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Original Article

Effect of carbodiimide cross-linking of decellularized porcine pulmonary artery valvular leaflets

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Abstract: Decellularization provides low immunogenicity and is only slightly subject to calcification in tissue engineering. However, the mechanical properties of the tissues are weakened after decellularization. We adopted cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to treat decellularized porcine pulmonary artery valvular leaflets to improve their mechanical properties. Twenty porcine pulmonary artery valvular leaflets were divided into three groups: the fresh control group A, group B treated with trypsin and Triton X-100 to remove cells, and group C cross-linked with EDC after decellularization. All samples were evaluated the physical and mechanical properties and were then subcutaneously embedded in rabbits. These valvular leaflets were removed after 1, 2, or 4 weeks and checked for pathological changes. The cells of the valvular leaflets were completely removed. The thickness of the valvular leaflets was thinner in group B than in group A ($P<0.01$). In the subcutaneous embedding of the group B samples, there was mild immunological response after 1-2 weeks, and parts of the scaffolds were degraded. After 4 weeks, fibroblasts had grown into the scaffolds. In group C, there was an increase in the tensile strength and thermal shrinkage temperature in group C compared with group B ($P<0.01$). In subcutaneous embedding of the group C samples, there was a mild immunological response after 1-2 weeks. The fibroblasts had grown into the samples. The EDC-based cross-linking procedure can enhance the tensile strength of decellularized pulmonary artery valvular leaflets and both decrease the valvular leaflets' rejection and promote tissue regeneration in vivo.

Keywords: Decellularization, EDC cross-linking, pulmonary artery valvular leaflets, mechanical properties

Introduction

Xenogeneic biological scaffolds are commonly used in various reconstructive surgery applications and are increasingly used in regenerative medicine strategies for tissue and organ replacement. The decellularization of porcine scaffold materials has been used because of this technique's preservation of the natural extracellular matrix [1-5]. However, cell removal impairs the mechanical properties of the valvular leaflet structure and exposes bare collagen fibres that are highly thrombogenic. In recent years, many reports have shown that the cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) can improve the properties of decellularized xenogeneic scaffold materials, including mechanical strength and immunogenicity [7-12]. We therefore adopted the cross-linking agent EDC to treat porcine pulmonary artery valvular leaflets in an effort to improve the mechanical properties and decrease the immunogenicity of decellularized valvular leaflets.

Material and methods

Materials

Twenty porcine hearts were purchased from Qian Xihe Food Co., Ltd. (Beijing, China). These hearts were obtained from male or female pigs that weighed from 100 to 150 kg and that were in good health. Triton X-100, trypsin, EDC,

2-(N-morpholino) ethanesulfonic acid (MES) buffer, and N-hydro-xysuccinimide (NHS) were purchased from Sigma (USA).

Collection and processing of the porcine pulmonary artery valvular leaflets

Under sterile conditions, pulmonary artery valvular leaflets were isolated from the porcine hearts. The myocardial tissue under the pulmonary artery valvular leaflets was removed, and each sample was cut into three parts and then randomly allocated to one of three groups: A, B, and C.

Grouping and processing methods

Group A included fresh porcine pulmonary artery valvular leaflets (control group). Group B was treated with trypsin and the detergent Triton X-100 to remove cells. Group C was treated with the cross-linking agent EDC after decellularization with trypsin and Triton X-100, as in group B.

Decellularization

The samples from groups B and C were decellularized by soaking in a 0.25% trypsin- ethylenediaminetetraacetic acid (EDTA) solution while agitating for 36 hours at a constant temperature of 37°C. The samples were then thoroughly washed with PBS and agitated for 48 hours in 1% Triton X-100 solution at room temperature. Next, the samples were persistently shaken in 20 mg/L RNase and 200 mg/L DNase solution for 24 hours at a constant temperature of 37°C. The samples were again thoroughly washed with PBS, placed in D-Hanks' solution containing penicillin and streptomycin, and stored at 4°C. After treatment, the group C samples were ready for EDC cross-linking.

