Original Article
Decellularized sheep internal carotid arteries as a tissue-engineered small-diameter vascular scaffold

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Abstract: Objective: This study aimed to contrast two different methods to decellularize sheep internal carotid arteries as a tissue-engineered small-diameter vascular scaffold and test histological and physiochemical properties. Methods: Blood vessels were divided into three groups: group A, fresh (control); group B, enzymatic decellularized (experimental I); and group C, enzyme-detergent mixed decellularized (experimental II). Hematoxylin and eosin (HE) staining, elastic and collagenic fiber dyeing, fluorescence staining, and scanning electronic microscopy were performed, and the thickness, tensile strength, burst pressure, moisture content, and DNA content were tested, recorded, and analyzed. Results: Group A revealed blue-stained cell nuclei, while groups B and C showed nuclei removal completely under HE staining and 4',6-diamidino-2-phenylindole (DAPI) immunofluorescence staining. In group A, the elastic and collagenic fiber staining revealed dense continuous without rupture, while the counterparts in groups B and C were more discontinuous and looser. The cells appeared orderly and compact in group A under a scanning electron microscope, while groups B and C showed only longitudinal fibers in the inner membrane. Group C showed the strongest tensile strength, lowest moisture content, and lowest DNA. Every group resisted pressure over 300 mmHg without burst. Conclusion: The enzyme-detergent mixed decellularization method was milder to damage the blood vessels compared with enzymatic decellularization, and demonstrated a higher level of decellularization and lower level of DNA content. Therefore, it could be used as a better decellularization method for tissue-engineered small-diameter blood vessel.

Keywords: Decellularization methods, sheep internal carotid arteries, small-diameter vascular scaffold, tissue engineering

Introduction

With the improvement in living standards, more people are now suffering from coronary artery diseases and related complications around the world, resulting in a wide use of coronary artery bypass grafting (CABG). Autologous blood vessels may be regarded as the best solution for coronary bypass. The main sources of autologous small-diameter (< 6 mm) vessels for CABG were internal mammary arteries, radial arteries, and great saphenous veins [1, 2], which were considered as the best solution. But owing to the limited sources, angitis, vascular lesions, amputation, etc., the large-scale use of autologous blood vessels is restricted [3]. Therefore, urgent manufacturing of tissue-engineered small-diameter vessels has become necessary to replace the aforementioned autologous ones. Artificial blood vessels, made from inorganic material, such as nylon, polyester, and polytetrafluoroethylene, have been used prevalently as great vessels with high flow capacity and low resistance, such as aorta, and have shown a good clinical effect [4]. However, these artificial non degradable in organic substitutions show a low long-term patency rate when applied to small-diameter vessels [2]. These materials cannot provide growth environment for the adherence of endothelial cells, resulting in thrombosis formation. They cannot simulate normal biomechanical changes of natural arter-
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ies in the host body, such as vascular elasticity, subsequently giving rise to endangium hyperplasia, stenosis, and occlusion on anastomotic stoma. Therefore, researches were more inclined to blood vessels made from natural materials from porcine and sheep.

As for allogeneic grafts, umbilical arteries have drawn the attention of researchers because of their advantages, such as great length, suitable diameter, high-degree sterility, and less change in caliber on both ends, which can avoid a severe rejection reaction [5]. But lack of sufficient sources, refusal of donors, and immunological rejection of human umbilical arteries has made their application restricted.

Therefore, tissue-engineered materials that tend to decellularize natural vessels with extracellular matrices (ECMs) left are the only alternative [6, 7]. Decellularization processes reduce the immunogenicity of ECMs. Derived from natural vessels, ECMs can also provide an ideal environment for cell adhesion and growth, and appropriate biological compatibility [8], in addition to the high degree of resemblance of morphology, specifications, and strength with host vessels.

