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Comparative study of the Triton X-100-sodium deoxycholate method and detergent-enzymatic digestion method for decellularization of porcine aortic valves

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Abstract. – BACKGROUND: Valve replacement is the primary surgical treatment for heart valve disease. However, the clinical benefit of biological valve substitutes is limited by their potential immunogenicity.

AIM: To seek a better method of preparing biological scaffolds for tissue engineering heart valves, we compared the ability of different decellularization procedures to remove cells and maintain the scaffold structure.

MATERIALS AND METHODS: Specimens of fresh porcine aortic valve leaflets were randomly divided into group I (n = 16), group II (n = 16) and group III (n = 16). The valve leaflets in group I were not decellularized; group II were treated with Triton X-100 and sodium deoxycholate; and group III were treated by a detergent-enzymatic digestion method. The valve leaflets were investigated by gross examination, hematoxylin-eosin staining, Masson's trichrome staining, and scanning electron microscopy to observe the valve structure and the integrity of collagen and elastin. The DNA content was measured to confirm the removal of cells.

RESULTS: The detergent-enzymatic digestion method completely removed cells and led to valve fiber structural alterations in group III. The Triton X-100-sodium deoxycholate method achieved both complete decellularization and preservation of the valve fiber structure in group II.

CONCLUSIONS: The detergent-enzymatic digestion method is a better technique for decellularization than the Triton X-100-sodium deoxycholate method, as it enables both complete decellularization and preservation of the valve scaffold structure.

Key Words:
Tissue engineering, Porcine aortic valve, Decellularization, Biological scaffold.

Introduction

Currently, valve replacement is the primary surgical treatment for heart valve disease; however, the clinical benefit of valve substitutes is limited by concomitant anti-coagulation therapy or tissue degeneration[1]. Mechanical valves offer a favorable durability, but require lifelong anticoagulation therapy and can lead to complications. Biological valves have favorable hemodynamics and lead to fewer thromboembolic complications. However, the potential immunogenicity of biological valves is associated with a poor durability[2]. In addition, pediatric patients require multiple valve replacement operations as they grow, as no valve substitutes have growth potential. With the development of tissue engineering, the concept of tissue engineering heart valve (TEHV) has been proposed by a number of researchers, in order to overcome the limitations of existing valve substitutes. Selection of the appropriate scaffold materials is key to the success of valve reconstruction[3]. Decellularized native biological valve scaffolds have been used as the most common scaffold material, and offer stability, a lack of cellular antigens and low immunogenicity[4]. At present, combined application of detergent, trypsin, a trypsin inhibitor and nucleic acid enzymes can be used to more effectively remove the cells, and better maintain the integrity of the extracellular matrix with a relevant proteoglycan and glycosaminoglycan content, collagen and elastic fibers, The knowledge of the composition and distribution of the various glycosaminoglycans/proteoglycans (GAGs/PGs) appears to be essential for under-standing the relationship between structure and mechanics of heart valve leaflets[5]. in order to obtain the ideal decellularized valve scaffold.

In this study, to optimize the preparation method for tissue engineering heart valve biological scaffolds, we carried out a comparative study of two methods of combined acellular combination for the

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decellularization of porcine aortic valves: the Triton X-100-sodium deoxycholate method, and a detergent-enzymatic digestion method.

Materials and Methods

Materials

Fresh porcine aortic valve conduits were obtained from a local slaughterhouse and transported in phosphate buffered saline (PBS) containing 100 U/ml penicillin and 100 µg/ml streptomycin. The valve leaflets were dissected from the conduits after washing repeatedly in physiological saline under aseptic conditions, and the valve leaflets were conserved in PBS after rinsing.

Trypsin, Triton (Triton X-100), ethylenediamine sodium tetracetate (EDTA), deoxycholic acid, deoxyribonuclease I (DNaseI), RNase I (RNasel).

Processing of Porcine Aortic Valves

Specimens of fresh porcine aortic valve leaflets were randomly divided into group I (not decellularized; $n = 16$); group II (Triton X-100-sodium deoxycholate method; $n = 16$); and group III (detergent-enzymatic digestion method; $n = 16$).

