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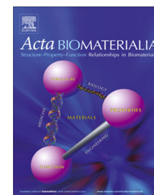

Full length article

Effects of combined cryopreservation and decellularization on the biomechanical, structural and biochemical properties of porcine pulmonary heart valves

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ABSTRACT

Non-fixed, decellularized allogeneic heart valve scaffolds seem to be the best choice for heart valve replacement, their availability, however, is quite limited. Cryopreservation could prolong their shelf-life, allowing for their ideal match to a recipient. In this study, porcine pulmonary valves were decellularized using detergents, either prior or after cryopreservation, and analyzed. Mechanical integrity was analyzed by uniaxial tensile testing, histoarchitecture by histological staining, and composition by DNA, collagen (hydroxyproline) and GAG (chondroitin sulfate) quantification. Residual sodium dodecyl sulfate (SDS) in the scaffold was quantified by applying a methylene blue activation assay (MBAS). Cryopreserved decellularized scaffolds (DC) and scaffolds that were decellularized after cryopreservation (CD) were compared to fresh valves (F), cryopreserved native valves (C), and decellularized only scaffolds (D). The E-modulus and tensile strength of decellularized (D) tissue showed no significant difference compared to DC and CD. The decellularization resulted in an overall reduction of DNA and GAG, with DC containing the lowest amount of GAGs. The DNA content in the valvular wall of the CD group was higher than in the D and DC groups. CD valves showed slightly more residual SDS than DC valves, which might be harmful to recipient cells. In conclusion, cryopreservation after decellularization was shown to be preferable over cryopreservation before decellularization. However, *in vivo* testing would be necessary to determine whether these differences are significant in biocompatibility or immunogenicity of the scaffolds.

Statement of Significance

Absence of adverse effects on biomechanical stability of acellular heart valve grafts by cryopreservation, neither before nor after decellularization, allows the identification of best matching patients in a less time pressure dictated process, and therefore to an optimized use of a very limited, but best-suited heart valve prosthesis.

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1. Introduction

With 29,672 heart valve procedures in Germany in 2013 [1], and the global figure on the rise, the search for an optimal valve replacement is ever more important. While mechanical valves bear the risk of biofilm formation [2] and require lifelong anticoagulation therapy [3], bioprosthetic grafts are prone to calcification, especially in young patients, leading to structural failure, which limit their durability to approximately 15 years [4]. Decellularized homogeneic heart valves might be a better alternative since they exhibit natural haemodynamics, do not require anti-coagulation and demonstrate decreased immunogenicity compared to valves containing cells [5], suggesting that glutaraldehyde treatment is unnecessary. Large animal studies have demonstrated

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repopulation with autologous interstitial cells producing ECM proteins in allogeneic [6–8] (ovine to sheep) and xenogeneic [9,10] (porcine to sheep) models. This remodeling suggests growth potential in pediatric patients, which has been confirmed by clinical studies in children [11]. Decellularized pulmonary homografts, implanted orthotopically in children, showed the lowest transvalvular pressure gradients, and the lowest insufficiency and explantation rates when compared to implanted cryopreserved homografts and bovine jugular veins [11]. Neumann et al. further demonstrated an absence of systemic cellular immune response in patients with decellularized homograft valves [12]. Until 2013, more than 600 decellularized heart valves had been implanted clinically [13].

The supply of decellularized homografts depends on tissue donation, but the ever growing discrepancy between donors and patient recipients highlights the need of a reliable storing method that would allow the efficient matching between donor graft and recipient. Cryopreservation is the most common method used in tissue banks for storing cells and tissues for long periods of time. Fahy et al. summarized the basic advantages of cryopreserved tissue for transplantation: i) flexibility of scheduling; ii) time for quality control; iii) liquid nitrogen storage is inexpensive and available in every hospital [14]. Cryopreserved vascular homografts have demonstrated superior performance, lower complication rates and better resistance to infection [15,16], while homograft valves have demonstrated lower infection, reoperation and structural degeneration rates compared to fresh grafts [17]. On the other hand, biomechanical studies have shown divergent results. Stemper et al. found similar biomechanical properties between fresh and frozen arterial grafts, whereas in refrigerated specimens the sub-failure stress and ultimate stress was significantly decreased [18]. Chow et al. observed structural and biochemical changes both in refrigerated and frozen tissue (−20 °C and −80 °C) [19]. The different outcome could be potentially explained by the different storing solutions [19–21] and storing temperatures used, as well as the use of dimethyl sulfoxide (DMSO) [22]. DMSO prevents intracellular ice formation to some extend and is, therefore, especially recommended for cellular tissues [23]. DMSO might also be important in acellular scaffold cryopreservation, since water redistribution and crosslink breaking by ice crystal formation in the extracellular matrix (ECM) during freezing can damage the tissue and result in impaired biomechanical properties [24].

