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Original Research Article

Effect of three decellularisation protocols on the mechanical behaviour and structural properties of sheep aortic valve conduits

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ABSTRACT

Purpose: To determine the best method for decellularisation of aortic valve conduits (AVCs) that efficiently removes the cells while preserving the extracellular matrix (ECM) by examining the valvular and conduit sections separately.
Material/methods: Sheep AVCs were decellularised by using three different protocols: detergent-based (1% SDS + 1% SDC), detergent and enzyme-based (Triton + EDTA + RNase and DNase), and enzyme-based (Trypsin + RNase and DNase) methods. The efficacy of the decellularisation methods to completely remove the cells while preserving the ECM was evaluated by histological evaluation, scanning electron microscopy (SEM), hydroxyproline analysis, tensile test, and DAPI staining.
Results: The detergent-based method completely removed the cells and left the ECM and collagen content in the valve and conduit sections relatively well preserved. The detergent and enzyme-based protocol did not completely remove the cells, but left the collagen content in both sections well preserved. ECM deterioration was observed in the aortic valves (AVs), but the ultrastructure of the conduits was well preserved, with no media distortion. The enzyme-based protocol removed the cells relatively well; however, mild structural distortion and poor collagen content was observed in the AVs. Incomplete cell removal (better than that observed with the detergent and enzyme-based protocol), poor collagen preservation, and mild structural distortion were observed in conduits treated with the enzyme-based method.
Conclusions: The results suggested that the detergent-based methods are the most effective protocols for cell removal and ECM preservation of AVCs. The AVCs treated with this detergent-based method may be excellent scaffolds for recellularisation.

1. Introduction

Valvular heart diseases are important causes of mortality. Congenital and acquired valvular dysfunction is frequently treated by heart valve replacement, which is a common procedure worldwide [1]. Aneurysmal rupture is one of the leading causes of death, with an incidence of 3–4% [2]. However, the current treatments for aortic valve defects in children are not satisfactory, and the incidence of regurgitation and stenosis are more common in children than in older patients. Children and neonates treated with mechanical valve prostheses can be confronted with significant postoperative complications due to the lack of appropriately sized prostheses. Therefore, to minimise probable postoperative complications, ideally, in terms of cell removal and mechanical properties, a decellularised aortic valve conduit (AVC) should be used as a substitute for juvenile patients. Surgical treatment of ascending aortic aneurysms accompanied with AV regurgitation is still challenging, and the management is a debatable matter. Therefore, alternative treatments are needed to reduce the incidence of this life-threatening event.

Mechanical and bio-prosthetic valves are the two conventional substitutes for dysfunctional heart valves. However, the application

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of these replacement valves is associated with several disadvantages, including their inability to grow, which is a particular problem in patients that are still growing, structural valve deterioration, the need for long-term anticoagulation therapy, immunological responses, and the absence of remodelling [3]. To overcome these limitations, decellularised heart valve conduits can be used as alternatives with physiological functions that are similar to those of natural valves, with the least immunogenic potential [4].

The process of engineering AVCs includes an effective method for decellularisation of both the valve and conduit sections for further in vitro or in vivo cell seeding. A critical point of the decellularisation process is the preservation of the extracellular matrix (ECM) in both sections. The goal of decellularisation is maximum cell and nuclei removal to minimise the cellular debris remaining in the AVCs and greatly lessen the adverse immune responses, inflammation, fibrous scarring, and calcification [4–7]. A practical scaffold should be capable of self-repair and remodelling, be durable, and not induce rejection. Therefore, it is necessary to determine the best decellularisation protocol for complete cell removal and preservation of the tissue microstructural features of the AVCs.

In this study, we compared three different protocols for the decellularisation of AVCs, and we introduced an ideal method that strikes a balance between maximum cell removal and preservation of tissue microstructure, with optimal biochemical properties to avoid possible complications encountered after transplantation.

2. Material and methods

2.1. Tissue preparation

The ethical committee of Tehran University of Medical Sciences approved this study. Twelve sheep, with a mean body weight of 47 ± 5 kg, were sedated by an injection of xylazine (0.15 mL per 10 kg body weight; IM). The animals were anesthetised by administration of atropine sulphate (11 mg per 10 kg body weight), propofol (5 mg per kg body weight), and diazepam (0.27 mg per kg body weight). Hearts were harvested under sterile conditions and placed in Hank's balanced salt solution (HBSS) containing antibiotics (penicillin, gentamycin, streptomycin, and amphotericin). AVCs were precisely dissected and decellularised. Harvested AVCs had a thin rim of subvalvular muscle tissue and a short arterial segment. A total of 12 AVCs were included in the study, and they were randomly divided into 3 decellularisation groups and a control group.