Group I porcine aortic valve leaflets were washed thoroughly in PBS, then conserved in preservation solution at 4°C. Group II porcine aortic valve leaflets were treated with 0.25% Triton X-100, 0.25% sodium deoxycholate, 0.02% EDTA, 20 mg/L RNase I and 200 mg/L DNase I with shaking for 48 h at 37°C[6]. The samples were washed thoroughly several times in PBS and then conserved in preservation solution at 4°C. Group III porcine aortic valve leaflets were treated with 0.05% trypsin and 0.02% EDTA with shaking for 12 h, washed thoroughly several times with PBS, then treated with 1% sodium deoxycholate, 20 mg/L RNase I and 200 mg/L DNase I with shaking for 24 h[7]. All decellularization procedures were performed at 37°C. The samples were washed thoroughly several times with PBS and then conserved in preservation solution at 4°C.

Testing of Valve Scaffolds

Valve leaflets from all three groups were fixed in neutral formalin, embedded in paraffin, sectioned and stained with Masson's Trichrome or hematoxylin and eosin (HE), and their morphology was observed by light microscopy. Valve leaflets from all three groups were also fixed in 2.5% glutaraldehyde at 4°C, and then observed by scanning electron microscopy.

DNA was isolated from the valve leaflets of all three groups ($n = 12$) using the DNA Extraction Kit. Briefly, the valve leaflets were frozen in liquid nitrogen, ground into a powder; 50 mg of the powder was vortexed with 3 ml Buffer CL and 15 µl protease K, and incubated for 3 h at 55°C until completely dissolved. The remaining steps were performed according to the manufacturer's instructions, and the absorbance values were measured at nm using a spectrophotometer.

Statistical Analysis

The statistical software SPSS version 17 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The experimental data is presented as ±SD and was compared using one-way ANOVA; $p < 0.05$ was considered significant.

Results

Gross Observations of the Valve Scaffolds

Samples from all three groups were subjected to gross observation. The valve leaflets from group I were milky white, soft and resilient (Figure 1A); the acellular valve scaffolds in group II were milky white with a glossy surface, flexible, transparent, and maintained a normal morphology (Figure 1B); the acellular valve scaffolds of group III were milky white with a glossy surface and slightly swollen. The elastic of the group III scaffolds was slightly diminished and transparent, and the valves had a similar morphology to normal valves (Figure 1C).

Light Microscopy Evaluation of the Valve Scaffolds

Samples from all three groups were subjected to HE staining: the fiber layer, spongy layer and ventricular structural layer of the values in group I were intact. The fiber structure was arranged in neat, close rows, with a wavy appearance. The surface of the fiber layer and ventricular layer were coated in a monolayer of endothelial cells. Mesenchymal cells were also visible in the spongy layer. Additionally, a large number of endothelial cells and stromal cell nuclei were visible within the leaflet structure of the valves in group I (Figure 2A). The fibers of the acellular valve scaffolds in group II displayed no structural changes, and were neat and compact; the wavy fiber structure was well preserved. Endothelial cells, mesenchymal cells and Aizen nuclei were not observed in the scaffold structures of group II (Figure 2B). In

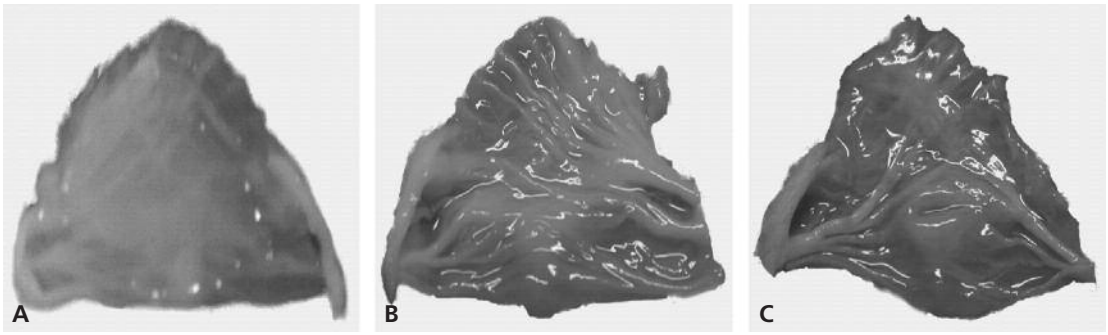

Figure 1. ***A,*** Native porcine aortic valve leaflet (group I). ***B,*** Scaffold from a porcine aortic valve after treatment by the Triton X-100-sodium deoxycholate Method (group II). ***C,*** Scaffold from a porcine aortic valve after treatment by the detergent-enzymatic digestion method (group III).

the valve scaffolds from group III, the fiber structure was loosely arranged, with a shallow fiber wave pattern. Endothelial cells and mesenchymal cells were not observed; however, a small number of Aizen nuclei were detected in the scaffold structures of group III (Figure 2C).

