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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,

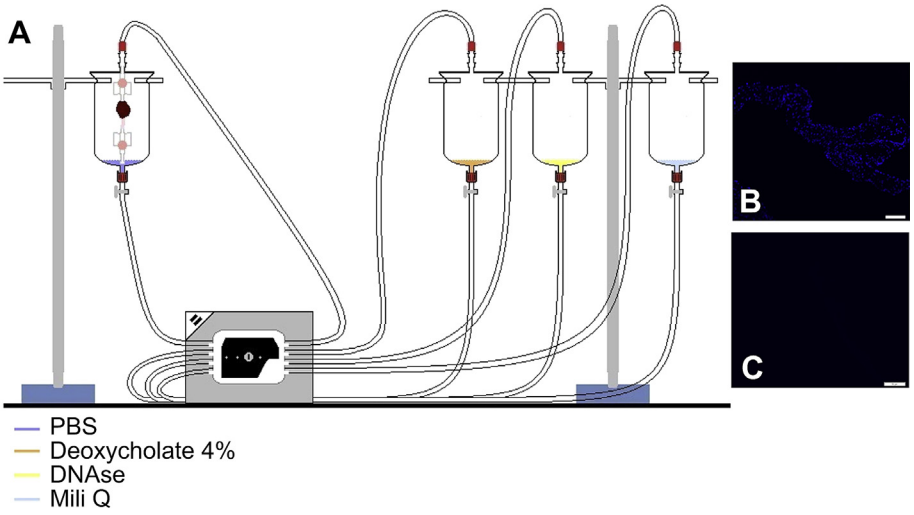

Fig. 1. Schematic overview of the procedure of decellularization. Sketch of the decellularization setup (A); DAPI staining of an aortic root before (B) and after decellularization (C).

Germany). The sections were placed onto attachment factor (Invitrogen, S-006-100, Sweden) coated plates and rat MSCs at passage 2 were seeded. Each cryosection was seeded with 120 000 cells and maintained in culture for 14 days, with media changed every three days. The samples were visualized using an inverted fluorescent microscope (IX71, Olympus, Japan). To reconfirm the *in vitro* seeding on the aortic valve: native, decellularized and reseeded aortic valves pieces (1×10^6 cells) were analyzed using scanning electron microscopy (SEM, JSM6490, JEOL, Japan); Tissue pieces were fixed in 2% glutaraldehyde and 1% paraformaldehyde (Histolab, Sweden) at room temperature. After rinsing in cacodylate buffer (Prolabo, France), the conduits were dehydrated through an ethanol gradient, critical point dried and sputter coated with gold. Then leaflets were visualized an imaged by scanning electron microscopy (SEM, JSM6490, JEOL, Japan).

2.12. *In vivo implantation*

Tissue samples of decellularized ($n = 6$) and native ($n = 6$) aortic roots were transplanted subcutaneously to immune-competent Sprague Dawley rats. Sham operations ($n = 6$) served as controls. Samples were harvested at day 7 and 14 and evaluated both macro- and microscopically. Samples were embedded in OCT, snap-frozen and cryosectioned at 8 μm (Microm, HM-500M, Germany). Sections were subsequently fixed in 4% formaldehyde for 10 min, and stained using H&E (Histolab, Sweden).

2.13. *Statistical analysis*

GraphPad Prism 5 (GraphPad Software, California, USA) was utilized for all statistical analysis, with significance levels of $p < 0.05$. All results were expressed as mean ± standard deviation. Data were compared using either an unpaired *t*-test or two-way ANOVA analysis. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparison test.

3. Results

3.1. *Morphological characterization*

We performed several decellularization methods and found the optimal one in terms of processing time reduction (4 h 20 min versus 24–154 h, $p < 0.05$), absence of intact cell nuclei based on DAPI staining (Fig. 1B and C) and a 70% reduction of total DNA.

The aortic root scaffold, obtained *via* this optimized decellularization protocol, was then further evaluated. The three-dimensional matrix architecture of the engineered tissue was well intact and almost unaltered, as indicated by H&E (Fig. 2A–D). The scaffold highly resembled the native tissue in terms of presence and configuration of glycosaminoglycan (Movat Pentachrome) (Fig. 2E–H), collagen (Masson's Trichrome) (Fig. 2I–L) and elastic fibers (Verhoeff van Gieson) (Fig. 2M–P).

