

## Original Article

# Decellularized porcine pulmonary arteries cross-linked by carbodiimide

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**Abstract:** The physical properties of the tissues are weakened after decellularization, and the exposed collagen fibers are prone to thrombogenesis. Several studies have proven that the use of carbodiimide (EDC) as a cross-linking agent can improve the properties of decellularized xenogeneic scaffold materials. We adopted EDC for the treatment of porcine pulmonary arteries in an effort to improve the physical properties of these arteries following decellularization. Twenty porcine pulmonary arteries were randomly divided into 3 groups. The control group (group A) consisted of fresh porcine pulmonary arteries with no further processing; group B was treated with trypsin and the detergent Triton X-100 to remove cells; and group C was cross-linked with EDC after trypsin and Triton X-100 treatment, as in group B. The pulmonary arteries were assessed based on water content, thickness, tensile strength, and thermal shrinkage temperature, to evaluate the physical properties of all of the samples. The scaffolds were then subcutaneously embedded in rabbits. These constructs were removed after 4 weeks and checked. The cells and matrix components of the arterial walls were removed and the fibrous scaffolds were retained. In group B, the moisture content of the pulmonary arterial walls was increased; and the thickness of the walls and the tensile strength of the pulmonary arteries were decreased in comparison with group A. In subcutaneous embedding of the group B samples in rabbits, after 4 weeks, fibroblasts had grown into the scaffolds and regenerated the tissue. The water content was decreased in the pulmonary arterial walls, there was an increase in the tensile strength and the thermal shrinkage temperature in group C compared with group B. The EDC-based cross-linking procedure can enhance the tensile strength of decellularized pulmonary arteries and decrease scaffold rejection and degradation and promote tissue regeneration in vivo.

**Keywords:** EDC cross-linking, decellularization, pulmonary arteries

## Introduction

The method of decellularized porcine scaffold materials has been widely used because of this technique's preservation of the natural extracellular matrix, which is difficult to imitate synthetically [1-4]. The cellular components of tissues are primarily responsible for the antigenicity and adverse response when implanted in nonautologous hosts. However, complete cell removal is not possible, and exposed xenogenic fibers still induce an immunological reaction and scaffold degradation in nonautologous hosts. In addition, the decellu-

larization procedure alters the tissues' physical properties and exposes collagen fibers that are prone to thrombogenesis [5]. In recent years, many studies 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, slow degradation time and reduce immunogenicity [6-8]. Therefore, we adopted the cross-linking agent EDC for the treatment of porcine pulmonary arteries, with the goal of improving the physical properties and quality of the decellularized scaffolds.

## Materials and methods

### *Collection and processing of the porcine pulmonary arteries*

Under sterile conditions, the pulmonary arteries were isolated from the porcine hearts (from male or female pigs that weighed from 100 to 150 kg and that were in good health. Qian Xihe Food Co., Ltd. Beijing, China). Each sample was cut into 3 parts in the longitudinal direction and then randomly divided into one of 3 groups: A, B, and C.

### *Grouping and processing method*

Group A included fresh porcine pulmonary arteries (control group) with no further treatment. Group B was treated with trypsin and the detergent Triton X-100 (Sigma, USA) to remove cells. Group C was treated with the cross-linking agent EDC (Sigma, USA) 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. The samples were vibrated in RNase 20 mg/L and 200 mg/L DNase solution for 24 hours at a constant temperature of 37°C and were again thoroughly washed with PBS, placed in D-Hanks solution containing penicillin and streptomycin, and stored at 4°C. After treatment, a part of each sample was removed for ordinary biopsy and electron microscopy scanning to evaluate the extent of the decellularization [9, 10].

### *EDC cross-linking*

EDC was used as a cross-linking agent to treat the decellularized scaffolds. The samples from group C were soaked in 50 mmol/L MES (Sigma, USA) buffer solution prepared in 40% ethanol for 24 hours, followed by the addition of EDC and NHS (Sigma, USA), 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.

### *Morphological observation*

The pulmonary arterial walls of the 3 groups were checked by microscopic observation. The samples were fixed in formaldehyde for HE staining and in 4% paraformaldehyde and 1% osmic acid for scanning electron microscopy. The fixed scaffolds were then observed by conventional HE staining and scanning electron microscopy.

