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Preservation of aortic root architecture and properties using a detergent-enzymatic perfusion protocol

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ABSTRACT

Aortic valve degeneration and dysfunction is one of the leading causes for morbidity and mortality. The conventional heart-valve prostheses have significant limitations with either life-long anticoagulation therapeutic associated bleeding complications (mechanical valves) or limited durability (biological valves). Tissue engineered valve replacement recently showed encouraging results, but the unpredictable outcome of tissue degeneration is likely associated to the extensive tissue processing methods. We believe that optimized decellularization procedures may provide aortic valve/root grafts improved durability. We present an improved/innovative decellularization approach using a detergent-enzymatic perfusion method, which is both quicker and has less exposure of matrix degenerating detergents, compared to previous protocols. The obtained graft was characterized for its architecture, extracellular matrix proteins, mechanical and immunological properties. We further analyzed the engineered aortic root for biocompatibility by cell adhesion and viability *in vitro* and heterotopic implantation *in vivo*. The developed decellularization protocol was substantially reduced in processing time whilst maintaining tissue integrity. Furthermore, the decellularized aortic root remained bioactive without eliciting any adverse immunological reaction. Cell adhesion and viability demonstrated the scaffold's biocompatibility. Our optimized decellularization protocol may be useful to develop the next generation of clinical valve prosthesis with a focus on improved mechanical properties and durability.

1. Introduction

The aortic valve replacement is one of the most common heart surgeries carried out in industrialized countries. Acquired diseases like aortic valve stenosis or regurgitation and congenital defects such as the bicuspid aortic valve led to more than 200 000 aortic valve replacement surgeries per year worldwide. As the incidence of age-related degenerative valve disease is increasing, the demand of aortic valve prostheses is predicted to rise significantly [1].

The common and most effective treatment is the surgical or percutaneous intervention replacement of the affected valve. Currently the valve is substituted either by a mechanical or bioprosthetic valve, made from glutaraldehyde fixed porcine aortic valve or bovine pericardium. However, despite improvements in the field of valve replacement, these substitutes still present limitations such as the need for long-term anticoagulation with its severe side effects and a restricted durability due to structural deterioration [2]. An alternative therapeutic intervention is to use decellularized or cryopreserved homografts, but those are limited by the donor shortage and their intermediate and long-term degeneration. Furthermore, young patients with their high immunologic competence may require repeated replacements, which are associated

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with additional intra- and postoperative risks and thus a poorer outcome [3]. Therefore, further research is required to develop and optimize substitute for the aortic heart valve.

This ideal valve replacement has to meet several requirements. Ideally, it provides life-long durability, has optimal hemodynamic and mechanical properties, is resistant to infection as well as calcification and does not provoke any adverse immune response nor thrombogenic tendencies. Furthermore, it should be a viable system with the ability to adapt, remodel, regenerate, grow and maintain homeostasis to avoid repeated replacement of the valve.

Tissue engineered valves may fulfill these demands. Indeed, there are several preclinical studies of decellularized heart valve replacements in large animal models with promising results [4–7]. Initial so-far successful clinical studies of a decellularized heart valve have been realized for the pulmonary position [8,9], and more recently even for the more mechanically demanding aortic position [3]. However, none of these studies have yet shown improved long-term results in comparison to current state-of-the-art valve prostheses.

The currently applied engineering method aims to provide a non-immunogenic prosthesis with cell adhesion potential [10]. But the extensive decellularization protocols employed presumably exhibit negative long-term effects on the mechanical integrity and function due to extracellular matrix (ECM) protein disruption.

We have previously shown that a shorter decellularization protocol can reduce the degenerative processes by preserving the ECM [11] potentially improving the *in vivo* long-term outcome. Therefore, we developed a rapid decellularization protocol that can yield a non-immunogenic and non-toxic aortic root scaffold, with specific mechanical properties.

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley rats (Charles River, Sweden) weighing 250–300 g were used as donors for organs and for mesenchymal stem cells (MSCs) isolation. The animals were treated in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the care and use of laboratory animals" prepared by the National Academy Press, revised 1996. Ethical permissions were granted by the Stockholm South Ethical Committee (S149-12 and S43-13).

2.2. Organ harvesting

Hearts with lungs and adjacent vessels were harvested *en-bloc* under sterile conditions using standard surgical procedures. The dissected organs were immediately stored and transported in phosphate buffered saline (PBS, Invitrogen, Sweden) containing 1% antibiotic (penicillin, streptomycin) and antimycotic (amphotericin B) (Invitrogen, Sweden) on ice.

2.3. Decellularization procedure

To prepare the organ for decellularization, the aorta was cannulated and ligated to a 20 G blunt cannula (BD, Sweden). The left ventricle was punctured with another cannula through the apex whilst flushing with PBS to ensure that there was no leakage. The aortic root was decellularized using a perfusion detergent-enzymatic method at a speed of 1 mL/min in room temperature (Fig. 1A). The protocol consisted of two cycles with sodium deoxycholate 4% (Sigma–Aldrich, Sweden), followed by PBS, deoxyribonuclease I (DNAse) (Sigma–Aldrich, Sweden), and MilliQ water (Purelab Ultra, Elga, Germany). In the second cycle, 2 mM ethylenediaminetetra acetic acid disodium salt solution (EDTA, Sigma–Aldrich, Sweden) was supplemented to MilliQ water. To wash out remaining detergent, the aortic root was perfused with DPBS (Invitrogen, Sweden) for 60 min at a constant flow rate of 1 mL/min.

