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Original Research

Preparation and characterization of a de-cellularized rabbit aorta as a promising scaffold in vascular tissue engineering

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Abstract: Development of scaffold is essential for promoting and supporting healing process. This study aims to establish a decellularized rabbit aorta for clinical vascular tissue engineering therapy. We successfully prepared decellularized small-diameter aortic scaffolds and investigated several properties of this engineered vascular tissue scaffolds, including cell adhesion, hydrophilicity, cytotoxicity, biocompatibility. Results showed that decellularized aortas have a porous structure with few cell remnants as examined by histochemistry and scanning electron microscopy. Both vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) cultured on decellularized aortas were adhered and proliferated well. Cell adhesion rates of rat VSMCs and ECs reached to 64.32±2.03% and 52.77±1.19%, respectively. VSMCs were able to migrate into outer surface of scaffolds. Hydrophilisity reached its maximal rate at 519±23% in 12 h. Importantly, no overt cytotoxicity was observed when grown in extraction solution of aortic scaffolds. Lastly, we also engrafted cell-scaffolds subcutaneously into nude mice *in vivo*. Implanted scaffold structure and proliferation of seeded cells were well maintained up to 8 weeks. In conclusion, we successfully prepared a decellularized rabbit aorta that not only largely maintains its extra-cellular structure, but also shows little toxicity. It may constitute a potential tool as a small diameter scaffold in vascular tissue engineering.

Key words: Tissue engineering, small-diameter scaffold, decellularized vessels, rabbit aorta.

Introduction

Coronary and peripheral vascular bypass grafting have been routinely performed on patients with organ failure or local tissue damage. To create an alternative path for blood flow during the surgery, either synthetic scaffolds that are made from biodegradable polymeric materials or autologous tissue graft will be used. Although the development of autologous vessels or organ tissues have been significantly improved during the last decade (1-3). However, there are also tremendous challenges. These vessels may not be suitable for patients who have experienced previous bypass surgery, lack of sufficient vessels of the suitable size, lack of desired mechanical properties or renewable sources of cells for using in the vascular bypass grafting. Therefore, there is urgent need for the development of clinically available substitute vessels by tissue engineering.

Decellularized vessels, either allogenic or xenogeneic largely containing or maintaining the extra-cellular matrix (ECM), which can not only promote cell adhesion and proliferation, but can also possess similar physicochemical properties and functions to that of natural vessels. Therefore, increasing attention have been paid on investigating of decellularized tissues as promising scaffolds in tissue engineering for organ transplantation or vessel graft. Indeed, decellularized organs, including human skin and veins, and bovine and porcine carotids, have been tested in preclinical or clinical trials (4-6). Although decellularized scaffolds from large animals. such as bovine or porcine aortas. have been extensively investigated, small diameter vessels (< 5 mm) are in practicality more useful in vascular tissue engineering. In addition, the migration of cells into decellularized pig aorta was shown to be inadequate (7). Therefore, development of decellularized materials with small diameter vessels (<5 mm) is important. However, small-diameter vessel scaffold as engineered tissues was not well described previously (8).

Decellularized vessel scaffolds can be prepared by removing the cellular components of the natural vasculature and which is characterized by low immunogenicity, well-kept mechanical properties, intact extra-cellular matrix contents and anti-degradation properties (9). It is expected that recipients' cells can be seeded and growth well *in vitro* and the decellularized vessels can be implanted *in vivo*. The challenges for decellularized organs are the unfavorable loss of ECM integrity, incomplete decellularization of the vascular cellular remnants or relatively high cell cytotoxicity (10,11). Previously studies suggest that treatment of bovine jugular veins with a combination of Triton X-100 and sodium deoxycholate can be successfully removing the cellular contents (12), but the stability of the vascular tissue was significantly comprised. Moreover, 0.025% trypsin plus 1% Triton X-100 are also reported to decellularize rabbit carotid arteries, but which led to significantly increased stiffness, decreased extensibility and decreased residual stress compared to the native arteries (13). In 1990s, Wilson et al. (14) have developed an acellular matrix vascular prosthesis (AMVP) made by detergent and enzymatic extraction of natural arteries, yielding a tissue framework of collagen and elastin from the ori-

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gional vessel, with preservation of the natural basement membrane at the blood flow surface. However, the previous studies have provided a promising method to establish the decellularized cells. Therefore, optimization of decellularized methods to remove cells while preserving ECM secreted by cells are essential for tissue engineering (14).

In this study, we developed a method using a PBS solution containing Triton X-100, sodium deoxycholate and nuclease to process decellularized rabbit aorta. We investigated its structural properties as a scaffold of vascular tissue engineering, which includes cytotoxicity, hydrophilicity and cell adhesion. The results showed that the de-cellularized rabbit aorta not only maintains its extra-cellular structure but also shows little toxicity. We also evaluated the properties of engrafted the cell-scaffolds subcutaneously in animals. Importantly, we successfully applied decellularized rabbit aortas vessel grafts in animals.

Materials and Methods

Reagents

Phosphate-buffered saline (PBS) was purchased from Gibco, USA. Dulbecco's modified Eagles' medium (DMEM) was purchased from Hyclone, USA. Fetal bovine serum (FBS) was obtained from Gibco, USA. Trypsin was obtained from INE, USA. Collagenase was got from Sigma, USA. CCK-8 cell proliferation kit was purchased from Dojindo, Japan. Triton X-100 was purchased from Sigma, USA. Sodium deoxycholate was purchased from Amresco, USA. EDTA (ethylene diamine tetraacetic acid) was purchased from Gibco, USA. Ribonuclease (RNase) and deoxyribonuclease (DNase) were obtained from Sigma. 3211 cell incubator was obtained from FORMA, USA. Nikon-III Inverse microscope and Nikon FDX235 imaging system were obtained from Nikon, Japan. 550 microplate reader was obtained from Bio-Rad, Japan. scanning electron microscope (AMRAY21000B) was obtained from USA.