### *Determination of water content*

Trypsin-EDTA and Triton-X100 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 walls of the 3 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 in the 3 groups was measured using a CH-B-type thickness gauge.

### *Tensile strength test*

Using a sharp tool, dissection of the pulmonary arterial wall was performed in the 3 groups. The resultant samples were 2 cm in length and 0.5 cm in width. We then tested the tensile strength using an electronic tensile testing machine RGW-0.1 at a speed of 50 mm/min.

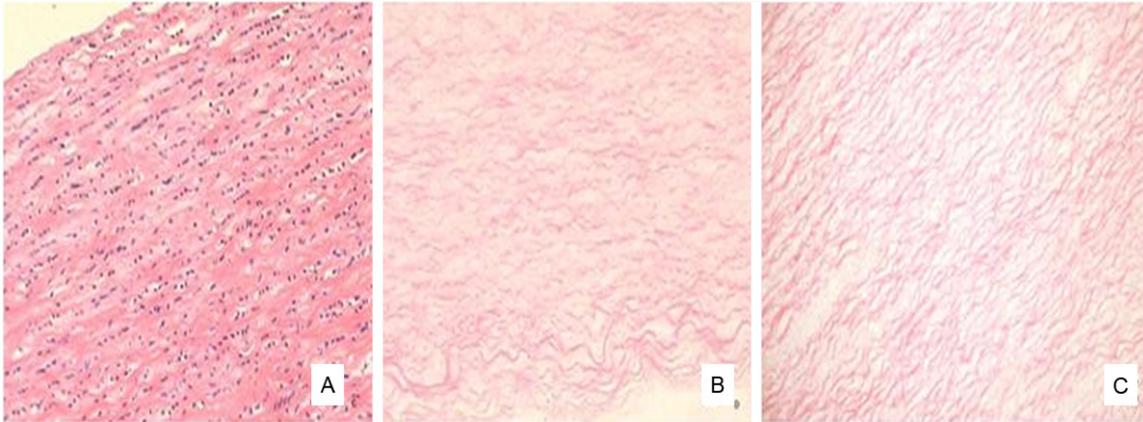
### *Thermal shrinkage temperature test*

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 experiment 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 Anzhen Hospital, Capital Medical University. All surgery was performed under anaesthesia, and all efforts were made to minimize suffering.

## Porcine pulmonary arteries cross-linked by carbodiimide



**Figure 1.** Representative H&E staining showing the porcine pulmonary arterial wall before and after decellularization. Magnification,  $\times 100$ . A: Fresh porcine pulmonary arterial wall; B: Decellularized porcine arterial wall; C: Decellularized porcine pulmonary arterial wall cross-linked by EDC.

Fifteen New Zealand rabbits (2.5-3.0 kg), supplied by the Beijing Anzhen Hospital, were used as experimental animals. After anesthesia, the skin on the back of the rabbits was cut, and the scaffold samples were embedded subcutaneously. The same types of samples were embedded in each rabbit.

### *Morphological observation in animal experiment in vivo*

The subcutaneously embedded tissue samples were collected at 1, 2, and 4 weeks after implantation to observe the scaffold morphology and to determine whether there were degradation and absorption. The fibrous tissues outside the samples were removed, after which the samples were fixed in formaldehyde for histopathology. The embedded samples were classified as inducing the following degrees of rejection: degree I, in which case there were no or few lymphocytes in the sample; degree II, in which a small quantity of lymphocytes was present; degree III, in which a moderate number of lymphocytes were present; and degree IV, in which a large quantity of lymphocytes, neutrophils, and phagocytes were present.

### *Statistical analysis*

All of the experimental data were analyzed using SPAA 11.5 software. The physical properties of the 3 groups were analyzed using the one-factor analysis of variance method. The data were considered statistically significant when  $P < 0.01$ .