The aim of this study was to assess the effect of cryopreservation prior and after decellularization on the biomechanics, histoarchitecture and biochemistry of porcine pulmonary valve scaffolds, and compare them to fresh, cryopreserved only, and decellularized only pulmonary valves. In its consequence, the question whether homografts already stored in cryobanks could possibly be decellularized and as thus pose a better substitute should be addressed.

2. Materials and methods

2.1. Tissue sample procurement

Fresh pulmonary heart valves were harvested at the local slaughterhouse from 80–120 kg landrace pigs, and assessed in five different groups, including: (i) fresh, non-treated, non-fixed (F); (ii) decellularized (D); (iii) cryopreserved (C); (iv) decellularized and subsequently cryopreserved (DC); (v) cryopreserved and subsequently decellularized after thawing (CD). Surrounding fat and loose tissue was removed from all valves, which were subsequently washed in an iodine solution (7.5% w/v) for 5 min, followed by a washing step with phosphate-buffered saline (PBS) supplemented with 1% (v/v) penicillin.

2.2. Decellularization

Decellularization of valve groups D, DC and CD was conducted on an orbital shaker at room temperature. The valves were treated with 0.5% (v/v) TritonX-100 for 24 h, followed by 0.5% (w/v) SDS for another 24 h, and 24 h in deionized water. All solutions were replaced by fresh solutions after 12 h. Finally, the valves were washed for 10 cycles in PBS (12 h each) to remove the detergents and cell debris.

2.3. Cryopreservation

Fresh (for groups C and CD) and decellularized valves (for group DC) were transferred into tubes with cryopreservation medium, containing 50% (v/v) fetal calf serum (FCS), 42.5% (v/v) Medium199 and 7.5% (v/v) DMSO, and subjected to a standardized 6-step cooling process to −100 °C that was developed by the German Society for Tissue Transplantation (DGFG; Feodor-Lynen-Str. 21, 30625 Hannover). The cryopreserved valves were stored at −150 °C for at least one week until further assessment. The valves were thawed by incubation in a 37 °C water bath until the cryopreservation medium had thawed. Subsequently, the valves were rinsed in PBS.

2.4. Tissue sample dissection

For the biochemical, histological and biomechanical assessment, the valves were divided into parts. One part, comprising half of a cusp together with sinus, valvular wall (proximal to sinotubular junction) and pulmonary trunk (distal to sinotubular junction), was fixed in 3.7% (v/v) formalin for subsequent paraffin embedding and histological staining. The remaining part of the valve was lyophilized separately. While the cusps were used for GAG analysis, the valvular wall was used for measuring DNA, hydroxyproline (HxP) and SDS content. A strip of the pulmonary artery trunk served as sample for biomechanical testing,

2.5. Histological analysis

Histological staining was conducted on 6 µm thick sections using Haematoxilin and Eosin (H&E) to assess the degree of decellularization and Movat-pentachrome for assessing the ECM protein distribution (collagen: yellow; elastin: red; GAGs: blue; nuclei: brown-red).

2.6. DNA assay

Three samples of lyophilized pulmonary trunk wall (2 mg, 5 mg, 10 mg) were incubated for 10 min with 200 µl 0.5% (w/v) SDS (Carl Roth) in triplicates at 100 °C. After adding 790 µl tris(hydroxyme thyl)-aminomethane (TRIS) (10 mM, pH7.5) and 10 µl Proteinase K (⩾600 m Anson-U/mL, Serva) the tissue was digested overnight at 60 °C. For analysis digested samples were diluted 1:10 with NaCl (200 mM)/TRIS (10 mM, pH7.5). Salmon sperm DNA diluted with NaCl (200 mM)/TRIS (10 mM, pH7.5) SDS (0.1%) were used to generate a standard curve. 100 µL of each sample dilution and 100 µL Hoechst 33258 solution (1 µg/mL) were placed in 3 wells of a 96-well-plate. Fluorescence was measured at λ = 465 nm.

2.7. Glycosaminoglycan (GAG) assay

Given the crucial role of GAGs in the integrity and performance of the valve cusps, only the lyophilized cusps were used for the quantification of chondroitin sulfate, one of four GAG-groups, according to the protocol established by Farndale et al. [25]. 1 mg lyophilized tissue was incubated at 60 °C for 3 h with

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