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PhD THESIS

INNOVATIVE BIOTECHNOLOGY FOR GENERATION OF CARDIAC TISSUE USING ANIMAL MODEL

SUMMARY

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KEYWORDS: cardiac decellularization, decellularized extracellular matrix (dECM), mesenchymal stem cells (MSCs), recellularization, deep convolutional neural networks (DCNN);

LIST OF PUBLICATIONS

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INTRODUCTION

The definitive treatment for end-stage heart failure is cardiac allotransplantation. Patients with this condition have a low quality of life and require repeated hospitalizations and interventions. There are fundamental limitations to heart transplantation for which scientists continually search for solutions. Firstly, the chronic organ donor deficit is a significant hurdle. Each year, the number of patients waiting for a heart transplant grows faster than the number of eligible donors. This regrettable circumstance has been observed in the United States and Europe. Second, the requirement for lifelong immunosuppression post-transplantation and the maintenance of a balance between rejection and infection substantially impact the long-term survival rate.

To address these obstacles, researchers in the field of cardiovascular tissue engineering initiated the development of innovative solutions. Some focus on expanding the availability of organs, whereas others aim to reduce the immunological reaction to donor's hearts. These complementary ideas inspired the development of biological substitutes capable of restoring, enhancing, or preserving tissue function. The aim is to create a scaffold capable of imitating the features of a native, healthy myocardium. This outcome promotes the tissue's survival and the development of cardiovascular cells.

Creating a functional bioartificial heart is a highly complex process, representing the cutting-edge area of medical research that has the potential to revolutionize the treatment of heart disease. The initial step implies the decellularization of the organ to generate a naturally derived three-dimensional (3D) extracellular matrix (ECM). Currently, tissue engineering offers a variety of decellularization strategies based on physical, biological, and chemical processes. Recellularization is the technique of repopulating the acellular scaffold with organ-specific cells to recreate its function. This is the second phase in the development of a bioartificial heart. Stem cell technology has gained increasing attention due to the multipotent cells capable of self-renewal and production of cardiomyocytes (CMs).

RESEARCH OBJECTIVES

The objectives proposed in the present research study are:

1. Critical analysis of data from the specialized literature with a focus on current techniques and challenges in cardiac decellularization.
2. Development, design, and implementation of an experimental device for rat heart decellularization:
   - modified pressure-controlled device based on the Langendorff principle;
   - modified Langendorff experimental device in the presence of an alternating rectangular electric field that presumptively allows faster decellularization of rat hearts, reducing the tissue's exposure time to the detergent since it has been proven aggressive toward the ECM;

3. Development of a software application based on a Deep Convolutional Neural Network (DCNN) model, trained to distinguish between different decellularization stages, establishing the precise moment of decellularization completion.
4. Assessment of the experimental model of decellularization using the following methods:
   - testing of the experimental device and mathematical processing of data obtained by physicochemical determinations;
   - description of complete decellularization using histological and electron microscopy analysis;
5. Development of the experimental settings for the generation of a complete bioartificial rat heart:
   - *in vitro* evaluation of morphological and immunophenotypical characteristics of human mesenchymal stem cells (hMSCs) as candidate cells for recellularization procedure;
   - *ex vivo* differentiation of hMSCs into cardiomyocytes (CMs) using 5-azacytidine for whole rat heart recellularization;
   - *in vitro* macroscopic and microscopic evaluation of the recellularized rat heart;

GENERAL PART – LITERATURE REVIEW

The engineering of a whole heart represents an incredible journey with the difficult goal of resolving end-stage cardiac failure by providing a biocompatible and living organ substitute. The challenge started in 2008 when Ott et al. pioneered the first attempt to develop a bioartificial heart.