A lot of decellularization methods are available, such as physical, enzymatic, and enzyme-detergent mixed decellularization, etc. Physical methods mainly refer to freezing-thawing, radiation, etc., via rapid repetition of freezing-thawing or irradiation to break off cellular membranes. However, these methods have the disadvantages of less cell removal, more intracellular material residual, and incomplete cellular membrane ruptures [9]. Therefore, physical methods were not considered in this study. Enzymatic decellularization methods apply one or more enzymes to damage ligandin and other protein macromolecular antigens among cells [10]. The enzyme-detergent mixed decellularization method usually employs the joint application of detergent and enzyme. The detergent destroys the cell membrane and exposes intracellular contents, and then one or more enzymes are used to pyrolyze corresponding intracellular remnants as a substrate, so as to realize decellularization. In this study, the enzymatic method was contrasted with the enzyme-detergent mixed method to investigate their histopathological, immunofluorescence, and biomechanical results, to develop a better decellularization protocol for a suitable tissue-engineered small-diameter vascular scaffold.

Ovine internal carotid arteries have advantages such as few branches, suitable inner diameter, and wide acquisition. Therefore, this article focused on the study of ovine internal carotid artery, with the purpose of finding a proper method for the potential substitutes of small-diameter vessel transplantation.

Material and methods

Reagents and instruments

The following materials were used: trypsin, TritonX-100, sodium deoxycholate, ethylenediaminetetraacetic acid (EDTA), magnesium chloride, DNase, RNase, myllicin, amphotericin B were from Sigma (St. Louis, MO, USA) or Beijing DongxuLingmei Biotechnology Ltd (Beijing, China).

Animals and groups

Internal carotid arteries from sheep of both genders weighing 50 kg each were supplied by Beijing Longhao Meat & Food Co., Ltd. (Beijing, China) for this study.

Preparation of the two kinds of methods to decellularize small-diameter sheep internal carotid arteries

Internal carotid arteries were cut into 10-cm pieces and divided into three groups (n = 10). The tunica externa was dissected by ophthalmological forceps, and the residual blood in the vessels was extruded. After washing three times, they were placed in phosphate-buffered saline (PBS) with myllicin (100 U/L) and amphotericin B (1 mg/L) at 4°C for future use.

For the enzymatic decellularization method group (group B), the blood vessels were placed in PBS with 0.25% trypsin, myllicin (100 U/L), and amphotericin B (1 mg/L) at 20°C and shook in a constant temperature rotating shaker at a speed of 200 rpm for 120 h. PBS was replaced every 8 h.

For the enzymatic decellularization method group (group B), the blood vessels were placed in PBS with 0.25% trypsin, myllicin (100 U/L), and amphotericin B (1 mg/L) at 20°C and shook in a constant temperature rotating shak-
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er at a speed of 200 rpm for 120 h. PBS was replaced every 8 h.

For the enzyme-detergent mixed decellularization method group (group C), the blood vessels were placed in PBS solutions with 0.25% sodium deoxycholate, 0.02% EDTA, 1% TritonX-100, myllicin (100 U/L), and amphotericin B (1 mg/L) and shook in a constant temperature rotating shaker at a speed of 200 rpm for 72 h. The blood vessels were then incubated in PBS with DNase (150 kU/L), MgCl₂ (0.05 mol/L), 0.1% RNase, myllicin (100 U/L), and amphotericin B (1 mg/L) and shook for 48 h. PBS was replaced every 8 h. The storage steps were the same with above.

Histological observation

After washing completely, the samples were fixed in formalin, dehydrated in an ascending series of alcohols, embedded in paraffin, and cut into serial sections (4 µm thick) before staining with hematoxylin and eosin (H&E). Collagen and elastic fibers were evidenced by taking advantage of Masson’s trichrome and Victoria Blue staining methods, respectively.

Immunofluorescence observation

The samples were cut into 5-mm long sections, then fixed with optimal cutting temperature compound (OCT, Sigma) and frozen for 30 min. The samples were cut into 7-mm thick sections using a freezing microtome (LeicaCM1950, Leica, Wetzlar, Germany). After fixing in 4% paraformaldehyde (Sigma) for 30 min, 50 mL 4',6-diamidino-2-phenylindole (DAPI; ZhongshanJinqiao Biotechnology Ltd. Co., Beijing, China) and 50 mL propidium diiodide (PI; Sigma) were applied on the sections to dye for 15 min. With PBS washing and 3% glycerol mounting, the samples were observed under an immunofluorescence microscope (LeicaDMI4000B, Leica, Wetzlar, Germany).

