucleases to the process, collagen, elastin, laminin, fibronectin, and GAGs ultrastructure and content may be significantly altered [176].

Trypsin, a serine protease, cleaves proteins hydrolytically at the arginine or the lysine amino acid residue on the carboxyl side, except when followed by proline residue and is used to digest cellular proteins in the decellularization process. The maximal enzymatic activity of trypsin occurs at 37°C and at a pH of 8 [123]. Because the proline is the main constituent amino acid in collagen and trypsin is not able to cleave proteins on this level, significant affectations on its amount in tissue during decellularization are not common. Nevertheless, surprisingly a few studies demonstrated a reduction in collagen after trypsin treatment [178, 185]. Besides, trypsin has adverse effects upon other extracellular components of tissue and organs, such as GAGs, laminin, fibronectin, and elastin, determined by their limited resistance to trypsin cleavage. So, visible histological damage to the ECM is often determined and, as a result, severe changes in the mechanical properties of the scaffolds can be observed [105, 147, 186]. In addition, controversial results about efficacy of trypsin in decellularization of cardiovascular tissue were observed [123]. For instance, Grauss R et al. (2005) established that by using trypsin for decellularization of normal aortic valve leaflets incomplete cells' removal can be obtained [178]. However, it is recommended to use trypsin within the initial DC step to improve subsequent DC agents' penetration, and thus to ameliorate removal of cells' nuclei from dense tissue [147].

As a conclusion, trypsin can degrade the extracellular matrix and cannot be considered a "perfect" strategy for decellularization of cardiac tissue where maintenance of ECM features is critical [105]. To reduce the duration of tissue exposure to this aggressive agent, thus minimizing the chemicals' negative impact and the disruptive effects of trypsin upon the

ultrastructure and composition of the ECM, elaboration of new efficient protocols with reduction of exposure time to trypsin treatment is necessary.

Collagenases have also been used for the purpose of decellularization; but, usually at very low concentrations unless maintenance of the structure is not required [24]. *Lipase*, an enzyme able to catalyze the hydrolysis of lipids, is used to digest the lipids, the process known as delipidation. But it is not sufficient to enzyme all lipids by itself [187]. *α-galactosidase* is used to remove the galactose-α-(1,3)-galactose, also known as α-Gal epitope, which is known to cause acute and hyperacute xenoreaction in humans [188].

Chelating agents, as EDTA (ethylenediaminetetraacetic acid, stabilizer and protease inhibitor), EGTA (ethyleneglycoltetraacetic acid) are commonly used in combination with other chemicals (e. g. applied in combination with trypsin [111, 147, 189] or detergents [147, 188] and are ineffective when used alone; however, not all possible combination and their effectiveness are studied already (e. g. a combination hyper- and hypotonic solutions and chelating agents) [24]. Intracellular proteases released as the cells are being trypsinized and inactivated by these agents. In this way degradation of extracellular matrix by proteases can be avoided, but, unfortunately, all the proteolytic activity of the intracellular proteases cannot be inhibited by it [24, 175]. Also, it has been shown that EDTA may diminish salt- and acid-soluble collagens by changes in collagen content and fiber orientation. In addition, increasing the concentration of EDTA in the digestion step may led to an increase in the pore size of the matrix [127].

Describing physical decellularization methods

Physical methods include sonication (ultrasound waves), freeze-thaw cycles (thermal shock), supercritical fluids, high hydrostatic pressures, pressure gradients, electroporation, vacuum technologies, and immersion, agitation, perfusion, as specific washing regimens [27, 105, 174, 190]. When applied independently, they are rarely sufficient and a great amount of remnant DNA is found in resulting scaffolds; thus, an immunogenic response *in vivo* may be produced once after implantation due to insufficient remove of genetic materials [105, 191, 192]. However, physical methods might be effective in facilitation cell membrane rupture [174]. Accordingly, application of a combination of physical technique with others described above demonstrates to be effectual tool in cell removing and development of acellular scaffolds without altering their mechanical properties [193]. This is due to reduction of the exposure time to chemicals action and limiting the needed treatment cycles and washing procedures.

For instance, the sonication effect is explained by the formation of cavitation bubbles that aids in the penetration of the chemical detergent by denaturating the cellular membrane and destroying the cellular components. The total amount of resulting bubbles depends on the

applied sonication power (SP). Thus, by increasing the SP the number of cavitation bubbles is increased and a faster removal of cellular debris is achieved, while too low SP will not aid in cellular removal [193, 194]. The feasibility of sonication with low frequency ultrasound (20kHz) is already demonstrated [195]. However, even the biological effects of ultrasound are obviously, the optimal sonication power for different types of tissue is not determined yet and further research in this field are necessary [190, 195-198].

Freezing therapy is another technique used in vascular grafts development, the cells' elimination being produced by alternating between freezing temperatures around -87°C and biological temperatures around 37°C applied to rupture cell membranes [105, 199]. Repeated cycles of freezing and thawing induce ice formation within the cytoplasm and cellular membranes. The existing practical models demonstrate that freezing does influence the mechanical stability of the tissue [11, 26]. The mechanisms of these changes may include the loss of smooth muscle cells, damage to ECM, bulk redistribution of water, or structural changes in alignment caused by ice crystal growth [200]. As result, the process should be controlled by using an adequate cryoprotectant, such as various sugars [201].

Ultra-high hydrostatic pressure (Ultra-HHP) assumes application pressures greater than 150 MPa till 1000 MPa to destroy cell membranes [105]; in addition, it may manifest sterilization effect against bacteria and fungi [202]. But it failed when applied alone in regard the complete DNA removal and may alter they structural and mechanical properties of the tissue because of deformation of collagen and elastin fibers. Therefore, pressure should be precisely controlled during the procedure. Supercritical carbon dioxide in another mechanical approach used in the DC of vascular tissue. It demonstrated to be nondeforming while allowing preservation of collagen and elastin content; however, its effectiveness in insufficiently studied [202, 203].

Immersion in chambers with decellularizing agents and agitation achieved with a shaker, agitator, ultrasound waves, or magnetic plate are commonly used protocols for hollow and less dense structures; these approaches are applied in practice for various types of tissue, including blood vessels. Perfusion regimens are utilized for homogenously distribution of the lysing agents throughout the organ via its vasculature. The flow rate is the critical parameter defining the efficiency of the method in terms of cells removal and avoiding elevated levels of pressure, which may have a severe negative impact on microvessels system [27].

Describing combined methods

Application of combined methods as a multistep process has the main goal to complete one another and to preserve the desired characteristics of the scaffolds in this way (e. g. mechanical methods and surfactants; enzymatic treatment and detergents) [105, 204-206]. This strategy is useful when thicker tissue should be proceeded, such as fat tissue or cartilage

disk.

In the field of tissue engineering, standards require the safety, reliability, and reproducibility of the process [131]. For development of an "ideal" DC protocol a deep and detailed comparison of different techniques is necessary [174]. According to Crapo et al. (2011), two main requirements for DC protocol should be accomplished: on the one hand, all foreign cellular antigens, including remaining DNA that may elicit immunogenic reactions, have to be removed (inflammatory reactions and therefore adverse short- or long-term results in clinical use); on the other hand, ECM components with their biochemical and biomechanical cues for cell migration and proliferation and normal tissue architecture have to remain intact, because its alteration inhibits recellularization and affects the scaffold durability [24]. So, a good balance generates biological scaffolds that do not elicit an immune response from the host while preserving the biological and mechanical properties of the conduit [131].

1.7 Investigating the Decellularized Scaffolds

Characterization of the resulting scaffolds is mandatory for identification of the optimal DC method. The methods applied for obtained matrices' evaluation may be classified according to the data provided as qualitative and quantitative and assume cellularity evaluation, extracellular matrix examination, mechanical profile testing, and residual cytotoxicity analysis [180, 206, 210].

Histological examinations are used to evaluate the efficiency of decellularization; it allows to determine the chemicals' penetration depth (PD) also. For instance, Hematoxylin and Eosin (H&E) staining is applied to evaluate the residual cells number in the tissues and in obtaining a general histological characterization of ECM scaffolds; while special stains, such as Verhoeff-Van Gieson (VvG), Masson's trichrome (MTC), Movat's Pentachrome, Picrosirius red or Safranin-O are used to examine tissues for the presence of various cytoplasmic and extracellular molecules [123, 211]. The VvG formulation is used to demonstrate normal or pathologic elastic fibers; MTC and Picrosirius red methods allow to detect collagen fibers in tissue, Movat's Pentachrome stains permits to highlight the preservation of ECM components – collagen, elastin, and amorphous ECM (such as fibronectin, glycosaminoglycans, and proteoglycan) and Safranin-O method is use for the detection of cartilage, mucin, and most cell granules [124, 175].

Immunohistochemical methods are utilized for selective identifying of specific intracellular cytoplasmic cytoskeletal proteins, such as α-smooth muscle actin and vimentin, as well as adhesion-related proteins, such as fibronectin, vitronectin, elastin, collagen type I, III and laminin by exploiting the principle of antibodies binding specifically to antigens in biological tissues [25, 157].

The *qualitative evaluation of remnant DNA* presence can be performed by 4',6-diamidino-2-phenylindole (DAPI), Propidium iodide, PicoGreen or Hoechst staining, while *quantitative DNA assessment* is realized by DNA quantification kits according to the manufacturers' protocol [73, 157, 211]. DAPI is a method of analytical fluorescence, which basic staining principle is determined by strongly reagent binding to adenine-thymine (A-T) regions in DNA. When compared DAPI, Hoechst fluorescence stain seems to be less toxic and stains DNA in living and fixed cells by dyes attachment to A-T regions with the next probe excitation by UV light (~360 nm). In term of quantitative evaluation of remaining genetic materials, suggested minimal criteria to satisfy the intent of DC is residual DNA content below 50 ng of double stranded DNA (dsDNA) per mg of dry weight, any DNA present should be in fragments less than 200 bp in length, and histological analysis with hematoxylin-eosin (H&E) and 4',6-diamidino-2- phenylindole should indicate no visible cell or nuclear material [24, 194, 202].

Gel electrophoresis is another method used as an efficient method for separation and analysis of macromolecules and their fragments, including DNA, based on their size and charge. It is used to identify and separate a mixed population of DNA fragments by length and to estimate the size of DNA fragments. The technique assumes running a current through a gel containing the molecules of interest. DNA fragments are negatively charged, so they move towards the positive electrode. Because all DNA fragments have the same amount of charge per mass, small fragments move through the gel faster than large ones [212].

Biochemical assays are available to confirm that desirable insoluble proteins of the ECM, such as elastin, collagen, and GAGs, are still present and to quantify it. For proteins quantification, following tissue extraction specific colorimetric assay kits are used, the procedure being performed according to the manufacturers' instructions [24, 180, 213].

Mechanical testing, and namely tension test, burst-pressure testing, stress relaxation, compliance evaluation, burst-pressure testing, suture retention strength test of the ECM, and vessel wall thinning after treatment provides insight into the presence and integrity of the structural proteins, such as collagen, elastin, fibronectin, and elastin within the scaffold [105, 123, 214]. Because of the lack of smooth muscle cells or ECM damage significant alterations in mechanical behavior of the developed grafts may be recorded [174].

Scanning electron microscopy (SEM) examination is focused on the either luminal endothelial or adventitial surfaces and 3D topography of the cross-section of the decellularized matrices [180, 215]. It allows to characterize the topography and ultrastructure of both the outer and luminal surfaces of the scaffolds, as well as to investigate the porous structure of the cross-section of scaffolds' samples.

Cytotoxicity evaluation is conducted to determine the presence of decellularization

agents' residue from decellularized ECMs. Obviously, the remaining detergents should negatively influence the scaffold cytocompatibility for seeding cells and proliferation. For toxicity evaluation MTT or MTS cell proliferation assay can be performed [127, 180, 216].

In vitro biocompatibility assay is used to simulate and predict biological reactions to DC vascular scaffolds before the material is placed in the living body [211], while *in vivo biocompatibility assay* assumes performing animal experiments before the material is placed in the human body. The investigation in this category must be in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Ethics Committee. The observation supposes subcutaneous implantation of decellularized tissue with further histological and immunohistochemical staining to detect fibroblasts, endothelial cells, macrophages, helper T lymphocytes, and cytotoxic T lymphocytes. In this way by measuring the number of inflammatory cells the host immune response may be evaluated and the result objectively organized [157, 216].

As conclusion, application of decellularized matrices in clinical practice have been attracting much attention in recent years. However, none of existing protocols is ideal and efficient enough in removal residual cellular components. When persisted, it may determine release of immunogenic reactions once *in vivo* use. In addition, there are fewer progressive results in DC of 3D organs, including blood vessels. So, it is necessary to develop and evaluate in more details some new realistic models and to determine the "gold standard" protocol for development of acellular scaffolds suitable in clinical "enactment".

2. MATERIALS AND METHODS

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 (control) 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 arteries were chosen because of their similarities to the human tissues and good and easy availability. Blood 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 the pigs and immediately transported to the laboratory in PBS without antibiotic-antimycotic 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 (Figure 7).

A.   B.   C.

Figure 7. **(A) Tissue harvesting. (B, C) Samples prior to proceeding.**

Porcine ascending aorta (B). Porcine common carotid artery (C)

Vessels were stored at -80°C; for adequately preservation of dimensional and mechanical properties aorta samples 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.

2.1 Chemical-Based *vs* Combined Approach in Aorta Decellularization

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

porcine vessels, namely, porcine aorta.

Initially, for decellularization the following solution were used:

- ✓ 1L 1XPBS: 100 mL of sterile 10XPBS added to 900 mL ultrapure water;

- ✓ 1M Tris-HCl buffer pH 7.5, as stock solution: 157.6 g of Tris-HCl dissolved in 750 mL of ultrapure water; pH adjusted to desired value using the 10M NaOH; the final volume filled to 1 L with ultrapure water;

- ✓ 0.5M of ethylenediaminetetraacetic acid (EDTA), as stock solution: 186.1 g of EDTA dissolved in 800 mL of ultrapure water; the solution was stirred vigorously using a magnetic stirrer; the pH adjusted to 8.0 using NaOH; next the solution diluted to 1L with ultrapure water;

- ✓ 1M Sodium Chloride (NaCl), as stock solution: 58.44 g of NaCl dissolved in 750 mL of ultrapure water; stir bar added to the baker and left on the stir plate until completely dissolved; next the solution diluted to 1 L with ultrapure water;

- ✓ Hypotonic buffer plus EDTA and NaCl: to prepare 500 mL of the specific hypotonic buffer 5.0 mL 1M Tris-HCl, 5.0 mL 1M NaCl and 10 mM 0.5 M EDTA added to 480.0 mL of ultrapure water;

- ✓ 0.5% SDS + 0.5% SDC (w/v): SDS (0.5 g) + SDC (0.5 g) added in a volumetric flask containing about 80 mL of buffer. Once the detergents dissolved completely, the volume made up to 100 mL;

- ✓ Triton X-100 (1% v/v): 1 mL Triton X-100 dissolved in 99 mL hypotonic buffer.

Chemical-Based Approach

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.

Combined (Chemical-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.

In both cases each decellularization step was carried out under rotation using rotating rollers (TRM50) with 35 revolutions per minute (Figure 8).
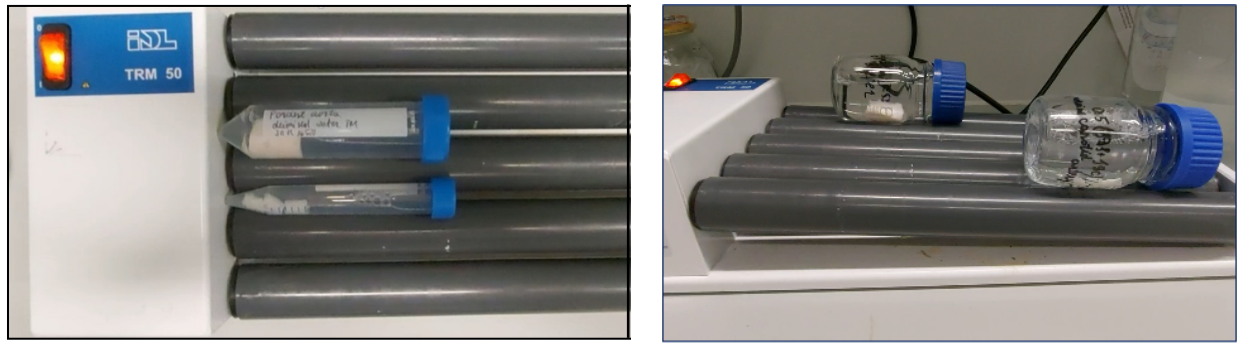
Figure 8. **Using the mechanical agitation for decellularization of blood vessels (device – rotating rollers TRM50)**

2.2 Evaluation of the Decellularization Protocol Efficiency Depending on the Vessel Diameter

The combined chemical-enzymatic decellularization approach was adapted to decellularize both porcine ascending aorta and porcine carotid artery. Four different exposure durations to ionic detergents were applied: 6 hours, 12 hours, 18 hours, and 24 hours. After each respective treatment time with the mentioned detergent cocktail, 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), the working solution being changed each 24 hours, and flushed for three times in PBS.

0.5-1 cm long pieces of vessel from each experimental group were cut off and fixed in 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 4ºC in PBS for additional analyses, if necessary (Figure 9).

Figure 9. **Macroscopic appearance of treated vascular samples with chemical-enzymatic protocol** (duration of SDS-SDC wash 12 hours)

2.3 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 decellularization, the resulting data being compared to the homologous data obtained from

frozen samples by performing H&E and DAPI staining.

2.4 Evaluation of Ultrasound Application for the Blood Vessels Decellularization

To evaluate the efficiency of ultrasound application in vascular tissue decellularization, two different combined working approaches were tested, namely association of sonication and osmotic shock or sonication and detergent treatment.

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 (+4ºC). In addition, the samples were placed in an ice bath to prevent tissue over-heating (figure 10).

Figure 10. **Working system used in sonication-assisted decellularization methods**

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, the samples were washed with phosphate-buffer saline (PBS). Vascular segments from each experimental group were fixed in 4% PFA for histological and fluorescent staining. Remaining vessel segments were stored at 4ºC in PBS for additional analyses, if necessary.

2.5 Characterization of Decellularized Porcine Matrices

The decellularized scaffolds, namely porcine carotid artery, 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;

Analysis was performed before and after treatment. The accomplished tests allowed to determine the efficiency of decellularization agents, their effect on the structure and nature of remaining matrix components, and to assess biomechanical properties of the scaffold.

Qualitative Evaluation of Decellularized Porcine Blood Vessels

Histological Staining: H&E

H&E stain was applied on paraffin-embedded cross sections (5 μm thickness), following standard protocols for dehydration, embedding, cutting, rehydration (Figure 11) and staining (Figure 12) for determination of cellularity and extracellular matrix features.

Samples fixed in 4% PFA overnight at room temperature were dehydrated with a dehydrator (HistoCore Pearl, Leica), that used series of xylol and ethanol dehydration steps. The dehydrated vessels were placed into molds containing hot paraffin and left to solidify on a frozen plate. Paraffin-embedded samples were sectioned into 5 μm thick sections using a semi-motorized rotary microtome (Leica RM 2245), placed on Microscope slides, and dried overnight. Rehydration was carried out by placing the sections in a glass rack to hold slides and moving this rack through a series of xylol and ethanol solutions (figure 11) [217].

For H&E stain of frozen tissue samples were cut into 7 μm sections using a cryotome (Microtome HM 560 Cryostat). Sections were then transferred on Superfrost Ultra Plus slides

and stored overnight at -80ºC, afterwards were fixed in acetone at -20ºC for 8 minutes and stained according to the established procedure (Figure 12).

30 min in a 60ºC oven
10 min in Xylol (2 TIMES)
10 min in 100% ethanol
2 min in 90% ethanol
2 min in 80% ethanol
2 min in 70% ethanol
Wash quickly in distilled water
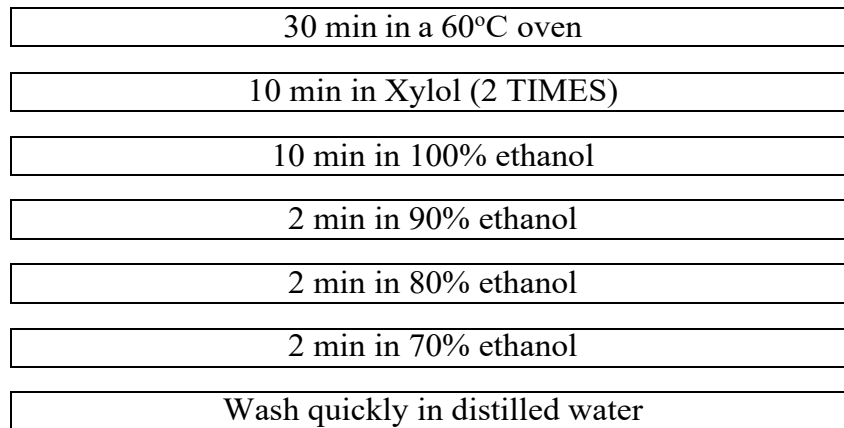
Figure 11. **Method of rehydration for paraffin sections**

H&E staining were conducted by placing the afore mentioned fixed slides in a staining rack and moving this rack through hematoxylin, eosin, and alcohol solutions. Stained sections were covered with slips using corbit balsam and left to dry overnight under the hoot. These stained samples were evaluated with a light microscope interfaced with an image analysis system [217]. Wall thickness was measured on histological sections on seven points before and after decellularization.

