Development and Evaluation of a Perfusion Decellularization Porcine Heart Model

– Generation of 3-Dimensional Myocardial Neoscaffolds –

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Background: Reports about the generation of 3-dimensional neoscaffolds for myocardial tissue engineering are limited. The architecture provided by perfusion decellularization of whole hearts would support the production of human-sized 3-dimensional living tissues from an acellular matrix. The aim of this study was to evaluate the potential of a perfusion decellularization model for whole heart tissue engineering.

Methods and Results: Hearts were obtained from 12 German Landrace pigs from a selected abattoir. After preparation, the hearts were mounted and perfused on a modified Langendorff decellularization model specifically constructed for this reason. Decellularization was achieved by an ionic detergent-based perfusion protocol. The quality of the decellularization process was quantified by histology and fluorescence microscopy. Data regarding the presence of residual DNA within the decellularized hearts was measured with spectrophotometric quantification and compared to controls. After histological examination, all hearts lacked intracellular components but retained various types of collagen, proteoglycan and elastin. Quantitative DNA analysis demonstrated a significant reduction of DNA in decellularized hearts compared to controls (84.32 ± 3.99 ng DNA/mg tissue vs. 470.13 ± 18.77 ng DNA/mg tissue ($P < 0.05$)).

Conclusions: The modified Langendorff perfusion decellularization model described here is applicable for whole porcine hearts by removing cellular content and DNA. The resulting 3-dimensional matrix provides an interesting tool for further studies in the field of whole heart tissue engineering. (*Circ J* 2011; **75**: 852–860)

Key Words: Cardiac tissue engineering; Decellularization; Organ perfusion

Total heart transplantation remains the only successful treatment for end-stage heart failure but the utility of heart transplantation is limited by donor shortage and many patients die while waiting on the shortlist.^{1,2} The human heart has a limited regenerative potential^{3,4} and therefore damage to the heart is mostly irreversible, as the mature contracting cardiac cells, the cardiomyocytes, are unable to divide, thus cardiac tissue engineering strategies are required for repair being a potential option.^{5–7}

The innovative fabrication and development of entire organ replacements requires sophisticated methods and an integration of basic scientific principles. However, it seems apparent

that organ perfusion decellularization models, which remove the majority of the cellular material, are mandatory for successful development of tissue engineered whole hearts. If hearts can be decellularized in one piece and preserve their 3-dimensional architecture with the abundant capillary network, the resulting construct would be the ideal target for tissue engineering—not only for parts of the heart but also for all components functioning together as one integrated unit that is able to respond to physiological stress like the natural heart.⁸

Although others^{9,10} already reported the encouraging results of the decellularization of whole hearts, as nature's platform, from rats and pigs, a detailed description of the perfusion

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Figure 1. Schematic diagram of the perfusion decellularization porcine heart model. The pump performs the detergent perfusion with possibility for variable flow and perfusion pressure. The inlet and outlet port permits easy and quick detergent change.

decellularization model was not provided.

The purpose of this study was to evaluate the application of an organ perfusion decellularization apparatus for whole heart tissue engineering of intact porcine hearts. Furthermore, the influence of the decellularization protocol on biochemical composition and ultrastructure of the decellularized neoscaffolds was characterized.

Methods

Preparation of Porcine Hearts

All together 12 porcine hearts were harvested from female hybrids of German Landrace pigs. The intact porcine hearts were obtained under clean conditions from a selected local abattoir. After the usual slaughtering process, the porcine hearts were excised within 5 min and immediately perfused via the aortic root with heparinized Locke-Ringer-solution at 25°C in order to remove coagulated blood. In the laboratory, the pericardial fat was removed from the hearts and the major vessels were trimmed appropriately.

After the preparation, the hearts were washed for 30 min in povidone-iodine-solution (Mundipharma, Limburg, Germany). Next antibiotic-containing phosphate-buffered solution (PBS, Sigma) enriched with 100 µg/ml penicillin-streptomycin (Biochrom) was perfused through the hearts at 1.5 L/min for 24 h, followed by an overnight incubation in Dulbecco's modified Eagle's medium (Invitrogen) at 4°C.