## Results

### *Morphological observation*

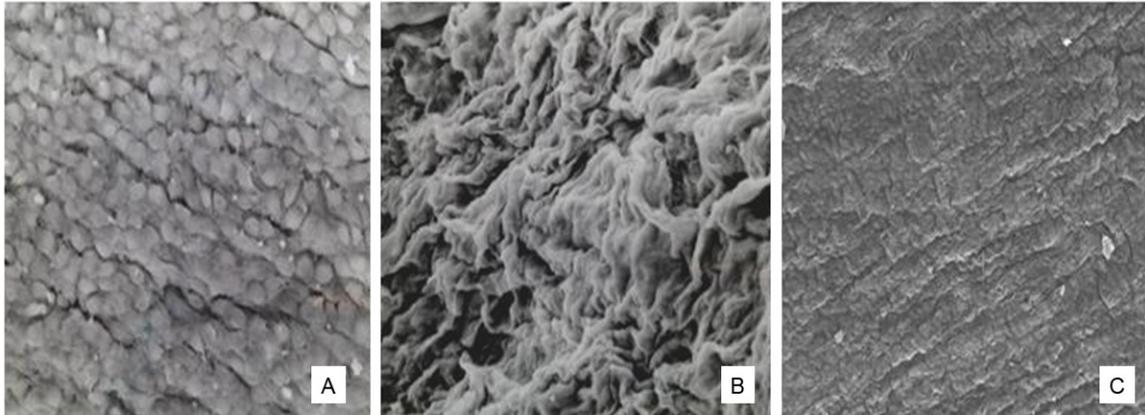
In group A, the color of the pulmonary arterial walls was reddish. After decellularization (group B), the color of the pulmonary arterial walls was white and transparent. The tissue structure was also loose, with a high water content. After decellularization and EDC cross-linking (group C), the color of the pulmonary arterial walls was opaque, and the structure was tighter than in group B but not tighter than in group A.

### *HE staining results*

By microscopy, it was observed that the combined trypsin-EDTA and Triton X-100 detergent treatment completely removed the cellular components of the pulmonary arterial walls. In the group B samples, we observed the tissue was looser than usual. The fiber orientation was normal in the tunica intima and tunica media of the pulmonary arterial walls, showing a typical wave, but the gap between the fibers was relatively larger than that in group C (**Figure 1**).

### *Scanning electron microscopy results*

Interstitial and endothelial cells inside the porcine pulmonary arterial walls were well organized in group A. After decellularization, the superficial cells were no longer present, and the fibers were exposed, leaving hollow gaps in group B, and in group C more compact fibers



**Figure 2.** Scanning electron microscopy showing the porcine pulmonary arterial wall before and after decellularization. Magnification,  $\times 1000$ . A: Fresh porcine pulmonary arterial wall; B: Decellularized porcine arterial wall; C: Decellularized porcine pulmonary arterial wall cross-linked by EDC.

**Table 1.** The physical properties of the porcine pulmonary arterial walls between the 3 groups

	Group A (n=10)	Group B (n=10)	Group C (n=10)	F Value	P Value
Water Content (%)	0.81 $\pm$ 0.02	0.91 $\pm$ 0.10	0.83 $\pm$ 0.03	42.34	<0.0001
Thickness (mm)	1.23 $\pm$ 0.06	0.79 $\pm$ 0.05	0.79 $\pm$ 0.04	238.645	<0.0001
Tensile Strength (MPa)	20.67 $\pm$ 1.66	18.63 $\pm$ 2.01	20.47 $\pm$ 1.98	3.523	0.044
Thermal Shrinkage Temperature ( $^{\circ}$ C)	72.31 $\pm$ 0.77	73.53 $\pm$ 0.87	78.03 $\pm$ 0.83	33.300	<0.0001

were shown after cross-linked by EDC (**Figure 2**).

#### *Water content of pulmonary arterial walls*

The water content in the pulmonary arterial walls in group C was similar to the water content observed in group A ( $P=0.058$ ). In contrast, the water content in group B was significantly increased after decellularization compared with the water content in groups A and C ( $P<0.0001$ ). This finding indicated that the EDC cross-linking process removed extra water from the tissues (**Table 1**).

#### *Thickness of pulmonary arterial walls*

The walls in groups B and C were significantly thinner than in group A ( $P<0.0001$ ), which indicated that decellularization had removed the cells and part of the matrix from the tissues. The tissue thickness of the pulmonary arterial walls in the decellularized group B scaffolds and EDC cross-linked group C scaffolds was not significantly different ( $P=0.695$ , **Table 1**).