8 min in Hemotoxylin
10 min in warm tap water (changing water every 2 min)
10 rinses in 95% ethanol
20 seconds in Eosin
5 min in 95% ethanol
10 min in 100% ethanol
5 min in Xylol (2 TIMES)
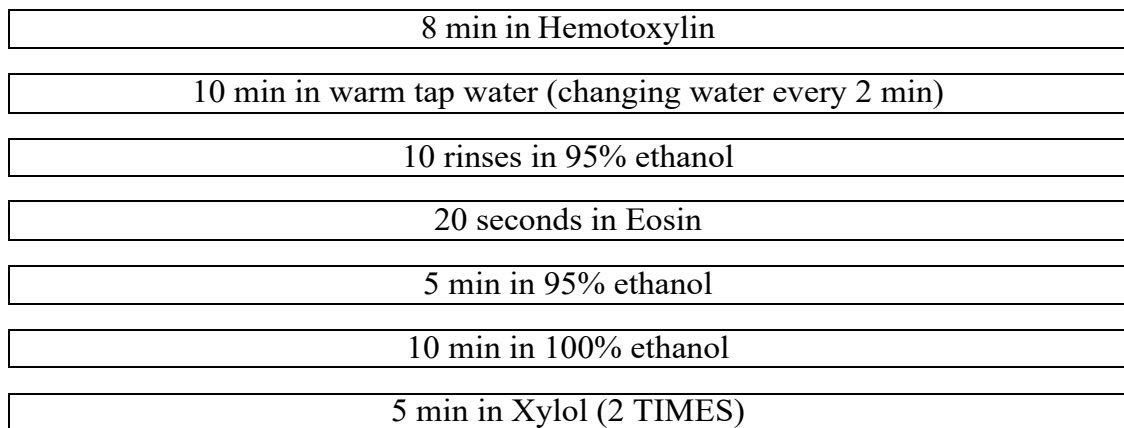
Figure 12. **Hemotoxylin and Eosin staining**

(a glass rack is used to hold the slides and move them through the solutions)

Fluorescent staining: DAPI

Frozen samples were sectioned into 7 μm thick sections using a Microtome HM 560 Cryostat, placed on Superfrost Ultra Plus slides, and stored overnight at -80ºC. Sections were washed in PBS. Sections were encircled using the Dako Pen and fixed with 4% PFA for 20 minutes in wet chamber. Sections were washed 3 times for 5 minutes each in a glass cuvette containing PBS. Then sections were incubated for 15 minutes with DAPI (0.33 μg/mL in PBS) to test the presence of remnant DNA. Sections were washed 3 times for 5 minutes each in PBS, protected with cover slips using Shandon Immu-Mount fluorescent mounting medium, and stored at +4ºC until medium was dried (48-72 hours). Stained samples were

analyzed with the Axio Observer A1 microscope [217, 218].

Basement membrane's evaluation by using immunohistochemistry

Collagen IV morphology was studied by immunohistochemical staining. A total 1 native control, 1 negative probe, and 1 decellularized artery were submitted for analysis. For immunohistochemistry, cross sections of frozen tissue samples (7 μm thickness) were treated for antigen retrieval. *Primary antibody*: Monoclonal Mouse Anti-Human Collagen IV. Clone CIV 22, Isotype IgG1, conc. used 1:25 (239M-18, Sigma-Aldrich, USA). *Secondary antibody*: Cy3 donkey anti-mouse. Clone N/A, Isotype IgG, conc. used 1:100 (AP192C, Sigma-Aldrich, USA). The immune complexes were then visualized with the Axio Observer A1 microscope.

Scanning Electron Microscopy (SEM)

Vascular segments (0.25-0.5 cm long) were cut from decellularized and native fresh vessel samples. Samples were processed for SEM following a standard protocol [217, 218]. They were cut open and anchored, luminal side up, onto pieces of cork (1 cm thick) using pins. The cork was placed with the samples facing the bottom of a 50 mL falcon tube that was filled with 5 mL of 2.5% glutaraldehyde in 0.1 M Sodium Cacodylate Trihydrate and left for 24 hours at room temperature (Figure 13). After the solution removal, it was replaced with 30% acetone for 10 minutes, 50% acetone for 10 minutes, 70% for 10 minutes, 90% acetone for 10 minutes, and 100% acetone (diluted with water). The samples were moved from cork to a sample holder and placed into the critical point dryer for drying. After drying, the samples were spattered with gold particles by Herr Price from the Institution of Electron Microscopy and visualized the following day on the scanning electron microscope. The luminal surface of control and treated samples were examined qualitatively for the presence of endothelial cells (cellularity assay) and characterization of basal lamina preservation.
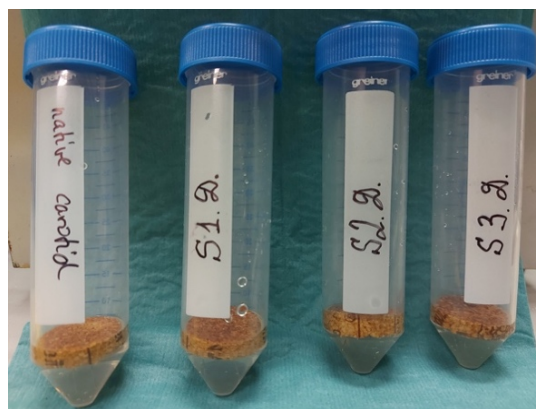
Figure 13. **Fixation the samples (control and experimental groups) with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for scanning electron microscopy**

Perfusion Decellularization

A perfusion system was set up using a perfusion pump. Alternatively, vessels were treated with similar solutions by perfusing decellularization solution into their lumen to improve the homogeneity of decellularization of long vascular segments (l=6.0 cm) (figure

14A).

The decellularization solutions covered the vessels and the decellularization solutions were pumped through the vessel at a rate of 25 mL/hour. At the end vascular segment was additionally incubated with DNase solution of 300 U/mL DNase I in 1 mM MgCl$_2$ in PBS at 37°C for 48 hours. Finally, the treated segment was washed 3 times with PBS and small pieces (end segments and middle portion, figure 14B) were cut off and fixed in 4% PFA or directly frozen in tissue tek O. C. T. compound or prepared for SEM analysis.

A.   B.
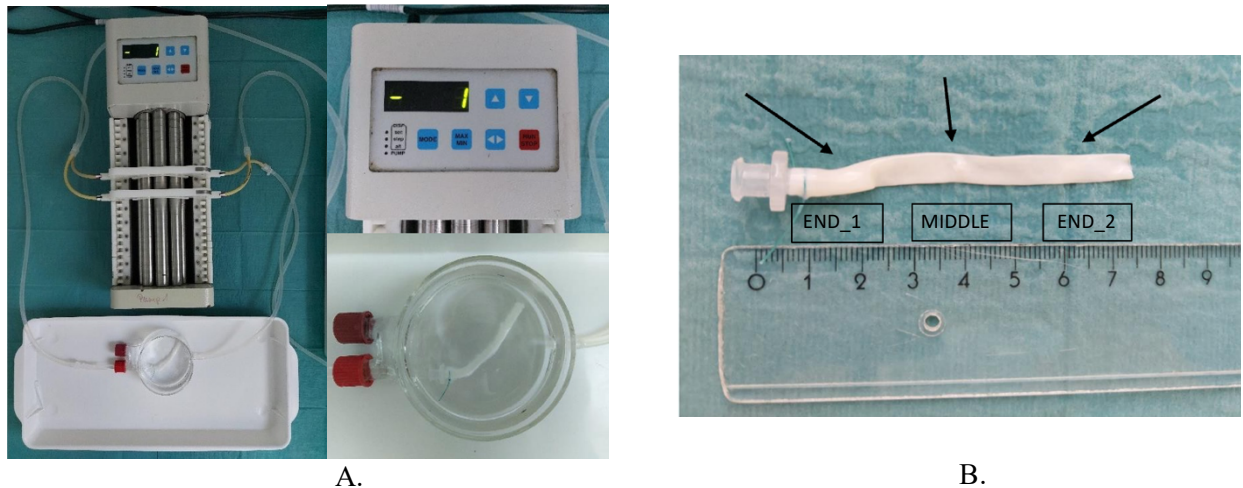
Figure 14. **Perfusion system for long vascular fragments' decellularization.** A. Porcine vessel (porcine carotid artery l=6.0 cm) attached to a perfusion system for perfusion-decellularization a 25mL/hour. B. Characterization of perfusion-decellularized matrix: middle segment and end portions

Quantitative Evaluation
DNA, Collagen, and GAG Quantification

The ECM components, collagen (insoluble and soluble) and glycosaminoglycans, and DNA were quantified. The procedures were performed according to MHH in-house protocol. Remnant double-stranded DNA was used as an indicator of remaining cellular material; quantification of hydroxyproline and GAGs allowed to observe the removal of common ECM proteins after DC and to briefly characterize ECM composition and morphology.

Pieces of control groups and decellularized porcine vessels (1-2 cm long) were freeze-dried for 22-23 hours at 0.018 mbar (Alpha 1-2 LD plus). The dried vessels were weighed (between 2.0 and 10.0 mg of freeze-dried product) and transferred to 1.5 mL safe-lock reaction tubes. 200 μL of sterile water was added to each sample and the samples were boiled for 10 minutes. The samples were left to cool down to room temperature and 790 μL of 10 mM Tris (pH = 7.5) was added with 10 μL of proteinase K (20 mg/mL in 50% Glycerol). The samples were incubated at 60°C and 1000 rpm overnight in a thermomixer compact (Eppendorf®) for digestion. Not completely digested samples were additionally treated with 10 μL Proteinase K and incubated further. Next, the digested samples were analyzed specifically to quantify

hydroxyproline, GAG, and DNA content (figure 15).

Hydroxyproline quantification	GAG quantification	DNA quantification
200 µL digest + 200 µL 6 M HCl: hydrolyzed at 105°C for 24h	50 µL digest + 950 µL PBE	100 µL digest + 900 µL DNAdilution buffer
Centrifuge for 15 min at 17000 g	Prepare Chondroitinsulfate A standard in blank buffer (95% PBE/5% TRIS) starting from 10 µL/ml	Prepare DNA standard dilution in DNA blank buffer starting from 20 µL/ml salmon sperm DNA
50 µL hydrolyzate + 450 µL of 2.2% NaOH-C/A buffer	Add 100 µL GAG standard and sample to separate wells in a 96-well plate	Add 100 µL DNA standard and sample to separate wellsin a 96-well plate
50 µL dilution + 950 µL C/A buffer	Add 100 µL of DMMB to each sample in the 96-well plate	Add 100 µL of Hoechst 33258 solution (1 µg/mL in 200 mM NaCl, 10 mM TRIS, pH = 7.5) to each sample in the 96-well plate
Prepare 500 µL Hydro-xyprolin standard in C/A buffer starting from 20 µL/ml	Measure absorbance at 595 nm	Measure fluorescence intensity at 465 nm with an excitation wavelength at 360 nm

Add 250 µL Chloramin-T solution to each sample and standard solution: Incubate for 20 min at room temperature

Add 250 µL of 6 M perchloric acid to each sample and standard solution: Incubate for 12 min at room temperature

Add 250 µL of p-dimethylaminobenzaldehyde solution to each sample and standard solution: mix and incubate for 20 min at 60°C

Add 250 µL of each sample and standard solution to separate wells of a 96-well plate. Read the absorbance at 565 nm

Figure 15. **Quantification method for decellularized tissue characteriza*tion***

Figure 16. **DNA standard curve for quantification analyses**.

DNA standard curve made from a known concentration of salmon sperm (starting conc. = 20 μg/mL) that was serially diluted with DNA blank buffer. A direct relationship between DNAand absorbance is expected and observed. This curve was used to interpolate unknown DNA concentration values of vessels for DNA quantification ($r^2 = 0.99$)
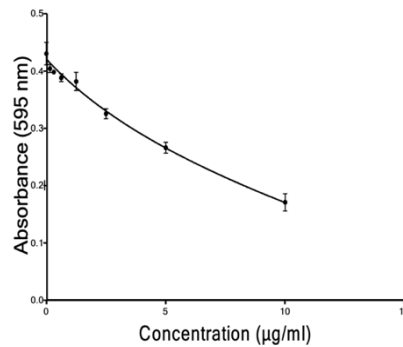
Figure 17. **GAG standard curve for quantification analyses**.

GAG standard curve made from a known concentration of chrondroitinsulfate A (starting conc. = 10 μg/mL) that was serially diluted with blank buffer. An inverse relationship between chrondroitinsulfate A and absorbance is expected and observed. This curve was used to interpolate unknown GAG concentration values of vessels for GAG quantification ($r^2 = 0.98$)
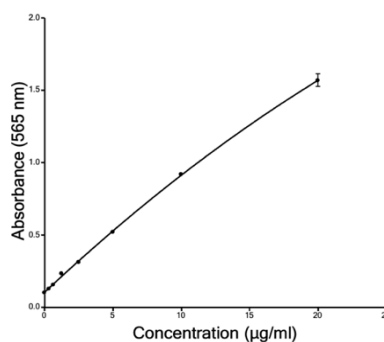
Figure 18. **Hydroxyproline standard curve for quantification analyses**.

Hydroxyproline standard curve made from a known concentration of hydroxyproline (starting conc. = 20 μg/mL) that was serially diluted with C/A buffer. This curve was used to interpolate unknown concentration values of vessels for collagen quantification ($r^2 = 0.98$)

Quantification values were interpolated from the standard curve (figure 16, 17, 18) and normalized to their dry weight. The percentage decrease was calculated from the average of each sample group, using the formula [217]:

$$\%\text{Decrease} = \frac{sample1 - sample\ 2}{sample\ 1} \times 100$$

Mechanical testing

Testing Suture Retention Strength of the Scaffold

Mechanical testing was performed using the INSTRON testing machine (Figure 19). Two groups, native (n=9) and decellularized (n=8) blood vessels, were tested for suturability outcomes. The measurement was performed according to the Niedersächsische Zentrum für Biomedizintechnik, Implantatforschung und Entwicklung (NIFE), MHH, in-house protocol, which is adapted from the methods described in ISO 7198:2016.
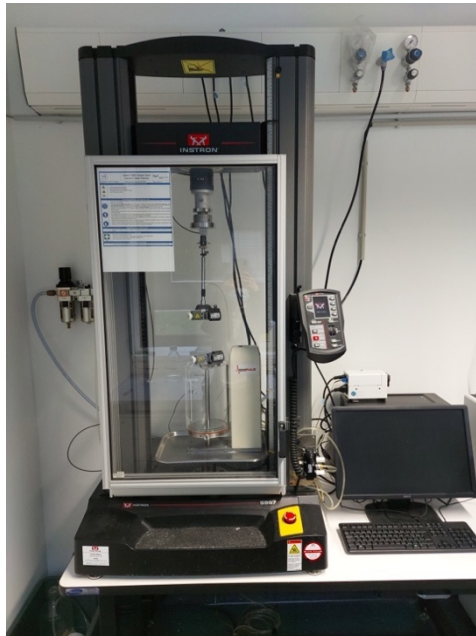
Figure 19. **INSTRON testing machine**

In this case, only the maximum force used to pull the suture out of the tissue was considered. The tests were performed with the sutures being pulled at the rate of 50 mm/min. The test was carried out only on blood vessels isolated perpendicular to the collagen fibers. For all tests, stripes of 10x5 mm (length (l) x width (w)) were cut out from non-treated and decellularized blood vessels. Their average thickness (t in mm) was measured using a Japanese thickness gauge mitutoyo, and the samples were mounted on a custom-made tissue holder (figure 20).

Briefly, for the suture retention test, only one end of the tissue was clamped in the holder of the tensile testing machine. On the other end a loop of a suture was passed through the middle of the sample, at a 2 mm distance from its free end as shown in figure 20A and clamped to one arm of the tensile tester. Non-absorbable, sterile and pigment blue 4-0 Prolene Suture was used to enhance visibility. The maximum force to pull the suture out of the tissue was

measured [219].

A.   B.   C.
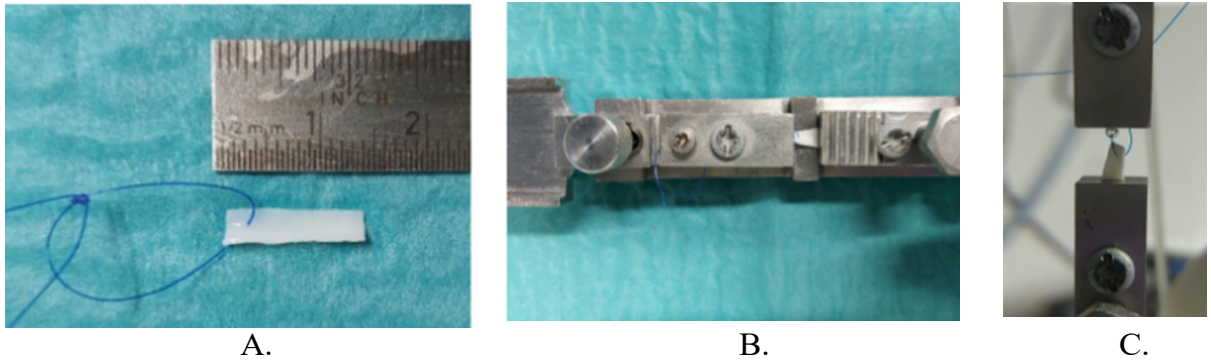
Figure 20. **Suture retention test.** A. Vessel sample prior to testing with one suture. B. Fixation of the blood vessel in the custom-made holder. C. Tissue probe during the testing process

Evaluating the Washing Method to Remove Residual SDS

Colorimetric assay with methylene blue is a straightforward and non-invasive method to detect residual SDS present in tissue was fulfilled according to MHH in-house protocol. SDS detection was performed by incubating with methylene blue and subsequent extraction with chloroform (figure 21, 22).

Figure 21. **Spectrophotometric evaluation of remnant SDS complexes**
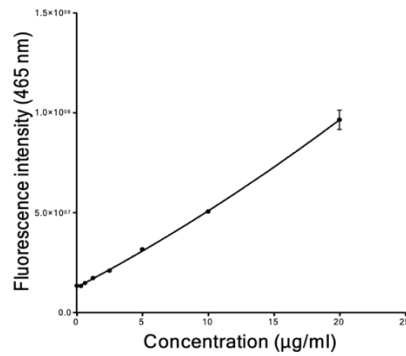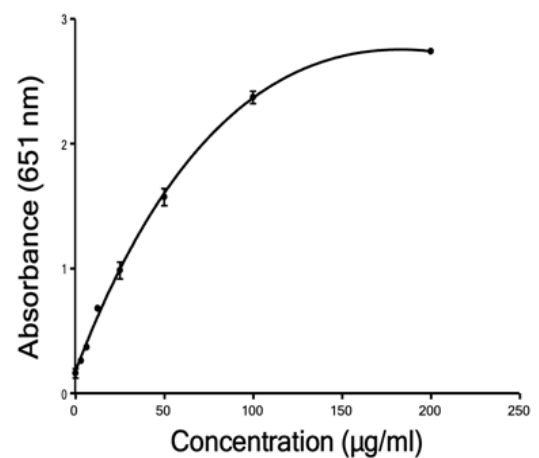

Figure 22. **SDS standard curve for quantification analysis**.

SDS standard curve made from a known concentration of SDS (starting conc. = 200 µg/mL) that was serially dilute with 10 mM TRIS pH 7.5. This curve was used to interpolate unknown SDS concentration values of vessels for SDS quantification ($r^2 = 0.99$)

Cell culture

HUVECs used in this study were purchased from Lonza and transduced by lentiviral transduction with GFP (GFP-HUVECs). After defrosting the cell suspension was moved to a T175 cell culture flask and incubated at 37°C and 5% CO_2. When a confluent layer was obtained, cells were detached from the bottom of culture dish with 10 mL TrypLE Select (Gibco™). When the cells were detached, 10 mL of EGM-2 was added, and the cells moved to a 50 mL falcon tube for centrifugation (5 minutes at 300xg). 3 mL of EGM-2 was added to the obtained cell pellet. Cells were counted using the Vi-cell™XR Cell Viability Analyzer [217, 220].