2.2. Decellularisation process

Nine AVCs were treated with 3 different decellularisation protocols (detergent-, enzyme-, and detergent and enzyme-based), with 3 AVCs per group. Before starting each treatment, all valves and conduits were placed in PBS for 90 min, and the solution was changed every 30 min to rinse the tissues. Then, each group was treated as described below. All materials were purchased from Sigma–Aldrich (Belgium) unless stated otherwise.

2.2.1. Protocol 1 (1% SDS + 1% SDC)

In this detergent-based decellularisation method, AVCs were treated with 1% (w/v) sodium dodecyl sulphate (SDS) and 1% (w/v) sodium deoxycholate (SDC) dissolved in distilled water for 48 h at room temperature. Then, they were washed with distilled water 2 times at 4 °C for 12 h each. In the final stage of the decellularisation procedure, the AVCs were washed in phosphate buffered saline (PBS) (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.78 mmol $Na_2HPO_4{\cdot}2H_2O$, and 2 mmol/L KH_2PO_4, pH 7.4) for 24 h by changing the solution every 12 h. This step was performed to eliminate the applied detergents.

2.2.2. Protocol 2 (1% Triton + EDTA + RNase and DNase)

In this detergent and enzyme-based method, the AVCs were placed in 1% (w/v) Triton X-100 with a combination of 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 20 μg/mL RNase, and 200 μg/mL DNase (dissolved in PBS) at 37 °C for 24 h. In the second step, samples were incubated in HBSS (137 mmol/L NaCl, 5.4 mmol/L KCl, 0.5 mmol/L KH_2PO_4, 4.1 mmol/L $NaHCO_3$, and 0.33 mmol/L Na_2HPO_4 without Ca, Mg, and phenol red) at 4 °C for 72 h. The solution was changed every 12 h (6 times).

2.2.3. Protocol 3 (0.1% Trypsin + RNase and DNase)

All the steps in this protocol were similar to those in protocol 2. The only difference was the use of 0.1% (w/v) Trypsin (TS) instead of Triton X-100.

All the above-mentioned steps were conducted under continuous shaking on a mechanical shaker at 50 revolutions per minute (rpm).

2.3. Histological examination and DAPI staining

To evaluate and compare the effectiveness of our decellularisation protocols in removing the cellular components of both the valvular and conduit sections, native and decellularised AVCs were cut into 3 mm × 4 mm sections and fixed in 10% neutral buffered formalin (Merck, Darmstadt, Germany) at pH 7.4 for 48 h at room temperature. Then, they were washed in distilled water, dehydrated in a graded series of ethanol, embedded in paraffin, and cut into 5–8-μm thick sections.

To visualise remnant deoxyribonucleic acids (DNA), the sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; Biotium, Inc.). To enhance fluorescence, the DAPI solution was diluted to 30 nM in PBS, and 300 μL was pipetted directly onto all samples. After incubation in the dark for 30 min and rinsing with distilled water, the slides were evaluated with a fluorescent microscope. Images were taken with a Nikon digital camera (DXM 1200).

Picrosirius red and Russell–Movat pentachrome staining were also used for collagen typing and histological evaluation of the ECM. For Picrosirius red staining, paraffin-embedded blocks were cut into 5-μm slices. The sections were deparaffinised and hydrated in graded series of alcohol. The slides were stained for 1 h in Picrosirius red solution (0.1% Sirius red in saturated picric acid), followed by two washes of 0.5% acetic acid without counterstaining. Under polarised microscopy, type I collagen fibres are seen as thick, strongly birefringent, yellow-orange fibres, and type III fibres are seen as thin, weakly birefringence, greenish fibres.

Different connective tissue constituents are highlighted by pentachrome staining. Using this technique, collagen, elastin, muscle, mucin, and fibrin can be differentiated. For Movat pentachrome staining, paraffin-embedded blocks were cut into 5-μm slices, deparaffinised, and hydrated in a graded series of alcohol. Briefly, mucin was stained with Alcian blue 8GS and converted to insoluble blue pigment by treatment with alkaline alcohol; nuclei and elastic fibres were stained black with Verhoeff's haematoxylin; fibrinoid, fibrin, and muscle were stained red with Crocein scarlet-acid fuchsin solution; and collagen and reticular fibres were stained yellow with Saffron.

2.4. Scanning electron microscopy (SEM)

Valvular and conduit sections of AVCs treated with the 3 decellularisation protocols as well as fresh control specimens were fixed with 2.5% glutaraldehyde (Merck, Darmstadt, Germany). Then, they were washed in PBS 3 times for 60 min each to remove the glutaraldehyde. Both native and decellularised samples were fixed with 1% osmium tetroxide, 0.8% potassium