We further analyzed the decellularized aortic root for ECM proteins and found the expression of ECM bioactive proteins, such as collagen I (Fig. 3A and B), collagen IV (Fig. 3C and D), laminin (Fig. 3E and F) and fibronectin (Fig. 3G and H). Notably, we detected a positive staining of the von Willebrand factor (vWf) at the surfaces of the valve and the aortic wall, without any presence of cell nuclei (Fig. 3I and J).

3.2. *Functional test*

We hypothesized that our optimized protocol preserved most of the native properties of the aortic root. We developed a biomimetic, retrograde pressure monitoring system to simulate *in vivo* settings. Using this setup, we made the following observations from the native aortic root (Fig. 4A): *a)* retrograde flow on the aortic surface led to valve closure; *b)* increasing the pressure over 50 ± 2 mm Hg led to a valve prolapse and leakage; *c)* a balance of continuous inflow and a physiological outflow leakage through the coronary vessels was indicated by a plateau at about 48 mm Hg. When we evaluated the decellularized aortic root, we found a similar pattern of the pressure curves, except for a lower peak pressure 22 ± 1 mm Hg. These monitored parameters displayed the properties of the decellularized aortic root with functional characteristics of the native tissue throughout the study.

3.3. *Rigidity testing*

Thereafter the decellularized tissue was evaluated by atomic force microscopy (Fig. 4B–H). The decellularization process resulted in general valve thickness reduction compared to the native tissue. The Young's modulus, calculated for each layer provided evidence that there was a general loss of elasticity in all three layers (fibrosa, spongiosa and ventricularis) after the decellularization process (Fig. 4H). Hence, the decellularization procedure led to an increase in stiffness of the aortic valve compared to native tissue. There was almost a three-fold increase in stiffness detected for the fibrosa layer (from 11 kPa to 30 kPa), a 6-fold increase for the spongiosa layer (15–92 kPa), and a 20-fold increase for the ventricularis layer (2–57 kPa). The ventricular layer of native valve showed the lowest Young's modulus and a considerable increase in stiffness after decellularization.

3.4. *In vitro biocompatibility assay*

In order to predict leukocyte response to the decellularized aortic valve tissue, we carried out a cell activation assay. We performed this