The first step in the process involves the decellularization of an animal or human organ, which results in the creation of a naturally derived three-dimensional (3D) extracellular matrix (ECM). Various physical, biological, and chemical techniques have been used to decellularize tissues with varying degrees of success. Ionic detergents, such as sodium dodecyl sulfate (SDS), are the most effective at removing cellular material, making them an essential component of most decellularization protocols. However, decellularization may cause ECM damage by removing critical cell adhesion, growth, and migration components. Because ECM degradation is a chemical process, reducing the decellularization time will leave the bioscaffold with a higher percentage of intact molecules required for recellularization.

The second step in developing a bioartificial heart is recellularization, which is the repopulation of an acellular scaffold with organ-specific cells to recreate its function. Stem cell technology has gained increasing attention because of the multipotent cells capable of self-renewing and generating heart muscle cells called cardiomyocytes. In several promising studies, embryonic stem cells, human mesenchymal stem cells (hMSCs), and induced pluripotent stem cells (iPSCs) were proven to induce cardiomyogenesis. Particularly, MSCs

are multipotent stem cells found in adult tissues with excellent self-renewal and differentiation capabilities and have been used to treat heart diseases.

However, effective recellularization of decellularized ECM has yet to be accomplished. Current recellularization strategies for cardiac dECM scaffold are limited by poor long-term cell survival, inhomogeneous cell distribution, and the correct mixture of cell types to generate native heart function.

SPECIAL PART – PERSONAL CONTRIBUTIONS

1. MATERIALS AND METHODS

1.1. Animals

The study was conducted following the ARRIVE recommendations and was approved by the Institutional Ethics Committees of the "Victor Babes" University of Medicine and Pharmacy Timisoara (No. 10/16.02.2021). The experiments adhered to the regulatory requirements for the study of laboratory animals and were carried out in an accredited facility.

1.2. Whole heart decellularization through coronary perfusion using a modified Langendorff experimental device

Twenty adult male Sprague-Dawley rats of 250–350 g (12–16 weeks old) were used for the generation of whole decellularized heart ECM. After explantation, the hearts (n=20) were decellularized using a modified Langendorff experimental device. The experimental device respects the principle described by Langendorff. The decellularization solution is aspirated from the decellularization chamber by the peristaltic pump and reintroduced into the heart via the cannula. The perfusion pressure of the heart is the pressure on the discharge branch of the peristaltic pump and it is monitored by the pressure transducer. The operation of the peristaltic pump is controlled by the automation system. The output of this system reaches a power amplifier and via a solid-state relay it controls the peristaltic pump function. Half of the hearts (n=10) were decellularized in the absence of the electric field (protocol 1 – control group) and the other half (n=10) in the presence of an alternating rectangular electric field (protocol 2 – electric field group). The hearts were perfused with 1% SDS in deionized water for 16 h at about 80 mmHg. A peristaltic pump was used for the perfusion of the heart, recirculating the decellularization solution during the entire course of the experiment. The heart was totally covered with solution within the glass container, constituting the electrochemical cell. Two stainless steel plate-type electrodes (the surface of each electrode was approximately 14 cm^2) were placed on the container to generate the electric field required for decellularization. The two electrodes were reachable from the exterior of the electrochemical cell. For generation of alternating electric field, a generator of functions was used, programmed to produce a rectangular electric signal, with

a constant frequency (20 kHz) and amplitude (100 mA), corresponding to 7.14 mA/cm^2 current density.

1.3. Spectrophotometric measurement of the DNA and total protein concentration from the decellularization solution

A kinetic analysis method of DNA and protein release from the heart during decellularization is proposed to compare the results of the protocols (protocol 1 - control group and protocol 2 - electric field group). The working hypothesis states that when DNA and protein concentration becomes constant, no more molecules are released in the decellularization solution, and the process can be considered complete.