EDC cross-linking

EDC was used as a cross-linking agent to treat the decellularized pulmonary artery valvular leaflets. The samples from group C were soaked in 50 mmol/L MES buffer solution prepared in 40% ethanol for 24 hours, followed by the addition of EDC (2 g/L) and NHS (0.5 g/L), which adjusted the MES concentration to 30 mmol/L. The samples were then agitated under ambient conditions for 12 hours and thoroughly washed to arrest the cross-linking process. Samples from the three groups were examined by ordinary biopsy, scanning electron microscopy to evaluate the extent of the decellularization and were utilized in the physical and mechanical testing.

Properties of the valvular leaflets

Haematoxylin-eosin (HE) staining and scanning electron microscopy: The samples were fixed in formaldehyde for H&E staining and in 4% paraformaldehyde and 1% osmic acid for scanning electron microscopy (the Olympus Company, Japan).

Determination of water content: Trypsin-EDTA and Triton X-100 detergent were used to decellularize the samples from groups B and C. The samples were then soaked in distilled water for 48 hours, and the surfaces were dried with filter paper. The valvular leaflets from the three groups were accurately weighed, followed by drying inside an oven at 80°C for 24 hours. The samples were then weighed again, and the percentage of unit weight was calculated.

Determination of thickness: The thickness of the samples from the three groups was measured using a CH-B-type thickness gauge (Shanghai Sophisticated Apparatus Limited Company, Shanghai, China).

In vitro testing of mechanical properties

Tensile strength: Using a sharp tool, dissection of the pulmonary artery valvular leaflets was performed, yielding samples that were 1.5 cm in length and 0.5 cm in width. We then tested the tensile strength using an RGW-0.1 electronic tensile testing machine at a speed of 50 mm/min.

Thermal shrinkage temperature test: The samples were placed in distilled water, and the thermal shrinkage temperature was tested using a JS981-B leather shrinkage temperature detector at a rate of 2°C/min.

Animal experiments in vivo: This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Beijing Anzhen Hospital, Capital Medical University. All surgery was performed under anaesthesia, and all efforts were made to minimise suffering.

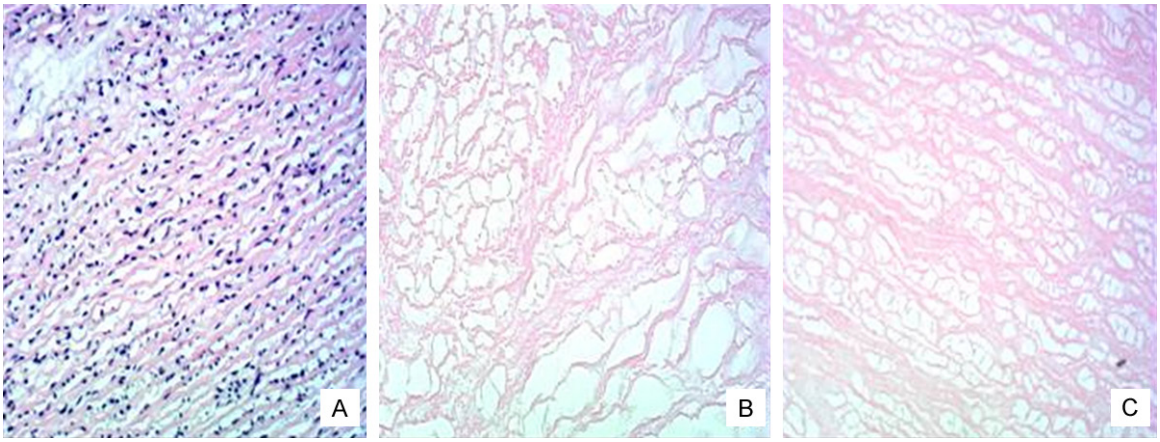

Figure 1. Representative HE-stained section showing the porcine pulmonary arterial valvular leaflets before and after decellularization. Magnification, A-C ×100. A: Fresh pulmonary arterial valvular leaflets; B: Decellularized pulmonary arterial valvular leaflets; C: Decellularized pulmonary arterial valvular leaflets cross-linked by EDC.