#### *Tensile strength of pulmonary arterial walls*

The tensile strength of the pulmonary arterial walls in group C recovered to the same level as

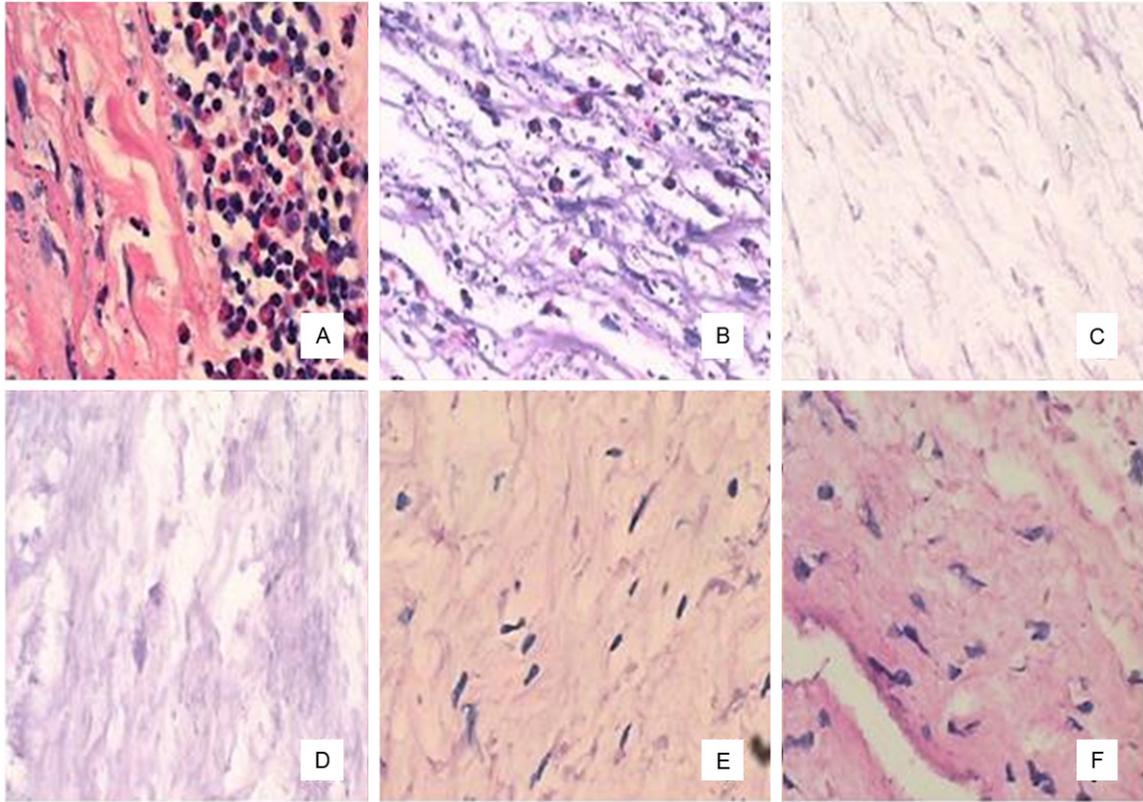
in group A, with no significant difference between the groups ( $P>0.01$ ). The tensile strength of the pulmonary arterial walls in group B, however, was decreased compared with the strength observed in groups A and C ( $P<0.01$ , **Table 1**), which indicated that the EDC cross-linking process increased the tensile strength of the decellularized tissues.

#### *Thermal shrinkage temperature of pulmonary arterial walls*

There was no difference in the shrinkage temperature between the pulmonary arterial walls in groups A and B ( $P=0.110$ ). Additionally, the shrinkage temperature was significantly increased in the pulmonary arterial walls 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 arterial walls in group A, and degree III-IV rejection was observed. In group B, there was a small quantity of lymphocytes in the pulmonary arterial walls, and degree II rejection was observed; in group C, a few lym-



**Figure 3.** H&E staining showing the porcine pulmonary arterial walls 1 week and 4 weeks after embedding. Magnification,  $\times 400$ . A-C: The porcine pulmonary arterial wall 1 week after embedding; D-F: The porcine pulmonary arterial wall 4 weeks after embedding. A, D: Fresh porcine pulmonary arterial wall; B, E: Decellularized porcine pulmonary arterial wall; C, F: Decellularized porcine pulmonary arterial wall cross-linked by EDC.

phocytes, a small quantity of fibroblasts, and degree I rejection were observed (**Figure 3**).