During all the decellularization experimental procedures, samples were taken from the decellularization solution at a preset protocol time interval for spectrophotometric measurement of the DNA and total protein concentration using NanoDrop ND-1000 Spectrophotometer. The samples (10 µl each) were collected using an automatic micropipette directly from the decellularization tank every 30 minutes. A quasilinear increase of the two parameters was presumed, followed by a plateau as the cells were progressively washed out, completing the process. The decellularization protocols using the modified Langendorff experimental device (protocol 1 - control group and protocol 2 - electric field group) could be compared according to the time needed for complete decellularization. It is, therefore, desirable to make the decellularization process faster so that the exposure time of the heart to the SDS decreases, knowing its potentially harmful action on the ECM. All hearts were weighed before the decellularization process, without notable differences between the two groups. The mean weight of rat hearts following dissection was 1.28 ± 0.12 grams.

1.4. The Monitoring System of the decellularization process

Each decellularization session needed to be supervised because of the sample collection at pre-defined time intervals for the spectrophotometric analysis. In response, we tried using artificial intelligence for image recognition teaching software to establish the exact time the rat heart is decellularized. A Monitoring System was developed to prove a strong correlation between the data collected from periodically sampling the decellularization solution and the state of the heart that can be visualized with an OpenCV-based spectrometer. The Monitoring System comprises the following elements: Desktop module, Arduino module, Stepper motor, Web Camera, Database, and Session folders.

Images were saved in the corresponding session folder, classified from 0% to 100%. The spectrometer service processed each image, obtaining a spectrometer metric by extracting the heart pixels. The mean value was calculated for each cycle because of the large number of images acquired. These metrics were stored in the database along with session-related data. It was established that when the visual appearance of the heart stops changing, the metric reaches a plateau, meaning the heart is decellularized, and the

process is complete. The decellularization times obtained were also normalized to the unit weight of each heart involved in the experiments.

The classification service was built with a DCNN model trained to classify the degree of completion of the images collected during the decellularization process. A decellularization session contained up to 50,000 collected images with 1,280×720 pixels resolution. Raw images collected were of this resolution. However, the heart area was cropped, removing unnecessary pixels and resizing the image to 200×200 resolution to reduce the resources needed to train a model on such a dataset.

After designing the spectrometer metric and detecting its plateau, we split the image dataset into 11 collections (equal segments of the session parts). The last collection contained images from the end of the session, where the spectrometer metric reached the plateau.

The DCNN was trained on 72,417 images using image augmentation to help generalize training with this dataset. Validation was achieved on 9,061 images that were not used during training. Testing was performed using 9,060 images that the model never met during training and validation. This process resulted in a model with 95% accuracy.

The metric was calculated using the classification result from each cycle by making a rolling window mean. Our theory was that every metric should start at 0% and end at 100% if complete decellularization. This metric showed a progress bar in the record session view to let researchers know when the process was complete.

1.5. Extracellular matrix characterization

In this study, decellularization was deemed complete when there was no trace of remaining myocardial tissue. Typically, decellularized heart scaffolds are assessed for completeness of decellularization using a combination of macroscopic and microscopic techniques. The decellularized hearts were fixed in 4% buffered formaldehyde solution, paraffin-embedded, and sectioned. Three standardized regions were analyzed: apical, mid-ventricular, and basis-near region. After rehydration, sections were first stained with hematoxylin and eosin (H&E). In order to validate the protocols' decellularization efficacy and the integrity of the remaining scaffold, Masson's trichrome, argentic impregnation, and orcein staining techniques were also used for visualization of connective tissue. Scanning electron microscopy (SEM) was used to characterize the morphological changes following decellularization.

1.6. Cell culture of human mesenchymal stem cells (hMSCs)

Human mesenchymal stem cells (hMSCs) were purchased from Lonza (Basel, Switzerland) and cultured at a cellular density of 10,000 cells/cm^2. Cells were expanded in α-minimum essential medium (α-MEM) containing 10% Fetal Calf Serum (FCS) and Penicillin/Streptomycin solution (Pen/Strep 10,000 IU/ml). Human MSCs proliferated as adherent cells in T75 culture flasks up to 80-90% cellular confluence when the cells were detached from the plastic surface using

Trypsin/EDTA solution. After counting, the cells were placed at the same cellular density in appropriate culture flasks for further expansion or other investigations. Two high-resolution visualization techniques were used for the morphologic analysis of hMSCs: scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Using immunocytochemistry (ICC), the hMSCs in culture were stained for surface and intracellular characteristic markers, revealing the expression of molecules involved in cellular functions. Further, flow cytometric evaluation was performed to validate the presence of hMSCs characteristic markers.