Biocompatibility Assay: *in vitro* cytocompatibility by contact test

Biocompatibility assay was performed by evaluating the cytoprotective effects of the decellularized vessels on the HUVECs' viability [217]. To test biocompatibility, decellularized porcine matrices (stored at 4°C in PBS) were prepared by cutting and placing the blood vessels luminal side oriented up onto 12-well cell culture plate (figure 23). The vessels were held down with two metal rings to prevent from floating and curling and were incubated for 48 hours with EBM-2 medium supplemented with antibiotics and fetal calf serum (FCS). For re-seeding, GFP-HUVECs detached from the flask were counted; 200000 cells/well were seeded onto vessels. HUVECs plated on plastic surface served as control group.
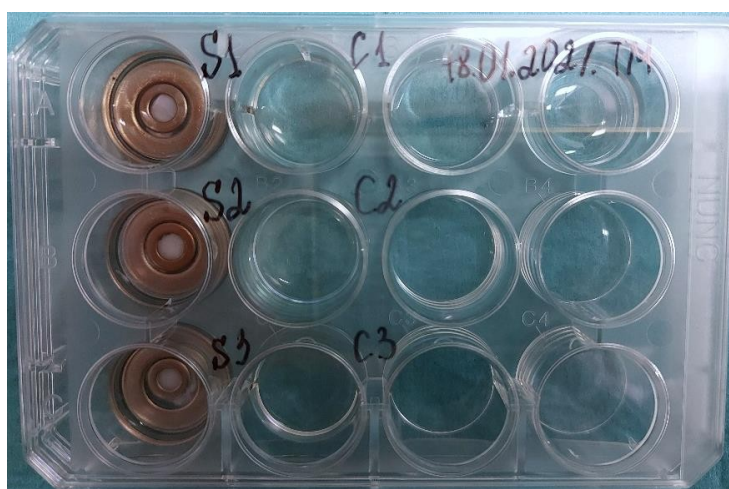
Figure 23. **Biocompatibility assay: fixation of the open vessels onto cell culture plate**

Cells were cultured at 37°C and 5% CO_2 for 5 days. Medium was changed every second day. Pictures were taken every day using the Discover V8 Stereomicroscope. On day 6th, samples were fixed with 4% PFA for 10 minutes, washed with PBS (3 times) for performing Live and Dead Cell Assay.

Live and Dead Cell Viability Assay (Calcein AM staining)

Fluorescent reagents, namely calcein acetoxymethyl (calcelin-AM) and ethidium homodimer-1 (EthD-1), were prepared separately in PBS. The solutions were added to each

well and incubated at 37°C, 5% CO_2, for 30 minutes. Pictures were taken using the Discover V8 Stereomicroscope for evaluating cells viability. Live cells stained with calcein-AM generated green fluorescence upon the excitation of their cytoplasm. Dead cells labeled with the EthD-1 generated red fluoresce [218].

2.6 Statistical Analyses

All statistical analyses and graphs were performed with the statistical softwares GraphPad Prism and SPSS. All numerical values, where applicable, were presented as mean±SD. 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 (vessel wall thickness, suturability strength, DNA, GAG, hydroxyproline content, and SDS analyses) 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 data or parametric data which do not meet normal distribution. Differences were considered significant at p-value lower than 0.05.

3. RESULTS

3.1 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 24).

NATIVE	CHEMICAL-BASED DC	COMBINED DC
H&E		

A.	B.	C.
DAPI		

D.	E.	F.
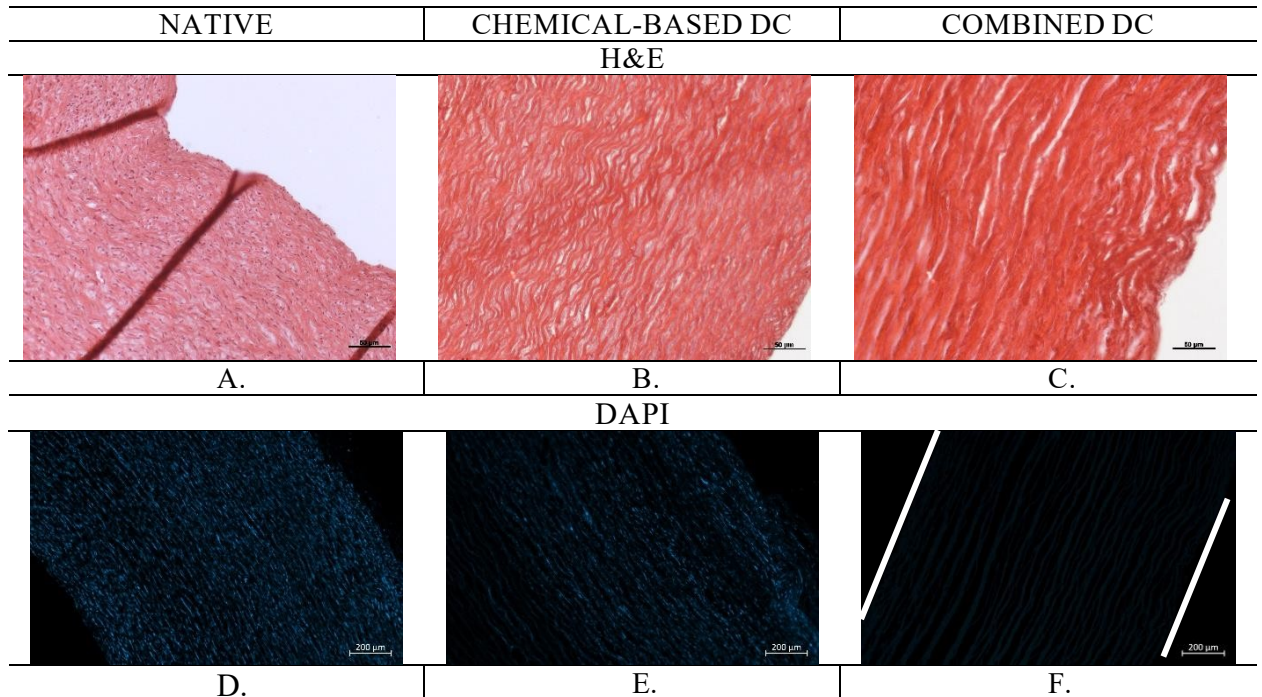
Figure 24. **Combined decellularization approach guarantee efficient cells' wash and preservation of the structure of the extracellular matrix.** H&E (A, B, C) and DAPI (D, E, F) showing cross-sections of native (A, D) and decellularized (B, C, E, F) porcine ascending aorta. **H&E**: The cytoplasm appears 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

The experiment showed the efficiency of chemical cocktails in combination with an enzymatic solution to prepare completely decellularized vascular grafts. The findings suggest that H&E staining cannot be used as a sole proof of DC and should be supplemented, at least, with a DNA stain, like DAPI [221].

3.2 Evaluation the Decellularization Protocol Efficiency Depending on the Vessel Diameter

Porcine aorta and porcine 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 25).

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 [222].

Figure 25. **Differences in decellularization efficiency of porcine vessels depending on vessels diameter [222].** 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.

3.3   Decellularization of fresh *vs* frozen porcine carotid arteries

The cross-sections of the scaffolds in each experimental group were examined by H&E staining and DAPI staining for cellularity assay and revealing the preservation of ECM (figure 26, 27). These qualitative methods provide a visual representation of the integrity of the remaining matrix, its gross architecture, and absence of any remaining cytoplasmic and nuclear materials after decellularization.

Figure 26. **Efficient decellularization of a porcine carotid artery visualized by H&E staining.** Native vessel (A, B, C). Decellularized vessel (D, E, F). Scale bar. A, D: 100 µm; B, E: 50 µm; C, F: 20 µm

Figure 27. **Efficient decellularization of a fresh porcine carotid artery visualized by DAPI staining.** Native vessel (A, B, C). Decellularized vessel (D, E, F). The outline of the vessel is highlighted with a white line. Scale bar. A-F: 50 µm

3.4 Evaluation of Ultrasound Application for the Blood Vessels Decellularization

The cross-sections of the scaffolds treated with ultrasound were examined by H&E and DAPI. 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 [194] (figure 28).

	3 hours		12 hours	
	H&E	DAPI	H&E	DAPI
20%	A.	B.	C.	D.
100%	E.	F.	G.	H.
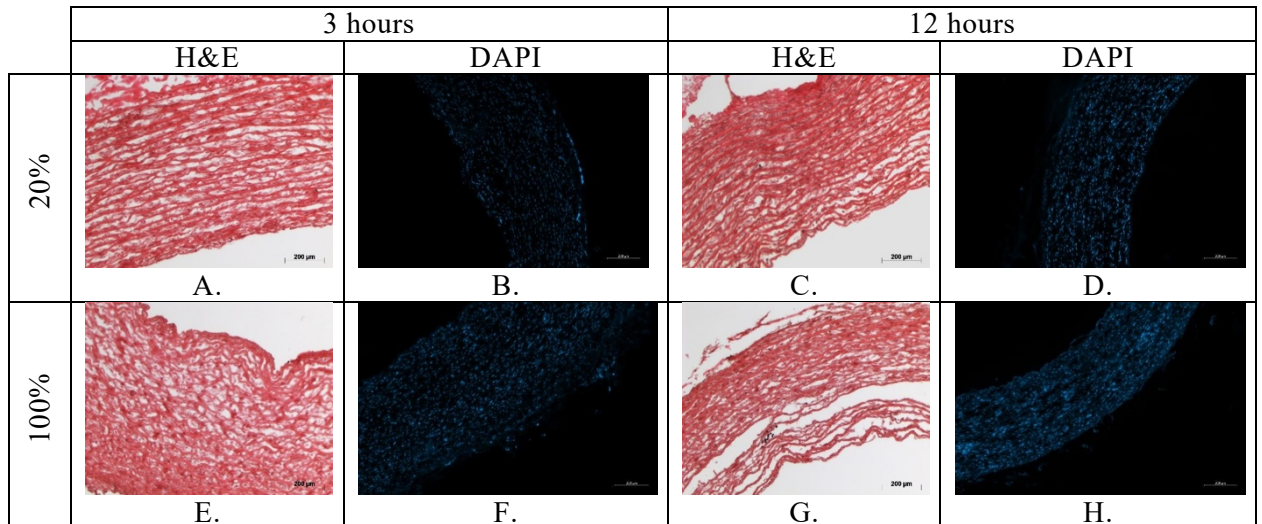
Figure 28. **Ultrasound application in blood vessels decellularization.** H&E staining (A, C, E, G) and DAPI staining (B, D, F, H). Scale bar. A, B, C, D: 200 μm

Ultrasonic waves used in combination with a non-ionic detergent offered the same result – lack of cellular elements elimination and DNA reduction. The presence of intact cellular elements suggested that reagents were not able to solubilize/destroy the membranes and to induce cell removal (figure 29).

	Native	Rotation + Triton X-100	Ultrasound + Triton X 100
H&E	A.	B.	C.
DAPI	D.	E.	F.
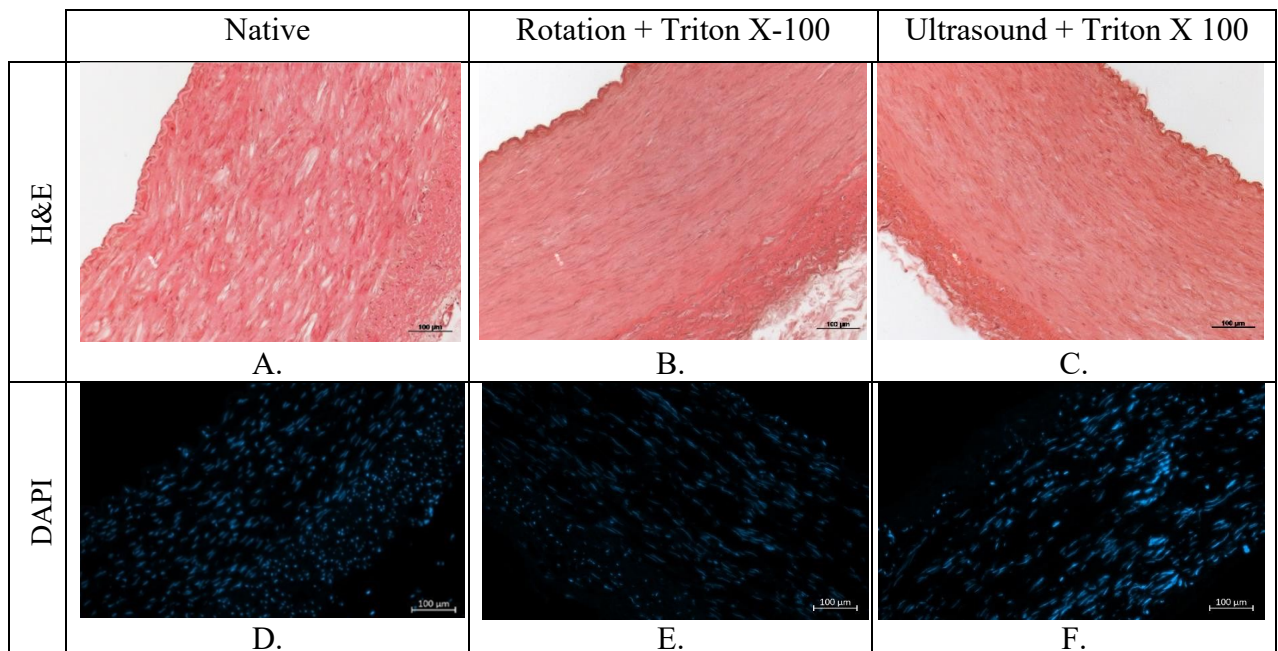
Figure 29. **Combined sonication-based approach in carotid artery decellularization.** H&E staining (A, B, C) and DAPI staining (D, E, F). Scale bar: 100 μm

3.5　Characterization of Decellularized Porcine Matrices

Decellularized porcine carotid arteries were characterized by different methods to analyze remaining components after decellularization and to recognize their quality, to evaluate mechanical integrity of the remaining matrix, and ability to host GFP-HUVECs. Each test has its own limitations and is focused on different aspects of the vessel structure, architecture, or composition. Therefore, applying a variety of evaluation techniques results in a better description of the resulting platform and understanding of the DC 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 (figure 30, A1.1, A1.2) and DAPI (figure 31, A2.1, A2.2) staining for visualization of decellularization efficiency. The histological inspection confirmed successful cell removal – blue-stained nuclei are clearly detectable in native samples, while no citoplasmatic and nuclear material was detectable in the whole treated vessels without harming the architecture of the ECM.

As measured on H&E-stained sections, statistic significant difference in arterial wall thickness (Table 4) before (**555.94±59.22 μm**) and after (**427.06±37.47 μm**) decellularization were detected (p=0.0001) with a reduction of 23.2% from initial value (Figure 32).

Table 4. **Arterial wall thickness measured on H&E-stained sections (μm).** T – type of tissue, N – native tissue, DC – decellularized tissue, M – number measurement

	T	M1	M2	M3	M4	M5	M6	M7	Mean±SD
S 1	N	492.83	494.90	517.16	499.19	483.56	478.28	498.90	**494.97±12.51**
S 1	DC	446.56	441.80	362.98	415.65	428.87	450.35	406.56	**421.82±30.57**
S 2	N	632.14	560.59	632.03	604.47	681.69	605.30	638.15	**622.05±37.38**
S 2	DC	484.99	478.50	399.59	443.60	466.40	403.43	510.75	**455.32±41.93**
S3	N	534.92	542.75	543.41	606.26	538.17	558.03	532.02	**550.79±25.68**
S3	DC	385.52	382.62	392.67	396.48	418.04	417.63	436.26	**404.03±20.13**
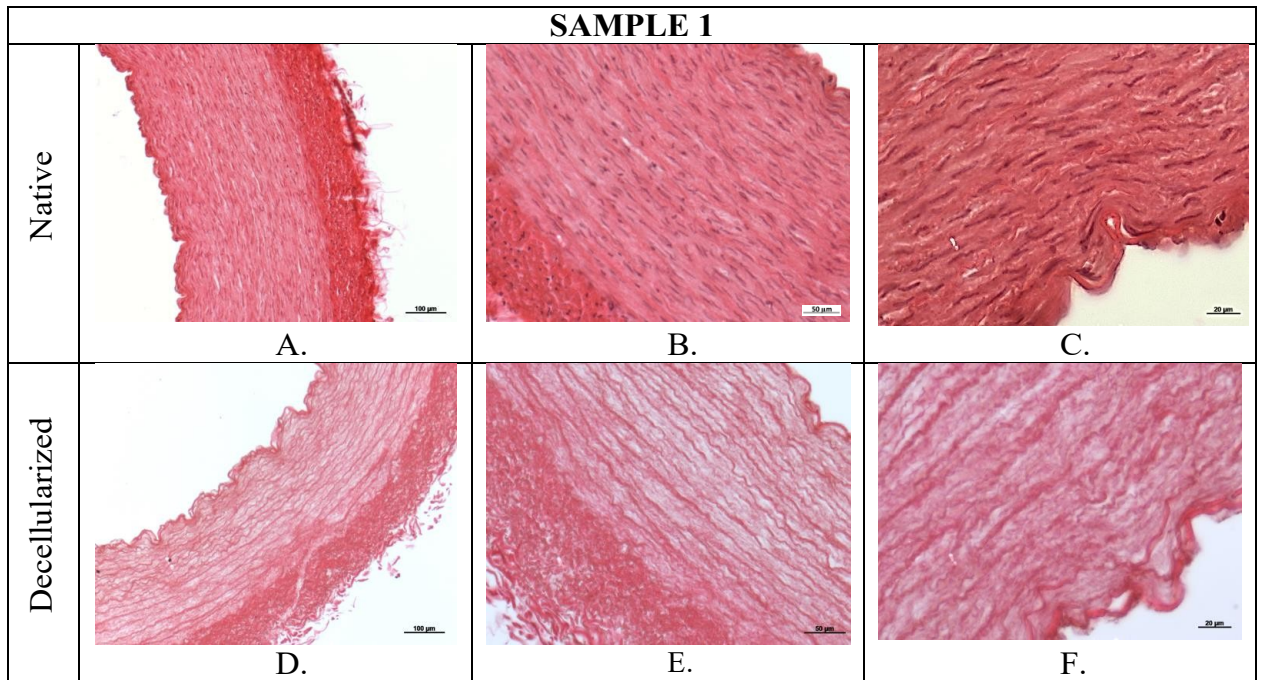
Figure 30. **Efficient decellularization of a porcine carotid artery visualized by H&E staining (SAMPLE 1).** Native vessel (A, B, C). Decellularized vessel (D, E, F). The cytoplasm appears pinky, nucleus is blue-purple, extracellular matrix have varying degrees of pink.