Samples from all three groups were also subjected to Masson's trichrome staining. The fibers of the valve leaf from the scaffolds in group I stained blue, and were neat, compact and wavy with a large number of blue and brown nuclei (Figure 3A). The fiber structure of the valve scaffolds from group II was arranged in close waves and was morphologically well preserved, with no damage and no blue brown nuclei (Figure 3B). The valve leaf fibers from the scaffolds in group III were arranged loosely, in shallow waves; however, no damage or blue brown nuclei were observed (Figure 3C).

Scanning Electron Microscopy Evaluation of the Valve Scaffolds

Samples from all three groups were observed by scanning electron microscopy. The surface of the valve leaf structures in group I was covered by a single smooth layer of endothelial cells; the endothelial cells maintained their typical pebblestone morphology and endothelial cell microvilli were visible (Figure 4A). The fiber structure of the acellular valve scaffolds in group II was intact, dense and orderly, with no obvious fractures; endothelial cells, mesenchymal cells and residual fragments were not detected (Figure 4B). The fiber structure of the acellular valve scaffolds in group III was scattered, without fractures and an absence of endothelial cells and mesenchymal cells; however, a small amount of residual cellular debris was observed (Figure 4A).

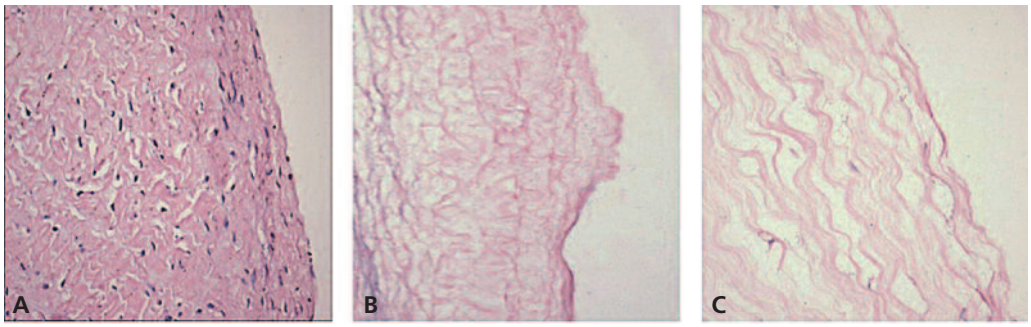

Figure 2. ***A,*** Native porcine aortic valve leaflet (group I). ***B,*** Structure of the scaffold from a porcine aortic valve after treatment by the Triton X-100-sodium deoxycholate method (group II). ***C,*** Structure of the scaffold from a porcine aortic valve after treatment by the detergent-enzymatic digestion method (group III). All images are light microscopy, HE staining, ×200.

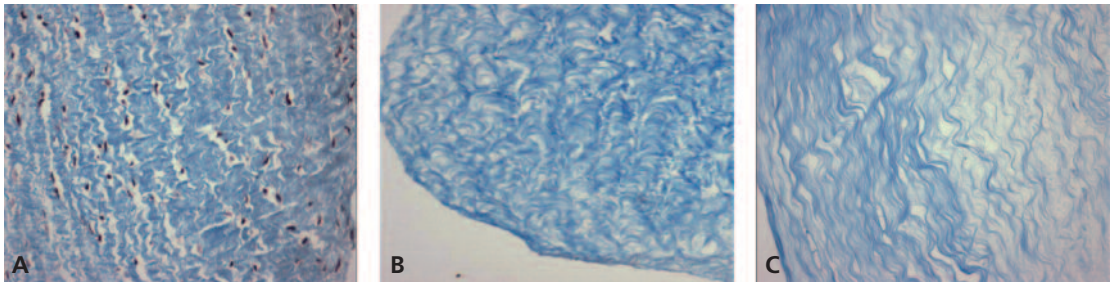

Figure 3. ***A,*** Native porcine aortic valve leaflet (group I). ***B,*** Structure of the scaffold from a porcine aortic valve after treatment by the Triton X-100-sodium deoxycholate method (group II). ***C,*** Structure of the scaffold from a porcine aortic valve after treatment by the detergent-enzymatic digestion method (group III). All images are light microscopy, Masson's trichrome staining, ×200.

DNA Content of the Valve Scaffolds

Note: the DNA content of group I and the other groups were significantly different ($p < 0.01$); the DNA content of group II and group III were not significantly different ($p > 0.05$).