Scanning electron microscopy

After washing completely, the samples were fixed for 4 h in 2.5% glutaraldehyde and rinsed in a PBS solution. They were then fixed in 1% osmium tetroxide for 1 h and dehydrated in an ascending series of alcohols and acetone in turn. Inner surfaces were inversed on a silici-fied glass at 45°C for 12 h. After located and sputtered with platinum, ultrastructures of the samples were observed by electron microscopy.

Thickness and tensile strength test

Ten samples of similar shape (2-cm long × 1-cm wide) from the three groups were randomly selected. After the surface moisture was fully absorbed using the neutral filter paper, each thickness was measured with a thickness gauge three times. The vessels were then pulled off at a speed of 20 cm/min after fixing them in a single-column vertical electronic tensile testing machine. The maximum tension of each sample was recorded, and the tensile strength was calculated using the following formula: tensile strength = maximum tension/cross-sectional area.

Moisture content test

Five samples with similar weight from the three groups were randomly selected. The samples were then soaked in distilled water for 48 h, and the surfaces were dried with a filter paper. The moist weights of were accurately measured first by a microelectronic balance, then sequentially the samples were placed in a freeze drier at -80°C to dehydrate for 24 h. At last, dehydrated weights were measured by the same microelectronic balance, and the moisture content was calculated using the following formula: moisture content = (moist weight-dehydrated weight) × 100%/moist weight.

DNA content test

Five dried samples of 1 g from the three groups were randomly selected. DNA was extracted from these dried samples with a commercially available kit (PureLink Genomic DNA Kits, Invitrogen, Carlsbad, California, USA) and eluted into 30 µL fluid. The DNA weight was measured and recorded with a spectrophotometer at 260 nm. The DNA content was calculated using the following formula: DNA content = DNA weight/1 g. Then, 5 mL of every 30 mg was taken out for DNA electrophoresis with 1% sepharose.

Burst pressure resistance strength test

Ten samples from the three groups were randomly selected. One end of the blood vessel
was ligated and the other end was located on one joint of a T-branch pipe, the other two joints of which were linked with a pressure detector transducer and a syringe. The syringe injected gas into the vessel to increase the intravascular pressure, and the pressure was digitalized on a screen. On the explosive rupture of the vessel, the burst pressure resistance strength was recorded.

**Statistical analysis**

The SPSS software (version 18.0, IBM SPSS, Chicago, Illinois) was used in the present study. Data were expressed as mean ± standard deviation, and analyzed using the two-way analysis of variance (ANOVA) to determine statistical significances between any two groups. The significance level was set at P < 0.05.

**Result**

**General observation**

Decellularized sheep internal carotid arteries were observed to be more pale and loose, while the fresh ones were reddish.

**Histological observation**

**HE staining:** Group A showed blue-stained cell nucleus and red-stained fibrous tissues, and fibrous tissue packed closely and neatly. Groups B and C showed no cell nucleus, but red-stained fibrous tissue was visible. The fibers of group C appeared loose but orderly, while the fibers of group B appeared loose and disorderly than the ones of groups A and C (Figure 1).

**Victoria Blue staining for elastic fibers:** Group A showed blue-stained elastic fibers, which were located closely and neatly. Group C showed compact and neat elastic fibers, without obvious fractures, while group B showed loose and more discontinuous elastic fibers than those of groups A and C (Figure 2).

**Masson staining for collagenic fibers:** Group A showed green-stained collagenic fibers, which were located closely and neatly. Group C showed compact and neat collagenic fibers, without obvious fractures, while group B showed loose and more discontinuous elastic fibers than those of groups A and C (Figure 3).