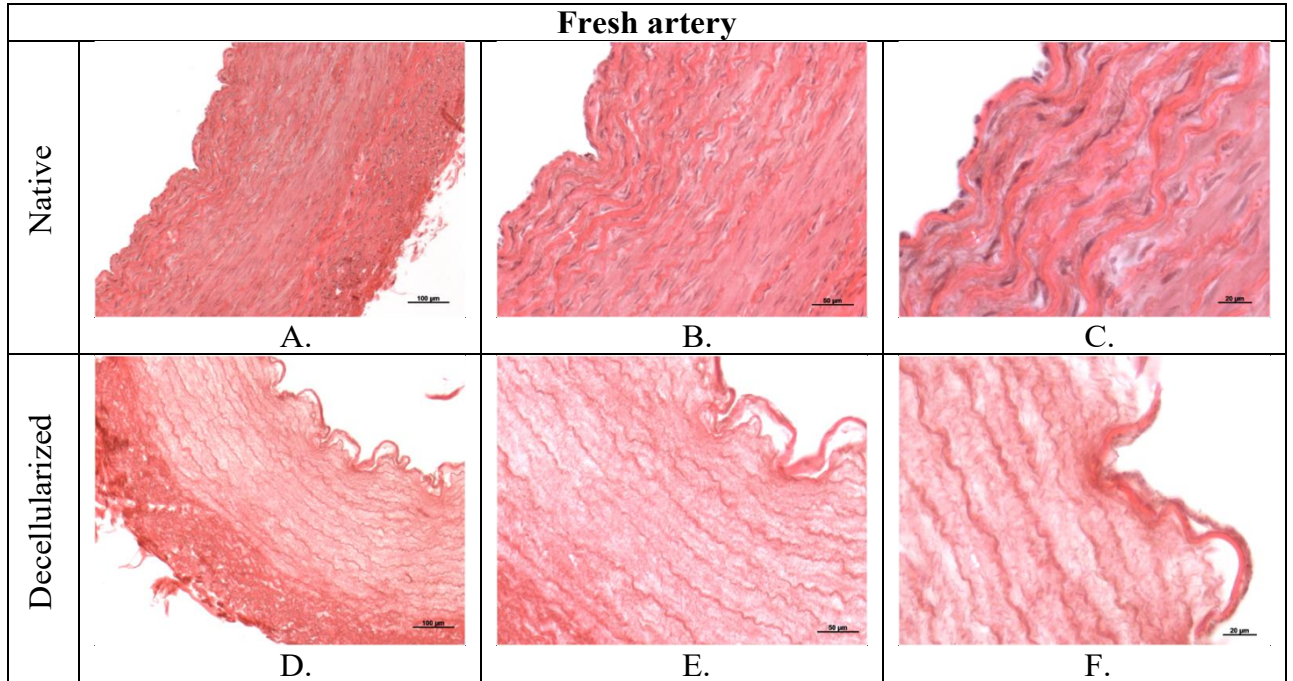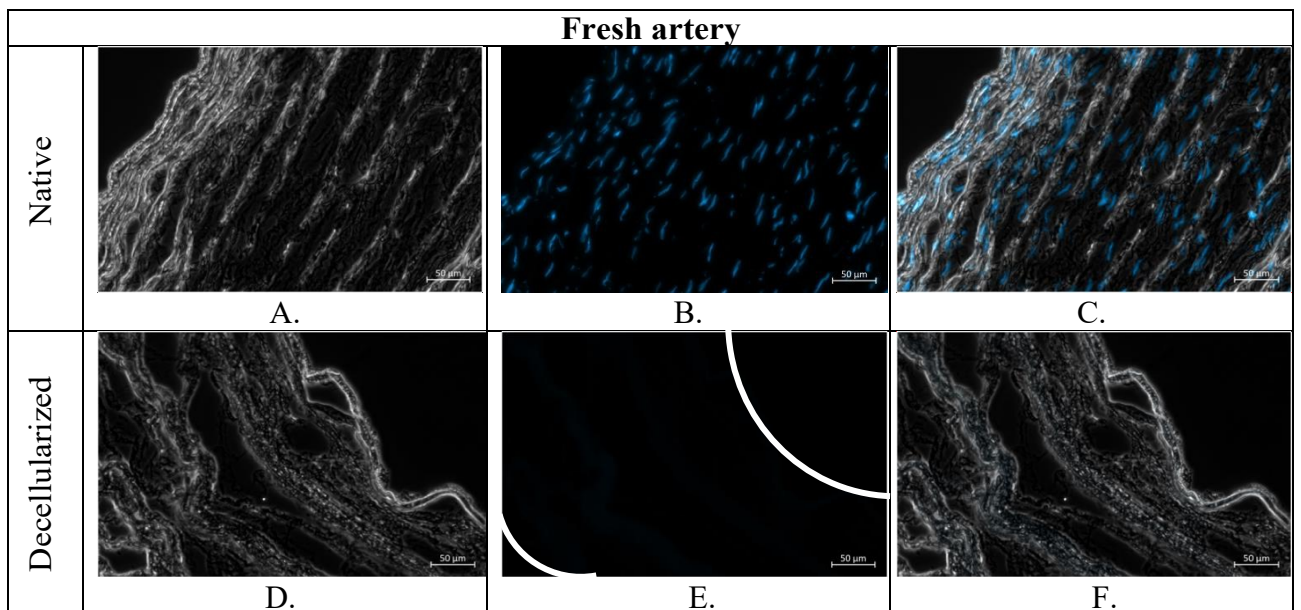Scale bar. A, D: 100 μm; B, E: 50 μm; C, F: 20 μm
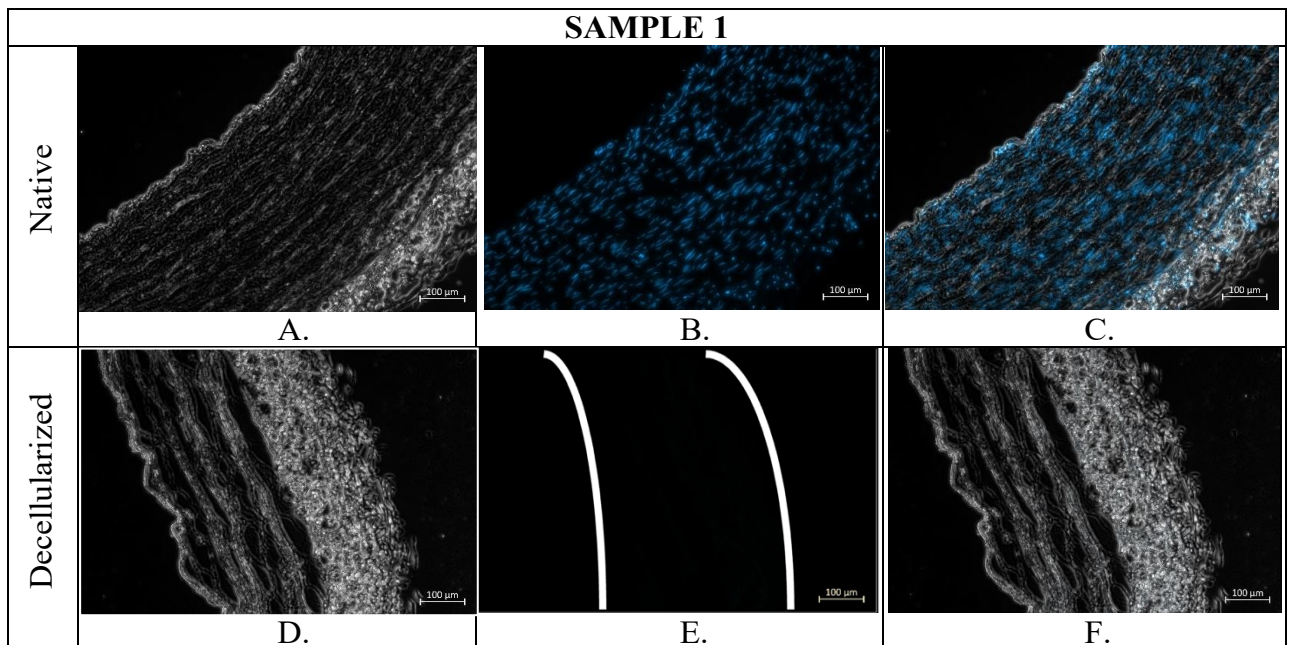
Figure 31. **Efficient decellularization of a porcine carotid artery visualized by DAPI staining (SAMPLE 1).** Native vessel (A, B, C). Decellularized vessel (D, E, F). Nuclei are blue, white lines represent the outer borders of the matrix. The outline of the vessel is highlighted with a white line.

Scale bar. A-F: 100 μm
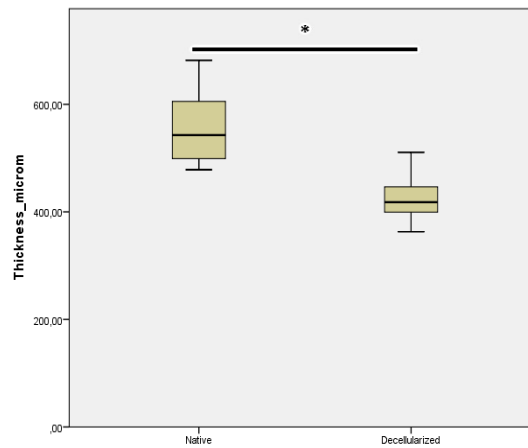
Figure 32. **Arterial wall thickness: native *vs* decellularized samples**. Data is presented as boxplots with (*) indicating the significance by independent t-test (significance is defined as p<0.05).

Basement membrane's evaluation by using immunohistochemistry

Basic histological stains, as H&E and DAPI, were used for determination of cellularity and ECM gross characterization, while 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 33).

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 34) [223].
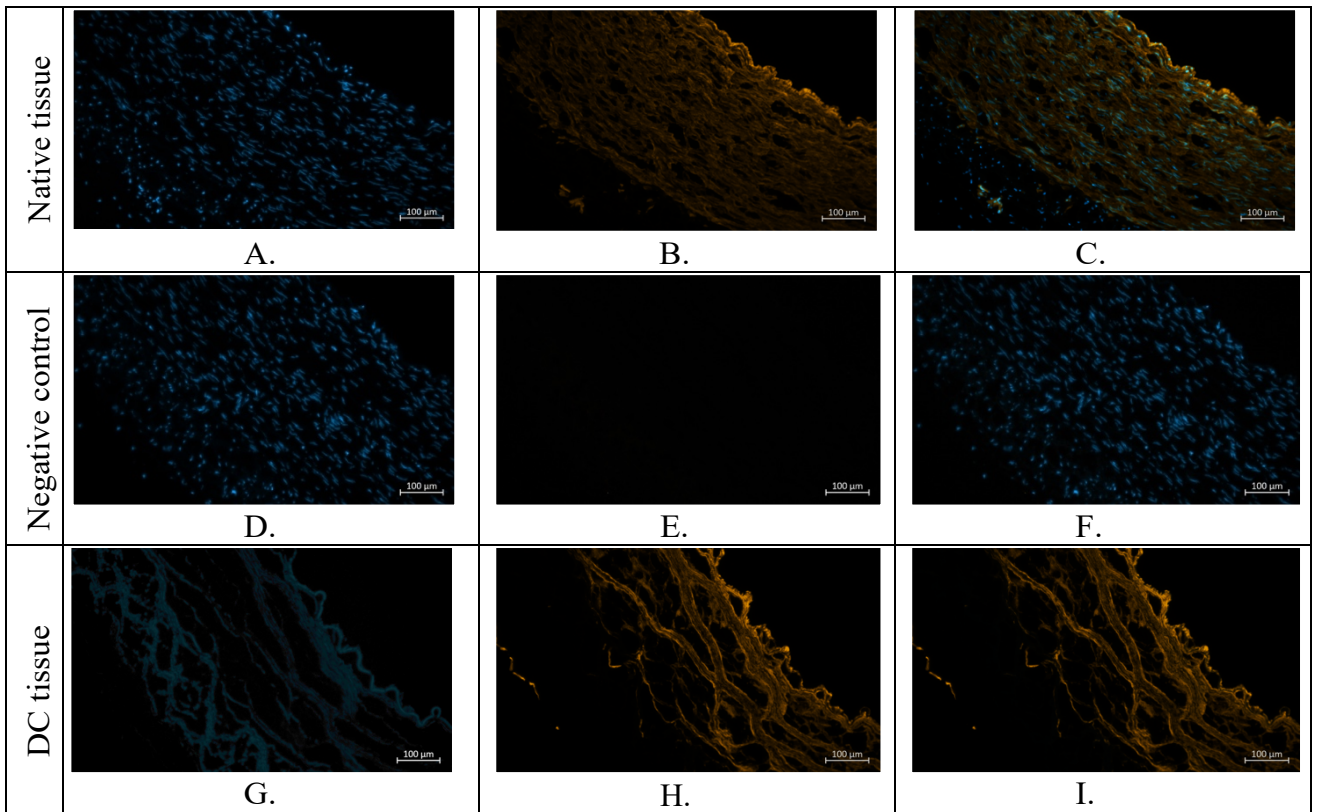
Figure 33. **Basement membrane's evaluation by using immunohistochemistry confirmed preservation the Collagen IV.** Native tissue (A, B, C). Negative control (D, E, F). Decellularized tissue (G, H, I). Scale bar: 100 µm
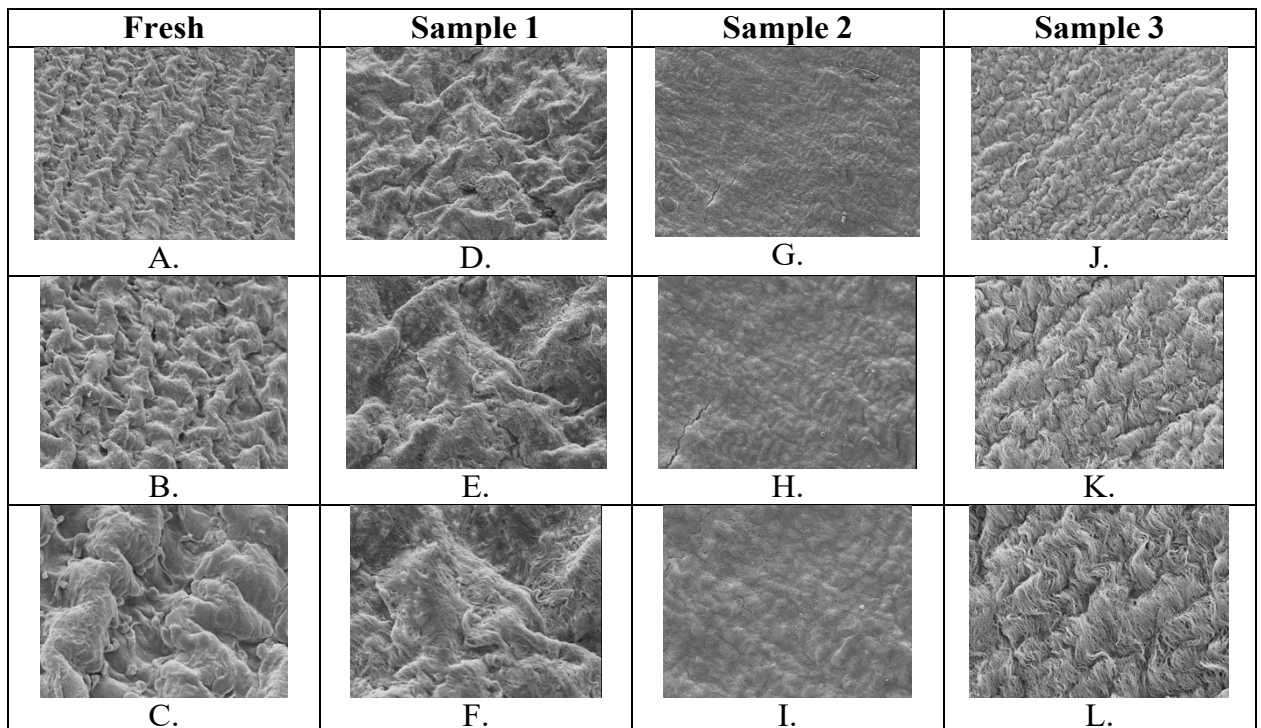
Figure 34. **SEM analysis of the luminal surface of porcine carotid vessels.** Fresh carotid artery (A-C). Decellularized carotid artery: SAMPLE 1 (D-F). SAMPLE 2 (G-I). SAMPLE 3 (J-L). The luminal surface of arteries appeared free of cellular remnants after DC (D-L). Scale bar. A, D, G, J: 50 µm. B, E, H, K: 20 µm. C, F, I, L: 10 µm

Perfusion decellularization

Effectiveness of combined decellularization approach was evaluated also for long vascular segments (6-cm piece of porcine carotid artery being used as testing model) by application of qualitative tests. The homogeneity of long vascular segments DC was improved by perfusion. To analyze if the vessels were decellularized evenly, middle and end segments of the perfusion-decellularized vessel were stained with H&E and DAPI. Additionally, the SEM analysis at decellularized matrices was performed (figure 35, 36).

On SEM analysis the luminal surface appeared free of cellular elements after decellularization, remnant debris on the luminal surface, indicating residual cellular and protein components, were not revealed. The basal lamina of treated sample appeared intact, exposed collagen fibers being visible accidentally in a few areas (figure 35).

A consistent and even elimination of cellular residues over the length of the vessel in the decellularized samples compared to native vessels was observed by basic histological staining. Elimination of nuclei and cytoplasmic particles was distinguished in treated vessels – no differences between the decellularized vessel segments (end portion *vs* middle portion) were detected when using the flow application of decellularization agents. In addition, the image showed that the vascular histoarchitecture (mainly the ECM structure) was apparently with great success preserved at the end of the decellularization process (Figure 36), suggesting satisfactory keeping of the mechanical properties of the scaffold.

These results of qualitative tests demonstrated that decellularization of long vascular segments can be achieved by perfusion-based incubation with DC chemicals with no detectable difference in cell removal between the end and middle part.

Native vessel	END_1	END_2
A.	B.	C.
D.	E.	F.
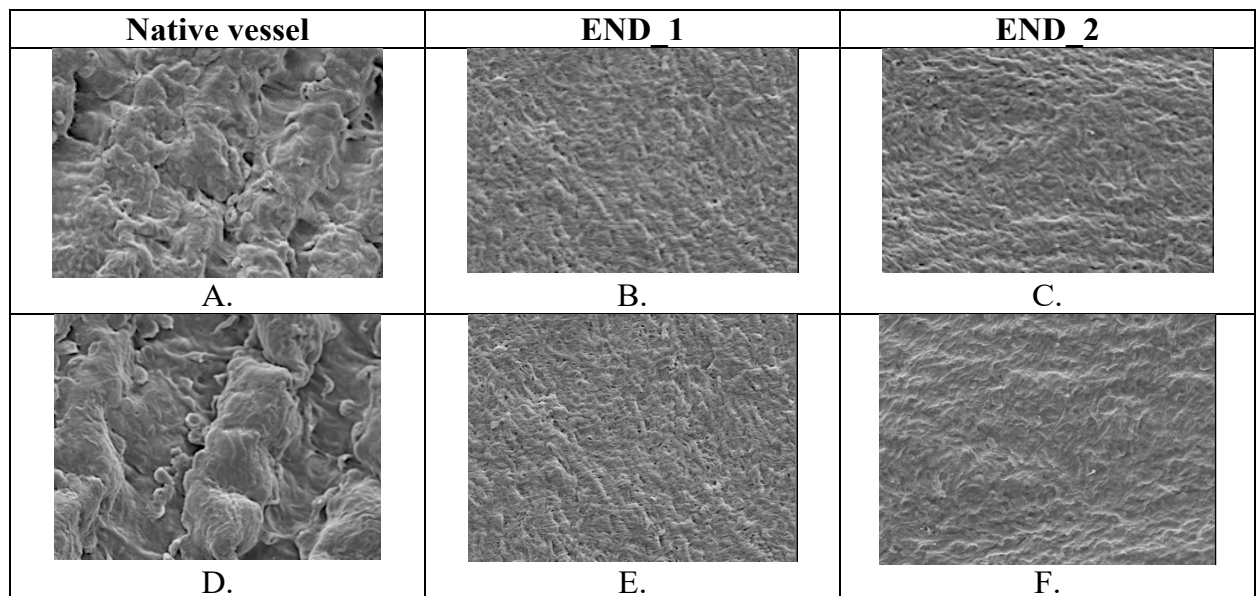
Figure 35. **Characterization of perfusion-based decellularization approach by scanning electron microscopy.** Native vessels (A, D). END_1 (B, E). END_2 (C, F). Scale bar. A-C: 20 μm. D-E: 10 μm

	Native vessel	END_1	MIDDLE	END_2
H&E	A.	B.	C.	D.
	E.	F.	G.	H.
DAPI	I.	J.	K.	L.
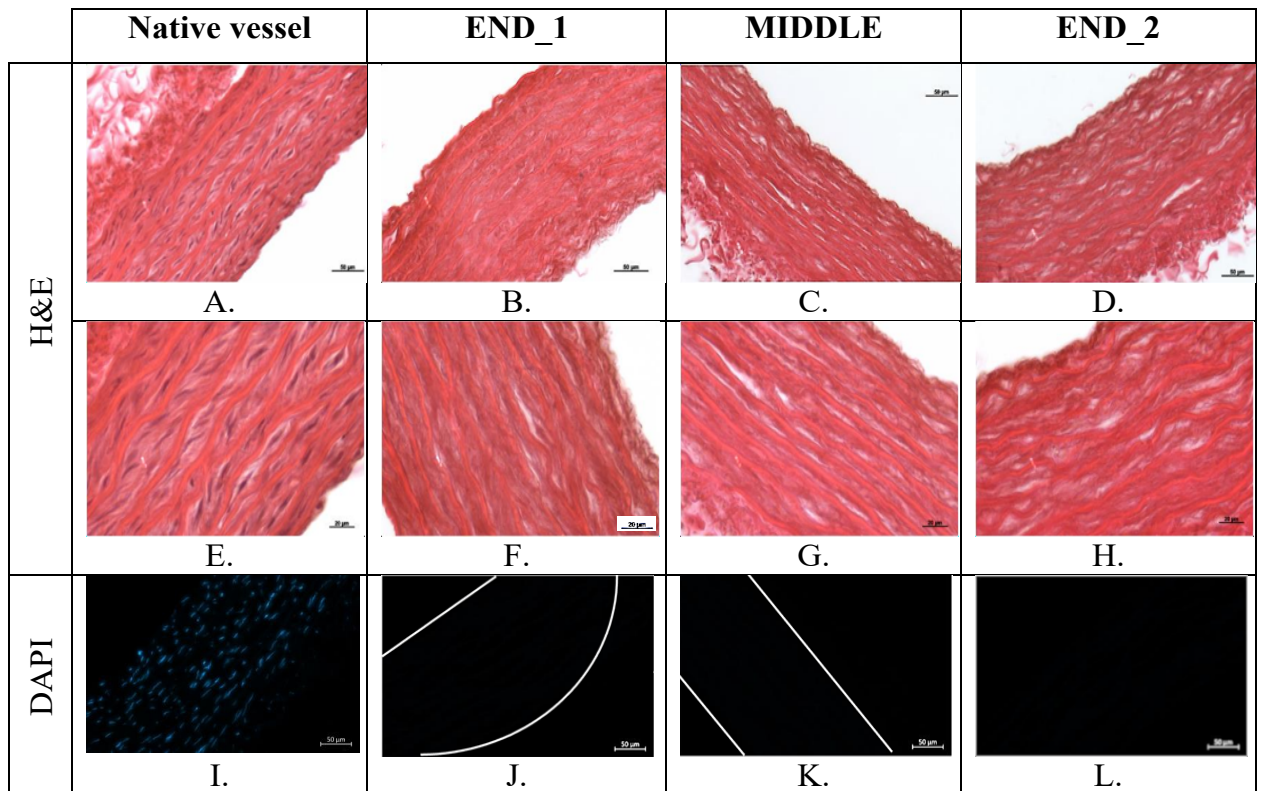
Figure 36. **Perfusion decellularization.** The efficiency of decellularization is evaluated by H&E staining (A-H, upper panel) and DAPIstaining (I-L, lower panel, nuclei are blue). Native vessels (A, B, I). END_1 (B, F, J). Middle section (C, G, K). END_2 (D, H, L). Scale bar. A-D: 50 μm; E-H: 20 μm; I-L: 50 μm

Quantitative Methods

Data are presented as mean ± SD. A student t-test analysis was performed to find statistically significant differences between the normally distributed homogeneous groups and Welch's test for normally distributed non-homogeneous values. Mann-Whitney U test was applied for non-parametric data or parametric data which do not meet normal distribution. $p < 0.05$ was considered statistically significant. and

A quantification of the nucleic acids before and after tissue digestion was performed to determine the DNA concentration in tissue, remaining DNA content being used as a numerical measure for decellularization efficacy. In this case the spectrophotometric quantification method was used according to MHH in-house protocol. DNA quantification confirmed the results from the basic staining with H&E and DAPI and showed DNA content in decellularized vessels is significantly reduced when combined decellularization protocol was applied.