Table I. DNA content of each valve leaf (n = 12, $\bar{\chi} \pm$ SD).

	Group I	Group II	Group III
DNA content (μg/50 mg)	27.000 ± 3.041	0.408 ± 0.085	0.422 ± 0.074

Discussion

The ideal acellular natural biological valve scaffold should have an absence of the major immunogenic cellular components, such as lipid membranes, membrane-associated antigens and soluble proteins, while retaining the integrity of the extracellular matrix proteins collagen and elastin[4]. It has been shown that the interaction of collagen and elastin in the aortic valve leaflet is crucial for maintaining tissue architecture during development and in tissue function[8]. A loss of GAGs has been described in both fixed tissues, during the preparation of bioprostheses, and in cryopreserve native tissues, with possible consequences on the graft performance[5,9]. However, residual cells or the residues of cells remaining within acellular porcine aortic valves can lead to a severe inflammatory reaction when the valves are implanted into humans[10]. Therefore, an effective method is required to remove tissue and cells, disinfect the tissue and reduce the immunogenicity of valve scaffolds, without destroying extracellular matrix components or the structural integrity of the scaffold.

Trypsin and detergents are both commonly used to remove cells; however, needs to be used at high concentrations and requires a longer processing time. High concentrations of trypsin can hydrolyze fibrin, thereby damaging the valve scaffold structure[11]. Cebotari et al [12] showed that residual toxicity can affect

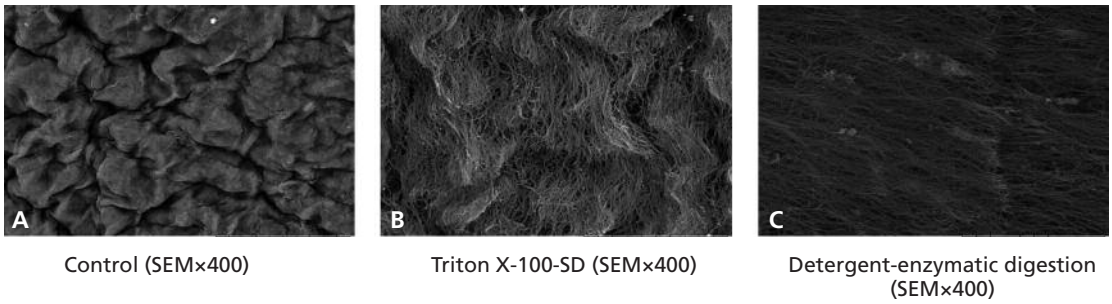

Figure 4. ***A,*** Native porcine aortic valve leaflet (group I), SEM ×1500. ***B,*** Structure of the scaffold from a porcine aortic valve after treatment by the Triton X-100-sodium deoxycholate method (group II), SEM ×4000. ***C,*** Structure of the scaffold from a porcine aortic valve after treatment by the detergent-enzymatic digestion method (group III), SEM ×400.

the cultivation of cells treated with high concentrations of detergent, but demonstrated that low concentrations of detergent below 0.5% left almost no residue and had no influence on cell culture after acellular. In addition, it has been shown that 0.01% define (SDS) could remove cells completely, but that sodium dodecyl sulfate (SDS) remained in the tissues and was difficult to release from the tissue elastin, which lead to degeneration of the collagen fibers due to swelling and fractures, reducing the hydrothermal stability, which exerted a strong cytotoxic effect on subsequent cell cultivation[13].

Based on the insufficiency of detergent treatments alone, a large number of researchers have demonstrated that the combined application of and detergent may be more advantageous. Courtman et al[7] previously used a detergent-enzyme digestion method for bovine pericardium, and showed that the heat shrink temperature of the three-dimensional collagen structure was not affected by decellularization, but observed a slight increase in stress relaxation. Cigliano A et al[5] used a detergent-enzyme digestion method for bovine pericardium, aortic and pulmonary roots,the result show that the extraction of the GAGs could affect the mechanical behavior of the valves by introducing flexural rigidity,thickness decrease and favor- ing an increase in tissue Ca^{2+} content. Dong et al[14] compared several acellular methods, and showed that the combined application of 1% Triton-X 100 and 0.05% trypsin could completely remove the tissue cells; 1% Triton-X 100 was superior to the single trypsin method or repeated freezing-thawing method. Li et al[15] showed that sodium deoxycholate combined with an enzyme digestion method was the optimal method, compared with two other types of detergent in contrast to three types of detergents are respectively combined with trypsin for decellularization.