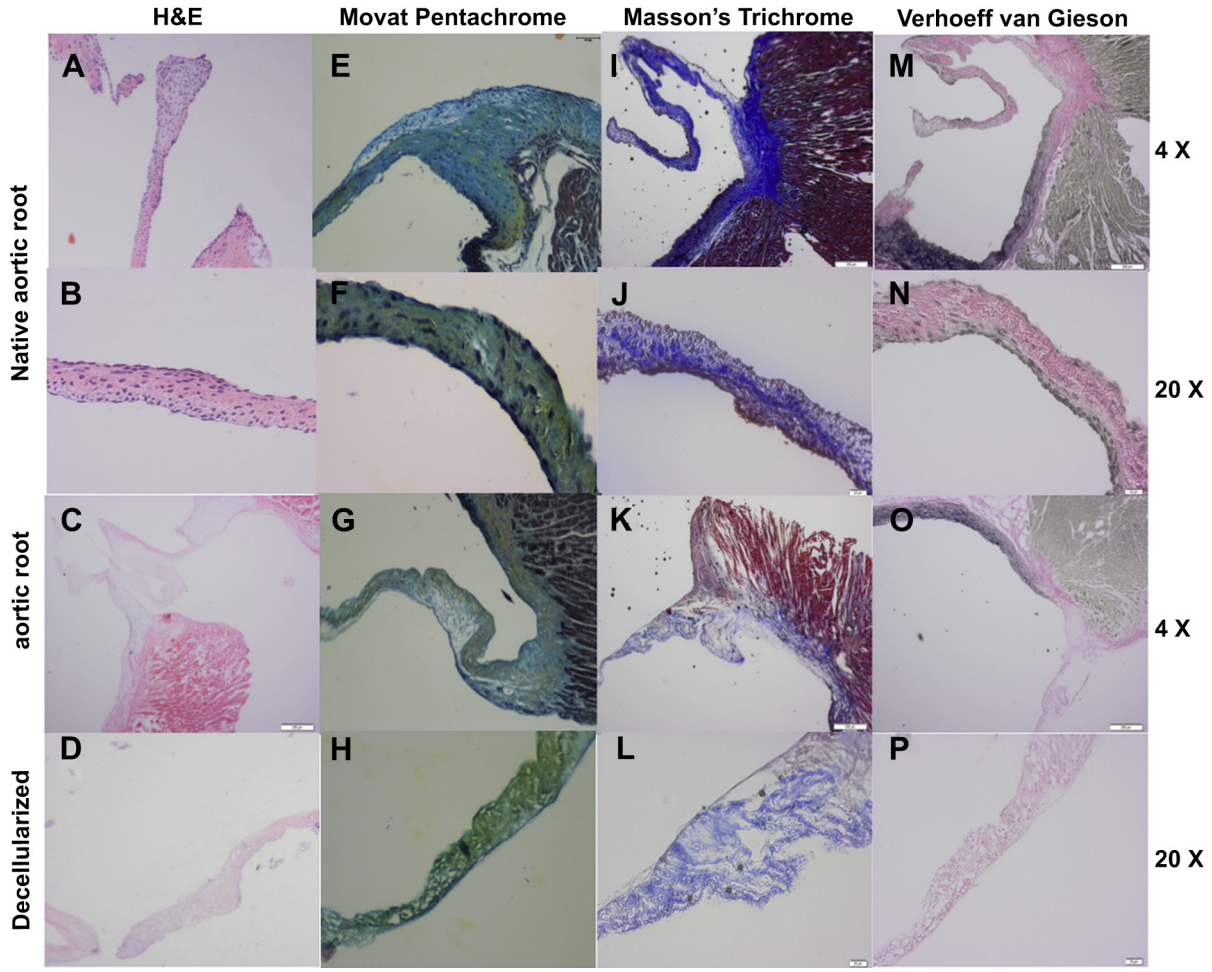

Fig. 2. The morphological and histological staining of native and decellularized aortic root. The native aortic root were assessed histologically for H&E (A and B), Movat pentachrome (E and F); Masson's trichrome (I and J); and Verhoeff van Gieson (M and N). The decellularized aortic root were assessed histologically by H&E (C and D); Movat pentachrome (G and H); Masson's trichrome (K and L); and Verhoeff van Gieson (O and P) stainings.

study by comparing our decellularization protocol with another established method for decellularizing small animal aortic root [12].

The first phase of the cell activation test (Fig. 5) displayed an initial emission of reactive oxygen species (ROS). The ROS emission was higher in on our decellularized scaffolds as compared to both control sample (PBS) and Kallenbach and colleagues' protocol [12]. This suggests that the scaffold we engineered did not contain antioxidant systems. This was reconfirmed when we detected least ROS production in samples prepared by a suboptimal protocol (our protocol but greatly reduced to 2 h 10 min). These results suggest that the levels of residual antioxidant systems in the decellularized tissues correlate to inefficient decellularization. In other words, a