DNA quantification showed that native vessels contained **23.56±4.73 μg/mg** DNA on average. Decellularization by combination of chemicals and enzymes decreased the DNA content in blood vessel' wall to **1.03±0.49 μg/mg** on average (table 5). Applied strategy allowed to reduce the DNA content to **95.6%** compared to native vessel samples (figure 37)

proving the efficiency of the established in-house protocol in terms of cell nuclei removal. A Welch's test for similarity showed that all treatment groups were significantly different compared to native vessels (p=0.0001) [224].
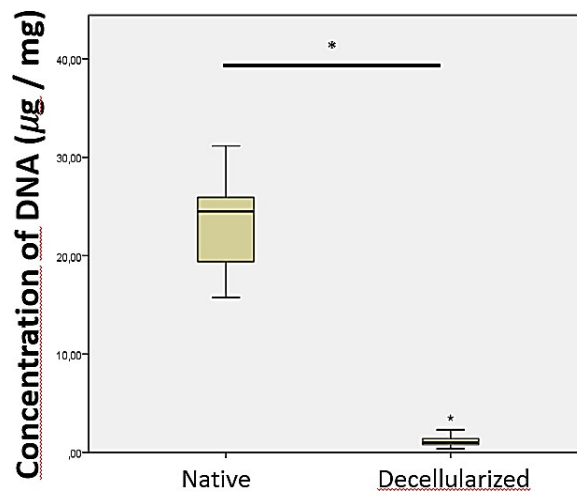
Figure 37. **DNA concentration: native *vs* decellularized samples.** DNA content is measured by fluorescent-spectrophotometric analysis with Hoechst 33528. Data is presented as boxplots with (*) indicating the significance by a Welch's test (significance is defined as p<0.05)

To observe the removal of common ECM proteins following decellularization, measurement was performed for collagen and glycosaminoglycans. Only small overall differences between controls and decellularized samples in terms of matrix composition and morphology were detected. GAGs content was significantly reduced, but hydroxyproline concentration associated with collagen as an indicator was retained after decellularization.

Quantification of GAGs revealed a decrease in GAGs content in all decellularized groups compared to controls (figure 38). GAGs quantification showed that native vessels contained **13.01±3.56 μg/mg** GAGs on average. Decellularization significantly decreased the GAG content to **1.30±0.72 μg/mg** on average (p=0.00001, table 5). The used decellularization strategy led to the GAG content drop to **90%** compared to native vessel samples (figure 39) [224]. A substantial loss of GAG content may suggest an alteration of mechanical properties of the graft by reducing its viscoelasticity and inhibit the ECs attachment on implantation or *ex vivo* reseeding.

In contrast, higher levels of hydroxyproline were observed in decellularized groups compared with native samples (figure 40). Hyp quantification showed that native vessels contained **54.26±10.68 μg/mg** Hyp on average. Decellularized vessels contained **69.70±7.60 μg/mg** Hyp on average (p=0.0744, table 5). There was **28.5%** increase in hydroxyproline content compared to native vessel samples (figure 41) [224] providing the dominant mechanical responses of tissue.
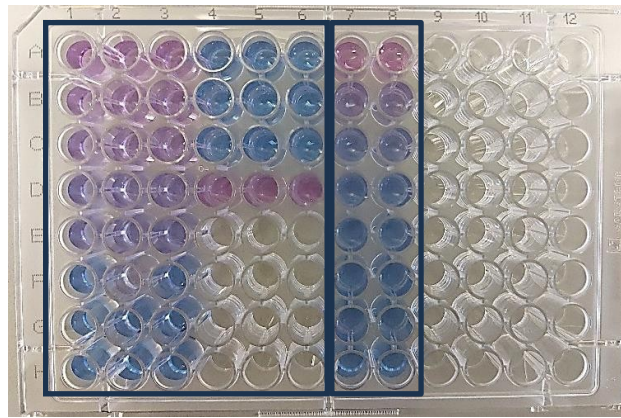
A.   B.

Figure 38. **GAG quantification microplate: experiment.**

A. Diluted study samples. B. Diluted samples to prepare of standard curve
(the GAG concentration uptodown in each well, starting from 10 µL/ml)
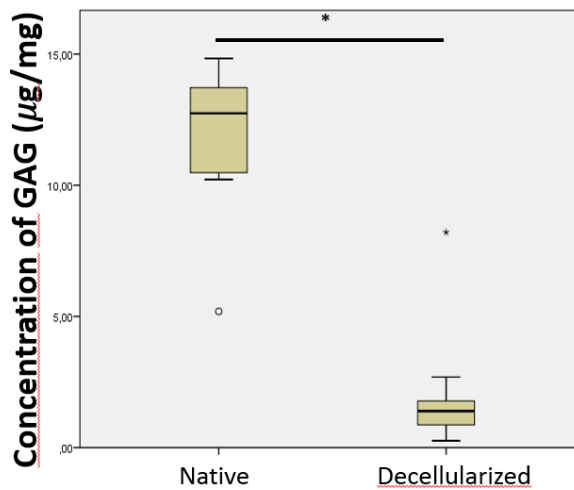
Figure 39. **GAG concentration: native *vs* decellularized porcine vessels.** GAG content is
measured by spectrophotometric analyses at 595 nm. Data is presented as boxplots with (*)
indicating the significance by a Mann-Whitney U test (significance is defined as $p<0.05$)
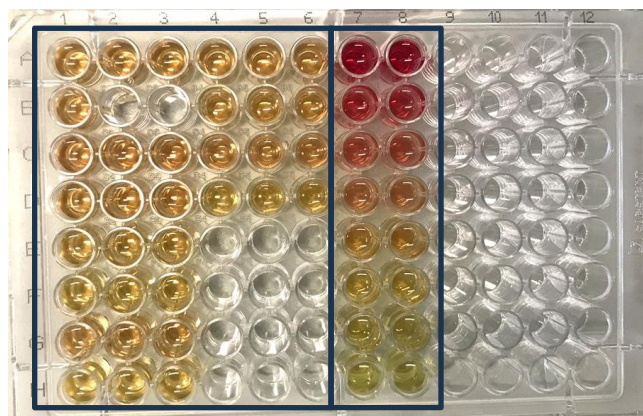
A.   B.

Figure 40. **Hydroxyproline quantification microplate: experiment.**

A. Diluted study samples. B. Diluted samples to prepare of standard curve (the
Hydroxyproline concentration up to down in each well, starting from 20 µL/ml)
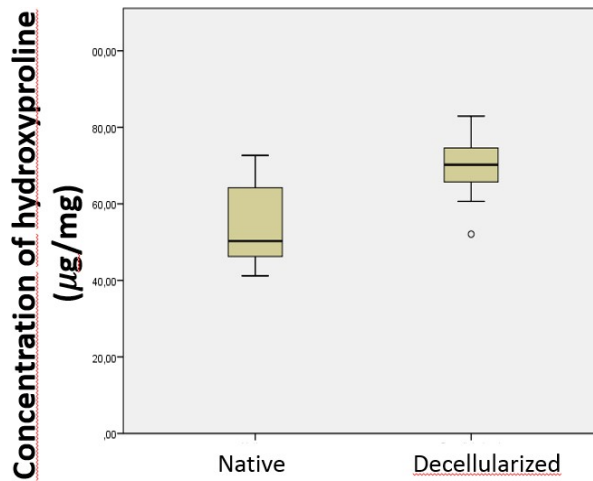
Figure 41. **Hydroxyproline concentration: native *vs* decellularized porcine vessels.**
Hydroxyproline level is measured by spectrophotometric analyses at 565 nm. Data is presented as boxplots. No significance is determined by Welch's test (significance is defined as $p < 0.05$)

Mechanical testing

Adequate initial strength is an absolute requirement for any arterial bypass graft. Decellularization of porcine vessels did not compromise the mechanical strength of the remaining matrix. 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 group were tested. To assess the effect of DC method on mechanical properties vascular fragments were strained until failure and analyzed for alterations (figure 42).
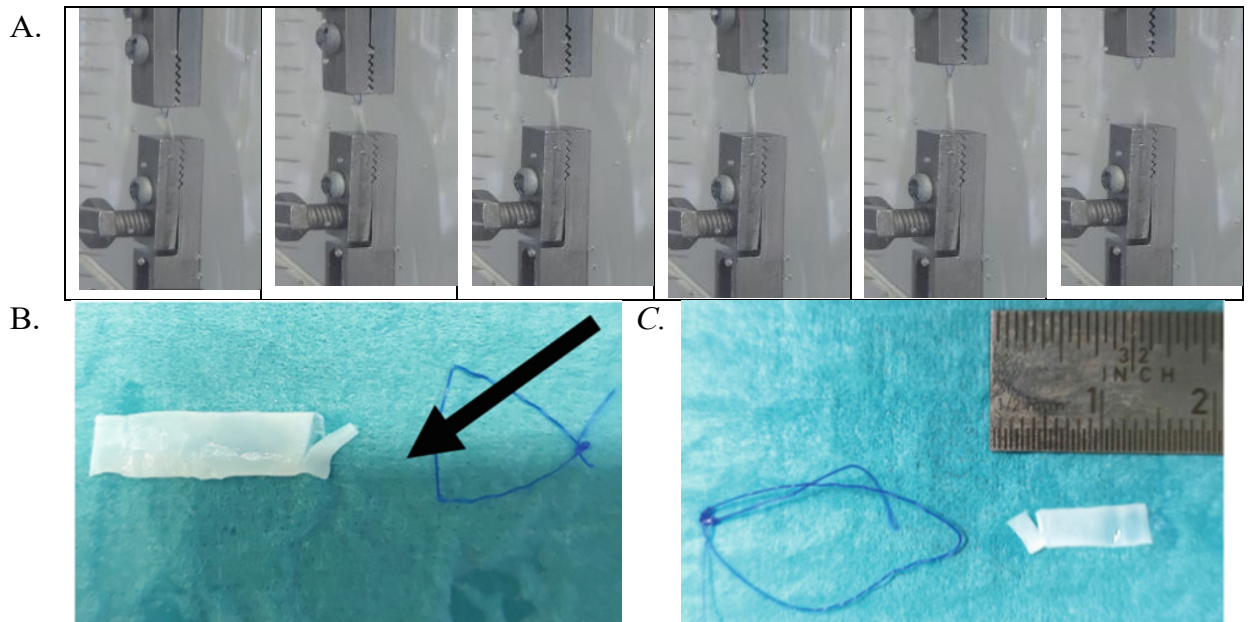
Figure 42. **Suture retention test.** (A) Time sequence of loaded sample from initial preload to near failure. Suture retention test for native (B) and decellularized (B) blood vessels

The average suture retention strength of native porcine vessels (n=9) was **1.08±0.39 N**. The average suture retention strength of decellularized vessels (n=8, table 5) was **1.14±0.38**

N. So, there was no statistically significant difference between untreated and decellularized samples (p=0.0731, figure 43). These findings suggested the graft scaffold had sufficient suture retention strength to withstand anastomotic forces [225]. From a functional standpoint, this corroborates the previous outcomes that no significant change was found in collagen morphology.
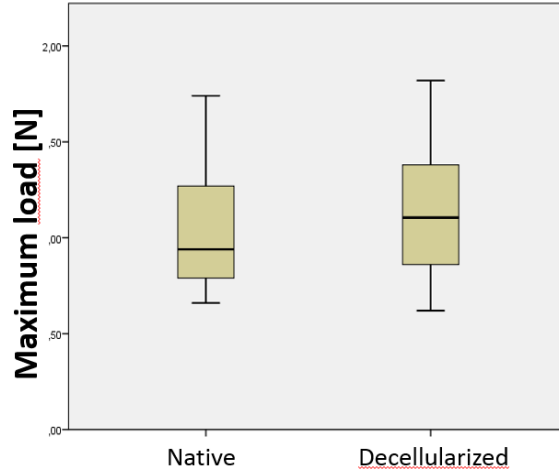
Figure 43. **Suture retention test: native *vs* decellularized samples.** Data is presented as boxplots; significance was not observed by independent t-test

Table 5. **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 scaffolds is 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 6) revealed that the combined decellularization method allowed to remove most of the SDS (figure 44, 45).

Table 6. **SDS quantification**

Type of tissue	SDS/tissue [μg/mg]										
NATIVE vessels	0.261	0.059	0.172	0.219	0.109	0.022	0.155	0.146	0.539	0.454	0.630
DC vessels	0.017	0.021	0.008	0.070	0.149	0.106	0.113	0.099	0.363	0.076	0.205	0.200
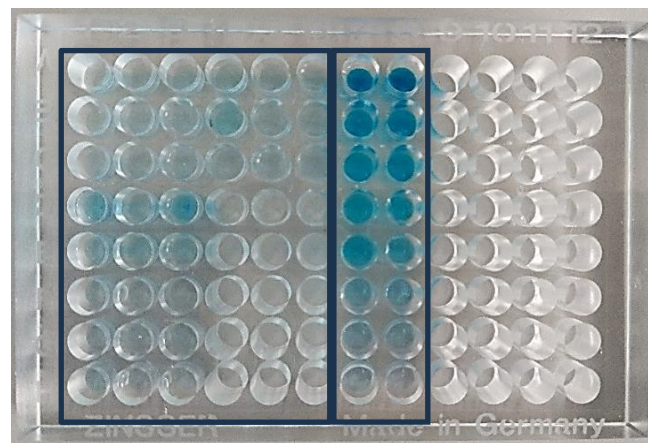
A.   B.

Figure 44. **SDS quantification microplate: experiment.**

A. Loaded wells with dilutes study samples. B. Diluted samples to prepare of standard curve starting from a known concentration of SDS (starting conc. = 200 μg/mL) that was serially dilute with 10 mM TRIS pH 7.5
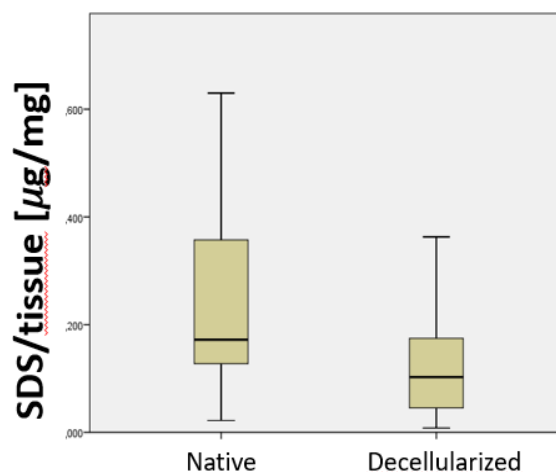
Figure 45. **SDS quantification.** Data is presented as boxplots;

Cell culture

For biocompatibility testing GFP-HUVECs were used. Cell culture procedure was described previously (Figure 46).

| A. | B. | C. |
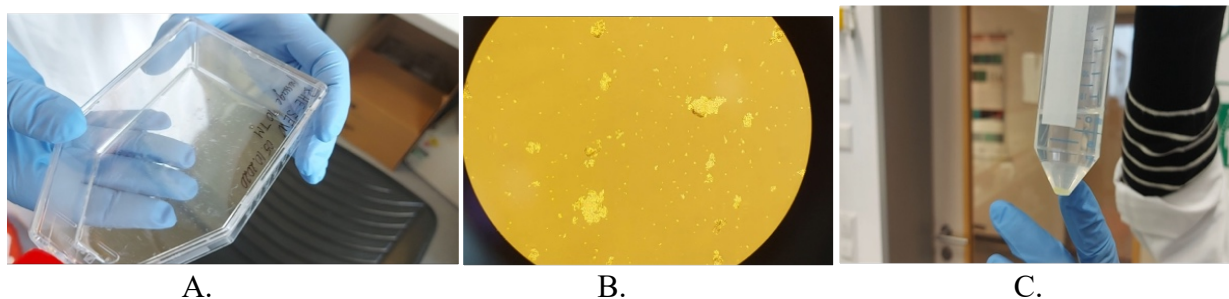
Figure 46. **Cell culture**. A, B. Cells detached from the surface of the culture flask using TrypLE Select. C. Cell pellet obtained after centrifugation

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 as previously described. All matrices retrieved after decellularization supported the attachment of HUVECs, as a confluent layer was observed after 5 days of cultivation (figure 47, 48); 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 [226].

CONTROL	SAMPLE 1
A.	B.
CONTROL	SAMPLE 2
C.	D.
CONTROL	SAMPLE 3
E.	F.

Day 6 (A, B)
Day 6 (C, D)
Day 6 (E, F)
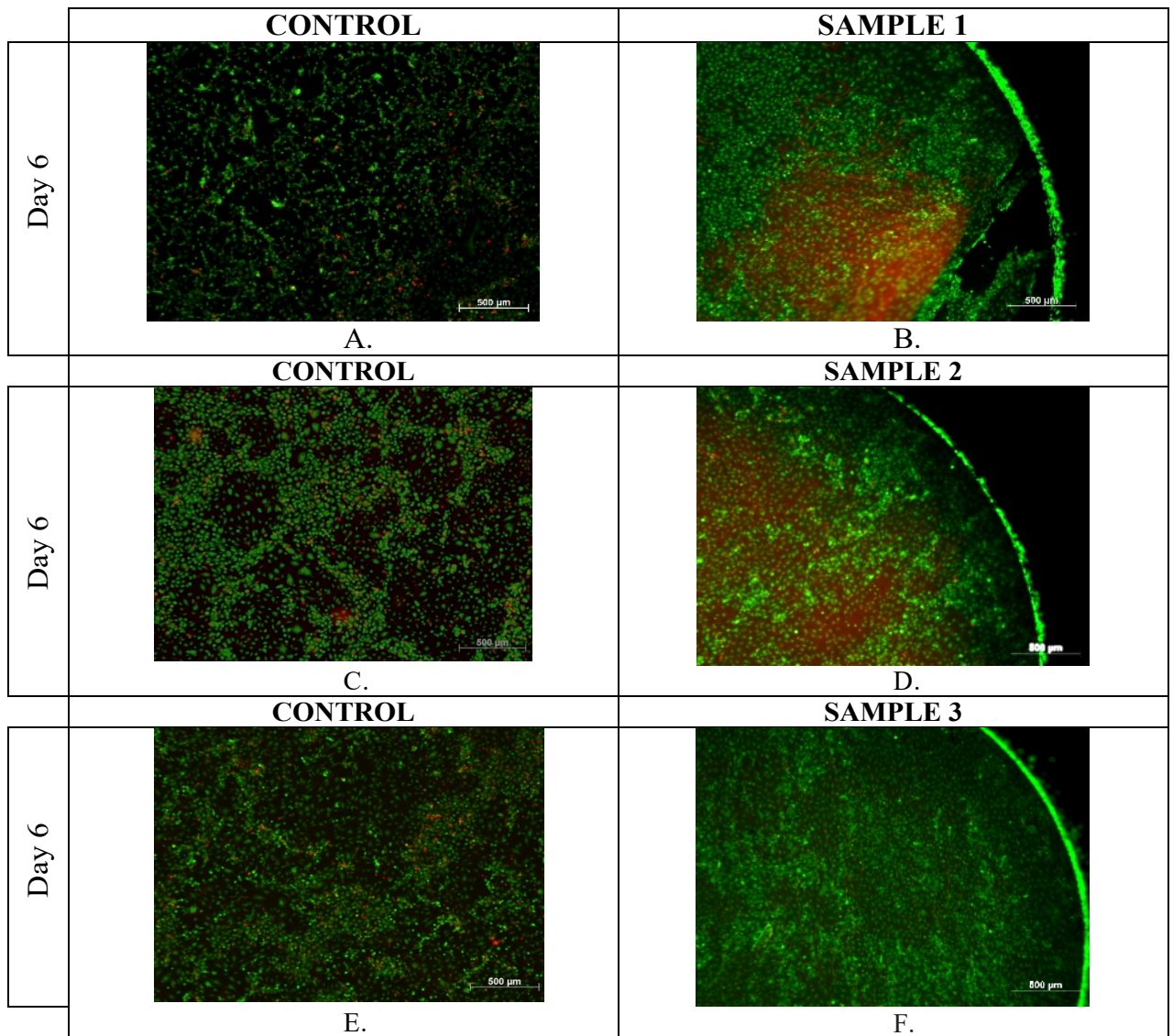
Figure 47. **Successful re-seeding of GFP-HUVECs onto decellularized porcine matrices.** 200000 cells/well seeded onto the luminal surface of matrix or plastic culture dish as control. Images were taken with a Stereomicroscope at 6th day post- seeding. Live/dead staining showed live cells evenly distributed in decellularized samples while dead cells were red (A-F). Control (A, C, E).