In this study, the values in group III were initially treated with 0.05% trypsin to dissolve matrix components in the valve leaflets, which may have loosened the cells and destroyed the integrity of the cells, and addition of the protease inhibitor EDTA prevented the destruction of collagen and elastic. Then, the ionic detergent sodium deoxycholate (1%) was used to dissolve the lipid bilayer of the cell membrane to lyse the cells completely, and treatment with the nucleases removed residual fragments of nucleic acid, which may reduce the ability of nuclear DNA to induce an inflammatory reaction after transplantation. After this treatment, under gross observation, the acellular valve scaffolds in group III had a glossy surface, were slightly swollen and transparent, with slightly diminished elastic, and retained a normal morphology similar to the control valve scaffolds treated with PBS. Under light microscopy, the fiber structure of the valve scaffolds in group III was arranged loosely, with a shallow fiber wave structure. Additionally, endothelial cells and mesenchymal cells were not detected; however, a number of blue-stained nuclei were observed in the scaffold structure in group III. Under scanning electron microscopy, the fiber structure of the acellular valve scaffolds in group III was not obviously fractured, endothelial cells and mesenchymal cells were absent, and a small amount of residual cellular debris was observed. The DNA content of the acellular valve scaffolds in group III was significantly lower than the control group. This study demonstrated that the detergent-enzymatic digestion method can remove completely cells from the valve leaf, but that the valve leaf structure underwent a certain degree of change, and a small amount of cellular residue remained.

Based on the moderate acellular effects and synergistic action of Triton X-100 and sodium deoxycholate, Samouillan et al[16] removed valve tissue cells using Triton X-100 combined with sodium deoxycholate; and reported that exposure to 1% Triton X-100 and sodium deoxycholate for 24 h could effectively remove tissue cells. Kasimir et al[6] showed that treatment with 0.5% Triton X-100 and 0.25% sodium deoxycholate for 24 h and 48 h could effectively remove porcine aortic valve cells and preserve the collagen/elastin ratio, compared with natural valves. In 2004, Rieder et al[17] successfully performed stent reendothelialization by seeding vascular endothelial cells onto acellular valve scaffolds prepared using 0.25% Triton X-100 and sodium deoxycholate, and the cells adhered and grew favorably. Sun et al[18] also studied biological characteristics of acellular valve scaffolds prepared using 0.25% Triton X-100 and sodium deoxycholate, and showed that the mechanical properties were similar to normal biological valves; additionally, the immunogenicity of the values decreased significantly, and endogenous porcine viral DNA was undetectable. At the same time, Ruan et al[19] seeded bone marrow mesenchymal stem cells onto scaffolds to achieve the initial construction of tissue engineered artificial heart valves.

In this work we treated the porcine aortic values in group II with 0.25% Triton X-100 and 0.25% sodium deoxycholate, and added a protease inhibitor to prevent cathepsin activation which impairs the matrix structure, and nucleases to digest intracellular nucleic acid, in order to prevent an in-

flammatory reaction after transplantation due to the presence of endogenous porcine retroviral nucleic acids. Gross observation demonstrated that the acellular valve scaffolds in group II had a glossy surface, were flexible and transparent, and maintained a normal morphology. The fibers of the acellular valve scaffolds in group II did not show any structural changes, and were neat and compact, with a well preserved wavy fiber structure. Endothelial cells, mesenchymal cells and blue-stained nuclei were not observed within the scaffold structure by light microscopy. Under scanning electron microscopy, the fiber structure of the acellular valve scaffolds was intact and arranged densely in an orderly manner, with no fractures; endothelial cells, mesenchymal cells or residual cellular fragments were not observed. The DNA content of the acellular valve scaffolds in group II was significantly lower than the control group. These results prove that low concentrations of Triton X-100 and sodium deoxycholate effectively removed the cells and maintained the integrity of the matrix in the porcine aortic valve leaflets.

Conclusions

This study demonstrates that a low concentration of Triton X-100 and sodium deoxycholate can effectively remove porcine aortic valve cells, does not lead to protease-mediated digestion, prevents the destruction of collagen fibers and elastic fibers, and preserves the structural integrity of the scaffold fibers. The Triton X-100 and sodium deoxycholate method produces an ideal scaffold for further studies on recellularization with recipient autologous human cells.

Conflict of Interest

The Authors declare no conflict of interest.

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