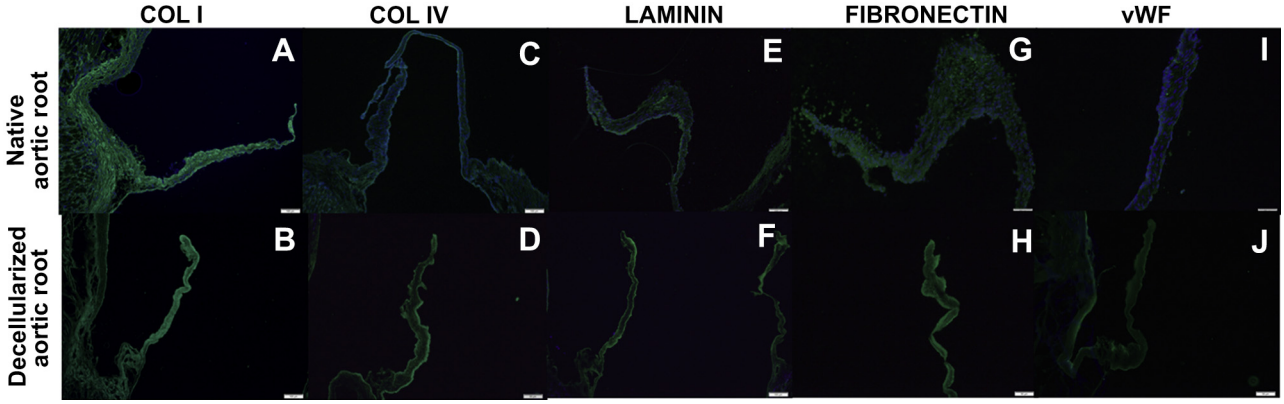

Fig. 3. Immunohistochemistry characterization of the extracellular matrix of the native and decellularized aortic root. Representative images of a native aortic root: Collagen I (Col I) (A); Collagen IV (C); Laminin (E); Fibronectin (G); and Von Willebrand Factor (vWF) (I). Representative images of a decellularized aortic root: Collagen I (Col I) (B); Collagen IV (D); Laminin (F); Fibronectin (H); and Von Willebrand Factor (vWF) (J).