Decellularization Protocol Described by Wainwright et al

The experimental model was described elsewhere.¹⁰ For decellularization the hearts were perfused by different solutions. Shortly, first by 0.02% trypsin/0.05% ethylenediaminetetraacetic acid (EDTA)/0.05% NaN₃, then by 3% Triton X-100/0.05% EDTA/0.05% NaN₃, followed by 4% deoxycholic acid and finally by 0.1% peracetic acid (PAA)/4% EtOH.

Perfusion Decellularization Model Used in the Present Investigation

The perfusion decellularization model consisted of a perfusion circuit and a pressure control module (Figure 1). The

whole circuit contained exactly 2,500 ml of volume. All components of the perfusion model were sterilized by a conventional autoclave and connected with silicon tubes (3/8 inch, Maquet).

The aorta was connected with a cannula (Teflon®, 25–27 mm diameter) for retrograde perfusion (Langendorff). Through the aortic cannula the perfusate was collected into a reservoir (Maquet, AR 28150) and pumped by a roller pump (Stöckert) into the circuit, where it passed a filter. Additionally an airtrap (gambro medical line) was interposed for an airfree perfusion.

A heat exchanger (D720 Helios C) warmed the perfusate to keep the temperature constant at 37°C. The roller pump was controlled via a pressure transducer (Medex Smith Medical) by a computer system (Engineo, Mainz, Germany), which continuously reported the perfusion pressure. An inlet and outlet port permitted easy and quick detergent change. Hearts were perfused at a constant perfusion pressure of 100 mmHg by Langendorff mode delivering a warm (37°C) solution of 4% sodium dodecyl sulfate (SDS) in PBS at 2.0 L/min for 12 h.

During this process, the hearts were washed with PBS for 15 min every 3 h to remove residual substances. The final washing step after SDS treatment included perfusion with PBS for another 24 h to remove remnant detergents and cell debris at 1.5 L/min.

Macroscopic Heart Morphology and Histology

The hearts were prepared using the classical approach of opening each of the 4 chambers to demonstrate the interrelations of cardiac valves and the structure of chambers.

In addition, hearts were fixed in 10% formalin, embedded in paraffin and sectioned following standard protocols. We cut heart tissue into 5 µm sections, stained them with Masson's trichrome stain to distinguish the cells from the surrounding connective tissue following the manufacturer's instructions (fomori, procedure HT10, Sigma-Aldrich).

4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Inc) and hematoxylin and eosin (H&E) stain were used as a first line of inspection to determine if remnant nuclear structures could be observed.

Figure 4. Representative H&E-stained (HE) and DAPI-stained (DA) samples of ventricular tissue with absence of nuclear staining after decellularization. (A) Native ventricle (HE), (B) decellularized ventricle (HE), (C) native ventricle (DA), (D) decellularized ventricle (DA) with absence of DAPI-positive nuclei (blue). Scale bars, 200 μ m.

Modified Movat's Pentachrome (Mastertechs, Lodi, CA, USA) stain was used to allow visualization of extracellular matrix components (ECM) such as collagen, elastin and glycosaminoglycans. Additionally, Herovici stain was used to present collagen I and III in the newly deposited matrix. The sections were analyzed by routine bright field and fluorescence microscopy (Olympus Optical Co, BX 51 and CKX 41 microscope). Images were acquired with the Cella Soft Imaging System (Olympus Soft Imaging Solutions®). Samples of decellularized ventricular tissue were processed for light microscopy and compared to native untreated cross-sections prepared with the above mentioned staining methods.

DNA Quantification

Sections out of 6 different regions of the heart (apex, left ventricle (LV), right ventricle, septum, papillary muscle, atrium) were processed for spectrophotometric quantification to determine the concentrations of residual DNA in the decellularized group compared to the controls. Samples (approximately 10–15 mg) were normalized according to equivalent dry weight in mg. The total amount of DNA was silica-membrane-based purified (QIAamp DNA Mini Kit, Qiagen, Basel, Switzerland) following the manufacturer's instructions and later quantified by spectrophotometry.