Two weeks after embedding, the tissues tightly adhered to the periphery and was therefore not easy to separate and removed. The tissues also exhibited unsmooth surfaces and dark red in color in group A. In group B, the pulmonary arterial walls were white and exhibited soft tissue, poor mechanical strength, severe adhesions, and peripheral tissues that were not easily separated. The pulmonary arterial walls in group C were also white, were easily separated, and exhibited good elasticity and moderate strength (**Figure 4**).

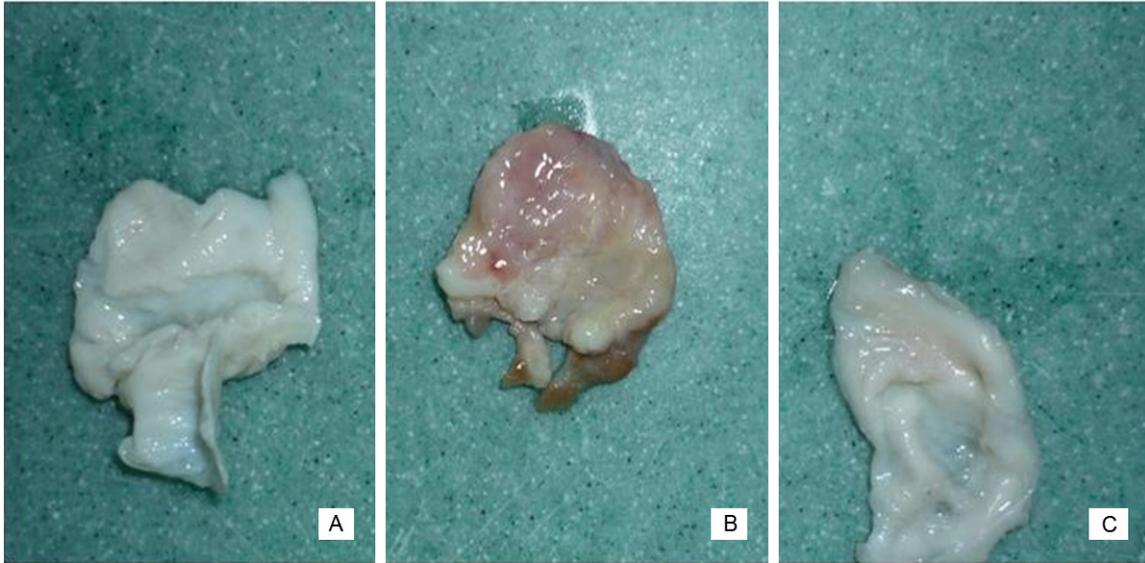
Four weeks after embedding, the group A tissue was difficult to separate; dark red in color; and lacking luster. The tissue also exhibited an unsmooth surface that was contracture-shaped, with part of the tissues not integrated. The group B samples were soft to the touch and had poor strength. The remaining tissue was

white and adherent. Finally, the group C samples were soft, bright white in color, and flexible. These scaffolds exhibited moderate strength, had no scleroses, and were easily separated. By microscopy, we observed that there were no cells in the group A samples and that a moderate number of fibroblasts had spread into the group B and C samples (**Figure 3**).

### Discussion

#### *Advantages and shortcomings of decellularization*

Decellularization can reduce the immunogenicity of allogeneic tissues [10-12]. However, previous studies have shown that after decellularization, the fibrous tissue matrix can still be immunogenic, and the mechanical strength of the materials also declines following excessive degradation. The physical properties of the fibrous matrix in decellularized tissues are also



**Figure 4.** General shapes of the porcine pulmonary arterial walls 2 weeks after embedding. A: Decellularized porcine pulmonary arterial wall cross-linked by EDC; B: Fresh porcine pulmonary arterial wall; C: Decellularized porcine pulmonary arterial wall.

different from this matrix's performance in fresh tissue, including increased water content and thickness [13-15]. Moreover, various factors during decellularization, such as concentration, method, time, and tissue thickness, can lead to poor decellularization (e.g., cell debris residue, incomplete cell removal), which can affect the survival of implants and tissues.