2.4. Tissue DNA quantification

Native ($n = 4$) and decellularized ($n = 4$) aortic tissues were processed for DNA isolation using the DNeasy Blood & Tissue Kit (Qiagen, Germany). All samples were processed according to the manufacturer's instruction. The purified DNA was eluted in RNase and DNase free water (Qiagen, Germany) and quantified using a Nano Drop® Spectrophotometer (ND-1000, USA).

2.5. Histological analyses

Native and decellularized aortic tissues were fixed overnight at room temperature in 4% formaldehyde (Histolab, Sweden) or embedded in OCT (Cryomount, Histolab, Sweden), snap-frozen and further processed. Paraffin embedded tissues were sectioned at 5 μm (Microm HM 350, Germany) and stained with: hematoxylin and eosin (H&E, Histolab, Sweden) to assess the architecture, 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, Sweden) to address the efficiency of the decellularization protocol, Masson's trichrome staining (Sigma–Aldrich, Sweden) to evaluate collagen, Verhoeff van Gieson Staining (Sigma–Aldrich, Sweden) to asses elastic fibers, and Movat pentachrome (Diapath, Bergamo, Italy) to visualize proteoglycans. All sections were visualized and imaged using an inverted microscope (Olympus IX71, Japan).

2.6. Immunohistochemistry

Cryosections of decellularized tissues sectioned at 8 μm (Microm HM 500M, Germany) were fixed in 4% formaldehyde (Sigma–Aldrich, Sweden) for 10 min. The sections were washed in PBS, and blocked using 5% goat serum (DAKO, Germany) for 1 h on a rocking platform at room temperature. Primary antibodies diluted in PBS: MHC 1 conjugated FITC (1:20, ab22367, Abcam, UK), MHC II (1:100, ab23990, Abcam, UK), fibronectin (1:100, ab6328, Abcam, UK), collagen I (1:100, ab34719, Abcam, UK), collagen IV (1:100, ab6586, Abcam, UK), von Willebrand Factor (abcam, ab6994; UK) and laminin (1:1600, ab6994, Abcam, UK) were applied and incubated at 4 °C on a rocking platform overnight, followed by a washing step using PBS with 0.1% Tween (Sigma–Aldrich, Sweden). The secondary antibody Alexa Fluor 488 (1:500, A11008, Invitrogen, Sweden) diluted in PBS was applied and incubated for 1 h at room temperature in a dark chamber. Slides were washed in PBS with 0.1% Tween and then counterstained with DAPI and mounted.

2.7. Functionality testing

To evaluate the functionality of the aortic root under physiological conditions, we determined the valve resistance to a retrograde pressure. The pressure curve of a retrograde flow applied to the valve was determined by connecting the aorta to a PBS-filled pump system (PHD 2000, Harvard Apparatus, USA) attached to a pressure transducer and amplifier module (TAM-D, Harvard Apparatus). A continuous flow of 5 mL/min was applied for 20 s followed by a 20 s pause and repeated for 20 cycles per tissue sample. The pressure and volume curves were monitored and recorded.

2.8. Rigidity testing

The decellularized and native tissue samples were embedded in OCT (Cryomount, Histolab, Sweden), snap-frozen and longitudinally sectioned at 10 μm (Microm, HM-500M, Germany). The sections were immersed in PBS and valve properties measured at room temperature using a CellHesion 200 atomic force microscope (JPK Instruments) mounted on a Zeiss Axiovert microscope (Germany) to determine the tissue stiffness. The triangular MLCT Microlever Probes (Veeco Probes) used had a spring constant of 0.03 N/m and were calibrated using a thermal noise method provided by the JPK CellHesion 200 control software V.3.3. The heterogeneous aortic valve was distinguished into three scan regions for evaluations (fibrosa, spongiosa and ventricularis). Young's Modulus was calculated from an average of five force–distance curves from 8 to 10 different positions along each layer using the JPK Image Processing software.

2.9. In vitro assay for biocompatibility

Decellularized aortic roots ($n = 9$) were evaluated using the Cell activation kit with Pholasin® (Knight Scientific, Plymouth, UK). Pholasin® is a highly sensitive chemiluminescent protein which detects reactive oxygen species (ROS). The samples were processed accordingly to manufacturer's instructions. Briefly, the kit can quantify antioxidant properties of the samples, and measure ROS production by added whole blood cells. The samples were analyzed by luminescence using a FLUOstarOptima plate reader (BMGLabtech, Germany). The kit includes two control chemicals (formyl-methionyl-leucyl-phenylalanine, fMLP, 10 μmol/L and Phorbol 12-myristate 13-acetate, PMA, 10 μmol/L)) that are strong inducers of ROS production. These were added during the experiment to prove the viability of the added cells.

2.10. Mesenchymal stem cells

Rat mesenchymal stromal cells (MSCs) were isolated from the bone marrow of hind limbs by cutting the femurs and tibiae at the metaphyses and flushing the marrow cavity with PBS (Invitrogen, Sweden) using a 23 G needle and syringe (BD, Sweden). Following centrifugation at 300 *g* for 5 min, the pellet was resuspended in Dulbecco's Modified Eagle Medium with 10% Fetal Bovine Serum and 1% antibiotic-antimycotic (all from Invitrogen, Sweden). The media was changed after 24 h to remove non-adherent cells, and the remaining adherent cell fraction was defined as MSCs at passage 0.

2.11. In vitro seeding of decellularized aortic valve

Decellularized tissue samples were embedded in OCT (Cryomount, Histolab, Sweden), snap-frozen and longitudinally sectioned at 10 μm (Microm, HM-500M,