Measurement of Mechanical Stability

For the measurement of mechanical stability $n=6$ porcine hearts in each experimental group were used. A latex balloon catheter was introduced into the LV via the aorta and was connected to a precision calibrated syringe for administration and withdrawal of fluid. A Millar micromanometer (Millar Instruments, Inc, Houston, TX, USA) was advanced into the ventricle via the apex. LV pressure was then determined by the Millar micromanometer at different LV volumes.

Statistical Analysis

All values in the figures and text are shown as mean $\pm$ SEM. A two-tailed Student's t-test was performed to determine significant differences of the native hearts (control) versus the decellularized hearts, considering P-values less than 0.05 as statistically significant. The commercially available SPSS statistical software package 13.0 for Windows (SPSS Inc, Chicago, IL, USA) was used for data analysis.

Results

Perfusion Decellularization Model

During our experiments, the function of the perfusion decellularization model was excellent with constant flow. No leakages from the compartments were observed. The observation

Figure 5. Photomicrographs of native and decellularized ventricular tissue stained with Movat's Pentachrome Stain (MP), Herovici's Stain (HS) and Masson's Trichrome Stain (MA) revealed stable preservation of extracellular matrix collagen and elastic fibers after the decellularization process. (A) Native ventricle (MP), (B) decellularized ventricle (MP), (C) native ventricle (HS), (D) decellularized ventricle (HS), (E) native Ventricle (MA), (F) decellularized ventricle (MA) with large preserved vascular conduit (black asterisk). Scale bars, 200 μ m. Movat's Pentachrome Stain: nuclei—dark purple to black, elastic fibres—purple to black, muscle—red, collagen—yellow, mucin—blue, cytoplasm—pink to brownish to red Herovici's Stain: young collagen and reticulin—blue, mature collagen—red, cytoplasm—yellow, muscle—purple, nuclei—black. Masson's Trichrome Stain: cytoplasm—red, erythrocytes—red, muscle—red, collagen—blue, nuclei—dark brown.

of the newly deposited myocardial neoscaffold through the reservoir wall showed good stability during the decellularization process.

The decellularization system was specially developed with standard equipment for heart surgery. It is inexpensive, safe

and simple to handle. The circuit volume was reduced to 2,500ml and an inlet and outlet port were used to easily exchange decellularization solutions.

Macroscopic Heart Morphology and Histology

During the decellularization process, the porcine hearts continuously lost their natural color until they became translucent beginning with the right ventricle, then the atria and finally the papillary muscles of the LV (Figure 2).

The overall morphology of the decellularized hearts compared to untreated controls was analyzed by examination and dissection of hearts with macrophotography. Normal heart morphology was present in all samples without recognizable defects after decellularization and preserved subvalvular area (Figure 3). Signs of adhesions, structural deterioration or leaflet thickening were absent. Further exploration of the heart valves revealed a smooth surface with translucent, decellularized leaflets without fenestrations or swelling.

The histological examination demonstrated that perfusion with SDS converted native porcine hearts into an almost acellular neoscaffold (Figure 4).

Light microscopy of decellularized hearts stained with Movat's pentachrome stain, Herovici's stain and Masson's trichrome stain indicated stable preservation of fiber orientation, composition of extracellular matrix collagen and elastic fibers (Figure 5).

DNA Quantification

Results of quantitative tissue analysis are shown in Figure 6. The total DNA content decreased significantly compared to controls (84.32 ± 3.99 ng DNA/mg tissue in the decellularized group vs. 470.13 ± 18.77 ng DNA/mg tissue in the control group ($P < 0.05$)). All samples are shown according to equivalent dry weight in mg.

Measurement of Mechanical Stability

Results of the pressure-volume relationships are shown in Figure 7. Native hearts and decellularized hearts using our protocol almost showed no major differences indicating an intact mechanical stability. In comparison to our protocol, the protocol developed by Wainwright et al shows a strong impairment of the tissue, which is shown in Figure 7 by a

curve that is less steep.