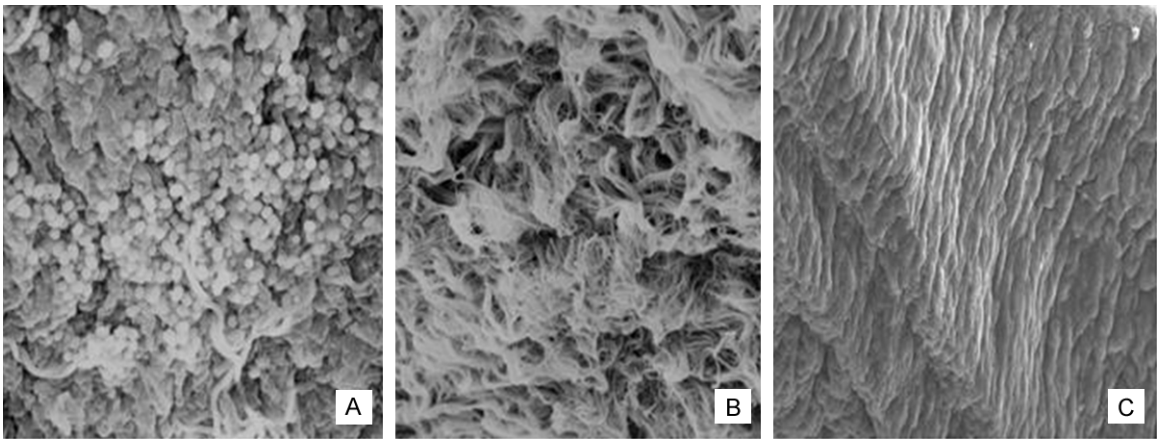

Figure 2. Scanning electron microscopy showing the porcine pulmonary arterial valvular leaflets before and after decellularization. Magnification, ×1000. A: Fresh pulmonary arterial valvular leaflets; B: Decellularized pulmonary arterial valvular leaflets; C: Decellularized pulmonary arterial valvular leaflets cross-linked by EDC.

Fifteen New Zealand rabbits, supplied by the Beijing Anzhen Hospital, were used as experimental animals. After anaesthesia, the skin on the back of the rabbits was cut, and the valvular leaflets were embedded subcutaneously.

Morphological observation: The subcutaneously embedded tissue samples were collected at 1, 2, and 4 weeks after implantation. The samples were observed to determine whether degradation and absorption had occurred. The fibrous tissue surrounding the samples was removed, after which the samples were fixed in formaldehyde for histopathology.

Statistical analysis: SPSS 13.0 software was used in the present study. All of the data were expressed as the mean ± SD ($\bar{x}$ ± S). Comparisons between the three groups were performed with one-way ANOVA. The results were considered to be statistically significant when $p<0.05$.

Results

Morphological observation

In group A, the colour of the pulmonary artery valvular leaflets was reddish. After decellularization (group B and group C), the colour of the pulmonary artery valvular leaflets was white and the tissue structure was loose.

HE staining

By microscopy, it was observed that trypsin-EDTA and Triton X-100 detergent treatment

Table 1. Comparison of the physical properties of the porcine pulmonary artery valvular leaflets between the three groups

	Group A (*n* = 10)	Group B (*n* = 10)	Group C (*n* = 10)	*F* Value	*P* Value
Water Content (%)	0.86 ± 0.03	0.87 ± 0.02	0.87 ± 0.04	0.085	0.919
Thickness (mm)	0.20 ± 0.02	0.14 ± 0.01	0.14 ± 0.02	42.822	<0.0001
Tensile Strength (MPa)	7.48 ± 0.59	5.32 ± 0.56	6.42 ± 0.53	37.216	<0.0001
Thermal Shrinkage Temperature (°C)	72.48 ± 0.31	72.86 ± 0.41	77.84 ± 0.88	25.711	<0.0001

completely removed the cellular components of the pulmonary artery valvular leaflets. In the group B and C samples, we observed that there was a three-layered structure in the artery valvular leaflets, similar to the group A samples. The orientation of the fibrous layers was effectively normal, but the tissue was looser than usual. Part of the small fibre structure was disordered, and a section of the tissues in the spongiosa layer was destroyed (**Figure 1**).