Decellularized carotid artery (SAMPLE 1 - B. SAMPLE 2 - D. SAMPLE 3 - F).
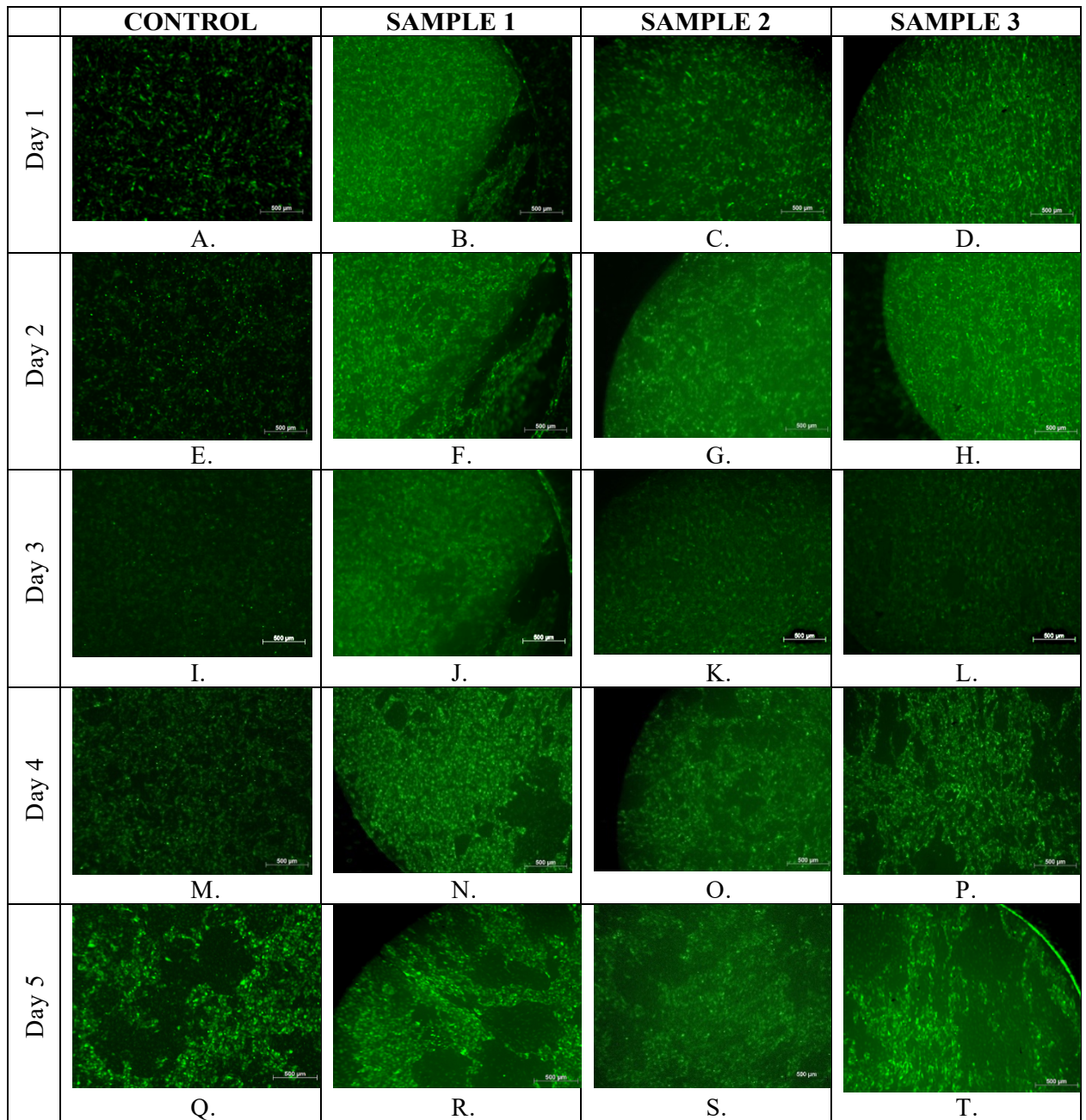
Figure 48. **Successful re-seeding of GFP-HUVECs onto decellularized porcine matrices. Day 1-5**: Cell viability evaluation by stereomicroscopy (A-T). 200000 cells/well seeded onto the luminal surface of matrix or plastic culture dish. Images were taken with a Stereomicroscope at 1st, 2nd, 3rd, 4th, and 5th. Plastic culture dish (control: A, E, I, M, Q). Decellularized carotid artery (SAMPLE 1: B, F, J, N, R. SAMPLE 2: C, G, K, O, S. SAMPLE 3: D, H, L, P, T).

4. DISCUSSIONS

Most research into vascular grafts' engineering has focused on the use of macromolecular synthetic materials. However, these materials yielded unsatisfactory long-term patency determined by thrombotic failure in small-diameter settings (<6 mm). The decellularized matrices took advantage of structure and mechanical properties, while avoiding adverse immunological reactions [83, 127, 218, 227, 228]. Defining a standardized and universal decellularization protocol for vascular tissue seems difficult given the diverse intended applications and tissue types.

The aim of the present experimental research was to investigate the effect of a novel combined in-house detergent based and detergent-enzymatic DC protocols on porcine arterial tissue (porcine aorta and porcine carotid arteries) in terms of cell removal, extracellular matrix preservation, and mechanical properties maintenance for development of acellular scaffold which could be used as a model for pre-clinical research. The morphological, biochemical, and biomechanical properties of obtained acellular scaffolds and their suitability for clinical application (maintenance of its processability properties) in bypass surgery were checked by a series of biological and mechanical tests, such as qualitative tests (determining the elimination of cellular content H&E, DAPI, and SEM; immunostaining against collagen IV), quantitative tests (thickness assessment, DNA quantification, and evaluation the preservation of ECM components, including GAGs and hydroxyproline content), biocompatibility assay (re-seeding of HUVECs onto decellularized matrices, SDS removal assay), and mechanical resistance evaluation (suturability), performed before and after treatment and compared to the native tissue with its natural structural conformation and specific chemical composition. The tested novel combined technology seems to be suitable for vascular bio-scaffold preparation.

Chemical-Based vs Combined Approach in Aorta Decellularization. Optimal Agents Combination for Vascular Tissue Decellularization. It is currently difficult to generate an ideal decellularized matrix by applying exclusively a single agent or technique [27]. Decellularization is typically accomplished by treating tissues with a combination of freeze-thawing, osmotic gradients, solvents, detergents, chelating agents, and physical methods, combinational techniques being considered the best and safest way forward to facilitate vascular tissue engineering allowing to preserve the general morphology and integrity of ECM [21, 24, 27, 31, 102, 229].

The agents that rely on tonicity as hypo- and hypertonic solutions (chemical methods) have limited utility as a sole DC method but can be applied to enhance the actions of detergents allowing to minimize the amounts of chemical agents and exposure time needed for effective cells elimination [230]. Detergent-based decellularization is the most extensively applicable approach to induce cell lysis [32, 161, 221]. The mentioned detergents play their

role in cell removal through cell degradation and realizing cell content to the environment [231]. Crapo et al. (2011) and White et al. (2017) indicated that ionic detergents, e.g., SDS and SDC, degrade the protein structure completely, by induce the breakage of elastin fibers a significant compliance alteration is registered, while non-ionic detergents, e.g., Triton X-100, maintain the proteins in their intact form [24, 181]. Fooladi et al. (2022) additionally demonstrated that collagen and GAG levels significantly decrease in tissues decellularized using SDS and CHAPS compared to native ones. Thus, using a combination of anionic or non-ionic detergents, as SDS and Triton X-100, would be a promising approach toward obtaining a suitable decellularized scaffold, which not only removes most tissue cells, but also maintains the natural tissue structure [231]. This approach has been successfully used till now to produce decellularized vascular grafts [15, 33].

Nucleases and other types of DNA degrading enzymes are used in combination with detergent-based approach to guarantee the removal of DNA from the scaffold [36, 217]. The appreciable effect of additional DNase use in combination with detergents for DC vascular tissue and complete DNA remnants extraction was demonstrated already by Koenig et al. (2019) for porcine aorta DC [22], Gui et al. (2009), and Row et al. (2017) for development of functional small-diameter vessels [232, 233]. Kajbafzadeh et al. (2018) could demonstrate successful DC of human mammary artery is not possible exclusively using detergents without enzymes as DNase [234]. An extra example from a recent thesis presented successful DC of porcine vena cava created by using a combinational approach (detergents: SDS, SDC, Triton X-100, CHAPS, and DNase) along with immersive/agitative-based incubation [21, 235]. Enzymatic treatment following detergent application for removal of cellular material can be also helpful, but negative effects on the ECM must be monitored [177]. Combined DC protocols seem to be efficient for successful vascular tissue decellularization to avoid single side effects for only one agent [83]. However, because of the experimental conditions' differences, the comparisons of the protocols described by different research groups are difficult [21].

To establish a protocol for the DC of porcine aorta as a prototype of large-diameter blood vessels two methods were compared: chemical-based *vs* multilateral approach where detergent wash was supplemented with enzymatic treatment; in both cases the process was carried out under rotation. Osmotic conditioning, having little impact on the ECM, was used as the initial step of the decellularization treatments to promote cell lysis through the stimulation of uncontrolled cellular swelling [236]. Hence, it was decided to combine non-ionic detergent (Triton X-100) with a low concentration of anionic detergents, namely hypotonic SDS and SDC (it was reported previously that hypotonic SDS and SDC is more effective than hypertonic or isotonic SDS solution [237]). It is recommended to avoid tissue exposure to high

concentrations of detergents, specifically SDS, because they have destructive effect to the ECM induced by denaturation of the matrix proteins and their total content decrease [238-240]. In addition, increasing SDS concentration exacerbates problems with poor cellular repopulation *in vivo* [5] on implantation.

The efficacy to remove cellular and nuclear material was tested by qualitative tests, namely, H&E and DAPI staining were performed. Compared with the chemical-based method were a DAPI positive smear was detected indicative of DNA remnants, only combined chemical-enzymatic technique with additional enzyme treatment resulted in a consistent and even elimination of cellular DNA and cells' remnants (figure 24). This finding suggests that further treatment with nucleases is required, this is in accordance with data from previous studies. In this analysis digestion with DNase reduced the DNA content, compared to native vessels, and DNA fragments were visually absent on DAPI staining in treated groups.

H&E-stained aortic samples did not match with the DAPI observations – the absence of nuclei in H&E staining pictures did not correlate with DAPI results. When comparing the staining as informativeness, nuclear remnants were clearly visualized by DAPI staining (no faiding of fluorescence) in the samples that were not treated by DNase, which were not readily visualized by H&E staining (figure 24). Thereby, H&E staining was found to be less sensitive than DAPI staining when analyzing cellular remnants in decellularized tissues. This finding suggest that H&E staining cannot be used as a sole proof of decellularization and should be supplemented, at least, with a DNA stain like DNA. Koenig et al. (2019) also reported that H&E staining was not a reliable method to characterize comprehensively decellularization efficiency. Taking into consideration this inconsistency between DAPI staining and H&E, a wide and multilateral quality control of decellularized tissue is required to be done, supplemented with quantitative assay as DNA quantification. Nevertheless, H&E staining revealed a well-maintained structure of the vessel with preserved and organized laminae, it is useful because it allows to do a preliminary screening of obtained decellularization matrix [22].

Evaluation the Decellularization Protocol Efficiency Depending on the Vessel Diameter. The choice of a porcine aorta or carotid artery as testing model is not surprising, based on widespread availability and relative size in comparison to human vascular tissue [241].

Reviewing the specialized literature, data including tissue thickness as DC factor efficiency have not been found so far. Experiment's results demonstrate that large-diameter blood vessels require an extended processing exposure than small-diameter blood vessels (Figure 25); no common decellularization protocol can be recommended. Further research is

necessary in the field to determine the optimal proper ratio tissue volume / decellularization solution volume / exposure duration.

Decellularization of Fresh vs Frozen Porcine Carotid Arteries. It seems the applied DC protocol is efficient in decellularization both native (fresh) and frozen tissue samples (Figure 26, 27). It is supposed that freezing step may damage the vessels – the fact that has not been proven in our experiments. However, to minimize the impact of ice crystals' formation impact on matrix preservation and its functionality while freezing, it is recommended to avoid long-term cry storage and repeated cycles of freeze-thaw.

Evaluation of Ultrasound Application for the Blood Vessels Decellularization. Azhim et al. (2011) demonstrated for the first time that using ultrasound (low frequency, high power) in combination with strong ionic detergent as SDS *vs* typical DC process (immersion or shaking with the same chemical solution) resulted in significant increase of decellularization efficiency and providing an effective short-term DC protocol for vascular tissue. The effect could be attributed to the cavitation effects which may facilitate the detergent penetration through the tissue [195].

Sonication has been shown to be a viable method for improving the quality of decellularization process. Results from a recent study demonstrated that sonication significantly increased the depth of decellularization agent penetration into the tissue, allowing quicker and easier removal of cells from scaffold in comparison to immersion treatment which could not achieve this effect using the same exposure time. This provides evidence that sonication is a promising method to be applied in tissue engineering [242-244]. In a second study the biocompatibility of obtained scaffold was demonstrated by confirmation of successful vascular smooth muscle cells adherence to it and cells infiltration into the tissues [245]. The same working group reported in 2019 the development of a closed sonication DC system utilizing a strong anionic detergent at different concentrations (0.1% or 2% SDS) for preparing acellular aortic scaffolds [246].

Other reports in the field demonstrated the efficiency of ultrasound application in combination with other chemicals in larynx [247], meniscus, and kidney DC [248-250]. Oliveira et al. (2013) showed the ability of ultrasound to effectively DC small intestine [251]. However, the report by Tchoukalova et al. (2018) did not confirm the efficiency of sonication combined with detergents (4% sodium deoxycholate) in washout of cells from the cartilaginous tissue [252]. The local expert group did not prove the utility of ultrasound application in tissue decellularization also, namely, it was found to be inefficient in amniotic membrane decellularization *vs* static treatment [253].

In the current study, the vascular tissue structure seemed to be significantly affected by high amplitude ultrasonic waves (figure 28). The negative impact of long exposure to high

US settings can be explained by prolonged subjection to the cavitation effect when excessive sonication is applied and microbubbles action that can puncture and destroy the matrix [188]. The same results were reported previously [242, 243, 248, 254-256]. Furthermore, it is recommended to use US carefully and with adequate limit to prevent damaging the tissue and altering the properties of the resulted post-procedural scaffold [18].

In addition, in the current experiment it was not possible to obtain proper DC by treating porcine vessels with ultrasonic waves too even in combination with a detergent (Figure 29). As Triton X-100 is considered a weak chemical, this might explain the inefficiency of the treatment compared to other studies. Moreover, Fooladi et al. (2022) also demonstrated that the DNA content in tissues decellularized using Triton X-100 is significantly higher than that in the other acellular groups [231]. To investigate if sonication may offer permeabilization of the matrix and easier access of the chemicals to the deep layers, it is suggested to apply stronger detergents such as SDS or SDC in the experimental activities.

Characterization of Decellularized Porcine Matrices. Complex characterization of the decellularized matrix is important for verifying the efficiency of the decellularization technique in terms of cytoplasmic and nuclear residue wash out and its safety with regards to recellularization ability. Better observing the effectiveness of decellularization implies a multilateral approach, including evaluation the degree of cellular removal and assessment the quality of the resulting matrix, because each test has its own limitations that should be taken into consideration [175, 221].

Qualitative Methods provide a visual representation of the integrity of the remaining scaffold and allow to identify any remaining cellular materials responsible for immune and inflammatory reactions post-implantation and specific ECM components. Each procedure, histological or immunohistochemical, focuses on a different property or aspect of the vessel structure; therefore, a wider variety of a qualitative procedures is used a better interpretation of the remaining matrix is obtained. Till now no standardized practices to characterize decellularization process is developed and implemented in experimental research.

H&E and DAPI Staining. Incomplete elimination of acellular antigens might lead to inflammatory reactions and therefore unsatisfactory short- or long-term results in clinical use [257]. Various microscopic and histological staining techniques are employed in practice till now to compare the characteristics between experimental decellularized scaffolds and fresh vascular controls [127]. Gilbert et al. (2006) recommended histological examinations such as H&E staining for initial evaluation of decellularized samples [113], and DAPI staining for detection the DNA presence [73].

To establish the current decellularization protocol, H&E and DAPI staining methods were used as independent preliminary screening tools to evaluate decellularization efficiency.

This is one of the mandatory evaluation parameters of DC efficiency set by Crapo et al. (2011) [24]. The histological inspection (H&E staining) of non-treated and blood vessels decellularized with the current DC protocol confirmed successful cell removing and revealed a well-maintained structure of the vessel with preserved and organized laminae (Figure 30, A1.1, A1.2). This condition was confirmed by DAPI analysis too. According to the DAPI staining, faiding of fluorescence were only visible in non-treated tissue (Figure 31, A2.1, A2.2).

Because of the removal of cellular elements from the vascular wall shown by means of histological staining there was detected a carotid wall thickness reduction (**23.2%**) compared to native arteries (Table 4). Probably, this variation is mainly due to the removal of smooth muscle cell from the tunica media. An overall decrease of vessel wall thickness in decellularized groups is likely to happen due to loss of some ECM components, such as GAGs (demonstrated additionally by spectrophotometric evaluation) also. However, the significant decrease of the wall thickness does not mandatory indicate alteration in the vascular graft function and mechanical resistance of the scaffold, and other tests are necessary.

Basement Membrane Evaluation: Immunohistochemistry, SEM Analysis, and Biocompatibility Assessment. The basal lamina is thought to be important for tissue physiology since it contains ligands responsible for a proper firm endothelial cells' attachment, retention, migration, growth and differentiation, biocompatibility of scaffolds, fostering future repopulation, immune response, and avoidance the early thrombi formation [35, 21]. In addition, elastic membrane plays the role of a physical barrier hindering the colonization of vascular smooth muscle cells into the scaffold's lumen, leading to intimal hyperplasia. Yu et al. (2019) showed that a luminal coating of the synthetically constructed vascular scaffolds mimicking the basal lamina, resulted in better endothelial cells fixation, growth, proliferation, and survival *vs* those without luminal coating [258].

Examination of the basement membrane demonstrated that combined decellularization approach did not significantly alter morphology. Collagen is the key protein factor of the decellularized vascular matrix responsible for vessel strength and remodeling *in vivo* post-implantation. Immunohistochemical staining (figure 34) revealed no significant evidence of alteration in collagen type IV distribution in matrix when considering the quality of decellularized carotids with combined protocol as compared to native samples, suggesting good biocompatibility of the implant material.

SEM allows the topology of a surface to be visualized on the nanoscale. Scanning electron microscopy is used to properly evaluate the quality of decellularized lumen, namely the degree of basal lamina preservation, critical for endothelial cells adhesion, resistance to thrombus formation, and atherogenesis prevention, and efficiency of cell removal, the balance

between those two being crucial [10, 22].

No cellular debris was visible from SEM analysis of decellularized carotid arteries, indicating successful decellularization. However, the basal lamina was partially fragmented, with collagen network visible in some areas of the luminal surface (figure 33). Nevertheless, it did not negatively affect the scaffold biocompatibility [223].

The utility of SEM photographs as qualitative tool for decellularization assessment was demonstrated by other researchers too. For instance, Meiring et al. (2017) examined by SEM the surface area of treated vascular specimens (baboons' arteries, namely carotid, radial, and femoral arteries) to clearly demonstrate the difference between normal and decellularized tissue in terms of cellularity. Additionally, SEM revealed the intact and integrous basal membrane and allowed to appraise the effects of re-endothelization, the decellularized scaffold being seeded with viable HUVECs forming complete confluent monolayer after seven days of cultivation [259].

In this study, ***biocompatibility*** was achieved and tested supplementary by performing *in vitro* contact test (figure 47, 48). The results of the test indirectly prove satisfactory preservation of basal lamina allowing for initial cell adherence direct after seeding, proliferation, and migration. As the cells matured, they probably start to secrete their own basal lamina which allowed them to form a confluent cell layer.

Additional assessment methods might be considered to ensure that a basal lamina is truly present; overview of the morphology of the luminal surface is not sufficient. Immunohistochemical staining for additional basal lamina proteins, e.g., laminin or fibronectin, are recommended for this purpose [55, 80, 88].

Perfusion Decellularization. In the recent years, the evaluation of perfusion-based protocols revealed the enormous potential of flow-dependent DC treatment. Fluid dynamics influence the surface contact and physical force of reagents to the tissue [34]. Perfusion regimens allow to enhance the surface contact and homogeneity of the detergent-tissue interface [7, 255]. Perfusion decellularization solution throughout the vasculature of tissues in the standard method to decellularize whole organs because it allows the constant decellularization solution distribution to deep areas [260].

With respect to vessels, perfusion allows the distribution of the decellularization solution to the center of the tubular structure, otherwise be difficult to reach [217]. In addition, the constant influx of fresh decellularization reagents allowed the reactants and degradation products to be constantly flushed out, improving the efficiency of the process in this way.

Previous studies reported that the rate of perfusion through the vasculature affects the efficiency of decellularization and important effect on mechanical properties of obtained decellularized scaffold are registered [131, 145]. Daugs et al. (2017) showed that perfusion at higher rates (300L/h) resulted in a better removal of cellular material that can be achieved in

a shorter amount of time [145]. A further illustration in this field showed that the perfusion method was superior for cell removal compared to the static immersion – creating submillimeter-diameter vascular scaffolds by decellularizing rat tail arteries using a combination of 0,3% isotonic peracetic acid and DNase I [261].

In this study, decellularization of vessels (6.0 cm in length) was realized at a perfusion speed of 25 mL/h. It was confirmed through middle and ends staining of porcine vessels, the performed qualitative assessment (H&E, DAPI, SEM) demonstrating the complete removal of any cellular debris (figure 35, 36).