1.7. Recellularization of cardiac dECM using hMSCs

Cardiac extracellular matrices (n=8) obtained through decellularization from the control group (n=4) and the electric field group (n=4) were used for whole-heart bioengineering. Initially, the decellularized hearts were perfused with PBS for 72 h.

The differentiation of hMSCs into cardiomyocytes (CMs) was conducted *ex vivo* using the nonspecific DNA methylation inhibitor 5-azacytidine (5-aza), observing its influence on cardiomyogenesis.

The protocol delivered the hMSCs (~5-7×10^9 cells) directly with 10 µM 5-azacytidine suspended in differentiation culture medium. Approximately half of these cells were delivered via a 24 h infusion through the patent aorta for cell seeding. The other half was delivered through 4 injections of 200 µl each into the ventricular walls thickness of the decellularized heart using a 27-G needle and a 1-cc tuberculin syringe. Subsequently, the medium was changed every 24 h with MSC standard culture medium. The hearts (n=8) were maintained for three weeks in a tissue-culture incubator with 5% CO_2 and humidified carbogen (5% CO_2 and 95% O_2) added to the medium reservoir. At the end of the experiments, we described the recellularized cardiac ECM using hematoxylin and eosin staining.

2. RESULTS

2.1. Generation of acellular rat heart matrices using perfusion decellularization

Decellularization was considered finished when there was no trace of myocardial tissue remaining. Macroscopic analysis of the ECM after the decellularization process showed an intact 3D geometry and the maintenance of the atrial, ventricular and valvular architecture. Photographs taken during decellularization showed a more efficient and rapid decellularization in the electric field group (~480 minutes) compared with the control group (~840 minutes). After decellularization, the coronary vessel tree's integrity was verified by injecting trypan blue solution through the connected cannula. The entire process was recorded, and the entry of the blue solution into the coronary

system was visualized without any signs of problems with the caliber of the vessels.

2.2. DNA and protein quantification

DNA and protein concentration in the decellularization solution was calculated using ultraviolet absorption spectrophotometry. In both decellularization protocols, the graphical method determined when the analytes (nucleic acids or proteins) reached a stationary value, i.e., the “plateau of the concentration curve”, demonstrating the working hypothesis exposed in the previous chapter, which states that when DNA and protein concentration becomes constant, no more molecules are released in the decellularization solution, confirming decellularization completion.

Good correlations were found between DNA/protein concentration mean values and the time of the procedure in the control group ($r=0.93$; $p<0.0001$; CI95% [0.872-0.968], and $r=0.92$; $p<0.0001$; CI95% [0.853-0.962], respectively). Similarly, when the electric field group was analyzed, the following correlations were found between DNA/protein concentration mean values and the time of the procedure: $r=0.88$; $p<0.0001$; CI95% [0.783-0.943], and $r=0.90$; $p<0.0001$; CI95% [0.821-0.954], respectively.

Based on the information provided using the spectrophotometric method, it can be concluded that using the Langendorff experimental device in the presence of an alternating rectangular electric field reduces the decellularization time by approximately 40%. This protocol is a promising approach for accelerating decellularization, ultimately leading to a faster and more efficient method for obtaining acellular scaffolds in tissue engineering applications.