**DAPI and PI immunofluorescence staining:** Group A showed blue granule-like cell nuclei and red-stained fibers, arranged compactly and neatly. Group B showed blue granule-like weaker cell nuclei, and loose and disorderly red-stained fibers. Group C showed blue gra-
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Figure 3. Collagenic fiber staining (× 100). A. Group A. B. Group B. C. Group C.

Figure 4. DAPI and PI immunofluorescence staining (× 100). A. DAPI staining of group A A2. PI staining of group A. A3. Light microscopy of group A. B1. DAPI staining of group B. B2. PI staining of group B. B3. Light microscopy of group B. C1. DAPI staining of group C. C2. PI staining of group C. C3. Light microscopy of group C.

nule-like weaker nuclei; however, the red-stained fibers were compact and neat. Under a light microscope, the fibers of group C were much more compact than the ones in group B (Figure 4).

Electron microscopy scanning: Group A showed endothelial cells in order on endangium, while groups B and C showed no endothelial cells but orderly arranged fibers (Figure 5).

Physical property test

Thickness test: The thickness of the groups was in the following order: group A (0.69 ± 0.06 mm) > group C (0.44 ± 0.04 mm) > group B (0.33 ± 0.05 mm). Statistical significances were noted among the three groups with P < 0.01. The data demonstrated that both decellularization methods could injure vascular walls, and the damage of group C was milder (Figure 6A). It was reasonable that this variation was due to the removal of ECMs.

Tensile strength test: The tensile strength of the groups was in the following order: group C (3.88 ± 1.12 N/mm²) > group B (3.71 ± 1.05 N/mm²) > group A (2.96 ± 0.60 N/mm²). A statistical significance existed between groups A and C (P = 0.039). No statistical significances were found between groups A and B (P = 0.089), and B and C (P = 0.690) (Figure 6B).

The data demonstrated that the enzymatic decellularization method would not affect the tensile strength of vascular walls, while the
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enzyme-detergent mixed decellularization method could enhance it by destroying fewer fibers that play great roles in maintaining tensile strength, and by condensing the residual fibers after decellularization.

Moisture content test: The moisture content of the groups was in the following order: group B (75.46 ± 0.85%) > group A (72.15 ± 1.24%) > group C (54.23 ± 0.96%). Statistical significances were observed among the three groups with \( P < 0.01 \) (Figure 6C).

The data exhibited that after decellularization, groups B and C both changed the moisture contents in vessels. Group B destroyed more ECMs
that contained less moisture, and increased the moisture contents relatively. Besides, the vessels of group B had loosened ECMs and stored more water in the reticular structure of ECMs with enzymolyzed hydrophobic lipoprotein of ECMs, and thus increased moisture content absolutely. This phenomenon was also explained by Courtman DW [11]. Group C had destroyed fewer ECMs and condensed them, thus decreased moisture contents relatively and absolutely.

**DNA contents test:** The DNA content of the groups was in the following order: group A (167.51 ± 40.33 mg/g) > group B (23.20 ± 2.32 mg/g) > group C (11.35 ± 1.32 mg/g). Statistical significances were found between groups A and B (P = 0.00), and groups A and C (P = 0.00). No statistical significance was found between groups B and C (P = 0.438) (Figure 6D).

DNA electrophoresis showed five bright lanes from lanes 11 to 15, fairly less bright lanes from lanes 6 to 10, and no bright lanes from lanes 1 to 5 (Figure 7). The results showed more complete DNA removal in group C than in group B.

**Burst pressure resistance strength test:** All samples of the three groups could withstand pressure up to 300 mmHg without burst (Figure 8). However, the samples showed slight pseudoaneurysm-like dilation in group B.

**Discussion**

With the improvement in living standards, vascular diseases show a trend of high incidence gradually, with coronary artery disease and peripheral vascular disease occurring most frequently. To satisfy the great demand for curing these diseases, developing tissue-engineered small-diameter vessel is an urgent requirement, owing to limited autograft substitute vessels derived invasively from internal mammary artery, radial artery, and great saphenous vein. Under the impacts of thrombosis formation, immunological rejection, cell hyperplasia, pseudoaneurysm formation, infection, and progressive atherosclerosis, tissue-engineered small-diameter vessels reveal disappointing results referring to long-term patency rates, which directly restrict the application of small-diameter vessels [12]. Thus, the improvement on histocompatibility of tissue-engineered small-diameter vessels has become more urgent.