Quantitative Methods. Depending on the applied decellularization approach, as reagents type (hypo- or hypertonic buffers, ionic and non-ionic detergents, proteases, and nucleases), their concentrations, and incubation times – they all may affect scaffold with respect to their degree of acellularity and the chemical and biomechanical properties of obtained 3D structure [74]. Quantitative methods allow to develop valuable insights about the DC process revealing correlations and trends relevant to stakeholders.

DNA Quantification. One of the main goals of decellularization is to remove antigenic components that may cause an adverse immune reaction upon implantation. Methods for a quantitative assessment of remaining DNA have been implemented in practice till know; even limitations in this field still exist and no standardization procedure is chosen yet [73]. It is assumed that DNA removal is indicative for antigen and cellular proteins removal. The current standard is based on DNA content based on dry weight [262].

In this study, DNA was quantified by a fluorescence analysis using Hoechst 33528. This method measures dsDNA with detection levels as low as 10 pg/µl [116]. However, Sedlackova et al. (2013) demonstrated DNA content may not be correctly reflected by the Hoechst method because it underestimates genomic dsDNA that have size <23 kpb [263].

Quantification showed that decellularization resulted in very low amount of DNA with an average of 1.03±0.49 µg/ng dry weight and a 95.6% decrease *vs* to non-treated vascular samples (figure 37, table 5). This finding corresponded with histological image analysis sections (figure 30, 31). However, by now it is unclear whether this few residual DNA fragments could elicit or not an adverse response from the host [264], for instance, many commercial products with positive clinical outcomes contain remnant DNA and no negative any host response was reported till now [73].

The quantification of DNA did not fall within the range of parameters set by Crapo et al. (2011) [24]. Nevertheless, Eyre (2019) could demonstrate even DNA remnants are still present in the scaffold, this DNA present only as very small fragments (<200 bp), fulfilling one of the criteria established by Crapo et al. (2011) previously [24, 217]. In addition, it is still under discussion and no confirmation data is available whether DNA itself is immunogenic

and a few remnants may negatively affect the biocompatibility of the scaffold upon implantation [264]. Contra dictionary, many studied have shown that efficiently decellularized matrices may remain immunogenic due to the presence of xenoantigens [24]. In addition, Gui et al. (2010) demonstrated that removal of DNA does not mandatory correlate with removal of cellular proteins, as β-actin: although DNA was efficiently removed in their study, the cellular protein was still present [265]. It indicates that analysis of DNA only is insufficient for characterizing of decellularization process and a complex post-decellularization characterization should be applied. More accurate results may be achieved by complementary testing using Polymerase Chain Reaction.

Collagen and GAGs Quantification. If one of the main goals of decellularization is to remove antigenic components that may cause on implantation an adverse immune reaction, preservation of ECM constitution is the second major demand of great importance in DC procedures due to their vital necessity for adequate biological behavior (function as a scaffold), cell signaling, and mechanical stability. Alterations in ECM composition inhibits scaffold repopulation and affect the scaffold durability in time [18, 21, 153].

The concentration and structure of ECM proteins will change after decellularization [266]. Collagen, GAG, elastin, fibronectin, and laminin are the most prominent proteins present in the ECM, all the components of the vascular wall being closely related to arterial biomechanics and vascular integrity [74]. Therefore, quantification of these proteins is an indicator for the remaining matrix integrity after decellularization.

Fibers of elastin and collagen mainly distributed in tunica media. Elastic and collagen fibers are responsible for the elastic behavior of blood vessels, their tensile strength, and stiffness [74, 113, 161, 267, 268]. GAGs found in the blood vessel wall as another key component contributes to biomechanical, biological and viscoelastic properties of the extracellular matrix, asretarding the rate of deformation of the collagen network when stress is applied suddenly (tolerance of pulsatile blood pressure), provide binding of many bioactive molecules as growth factors and cytokines in the ECM, and regulates water holding in tissues and diminish calcification [74, 113, 161, 231, 267, 269, 270]. Additionally, ECM proteins play a role in interacting with cells by affecting the cellular behaviors through signaling factors to support their adhesion, proliferation, migration, differentiation, and degradation [231].

Different chemicals used for DC may cause significant alteration of the scaffolds' components [160]. Biochemical assays are available to confirm that desirable components of the ECM in the treated scaffold [24].

Since GAGs are in both cells and ECM, it is expected that decellularization will lead to some decrease in GAG content. GAG quantification indicated preservation of GAG content after DC. The dimethyl methylene blue (DMMB) assay was used to quantify remaining GAGs

in the matrix after decellularization. It works on the property of metachromasia phenomenon: a color change from blue to pink, determined interacting of the positively charged thiazine molecule in the dye with the negatively charged components on the GAG molecules [271].

In this study, quantification of GAG content showed a reduction of GAG in all decellularized groups *vs* non-treated vessels (figure 39, table 5). GAGs are abundant on cell membranes and part of the GAG loss may thus be related to cell removal in the DC process, but it is unclear whether retention of GAG is critical for the biocompatibility of decellularized scaffolds and how much tensile stresses resistance is affected without the presence of GAG and at what GAG loss value significantly alter the mechanical integrity of the matrix.

Collagen retention is critical for the biocompatibility of decellularized scaffolds. A major and unique component of collagen is hydroxyproline. This amino acid is quantified by its reaction with p-dimethylaminobenzaldehyde forming a chromophore. Hydroxyproline abundance is assumed to be directly correlated to the abundance of collagen in the tissue [272, 273].

Higher levels of hydroxyproline were observed in decellularized samples *vs* non-decellularized samples (figure 41, table 5). As the hydroxyproline content is calculated relative to the dry weight of the tissue – and decellularized tissue lack cellular content - retention of collagen fibers would result in a higher amount-to-mass ration (concentration) in decellularized samples compared to native samples (this result is due to the increased relative percentage of ECM to total weight after cell removal).

Mechanical testing. Engineered vessels should contain sufficient tensile strength to provide mechanical resistance to tension, sufficient elastic support to provide recoil, and viscoelastic strength to maintain a resistance to constant shear flow and strain. The modification in the concentration and structure of ECM proteins may lead to changes in the mechanical properties of the scaffold [266]. In order to evaluate the variations caused by the decellularization process that should be effectively studied prior to clinical use, several mechanical properties of the potential blood vessels substitutes should be tested in order to betterunderstand their acute functionality and functionality over time, including compliance, Young's modulus (measuring the stiffness), burst pressure, circumferential ultimate stress, biaxial tensile tests (measuring anisotropic characteristics), and creep test (or stress-relation test, measuring theviscoelastic properties over time) [162]. Mechanical testing of ECM after treatment provides insight into the presence and integrity of the structural proteins within the scaffold [49]. Preservation of good biomechanical properties are important meet the minimum required value necessary for intraprocedural manipulations and to avoid aneurysms formation post-implantation [274].

Pellegata et al. (2013) tested the mechanical properties of porcine arteries decellularized with deoxycholate solution and treated with DNase I. Among all mechanical tests performed for decellularized tissue and native samples inclusive compliance and Young's moduli they found statistically significant differences for only two properties; lateral ultimate strain and stressrelaxation [10]. In conclusion, the studies experimentally showed the various chemical decellularization treatments do not significantly affect the mechanical properties of the decellularized matrices [10, 162]. Abousleiman et al. (2008) tested SDS-decellularized human umbilical veins, and determined the elastic moduli for decellularized and native vessels were similar [275].

However, discrepancies among the results reported in the literature (various studies reported changes in the mechanical properties of decellularized tissues) exist and the data are hardly comparable because of the different testing models applied and the diverse setting parameters.

As a measure of scaffold strength and stability it was decided to evaluate matrix suture strength – no significant and detrimental effect on the stability of decellularized matrix was fixed (figure 43, table 5). Suture retention strength did not alter in comparison to native blood vessels. We claim no differences between native and decellularized arteries; however, the reference native vessel on our study were stored at -80ºC prior to characterization, a condition that can alter SMCs functionality. These findings corroborate the results presented by Williams et al. (2009) [272] (no statistically significant differences were found in defrozen/native comparisons) and Pellegata et al. (2013) [10] (no statistically significant differences were found in both native/defrozen and defrozen/decellularized comparisons).

Evaluating the Washing Method to Remove Residual Detergents. Most current DC protocols are detergent based. With the increasing popularity of utilization of biological scaffolds obtained by decellularization, it is essential to understand the effects of chemical agents' remnants on cell viability during the recellularization process. Despite numerous studies being conducted, only a limited number have investigated thecytotoxic effects of DC agents on cell viability [180, 231, 273]. However, prior to human use it is indispensable to determine whether a given implant material has potential cytotoxic properties to living cells or may cause undesirable host response *in vitro* post-implantation.

Consequently, intermittent washing periods are included in all currently used protocols as terminal process step to prevent adverse effects of DC agents and increase DC efficiency [18, 74, 267, 238, 276]. For instance, Pu et al. (2008) could demonstrate that cytotoxic effect of detergents may be reduced by increasing the number of wash cycles [179]. Perea-Gil et al. (2015), for example, described successful myocardial DC protocol with extensive washing between two periods of detergent exposure [277]. The intermittent cyclic washing period was

applied to remove any residual agents as well as detritus, additionally facilitating further penetration of fresh agents and maximizing the efficiency of DC procedure. By using repeated cycles of DC and washing steps the penetration depth increased up to 43% ($p<0.001$), providing the cyclic treatments were significantly more effective than continuous incubation [18].

SDS, commonly used in DC protocols, is recommended for DC because of its highly efficient in removal of cellular and genetic components and debris; however, it is cytotoxic and damages tissues and organs' structure and their mechanical properties when used in high concentrations – using low concentrations being recommended to overcome the potential drawbacks. Removing the residual SDS is one of the critical problems in decellularization method to reduce its adverse effects and promote cellular ingrowth after implantation. For instance, according to Zvarova et al. (2018) endothelial cells have a very high susceptibility even to low concentrations of SDS detergent [278]. The importance of SDS residual monitoring in decellularized matrices was demonstrated by Massaro et al. (2022) also [267]. As a result, efforts are needed to ensure its careful removal from scaffolds at the end of decellularization to ensure the viability of reseed cells and cellular ingrowth [27].

Removal of detergents, especially anionic detergents, from proteins and large acellular scaffolds is known to be a difficult task, determined by its strong interaction with ECM proteins, but mandatory. Referring to Cebotari et al. [185] it is demonstrated that a restricted number of washing cycles after SDS-assisted decellularization induces a cytotoxic effect on endothelial cells. It was stated that Triton X-100, a non-ionic agent, can be helpful for SDS removal and was supposed to be more efficient than PBS washing used in many studies [105, 279, 280].

How does Triton X-100 act to remove/replace SDS? Detergents like SDS occur in micelles, that is aggregates where the hydrophobic tail points inward and the hydrophilic head group points out, to interact with water. If you add another detergent like Triton, its molecules intercalate into these micelles and with increased concentration replace the SDS [21].

Recent advances in tissue engineering have enabled the development of three-dimensional scaffold that can be populated with cells to create functional tissues. However, the success of these scaffolds depends on their ability to provide an environment conductive to cells attachment, proliferation, and remodeling of ECM. To achieve this, it is crucial to ensure that SDS residues are completely removed, allowing successful cells integration [280, 281].

Chloroform extraction and quantification of SDS complexes were performed. Methylene blue (MB)-based detergent assay was applied. There was no cytotoxic effect related to detergent-enzyme DC protocol (Figure 45, table 6).

Biocompatibility Assay: in vitro cytocompatibility by contact test with cells. Requirement for ideal vascular replacement is biocompatibility. Biocompatibility of engineered scaffolds is important for further clinical usage. Decellularized biological scaffolds have the potential to revolutionize vascular restore and restoration. By supporting endothelial cell growth, decellularized biological scaffolds can reduce thrombogenicity and restore vascular function in a safe and efficient manner [267, 282]. The use of such scaffolds could potentially reduce the surgery impassivity and improve patient's outcomes while also reducing costs associated with healthcare delivery [267, 281].

In addition, a continuous lining of endothelial cells on the luminal surface of tissue-engineered blood vessels seem to be essential for avoidance the intimal hyperplasia and graft occlusion [31]. *In vitro* it has been shown that decellularized biological scaffolds can support endothelial cell adhesion and growth [35, 134, 232, 245, 283-285].

Therefore, it has been necessary to check the ability of the decellularized carotids to bear cellular adhesion. The *in vitro* contact test with cells demonstrated all scaffolds retrieved after decellularization allowed HUVECs survival and supported the formation of a confluent GFP-HUVEC layer on the luminal surface (figure 47, 48). Previous studies have also found that decellularization with combined detergent-enzyme DC protocol supports *in vitro* subsequent adhesion, growth, and function of cells onto the remaining matrix [185, 245, 286, 287]. These findings support the usage of combined detergent-enzymatic approach as a decellularization strategy.

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* performance of 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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Information value and weight of research results

LIST OF SCIENTIFIC PUBLICATION AND EVENTS

at which the results of the research for the doctoral thesis in Medical Sciences 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).

3. Visnevschi S., **Malcova T.,** Calistru A., Nacu V. Stem-cell therapies in critical limb ischemia. In: *The Moldovan Medical Journal*. 2021; 64(1): pp. 63-67. ISSN: 2537-6373 (Print) / ISSN: 2537-6381 (Online).

  - ✓ **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.

5. Popescu V., Jian M., **Malcova T.** Procedures for in situ monitoring of gene methylation in cancer prediction (literature review). In: *Revista de Ştiinţă, Inovare, Cultură şi Artă „Akademos"*. 2021; 61(2), pp. 89-95. ISSN: 1857-0461 / E-ISSN: 2587-3687.

6. **Malcova T.** Tissue-engineered small-diameter vascular grafts: background and new technology trends. In: *Arta Medica*. 2023, 87(2): pp. 93-95. ISSN: 1810-1852.

- **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 scaf-

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folds. 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. Timisoara Anatomical Days. In: *Abstract Book. Research and Clinical Medicine*. Timisoara, Romania: 2019; 3(1), p. 49. ISSN: 2360-1124.

11. **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. In: *Abstract Book. International molecular medicine symposium*. Istanbul, Turkey: 2019; p. 76.

12. **Malcova T.,** Nacu V., Rojnoveanu Gh., Andrée B., Hilfiker A. Protocolul de decelularare a vaselor sanguine este dependent de diametrul acestora. In: *Chirurgia (Bucur). Conferința Națională de Chirurgie 2021*. Online Edition: 2021; 116(Suppl.1), pp. 118-119. ISSN: 1221-9118. ISSN (online): 1842-368X.

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.

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✓ **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 alogrefei osoase vascularizate. Etapă de studiu experimental *in vivo*. In: *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

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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 colaagen 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 September 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

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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. Chisinau, 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., Tugui 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.,** Băluțel 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., Andree 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 alogrefei 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.

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

40. **Malcova T.,** Băluțel 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 (01.04.2022-30.11.2023).

(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 June 2018.

43. Exchange program at Hannover Medical School, Hanover, Germania, programme Horizon 2020 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 Octomber 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.

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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.

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Qualitative characterization SAMPLE 2 and SAMPLE 3 (H&E)

SAMPLE 2		
Native		
A.	B.	C.
Decellularized		
D.	E.	F.

Figure 1. **Efficient decellularization of a porcine carotid artery visualized by H&E staining (SAMPLE 2).** Native vessel (A, B, C). Decellularized vessel (D, E, F). Scale bar. A, D: 100 µm; B, E: 50 µm; C, F: 20 µm
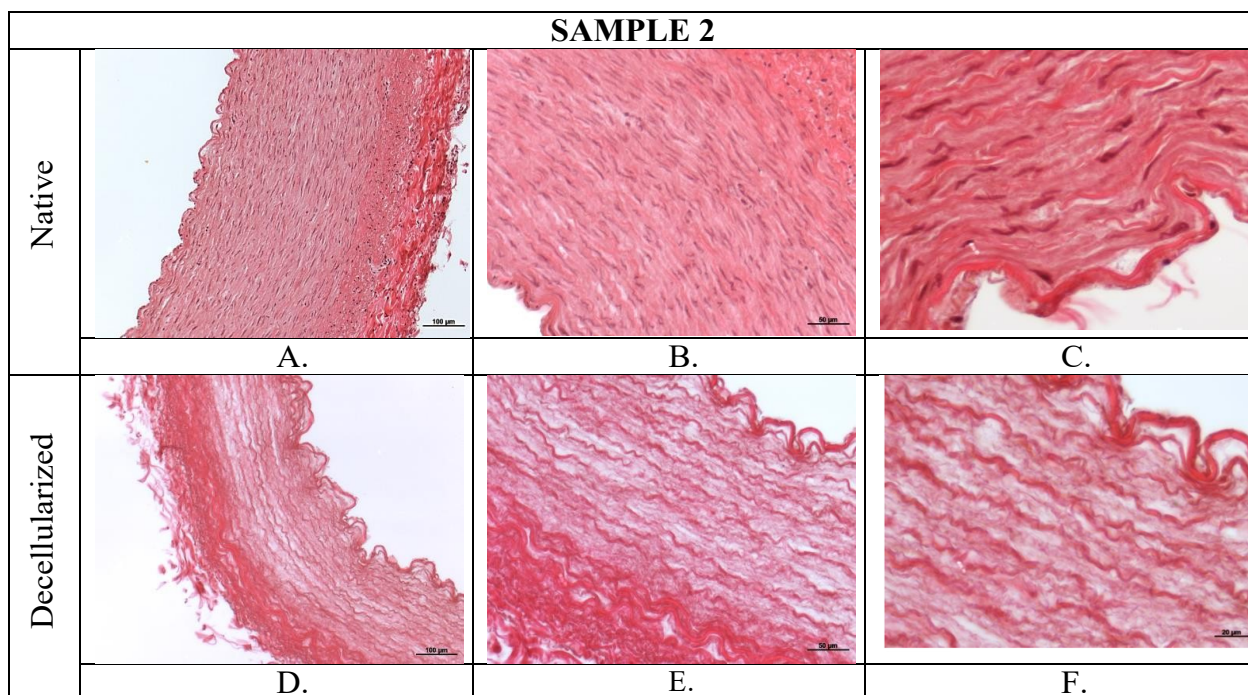
SAMPLE 3		
Native		
A.	B.	C.
Decellularized		
D.	E.	F.
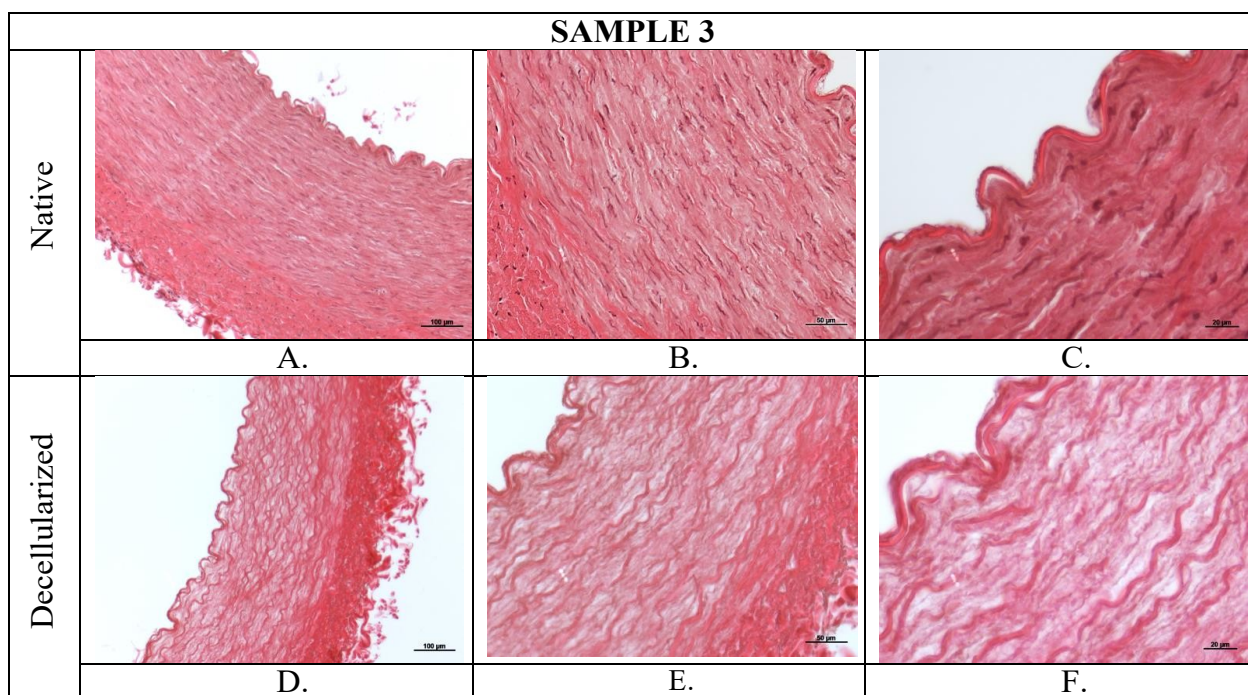
Figure 2. **Efficient decellularization of a porcine carotid artery visualized by H&E staining (SAMPLE 3).** Native vessel (A, B, C). Decellularized vessel (D, E, F). Scale bar. A, D: 100 µm; B, E: 50 µm; C, F: 20 µm