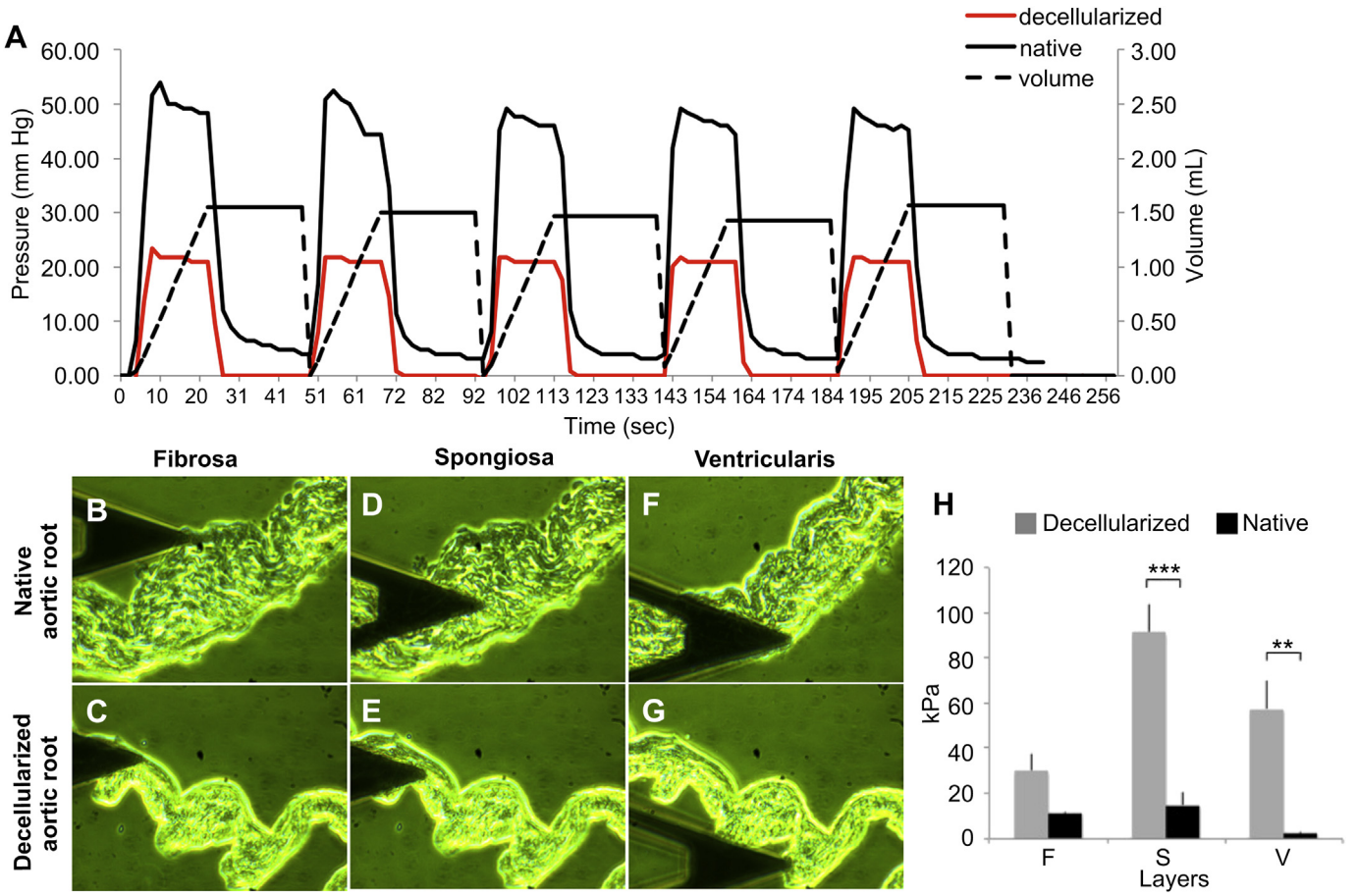

Fig. 4. The mechanical and functional evaluations for the aortic root. The retrograde pressure measurement recordings for a native and decellularized aortic root for pressure (mm Hg), time (sec) and volume (mL) (A). Morphology of all three layers of the aortic root by atomic force microscopy: native aortic root's fibrosa (B); spongiosa (D), and ventricularis (F) and decellularized aortic root's fibrosa (C); spongiosa (E), and ventricularis (G). The Young's modulus comparison for the stiffness of each layer in the aortic root (H).

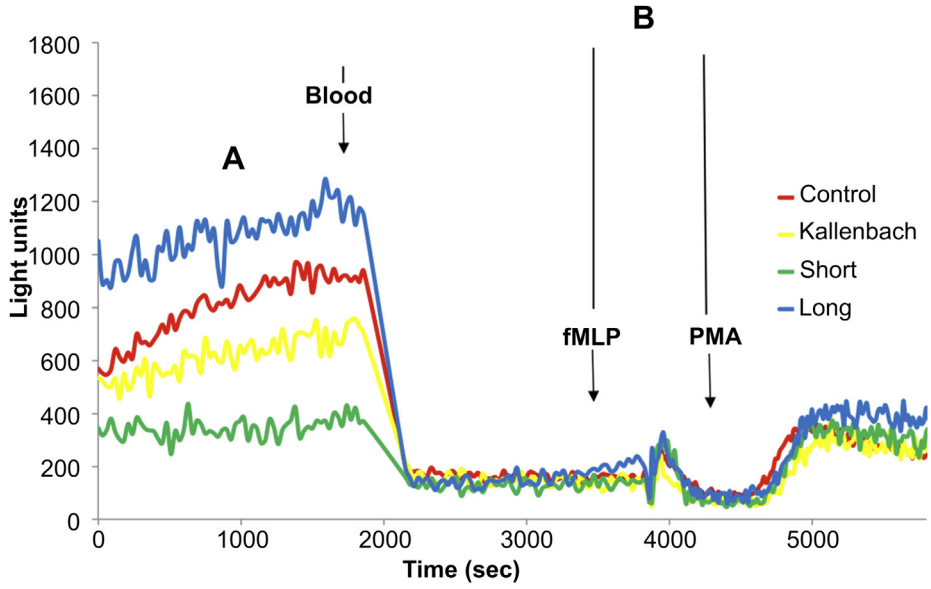

Fig. 5. *In vitro* analyses for biocompatibility of the aortic root. Measurement for the tissue emission of reactive oxygen species (A). Cell activation assay to predict leukocyte responses when formyl-methionyl-leucyl-phenylalanine (fMLP, 10 μmol/L) and Phorbol 12-myristate 13-acetate (PMA, 10 μmol/L) were added (B). The samples used were PBS (negative control, red); Kallenbach's protocol (trypsin protocol, yellow); short (Our developed protocol with reduced processing timing, green) and Long (Our developed protocol, blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

high production of ROS during this assay would indicate a well decellularized tissue. The decellularized tissue engineered with our protocol showed higher ROS production than any of the engineered tissues, indicating the lowest number of residual antioxidant systems. However, when whole blood (human) was added to the scaffolds, the activated leukocytes did not respond differently to any of the decellularized tissues than to the control (PBS), suggesting that neither of the samples would evoke a hyperacute rejection (humorally mediated). This proved the biocompatibility of the engineered aortic root.