2.3. Optimization of rat heart decellularization using a Deep Convolutional Neural Network (DCNN) model

The present study developed a classification service using a DCNN model trained to classify the degree of completion of images collected during decellularization. The model has trained on heart ECM from male rats (n=10) obtained using decellularization protocol 2 (1% SDS coronary perfusion in the presence of an alternating rectangular electric field). The system provided acquisition, processing, and analysis of the rat heart during decellularization. The experimental data made it possible to establish the exact time the organ became translucent, ending the process. Correlating the graphics from the spectrophotometry with the metrics produced by the spectrometer and classification services, we could state that the model optimized the decellularization process providing an estimation of progress. As a performance measure, the metrics of the classification system for hearts around the same age and weight were precision = 0.95, recall = 0.95, f1-score = 0.95, and accuracy = 0.95.

To summarize, we developed and validated an artificial intelligence-assisted software application to determine through classification when the heart is fully decellularized and, during the process, provide an estimation of progress.

The correlation between spectrophotometry and the metrics generated by the spectrometer and classification services confirmed our theory.

This study demonstrates, for the first time, the relationship between cell removal in perfusion decellularization and the two metrics produced by an OpenCV-based spectrometer and a DCNN based classifier model. This is a novel assessment of decellularization efficiency using a non-invasive technique. Also, instead of collecting samples from the decellularization solution, the spectrometer and classifier can estimate the decellularization state in real-time, thereby optimizing the old process. This research could lead to a new standard for heart decellularization, thus encouraging significant advances in regenerative medicine.

2.4. Assessment of cellular material removal and ECM architecture integrity

Assessment of cellular material removal and ECM architecture integrity was performed by basic macroscopic analysis, histological and electron microscopy assessment. The decellularized rat heart was translucent, with all cellular components removed during decellularization. The acellular scaffold maintained its overall shape and size, with all major anatomical features intact. The efficiency of decellularization was validated through a number of different stainings. As an initial step, the simple H&E staining method demonstrated the removal of cells, while preserving the 3D ECM. The preservation of collagen fibers and clearance of nuclear content from the decellularized scaffolds was highlighted using Masson's trichrome staining. Orcein staining and argentic impregnation visualized the elastic fibers and the reticular fibers, respectively. Scanning electron microscopy of the 3D cardiac framework strengthened the results of the optical microscopy, showing preserved collagen fibers with no cellular components remaining. The evaluation of the matrix content using microscopy showed no significant differences between the scaffolds generated by the two decellularization protocols.

2.5. In vitro characterization of hMSCs

The present study performed a detailed *in vitro* characterization of hMSCs using SEM and TEM as high-resolution visualization techniques. The results showed intensely metabolically active cells with good structural organelles, creating the premise for further differentiation towards CMs. We also conducted an immunophenotypic analysis, staining the hMSCs for surface and intracellular characteristic markers as follows: CD105 - expressed on the surface of 100% hMSCs, CD117 - expressed on the surface of 90% hMSCs, vimentin - expressed on the surface of 100% hMSCs, Ki67 -expressed at nuclear level of 60% hMSCs. Flowcytometric evaluation showed expression of hMSCs characteristic markers: CD90, CD44, CD29, CD73, and CD105. These markers have a constant expression during multiple cellular passages (P1-P4), while other markers decrease or lose their expression, as the cells are suffering *in vitro* proliferation and expansion (CD31, CD117, CD95). However, these pattern of

expression from passage 1 to passage 4 is indicative for loss of some hMSCs functions, required for regenerative medicine experiments, and suggests to be used at early passages for improved results in regeneration of various tissues.

2.6. Formation of bioartificial whole rat heart

This extensive research also achieved *ex vivo* recellularization of decellularized rat hearts (n=8) using hMSCs (~5-7×10^9 cells) differentiated into CMs using 5-azacytidine. From a macroscopic perspective, both the atria and ventricles from the recellularized heart were observed to have well-defined cardiac walls. The blood vessels were visible through the thickness of the heart. At the base of the ventricular mass was a concentration of fibrous bands, which could be the heart's fibrous cytoskeleton. Different regions of the recellularized hearts were stained with hematoxylin and eosin to confirm the presence of numerous cells disseminated throughout the acellular scaffold. The examined tissue contained loosely arranged cardiomyocyte-like cells (CLCs) with visible ramifications, cross-striations, and discernible nuclei. Even though the histological architecture was atypical, a structural organization was still discernable. On the examined material, approximately 20% of the matrix was composed of cells with visible nuclei interspersed with acellular areas and zones of dense cellularity.