For heterogeneous graft, swine, ovine, and canine get more attention. Given the taboos of religion and animal conservationism, this paper excludes the study of pigs and dogs. Referring to the anatomical inner diameter, splenic, limb, and internal carotid arteries are all suitable. However, the splenic and limb arteries extend more branches, so internal carotid arteries extend more branches, so internal carotid arteries caught researchers’ eyes in this study.

Nowadays, decellularized ECMs are being used, which include complete removal of cellular material from tissues via physical, chemical, or enzymatic agents without damaging the composition, mechanical integrity, and biological activity of native ECMs [13]. The ECM structure is preserved so that subsequent adhesion, migration, proliferation, and differentiation of host cells may be supported [14]. ECMs after decellularization have been widely used in biological territory, such as cardiac valve, and have harvested a good clinical effect.

Through the decellularization process, the ECMs of natural vessels can minimize immunological rejection to the greatest degree after
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Decellularization methods on small-diameter vessels at present focus on dissolving cells and rinsing cell residuals. Finally, the effect and feasibility of methods are evaluated through physicochemical property measurements and biological characteristic tests of ECMs. But, all methods cause damage to ECMs more or less, which in turn affect their functions indirectly, according to the damage level [17]. Thus, a less ECM-damaging decellularization method is useful for tissue-engineered small-diameter vessels. After comparison of two different decellularization methods, this study found that the enzyme-detergent mixed decellularization method can preserve ECMs, and the tissue-engineered small-diameter vessels resemble natural blood vessels more, in aspects such as vascular strength. The mixed decellularization method can decompose cellular membrane completely with detergent and expose intracellular DNA and RNA fully, and then enzymolize DNA and RNA with nuclease, so as to realize antigen removal after rinsing, while maximizing ECM retention. However, in the enzymatic decellularization method, trypsin disintegrates proteins without obvious specificity. When trypsin destroys proteins on cellular membrane and among cells, it can decompose proteic components of ECMs unintentionally, which contains less moisture, and loosens the ECM structure to store more water, leading to an increase in water content both relatively and absolutely. Besides, the method can lead to more thickness loss.

ECMs after decellularization can trigger thrombosis formation and impair long-term patency rates without protection of endothelial cells. Therefore, recent studies focus on recellularization [18], which can prevent thrombosis or retard it, at least. With little immunogenicity to activate immunological rejection and great potentials to differentiate into smooth muscle cells (SMCs) in hosts, smooth muscle progenitor cells (SMPCs) draw researchers’ attention on recellularization. Heterogeneous or allogeneic SMPCs can be cultivated within the ECMs to smoothen the inner surface of vessels, contributing to migration and adhesion of endothelial cells. After differentiation into SMCs, elastic fibers secreted by SMCs devote to enhancing strength and elasticity of ECMs [19, 20], making synthetic vessels more similar to normal vessels. Re-endothelialization can avoid thrombocyte coagulation and thrombosis formation, and reduce hemadostenosis effectively.

Histological observations demonstrate complete removal of nuclei and cellular components in treated vessels by two kinds of decellularization methods, which were also confirmed by significant reduction on vascular thickness and DNA content. Essential ECM proteins such as collagen and elastin were well preserved by the enzyme-detergent mixed decellularization method, which may provide a suitable environment for adhesion, proliferation, and migration of host cells.

**Conclusion**

Both methods of decellularization could remove vascular cells. The enzyme-detergent mixed decellularization method demonstrates milder damage to vessels, higher level of decellularization, and lesser DNA residual compared with the enzymatic decellularization method. However, physicochemical properties were more similar to fresh vessels. So the enzyme-detergent mixed decellularization method could be used as a better decellularization method for tissue-engineered small-diameter vessels.

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Disclosure of conflict of interest

None.

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