3.5. Cell adhesion study

MSCs from rodents were seeded *via* the aortic root and were maintained in culture for 14 days in order to study the tissue's biocompatibility. The surface of the native aortic leaflet displayed cobblestone-like endothelial cell-layer on both sides (Fig. 6A and B). We did not detect any presence of cells in the decellularized aortic tissue on either the aortic or the ventricular surfaces (Fig. 6C and D). However, seeded scaffolds showed cells on both the aortic and ventricular using SEM (Fig. 6E–H).

3.6. In vivo implantation

Animals receiving decellularized tissue did not display any symptoms of health impairment or adverse immune response up to 14 days post-implantation (Fig. 7A–F). We confirmed by histological analyses that there were no signs of increased immunoreaction when compared to sham. Specimens of animals that were implanted with native aortic root showed a distinct inflammatory response (Fig. 7E and F). There was an extensive presence of granulocytes when compared to both experimental groups i.e. decellularized tissue and sham surgery (Fig. 7A–D). The inflammatory reaction in animals that received the decellularized tissues was almost similar to the sham operations. These findings suggest that these engineered scaffolds are non-immunogenic.

4. Discussion

The aortic valve replacement is one of the most common heart surgeries with a predicted rising demand due to demographic changes. Hence, there is an immense interest of providing an ideal valve substitute not only for optimal health care but also for economical issues. The currently available aortic valve substitutes still have their limitations such as the need for anticoagulation in mechanical and limited durability in biological valve replacements.

Tissue engineered valve replacement seems to be the next promising solution and more efforts must be done to further understand the relevant aspects, such as mechanical, immunological and functional properties. One of the major reasons for graft failure is the degenerative process, which may be induced by immunological responses [3]. Currently, various protocols investigated aortic valve decellularization and subsequent recellularization in small [12–15] and large [4–7] animals. Cell adhesion does not appear to be the only factor to obtain an optimal engineered valve for a successful clinical application. We believe that in order for one to provide durable valve prosthesis, mechanical properties are relevant. Mechanical properties of the decellularized valve are impaired when ECM is disrupted and alteration of the microstructure occurs. Long lasting decellularization protocols for 24–154 h [12–14], currently used in clinical and experimental settings, have a significant impact on the scaffold's properties, mainly on the rigidity and mechanical strength to withstand the retrograde pressure.

Here, we aim to develop a rapid method that protects the biomechanical properties whilst yielding a non-immunogenic scaffold. In the long-term we hypothesize that this can provide an improved durability with optimal hemodynamic. We obtained a completely decellularized aortic root *via* perfusion method within 4 h 20 min. The yielded scaffold preserved native-like microstructure and the essential ECM proteins that are relevant for cell attachment and proliferation [16]. These data suggest that the obtained scaffold is non-toxic and allows for cell adhesion. We further investigated the mechanical and functional characteristics of the decellularized aortic root and found trends similar to the native tissue for all monitored parameters, such as pressure related time points of valve closure, leakage and plateau phase. Notably, the measured peak pressure was reduced in decellularized tissues. In order to define the morphological region that contributed to this alteration, the rigidity of the tissue was evaluated in detail using atomic force microscopy [17] and found a significant increase in stiffness to all three layers of the valve.

Despite the significant reduction in processing time with preserved biomechanical properties, the engineered aortic root did not show any signs of immunogenicity as indicated by the absence leucocyte *in vitro* response to the tissue [18–20]. Although approximately 30% of total DNA remained in the tissue after processing, MHC I and II were not expressed (*data not shown*). Our *in vivo* heterotopic transplantation model did not show any adverse immunological response to the decellularized tissue, which