CONCLUSIONS

- The modified experimental pressure-controlled device allows complete decellularization of the perfused rat hearts with a surfactant solution of 1% sodium dodecyl sulfate (SDS).

- Applying an alternating rectangular electric field (20kHz) to the Langendorff decellularization system significantly increases the efficiency of the process (the decellularization time is reduced by approximately 40% compared to the control group). Choosing the proper decellularization agent with a short exposure time is crucial for generating highly bioactive scaffolds. This study outcome represents an essential milestone in organ bioengineering using a small-animal model scaffold.

- The artificial intelligence-assisted software application created as part of this thesis is a non-invasive, non-destructive method to assess through classification when decellularization is complete. Correlating the graphics from the spectrophotometry (DNA/protein concentration in the decellularization solution) with the metrics built by the spectrometer and classification services, the model optimized the decellularization process delivering an estimation of progress.

- Perfusion decellularization of rat hearts with 1% SDS resulted in a 3D matrix free of visible xenogenous cellular material. The process was validated through extensive histological analysis and SEM, confirming the complete removal of cellular remnants and the preservation of native ECM.

- Human MSCs are promising candidate cells for recellularization procedures. The *in vitro* evaluation of hMSCs' morphological and immunophenotypical characteristics provided a foundation for their subsequent use. The *ex vivo* differentiation of hMSCs into CMs using 5-azacytidine demonstrated their ability to adopt a cardiac lineage. At the same time, the *in vitro* evaluation of the recellularized rat heart validated the successful integration of hMSCs-derived CMs within the cardiac matrix.

PERSONAL CONTRIBUTIONS

- Meticulous refinement of the anesthetic-surgical protocol and heart cannulation technique for whole organ decellularization;

- Development of an experimental design and implementation protocol for whole heart decellularization through coronary perfusion using a modified Langendorff device;

- Successful assessment of the decellularization efficiency using spectrophotometry as a non-invasive technique but also through histological analysis and a high-resolution visualization technique (SEM);

- Participation in creating a Deep Convolutional Neural Network (DCNN) algorithm to predict the decellularization progression and end point as a non-destructive method of scaffold assessment;

- Preparation of human mesenchymal stem cells for cardiac matrix recellularization; *in vitro* morphological (SEM and TEM) and immunophenotypic (immunocytochemistry and flow cytometry) analysis; generation of characteristic images and graphs and interpretation of results;

- Optimization of recellularization protocols by retrograde infusion of cell suspension and intraventricular injection into decellularized rat heart matrix;

- *Ex vivo* analysis of recellularization efficiency by H&E staining;

FUTURE RESEARCH DIRECTIONS

Although this Ph.D. study includes a rigorous and complex research process, there are future research directions that we would like to pursue:

- Implementation of a more comprehensive characterization of the decellularized heart by quantifying residual SDS, IHC analysis of the main ECM proteins, mass spectrometry-based proteomics, and biomechanical performance (e.g., atomic force microscopy, single or bi-axial mechanical tests);

- Employment of other cell types (e.g., human induced pluripotent stem cells) for recellularization which could result in an increased proportion of cell populations in the matrix;

- In-depth study of targeted genome editing, such as the CRISPR platform, which has proven to be a powerful instrument for manipulating patient-derived hiPSCs, allowing the generation of autologous corrected cells eligible for human tissue grafts;

- Improvement of cardiomyocyte differentiation, followed by electrical and mechanical testing of the bioartificial heart;

As a closing remark, a future perspective is to link technical domains, such as IT and engineering, with medicine. Together they can lead to great advancement in the tissue engineering field.