Scanning electron microscopy

Endothelial cells inside the porcine pulmonary artery valvular leaflets were well organized in group A. After decellularization, there were no cells present, and the collagen fibres were exposed, forming hollow gaps in group B. In group C, more compact fibres were observed after cross-linking by EDC, and there were no hollow gaps (**Figure 2**).

Physical properties of the pulmonary artery valvular leaflets

Water content: There was no significant difference in the water content of the pulmonary artery valvular leaflets between the three groups (group A vs B, P>0.05; group A vs C, P>0.05; group B vs C, P>0.05, **Table 1**).

Thickness: The valvular leaflets in groups B and C were significantly thinner than in group A, which indicated that decellularization had removed the cells from the tissue. The tissue thickness of the pulmonary artery valvular leaflets in the decellularized scaffolds in group B and in the EDC cross-linked scaffolds in group C was not significantly different (P>0.05, **Table 1**).

Tensile strength: The tensile strength of the pulmonary artery valvular leaflets in group B decreased compared with the strength observed in groups A and C (P<0.01, **Table 1**), which indicated that the EDC cross-linking process increased the tensile strength of the decellularized tissue.

Thermal shrinkage temperature: There was no difference in the shrinkage temperature between the pulmonary artery valvular leaflets in groups A and B (P>0.05). Additionally, the shrinkage temperature significantly increased for the pulmonary artery valvular leaflets in group C compared with groups A and B (P<0.01, **Table 1**).

Effects of subcutaneous embedding: One week after embedding the samples, there was a large quantity of lymphocyte infiltration in the pulmonary artery valvular leaflets in group A. In group B, there was a small quantity of lymphocytes in the pulmonary artery valvular leaflets, and in group C, few lymphocytes and a small quantity of fibroblasts were observed (**Figure 3**).

Two weeks after embedding, altered colour in the pulmonary artery valvular leaflets and constricted and obstructed valvular leaflets were observed. The tissue tightly adhered to the periphery and was therefore not easy to separate and remove in group A. Microscopy shown that parts of cells had undergone necrosis and were then absorbed, so there were only small amounts of lymphocyte aggregation in group A. In group B, the pulmonary artery valvular leaflets were white and exhibited soft tissue, poor mechanical strength, severe adhesions, and peripheral tissues that were not easily separated. Additionally, there was a small quantity of lymphocyte infiltration and fibroblasts. The pulmonary artery valvular leaflets in group C were also white and easily separated and exhibited good elasticity and moderate strength. Microscopy revealed few inflammatory cells and spindle-shaped fibroblasts that had spread parallel to the scaffolds (**Figure 3**).

Four weeks after embedding, the group A tissue was difficult to separate, dark red in colour, and lacking in lustre. The tissue also exhibited