Discussion

To our knowledge, this is the first detailed report about the construction of an organ perfusion decellularization circuit for whole heart tissue engineering. Our described protocol does not only provide an easy to manage procedure but also allows to achieve good decellularization results and good mechanical stability of the decellularized tissue. The present study reports our initial methodological efforts for the investigation of a biocompatible decellularized whole porcine heart. The use of a decellularized heart was preferred because synthetic-based scaffolds are not only expensive and potentially immunogenic, they also suffer from toxic degradation and inflammatory reaction.

Our group already reported the clinical experience of in

vivo created tissue engineered pulmonary heart valves based on decellularized xenograft matrices.¹¹ Now, our ultimate goal was to create an “off the shelf” 3-dimensional myocardial neoscaffold that can subsequently be perfused with living cells prior to implantation. In practice, the porcine hearts were harvested from slaughterhouse pigs, perfusion-decellularized to reduce antigenicity and prepare it for future seeding and use. The use of porcine hearts as a biological-based scaffold offers several potential advantages: (1) ease of harvest and large available quantities from slaughterhouse pigs; (2) size appropriate for transplant procedures; (3) human and porcine hearts show remarkable anatomical similarity; (4) strong cellular adhesive property of the porcine tissue surface;¹² and (5) a 3-dimensional supporting matrix including vasculature and basement membrane structures that allow for migration of living cells with important extracellular matrix proteins such as collagen, elastin and proteoglycans, which are missed in synthetic polymer material. This finding is important as collagen is responsible for vessel strength, and elastin imparts distensibility and recoil.¹³ Collagen is mainly found in the LV where it provides strength and stiffness to maintain coaptation during diastole. These proteins are well known as critical elements for the structural integrity and biomechanical profile of tissue engineered constructs formulated by Bader and colleagues.¹⁴

Various methods of decellularization treatments have been employed in cardiac tissue engineering. The most commonly used decellularization elements are divided into the following: (1) non-ionic detergents such as Triton X 100;^{9,10,15} (2) ionic detergents such as SDS,^{9,10,16,17} deoxycholic acid;^{16,18–20} (3) enzymes such as Trypsin,^{10,21,22} nucleases of deoxyribonucleic and ribonucleic acid (RNase, DNase);^{23,24} and (4) chelating agents such as EDTA.^{25,26} Detergents are water-soluble molecules that are divided into an ionic and non-ionic group according to their hydrophilic/hydrophobic character and ionic groups.¹⁵ Most frequently, a combination of the above mentioned decellularization treatments is used.

SDS^{16,17} or a combination of ionic- and non-ionic detergents²⁰ showed excellent cell removal capacity with preservation of the major structural ECM molecules. It was also shown that treatment with SDS resulted in disintegration of collagen fibers²⁰ as well as fragmentation and swelling of the collagen.²⁷ Other influences on ECM preservation could be: (1) detergent concentration; (2) duration of treatment; (3) presence of protease inhibitors; and (4) differences in species.^{1,15}

In these initial investigations, we developed a perfusion based technique for decellularizing porcine hearts using SDS, a common tissue detergent. In our study, decellularization with SDS resulted in a biological matrix free of any visible xenogenous cellular material, which was validated by histological examination and DNA spectrometry. Furthermore, warm detergent solution (37°C) was used because temperature strongly influences the velocity of the decellularization process in a positive way. As indicated in **Figure 7**, comparing our protocol of decellularization with the one of Wainwright et al,¹⁰ the results in terms of mechanical stability are superior and even comparable with those of native hearts. This again indicates that the ECM is intact which is essential for recellularization and tissue engineering.

Gilbert et al²⁸ described the impossibility that any combination of decellularization methods will remove 100% of all cell components. They concluded that methods which remove most or all of the visible cellular material result in biologic scaffold materials that are safe for implantation.