Qualitative characterization SAMPLE 2 and SAMPLE 3 (DAPI)
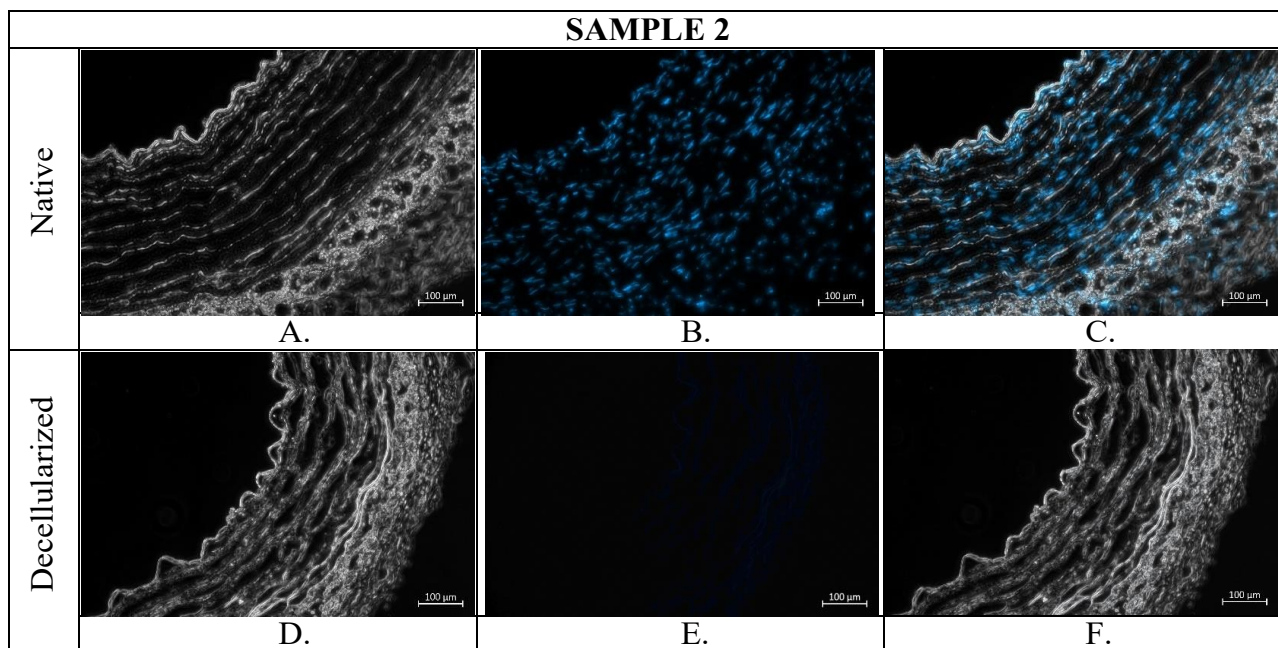
SAMPLE 2		

Figure 1. **Efficient decellularization of a porcine carotid artery visualized by DAPI staining (SAMPLE 2).** Native vessel (A, B, C). Decellularized vessel (D, E, F). The outline of the vessel is highlighted with a white line. Scale bar. A-F: 100 µm

SAMPLE 3		

Figure 2. **Efficient decellularization of a porcine carotid artery visualized by DAPI staining (SAMPLE 3).** Native vessel (A, B, C). Decellularized vessel (D, E, F). The outline of the vessel is highlighted with a white line. Scale bar. A-F: 100 µm

Republica Moldova
Ministerul Sănătăţii

CERTIFICAT DE INOVATOR

Nr. 5937

Pentru inovaţia cu titlul

PROCEDEU DE DECELULARIZARE A VASELOR SANGUINE DE CALIBRU MIC

Inovaţia a fost înregistrată pe data de
la Universitatea de Stat de Medicină şi Farmacie
"Nicolae Testemiţanu"

Se recunoaşte calitatea de autor(i)

MALCOVA Tatiana, NACU Viorel

Data eliberării   **12 August 2022**

(Semnătura autorizată)

INSTITUȚIA PUBLICĂ
UNIVERSITATEA DE STAT DE MEDICINĂ ȘI FARMACIE
"NICOLAE TESTEMIȚANU" DIN REPUBLICA MOLDOVA

Institutul Național de Cercetare în Medicină și Sănătate

Pag. 6 / 7
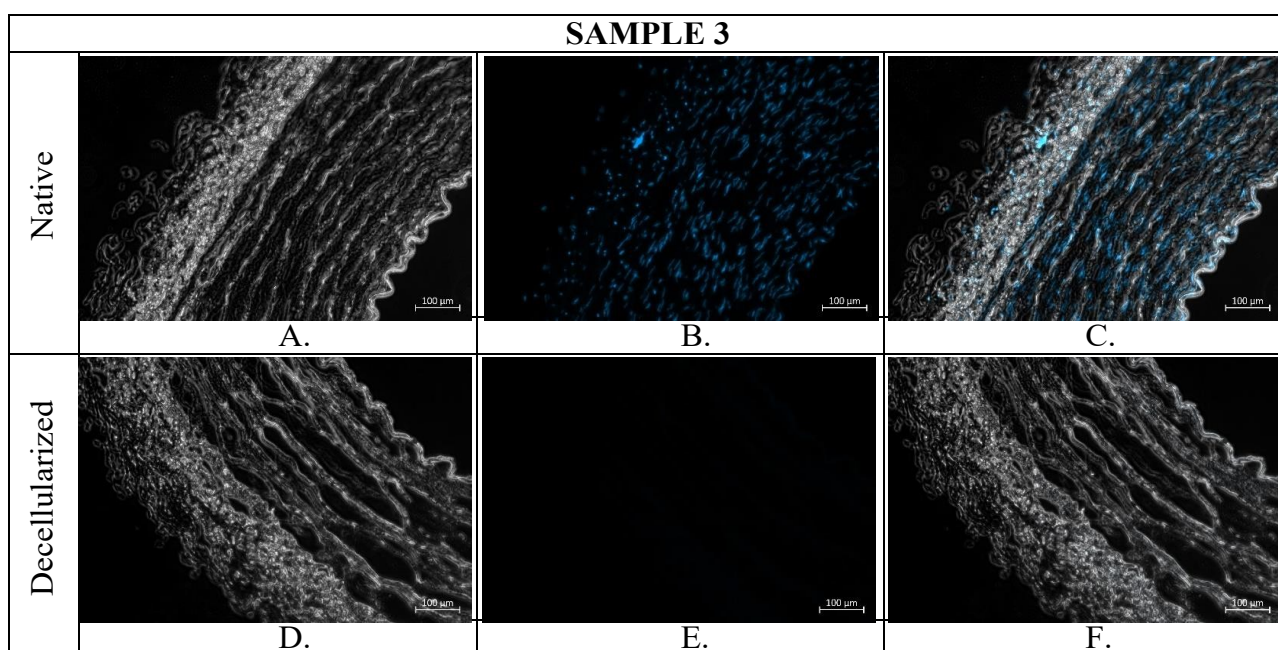
APROB
Prorector pentru activitate de cercetare,
USMF „Nicolae Testemițanu" din RM
academician al AȘM,
prof. univ., dr. hab. șt. med.
_____ **Stanislav GROPPA**
"___" _____ 2023

ACTUL nr.60
DE IMPLEMENTARE A INOVAȚIEI
(în procesul științifico-experimental)

1. **Denumirea ofertei pentru implementare:** "PROCEDEU DE DECELULARIZARE A VASELOR SANGUINE DE CALIBRU MIC"

2. **Autori:** MALCOVA Tatiana, asistent universitar, cercetător științific, studentă-doctorandă; NACU Viorel, prof. univ., dr. hab. șt. med

3. **Numărul inovației:** *Nr. 5937 din 12 august 2022*

4. **Unde și când a fost implementată:** Laboratorul de Inginerie tisulară și culturi celulare al USMF„Nicolae Testemițanu", perioada 2020-2023.

5. **Eficacitatea implementării:** Problema pe care o rezolvă lucrarea constă în obținerea matricei decelularizate ale arterelor carotide de porc, prototip al vaselor sanguine umane de calibru mic ($d < 6.0$ mm).

6. **Rezultatele:** Rezultatul constă în obținerea matricei sanguine acelulare potrivită pentru a fi utilizată ca scaffold în Ingineria Tisulară pentru obținerea vaselor sanguine artificiale. Propunerea este utilizată în practică experimentală în cadrul Laboratorului de inginerie tisulară și culturi celulare.

Prezenta inovație este implementată conform descrierii în cerere.

Șef laborator de inginerie tisulară și culture celulare
dr. hab. șt. med., prof. univ. _____ **Viorel NACU**

Șef centru, CCDSB,
dr. hab. șt. med., prof. univ. _____ **Mihail TODIRAȘ**

Șef Departament Cercetare,
dr. hab. șt. med., conf. univ _____ **Elena RAEVSCHI**

Coordonat: _____ E. Groza

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UNIVERSITATEA DE STAT DE MEDICINĂ
ȘI FARMACIE «NICOLAE TESTEMIȚANU»
DIN REPUBLICA MOLDOVA

NICOLAE TESTEMITANU STATE UNIVERSITY
OF MEDICINE AND PHARMACY
OF THE REPUBLIC OF MOLDOVA

INSTITUTUL NAȚIONAL DE CERCETARE
ÎN MEDICINĂ ȘI SĂNĂTATE

NATIONAL INSTITUTE FOR
MEDICINE AND HEALTH RESEARCH

MD-2004, Chișinău, bd. Ștefan cel Mare și Sfânt, 165; **tel.:** 022 205-706, 022 205-398; **e-mail:** incms@usmf.md

Nr. 23/03 din 14.03.2023

CERTIFICAT DE CONFIRMARE

Prin prezenta, se confirmă că Doamna Tatiana Malcova, asistent universitar, Laboratorul de inginerie tisulară și culturi celulare, Catedra de anatomie și anatomie clinică, USMF "Nicolae Testemițanu" din Republica Moldova a participat la realizarea cercetărilor în cadrul următoarelor proiecte științifice:

1. Titlul proiectului: „Ingineria tisulară în crearea și restabilirea țesutului hepatic"

Tipul: național
Programul: proiect instituțional
Codul/cifrul proiectului: 15.817.04.04. F
Finanțatorul: Academia de Științe a Moldovei
Funcția: executor
Perioada: 2015-2018

2. Titlul proiectului: „Grefe combinate: pielea decelularizată și celule stem în tratamentul leziunilor postcombustionale și posttraumatice".

Tipul: național
Programul: Programul de stat
Codul/cifrul proiectului: 16.00354.80.03A
Finanțatorul: Academia de Științe a Moldovei
Funcția: executor
Perioada: *2016-2017*

3. Titlul proiectului: „Nanoarhitecturi în bază de GaN și matrici tridimensionale din materiale biologice pentru aplicații în microfluidică și inginerie tisulară"

Tipul: național
Programul: Programul de stat
Codul/cifrul proiectului: 20.80009.5007.20
Finanțatorul: Agenția Naționala pentru Cercetare și Dezvoltare
Funcția: executor
Perioada: 2020-2023

Proiectele date sunt luate la evidență în registrul proiectelor de cercetare din cadrul Institutului Național de Cercetare în Medicină și Sănătate al USMF „Nicolae Testemițanu".

Centrul de suport al proiectelor,

Specialist principal
*Diana Cucoș*_____

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QUALITATIVE EVALUATION OF DETERGENT-ENZYMATIC DECELLULARIZED SMALL-CALIBER BLOOD VESSELS

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Certificate
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Hereby certifies that

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Prof. Victor SONTEA
Co-chair of ICNBME-2023

Acad., prof. Ion TIGINYANU
Co-chair of ICNBME-2023

UNIVERSITATEA DE STAT DE MEDICINĂ ŞI FARMACIE „NICOLAE TESTEMIŢANU" DIN REPUBLICA MOLDOVA

DIPLOMĂ

Se decernează

Dnei Tatiana Malcova

rezident, anul V, *Chirurgie generala*

Laureat al Concursului „Performanţe în cercetare"

**pentru ciclul de lucrări în domeniul Chirurgiei generale.
Inginerie tisulară şi culturi celulare**

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AUTHORS:

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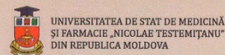
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Prof.Univ.Dr.Habil. DANIEL COCHIOR

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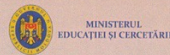
DIPLOMA of GOLD MEDAL

Is adwarded to: New methods in tissue engineering: decellularization of small-caliber blood vessels and collagen concentration

Autor/s: Malcova Tatiana, Jian Mariana, Cobzac Vitalie, Mostovei Andrei, Bujor Mihaela, Nacu Viorel

Nicolae Testemițanu State University of Medicine and Pharmacy of the Republic of Moldova

President of Jury
Profesor doctor ISTUDOR Nicolae

President of Organization Commite
Correspondent Member of ASM, Habilitation in economics,
Professor STRATAN Alexandru

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OFFER LETTER

Tatiana Malcova
100 Miciurin Str
Corten
Taraclia
MD-7421
Moldova

4th October 2017

Dear Tatiana,

We are pleased to inform you that you have been accepted on the Erasmus+ International Credit Mobility programme at the University of Chester for the following mobility:

> Level of study: PhD
>
> Duration: 7 months
>
> Dates: 6th November 2017 – 6th June 2018

You will receive a mobility grant from the European Union's Erasmus+ programme International Credit Mobility (agreement number: 2016-1-UK01-KA107-024078). Please read the following information carefully.

Travel: Your travel costs will be covered **within the limits of the travel grant.** You can either book your travel directly yourself and then claim the expense back from us or the University of Chester can book flights and transfers on your behalf **only** upon confirmation that you have received any necessary Visa or other required documentation to travel. You will therefore be required to send us this information (e.g. a copy of your Visa and acknowledgement letter) as soon as you receive it to the following email address; InterMed@chester.ac.uk . If you do not need to apply for any additional Visa or travel permissions, please confirm this via email to the address above. Please note that you will need to organise your own travel insurance to cover your return journey.

Accommodation & Subsistence: Upon arrival you will receive a subsistence grant (the exact amount will be confirmed in your Grant Agreement along with the payment schedule) to cover your living costs. This will be paid directly into a UK bank account which you will be required to set up on arrival. As this may take a few days to be operational, you may wish to bring sufficient funds to support you for those first few days. Tuition fees will not be charged.

In order to accept this offer, please sign and date the enclosed documents:

1. Grant Agreement – this outlines the Terms and Conditions of the award
2. Learning Agreement- this outlines the modules that you will be undertaking, institutional details and information relating to the components of your home institution course which will be replaced by the study undertaken in the UK.

Once you have signed these documents, please send a scanned copy to InterMed@chester.ac.uk

We look forward to welcoming you to the University of Chester.

Yours sincerely

Prof. Nick Avis

Pro-Vice-Chancellor (Research & Knowledge Transfer)

Agreement number: 2016-1-UK01-KA107-024078

DIPLOMA DE ONOARE
A ACADEMIEI DE ȘTIINȚE A MOLDOVEI

se conferă

dnei Tatiana MALCOVA

doctorand, cercetător științific la Universitatea de Stat de Medicină și Farmacie „Nicolae Testemițeanu", pentru rezultate relevante în activitatea de cercetare științifică, implicarea activă în activități de promovare a științei, precum și cu prilejul Zilei Internaționale a Femeilor și Fetelor cu Activități în domeniul Științei

Academician Ion TIGHINEANU
Președinte al Academiei de Științe a Moldovei

Hotărârea Prezidiului AȘM nr. 37 din 3 februarie 2023

PERSONAL INFORMATION   Tatiana Malcova

📍 Chisinau, Nicolae Costin str., 66/50, MD-2071, Republic of Moldova

📱 +37360211190

✉ tatiana.malcova@usmf.md
malcovatatiana92@mail.ru

Date of birth 25.08.1992 | Nationality Republic of Moldova

WORK EXPERIENCE

15.10.2021-present	Assistant professor, Department of surgery no. 1 "Nicolae Anestiadi", *Nicolae Testemitanu* State University of Medicine and Pharmacy, Laboratory of Tissue Engineering and Cell Culture
01.01.2020-30.06.2023	Scientific researcher, Laboratory of Tissue Engineering and Cell Culture, *Nicolae Testemitanu* State University of Medicine and Pharmacy
01.01.2020-present	Scientific researcher, Laboratory of hepato-pancreato-biliary surgery, *Nicolae Testemitanu* State University of Medicine and Pharmacy,
08.11.2016-08.11.2021	Resident doctor, Specialty General Surgery, Institute of Emergency Medicine, Chisinau

EDUCATION AND TRAINING

06.02.-11.02.2023	*Educational course* "Basic Emergency Care", Chisinau, Republic of Moldova
09.01.-13.01.2023	*Educational course* "Hostile Environment Surgical Training", Chisinau, Republic of Moldova
02.10.2017-02.10.2022	PhD-student, Specialty Tissue Engineering and Cell Cutures Nicolae Testemitanu State University of Medicine and Pharmacy, Chisinau
05.06.-13.06.2021	*Summer school* "Nanotehnologii și biomedicină în contextul provocărilor secolului XXI", Chisinau, Republic of Moldova
01.09.2020-28.02.2021	*PhD mobility* at the Leibniz Research Laboratories for Biotechnology and Artificial Organs și Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany
19.10.2019-16.05.2020	*Advanced course* in Nanotechnologies and Biomedical engineering (project Horizon2020 *NanoMedTwin*), Chisinau, Republic of Moldova
14.09.-17.09.2019	*Autumn School* in Bioengineering, Chisinau, Republic of Moldova
16.05.2019	*Workshop* Polymerase Chain Reaction and Cell Culture, Istanbul, Turcia
14.03.2019	*Course* Standarte actuale în managementul cancerului de rect jos situate, Iasi, Romania
22.01.2018-15.06.2018	*PhD mobility* at the University of Chester, Erasmus + mobility programme between the University of Chester and "Nicolae Testemitanu" State University of Medicine and Pharmacy
20.10.2016-22.10.2016	*Workshop* Basic Plastic and Reconstructive Surgery Skills, 9th Edition, Chisinau, Republic of Moldova
07.09.2016-08.09.2016	*Workshop* Termal burn wound treatment and management, Chisinau, Republic of Moldova
02.12.2015-05.12.2015	*Workshop* Basic Surgical Skills, 7th Edition, Chisinau, Republic of Moldova
11.11.2015	*Workshop* Noduri și Suturi, Cluj-Napoca, Romania
09.11.2015-13.11.2015	*Clinical Trainigs* at the University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania (TransMed programme)
09.2010 - 06.2016	Faculty of General Medicine, certificate ALM nr. 000001075 from 16.06.2016 "Nicolae Testemitanu" State University of Medicine and Pharmacy
09.2007 - 06.2010	Baccalaureate, certificate AB nr. 000115117 from 13.07.2010, Theoretical lyceum „M. Guboglo", Ceadir-Lunga, Republic of Moldova
09.1998 - 06.2007	Gymnasium, certificate ASG nr. 0682234 from 19.06.2007 Theoretical lyceum Corten vill., Taraclia, Republic of Moldova

Mother tongue(s)	Romanian, Bulgarian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
Russian	C2	C2	C2	C2	C2
English	B2	B2	B2	B2	B2

PERSONAL SKILLS

Communication skills	Good communication and teamwork skills

Digital skills

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem solving
Basic user	Basic user	Basic user	Basic user	Basic user

- good command of office suite (word processor, presentation software), Internet applications (Google Chrome), on-line database (PubMed), statistical software (EpiInfo, SPSS)

Other skills	Capacity for analysis and synthesis
	Communicability
	Responsibility
	CreativityDiscipline

Driving licence	B

Publications

Articles

Rojnoveanu G, Gurghis R, Gagauz I, **Malcova T**. Thoracic Penetrating Wounds with Cardiac Injury: A Single-Center Experience. Chirurgia (Bucharest, Romania: 1990). 2022,117 (6);660-670. ISSN: 1221-9118. ISSN (online): 1842-368X

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International Molecular Medicine Symposium by the Bosphorus, Istanbul, Turkey (May 2019)

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Annual Scientific Conference dedicated 20 years of independence of Republic of Moldova, Chisinau, Republic of Moldova (October 2011)

STATUTORY DECLARATION

I hereby assure that I have composed the present thesis entitled "*Morphological and biomechanical modifications in blood vessels decellularization*" independently and have used no other appliances than indicated. Parts being gathered from other works according to wording or meaning I have indicated in the reference list. I am aware that, otherwise, I will bear the consequences in accordance with the law.

Name: MALCOVA Tatiana

Date 20th of November 2023

Signature

DECLARAȚIA PRIVIND ASUMAREA RĂSPUNDERII

Subsemnata, declar pe răspundere personală, că materialele prezentate în teza de doctorat *„Modificările morfologice și biomecanice în decelularizarea vaselor sanguine"* sunt rezultatul propriilor cercetări și realizări științifice. Conștientizez că, în caz contrar, urmează să suport consecințele în conformitate cu legislația în vigoare.

Nume: MALCOVA Tatiana

Data 20 noiembrie 2023

Semnătura

DÉCLARATION SUR LA RESPONSABILITÉ

Je déclare la responsabilité personnelle que les informations présentées dans cette thèse sont le résultat de mes propres recherches et réalisations scientifiques. Je me rends compte que, sinon, en subiront les conséquences conformément à la loi.

Nom: MALCOVA Tatiana

Date 20 novembre 2023

Signature