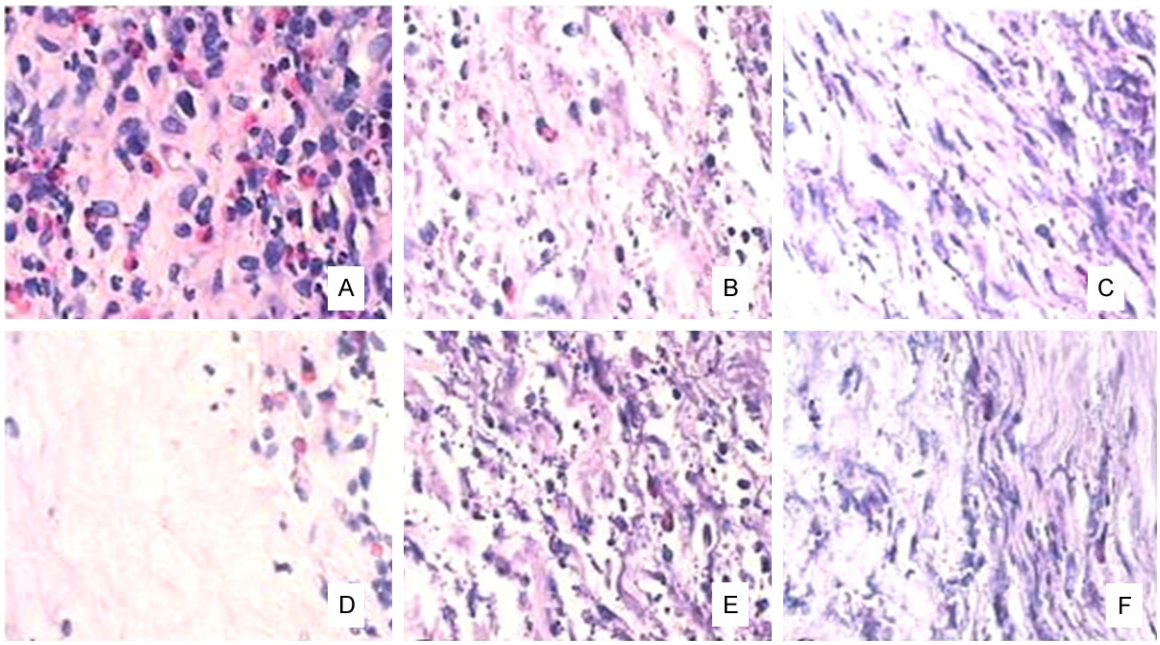

Figure 3. HE staining showing the porcine pulmonary arterial valve 1 week and 2 weeks after embedding. Magnification, ×400. A-C: HE staining 1 week after embedding. D-F: HE staining 2 weeks after embedding. A, D: Fresh porcine pulmonary arterial valvular leaflets; B, E: Decellularized porcine pulmonary arterial valvular leaflets; C, F: Decellularized porcine pulmonary arterial valvular leaflets cross-linked by EDC.

an unsmooth surface that was contracture-shaped, with part of the tissues not integrated. The group B samples were soft to the touch and had poor strength. Parts of the valvular leaflets had disappeared, and the remaining tissue was white and adherent. Lastly, the group C samples were soft, bright white in colour, and flexible. These valvular leaflets exhibited moderate strength, had no scleroses, and were easily separated. By microscopy, we observed that there were similar results to that at two weeks.

Discussion

Valve replacement represents the most common surgical therapy for end-stage valvular diseases. The increased risk of postoperative haemorrhage, thromboembolism, and drug-drug interactions due to commonly used artificial mechanical heart valvular leaflets affect patients' quality of life. Biological valvular leaflets are accompanied by a low risk of thromboembolism and endocarditis and offer growth potential for paediatric patients. However, biological valvular leaflets are associated with different major complications, such as deterioration of the valve structure, graft calcification, limited durability, and liability to an immunological response [13]. Patients are at risk of valve-related complications and reoperation. The use of tissue engineering methods to create heart valve constructs has the potential to overcome the fundamental drawbacks of more traditional valve prostheses [14-18]. The aim of this study was to determine the effect of EDC cross-linking on scaffolds' physical and mechanical properties and in vivo pathological changes following heterogenic transplantation.

Decellularization techniques have led to the development of scaffolds for multiple organs and tissues, including the heart, heart valvular leaflets, blood vessels, liver, lung, and kidney. Decellularized xenogenic biological scaffolds have been successfully used in the clinic for tissue engineering and regenerative medicine applications [15, 18]. The clinical effect of decellularized organ and tissue scaffolds is encouraging. Although the decellularization process significantly reduces cell-mediated immunorejection of acellular xenogenic scaffolds, the process may induce a mild immune reaction [5, 19-21]. Additionally, the mechanical strength of acellular xenogenic scaffolds also declines following excessive degradation. The physical properties of the fibrous matrix in decellularized tissue are also different from this matrix's performance in fresh tissue,

including decreased thickness and tensile strength. Moreover, various factors during decellularization, such as concentration, method, time, and tissue thickness, can lead to poor decellularization (e.g., cell debris residue and incomplete cell removal), which can affect the survival of implants and tissues. In this study, decellularization was confirmed by H&E staining, scanning electron microscopy. Trypsin was first used to cause the stromal cells to detach from the gaps in the fibres and to undermine the integrity of the cells. The nonionic detergent Triton X-100 was then used to extract cell debris and to completely remove the cellular components through sustained agitation and washing. General histopathology, scanning electron microscopy confirmed that the cells had been completely removed and that the fibrous scaffolds were integrated.