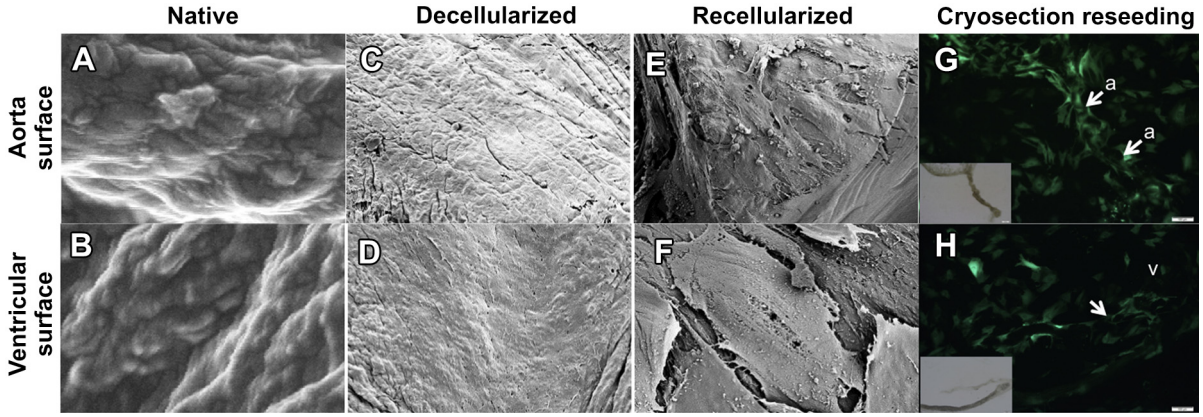

Fig. 6. *In vitro* biocompatibility through cell seeding. The aortic surfaces of the native (A); decellularized (C); recellularized (E) and reseeding GFP-labelled rat MSCs on cryosections (G), arrows and "*a*" indicate the cell attachment along the aortic side of the valve. The ventricular surfaces of the native (B); decellularized (D); recellularized (F) and reseeding GFP-labelled rat MSCs on cryosections, arrows and "*v*" indicate the cell attachment along the ventricular side of the valve (H).

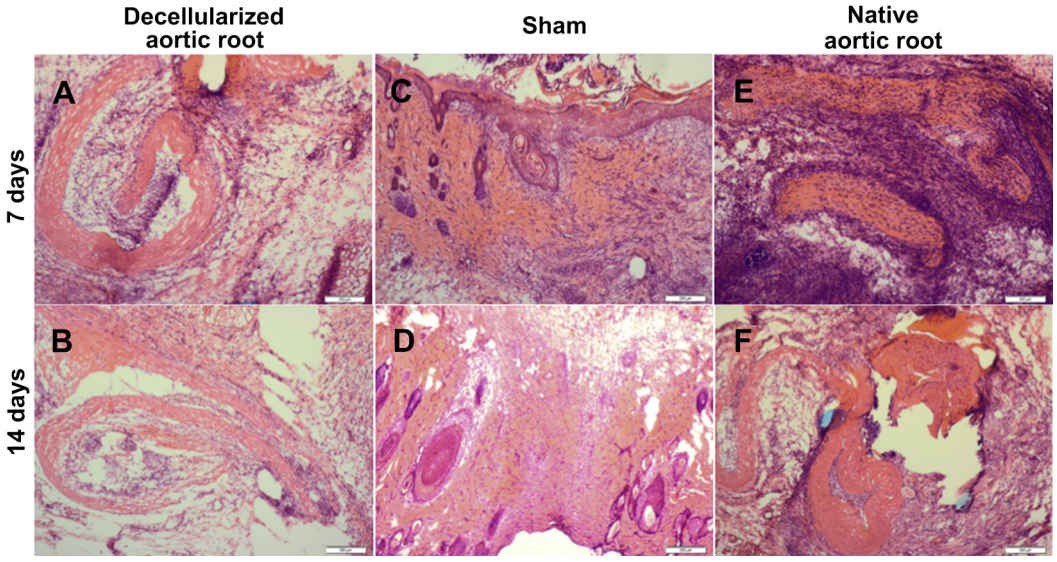

Fig. 7. Histological analyses of the heterotopical implanted tissues. H&E staining of decellularized (A and B); sham (C and D) and native (E and F) aortic root after 7 and 14 days post-implantation.

suggests that the significance of remained DNA might not be as crucial as assumed previously. The significance of remained DNA is currently controversial and widely discussed [21–23].

5. Conclusions

The process of degeneration and functional loss of biological aortic roots may be due to the aggressive and long-lasting decellularization protocols. This could attribute towards an unfavorable impact on the biomechanics of the obtained scaffold in spite of a successful recellularization of the aortic root, with subsequent impaired *in vivo* outcomes. We have shown in this study that the shortened decellularization protocol using a perfusion setup preserved the organ's architecture, ECM composition, biomechanics, and yielded a non-immunogenic scaffold. Further *in vivo* studies will be necessary to determine the impact of the developed decellularization method for the durability of the aortic root.

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