#### *EDC cross-linking*

It is important to completely restore the physical properties of decellularized tissues while eliminating the immunogenicity of the cellular remnants, making the tissue more relevant for clinical applications. To achieve this goal, in the current study, the EDC cross-linking method was applied to a decellularized pulmonary arteries to improve the physical properties, slow the degradation time, and reduce the immunogenicity of the materials. EDC, which is a cross-linking agent, has low cytotoxicity and can bind glutamic acid in a polypeptide chain or a carboxyl group in aspartic acid. EDC then cross-links with other free amino groups in the polypeptide chain to form so-called "zero-length cross-linking" [16]. During this cross-linking, no new material is introduced. Moreover, *in vitro* studies have confirmed that EDC is hypo-cytotoxic or not cytotoxic [14, 15]. EDC cross-linking also improves the physical

properties and biological stability of the materials.

In the current study, trypsin was first used to dissolve part of the matrix within the blood vessels to make the stromal cells detach from the gaps in the fibers and to undermine the integrity of the cells. The nonionic detergent Triton X-100 was then used to extract cell debris and to remove the cells and several of the decellularization matrix components through sustained agitation and washing [10]. General histopathology and scanning electron microscopy confirmed whether the cells had been completely removed and whether the fibrous scaffolds were integrated.

The determination of the scaffolds' water content exhibited that after decellularization, the water content of the pulmonary arteries was significantly increased, indicating that with the removal of the cells, hydrophobic lipid components were also removed, whereas the collagen fibers, elastic fiber network, and hydrophilic materials attached to the mesh structure were retained. These hydrophilic substances can accumulate water in reticular formation and significantly increase a scaffold's water content. The phenomenon was also explained by Courtman DW [17]. In contrast, after EDC cross-linking, the water content in the pulmonary

arterial walls decreased to a level similar to the content of fresh pulmonary arteries. Because edema is not conducive to tissue healing and surgical sutures, EDC cross-linking may be a particularly important facet of scaffold preparation.

In the current study, the thickness of the pulmonary arterial walls in groups B and C was significantly thinner than in group A [18-20]. No significant difference was found in the wall thickness between groups B and C. These results indicated that during decellularization, the cellular and matrix components of the tissue were removed, causing changes in thickness. The decellularized tissue area was increased in group B, which might have been due to the destruction of connections between the fibers, resulting in a relatively loose tissue structure.

In the present study, tensile strength analysis shew that the decellularized pulmonary arterial wall tensile strength were significantly lower than in the fresh pulmonary artery. However, EDC cross-linking after decellularization increased the tissue's tensile strength, restoring the strength to the level of normal tissues. This finding indicated that cross-linking can enhance connectivity between the fibers.

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 pulmonary arteries. However, after EDC cross-linking, the thermal shrinkage temperature increased significantly, to approximately 78°C, compared with the control group, which indicated that EDC processing successfully triggered a cross-linking reaction between the fibers.

In the tissue embedding experiment, the lowest level of rejection (degree I) occurred 1-2 weeks after implantation of the decellularized porcine pulmonary arteries cross-linked by EDC. Degree II rejection was noted for the decellularized porcine pulmonary arteries, and degree III-IV rejection was observed for the fresh porcine valves. These results demonstrated that cross-linking by EDC can reduce the immunogenicity of

decellularized tissues, which may prevent rejection-induced tissue degradation and calcification. Four weeks after embedding, by microscopy, we observed that there were no cells in the group A samples and that a moderate number of fibroblasts had spread into the group B and C samples. The cross-linked scaffolds may be prone to the renewal cell growth and renewal stromal-matrix synthesis. In contrast, contractured deformities, the disappearance of the original cells, the blurred fiber texture, and the few stromal cells growing in the fresh samples shew that non-decellularized, fresh heterologous tissues were difficult to regenerate.

In conclusion, the EDC cross-linking method can enhance the tensile strength of decellularized pulmonary arteries, decrease the scaffolds' rejection and degradation, and even promote tissue regeneration in vivo. Cross-linking increases material stiffness and slows degradation times, which are thought to be beneficial effects, although slowed cell migration may hinder tissue regeneration, as suggested by certain reports [21, 22]. In the future, we should observe the decellularized, cross-linked scaffolds for a longer period of time and further explore the constructs' cell attachment and regenerative capabilities. In addition, the exact incubation time and EDC concentration required for optimal cross-linking should be further explored.

### Abbreviations

EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; PBS, Phosphate-buffered saline; MES, 2-(N-morpholino)ethanesulfonic acid; NHS, N-hydroxysuccinimide.

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