The results of our study compare favorably with those re-

ported by the Wainwright group¹⁰ and illustrate the latest developments in whole heart tissue engineering. Unlike the time-consuming and expensive perfusion of hearts with different decellularizing solutions and freezing at –80°C for cell lysis,¹⁰ our single perfusion under constant pressure with SDS resulted in a comparable reduction of cellular content with minimal damage to the extracellular matrix. In this context it is indispensable to highlight our simple disinfection concept before the decellularization process compared with other findings in this field.^{9,10}

However, in our study, we achieved a smaller reduction of nuclear material compared to others,^{9,10} the role of small amounts of nuclear material or cytoplasmic debris within the remaining scaffold is unclear. There are no reports to date showing a direct cause-effect relationship between such cellular remnants and an adverse host response to date.²⁸ Nevertheless, residual cellular material within the neoscaffold might lead to calcification and will have to be further reduced to a minimum in our following studies by adapting the perfusion decellularization treatment.

The mechanical stability of whole porcine hearts on the decellularization process is a critical point of primary function. Any decellularization regime could make the scaffold more susceptible to enzymatic degradation in vivo, which would lead to a rapid decrease in the strength of the scaffold. Using the minimal SDS concentration necessary to render hearts acellular, we subsequently demonstrated retention of extracellular matrix morphology, which might also provide signals to seeded cells to enhance their differentiation and function.^{29,30} It is recognized that every tissue-engineered scaffold will result in a different cellular response in terms of degradation.³¹ As a consequence the selected scaffold should meet the mechanical properties and degradation times required.

Together, these studies serve as proof of concept for the generation of perfusion decellularized whole porcine hearts as a potential neoscaffold for myocardial tissue engineering.

In this work, the presented perfusion decellularization system has several technical and practical advantages. It is compact in size, has a low perfusion volume, little production costs and it is easy to handle. The components were constructed from materials that withstand the thermal stress of autoclaving for sterilization. Additionally, through the transparent texture of the reservoir an observation of the decellularization progress is enabled. Sterilization and detergent change was easy to perform through the outlet and inlet port. Hence, our system is applicable in most laboratories.

Additionally, the present protocol which uses SDS (4%) only as a detergent and avoids the use of other physical, enzymatic and chemical treatments such as freezing, trypsin and EDTA shows better preservation of the extracellular structure. It has been shown that especially this kind of treatment causes severe disruption of the normal ECM structure. Therefore it is essential to avoid a multimodal aggressive decellularization treatment to preserve mechanical stability for future reseeding protocols.²⁸

In conclusion, our presented model serves as a useful and simple tool with excellent functionality for different studies that allows production of 3-dimensional myocardial neoscaffolds. Modifications of the here described model could easily be used in perfusion decellularization systems of other tissues and organs.

Study Limitations

The mainstay of our structural analysis was digital image analysis following histological staining. Although our methods

suggest stable extracellular matrix fiber morphology, quantitative molecular analysis of collagen and elastin was not performed to assay structural changes within the fibers themselves.

One shortcoming of our study is the question, whether the porcine matrix carries potential immunogenic reactions and infectious risks through the transmission of porcine endogenous retrovirus^{32,33} or α -Gal epitopes. For this reason further studies are required to confirm or exclude this problematic issue.

Conclusions

The herein described method showed that perfusion mimics the transport conditions within native cardiac muscle and enables in vitro engineering of acellular constructs with uniform 3-dimensional tissue architecture.

The data collected suggest that porcine hearts decellularized by using SDS perfusion represent an important and potential scaffold for further efforts in the field of whole heart tissue engineering.

Our perfusion decellularization method produces a well preserved xenogenic 3-dimensional neoscaffold that fulfills the requirements of acellularity and reported susceptibility to recellularization.¹⁰ Additionally, our decellularization protocol shows the capability to effectively preserve mechanical stability of the ECM. This is important for preserving the mechanical integrity of the decellularized heart as whole organ for future reseeding steps. Cell extraction by using SDS is simple and almost complete, without significant disturbance of the extracellular matrix morphology. Whether these 3-dimensional neoscaffolds are able to show intact muscle tissue with living contracting cells, including force development and conduction velocity as it was already reported in a smaller model,⁹ is now the subject of ongoing studies.

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Disclosures

None.

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