Carbodiimide has been used as alternative cross-linking agent because this agent contains no potentially cytotoxic aldehyde residuals. Carbodiimide non-specifically cross-links all proteins by activating the carboxylic acid groups of glutamic and aspartic acids to form an O-acylisourea intermediate. Carbodiimide is an example of a zero-length cross-linking agent that cross-links peptides to one another without introducing additional linking groups [7-10]. EDC presents very low cytotoxicity compared with glutaraldehyde because the urea derivative released when the cross-link is generated is easily rinsed away, leaving no residual chemicals [22]. Several reports demonstrated that the proliferation of seeded endothelial cells significantly increased on EDC/NHS-cross-linked collagen compared with non-cross-linked type I collagen. Furthermore, higher cell numbers were found at increasing cross-link densities using EDC in combination with NHS to examine various cellular functions of human umbilical vein endothelial cells in vitro [23].

The valvular leaflets were thin. There was no significant difference in the water content of the valvular leaflets between the three groups. Because oedema is not conducive to tissue healing and surgical sutures, EDC cross-linking may be a particularly important facet of the preparation of scaffolds from valvular leaflets.

In the current study, the thickness of the pulmonary artery valvular leaflets in groups B and C was significantly thinner than in group A. No significant difference was found in the valvular leaflet thickness between groups B and C. These results indicated that during decellularization, the cellular components of the tissue were removed [24], causing changes in thickness. The decellularized tissue area increased in group B, which might have been due to the destruction of connections between the fibres, resulting in a relatively loose tissue structure.

In the present study, tensile strength analysis demonstrated that the decellularized pulmonary artery valvular leaflets' tensile strength was significantly lower than in group A. However, EDC cross-linking after decellularization increased the tissue's tensile strength, restoring the strength to a level close to that of normal tissues. This finding indicated that cross-linking can enhance connectivity between the fibres.

Thermal shrinkage temperature is regarded as a good indicator of cross-linking in collagen materials. A high thermal shrinkage temperature indicates that cross-linking is more complete and that the material's thermal stability is high. In the current study, after decellularization, there were no significant changes in the thermal shrinkage temperature of the valvular leaflets. However, after EDC cross-linking, the thermal shrinkage temperature increased significantly, to approximately 78°C, compared with groups A and B, which indicated that EDC processing successfully triggered a cross-linking reaction between the fibres.

In the tissue embedding experiment, the results illustrated that cross-linking by EDC reduced the immunogenicity of decellularized tissues, which might prevent rejection-induced tissue degradation. Two and four weeks after the implantation a moderate number of fibroblasts may have spread into the group B and C samples. In group A, there was a small quantity of inflammatory cell infiltration, and gradual necrosis was observed. These results showed that non-decellularized, fresh heterologous tissues may display the onset of a foreign body response, with encapsulation of the tissue and its partial digestion. Additionally, there may be renewed cell growth in decellularized or cross-linked valvular leaflet tissues. We plan to perform deeper investigation of the immune response pattern, the cell infiltrate distribution, possible fibroblast penetration, and/or matrix mineralisation.

In summary, the EDC cross-linking method can enhance the tensile strength of decellularized pulmonary artery valvular leaflets, decrease the immunological response to the valvular leaflets and the leaflets' degradation, and even promote tissue regeneration in vivo. Cross-linking increases material stiffness and slows degradation times, which are thought to have beneficial effects. In the future, we should observe the decellularized, cross-linked valvular leaflets for a longer period of time and further explore cell attachment to and the regenerative capabilities of the tissue-engineered valvular leaflets. In addition, the exact incubation time and EDC concentration required for optimal cross-linking should be further explored.

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Disclosure of conflict of interest

None.

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