Animals

Sex-mismatched New Zealand white rabbit (~3 months old), BALB/c nude mouse (19 to 23 g, ~6 weeks old) and Wistar rats (~2 months old) were obtained from the local animal breeding facility (Daping animal house, Third Military Medical University, China). A canine model was used for this study (4 mongrel dogs, weight 20 to 30 kg, age 2 to 3 years). All animal work was approved by the regulatory authorities of Third Military Medical University of Chongqing and complied with the Chinese government guidelines.

Harvesting of tissues and preparation of decellularized rabbit aortas

Descending aorta of New Zealand white rabbit (length of 5 cm) was collected freshly in a local animal facility under sterile conditions. After cleaning off fat and adherent tissues, aortas were rinsed 5 times with sterile PBS. The aorta was then transferred into 50 ml Greiner tubes containing 40 ml decellularizing PBS solution with 0.25% Triton X-100, 2.5 g/L Sodium deoxycholate, 0.2 g/L EDTA, 0.1 g/L RNase and 0.1 g/L DNase. Deceullarization was processed at 37 °C shaking at 200 r/min with a shaking bed for 48 h and the solution was changed every 24 h. The decellularized aorta was lyophilized as described before (4) and sterilized with ^{60}Co at a dose of 25000 Gy.

Characterization of decellularized rabbit aortas

Decellularized rabbit aortas were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were dewaxed, rehydratred and stained by using hematoxylin and eosin (H&E), Masson's trichrome (MT) and Verhoeff van Gieson's (VVG) for assessment of histological morphology, collagen and elastic fibers, respectively.

Scanning electron microscopy (SEM) to assess the ultra-structure of decellularized rabbit aortas

Luminal and external surface ultra-structure of decellularized rabbit aortas were examined by by SEM. Lyophilized aorta sections were cut into small pieces (5 mm × 5 mm). The constructs were fixed overnight with 4% glutaraldehyde and then dehydrated by dipping in a subsequent series of ethanol solutions (50%, 60%, 75%, 99% and 100%, respectively). The constructs were dried coated with gold and imaged with scanning electron microscope.

Cell culture

Rat aortic vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) were freshly isolated as previously described (15). Cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Animals were anesthesized by injection of atropine sulfate (0.05 mg/kg), xylazine (5 mg/kg), and ketamine hydrochloride (12 mg/kg) and pentobarbital sodium (8 to 10 mg/kg). The segments of the greater saphenous vein of the dog were obtained under sterile conditions. After rinsed with PBS, the harvested segments were filled with 0.1% collagenase (Sigma, USA). With both ends occluded, vessel were placed in a petri dish filled with HBSS and incubated in a 5% CO_2/95% air atmosphere at 37 °C for 20 min. Afterwards the vein was flushed with 50 ml M-199 medium with L-glutamine containing 10% fetal bovine serum. The ECs were removed from the inner surface of the vessel by gentle mechanical scratching.

The remaining greater saphenous vein was then cutting into small pieces and incubated in a solution containing 0.2% collagenase and 0.02% EDTA for another 3 h for VSMCs digestion. The digestion was also stopped by adding 10% FBS and VSMCs were separated by washing several times. VSMCs were suspended into DMEM medium containing 10% FBS and antibiotics. Both cells were cultured in an incubator at temperature of 37 °C and atmosphere containing 5% CO_2. After the cells growth into 80% to 90% of confluence, they were used for experiments.

Immunofluorescent staining of cells

Immunohistological staining of cells were conducted as previously described (16). Briefly, after cells were fixed by 4% PFA and permeablized with Triton-X100, the cells were then blocked with BSA and incubated

with primary antibody against of EC marker Factor VIII and fluorescence secondary antibody. The nucleus were staining by DAPI. The expression of Factor VIII then visualized by a fluorescence microscope.

Mechanical assessment of engineered tissue scaffold

Decellularized rabbit aortas with or without rat VSMCs co-culture were cutting into 4 mm long segments for mechanical testing. Freshly isolated normal rabbit aortas were used as a control. The mechanical properties were evaluated by testing the failure of cross section segments using an Instron 5842 uni-axial mechanical tester equipped with a 5.0 N load cell at room temperature. Before testing, the rehydrated scaffold materials were soaked into PBS for 2 h at room temperature. Video capture of mechanical testing was collated with force-extension data and segment dimensions to calculate ultimate tensile stress, strain at failure, and linear elastic modulus. Instrumental control and data collection were performed using LabVIEW (National Instruments).

Cytotoxicity of decellularized rabbit aorta

Decellularized rabbit aortas were evaluated *in vitro* by assessment of the cytotoxicity of cultured rat ECs or VSMCs on decellularized rabbit aortas. Briefly, 200 mg lyophilized sterile decellularized rabbit aorta section was mixed with 10 ml DMEM medium containing 10% FBS, incubated at 37°C in a cell culture incubator for 48 h. The supernatant was then sterilized by passing through a 0.22 μm Millipore filter and then was serial dilution with DMEM medium containing 10% FBS to 100%, 50%, 25%, 12.5% and 0 (negative control) of original extracts. DMEM medium containing 10% FBS plus 0.64% phenol was used as a positive control. Cell cytotoxicity was evaluated with a CCK-8 (cell counting kit 8) method. Briefly, rat ECs and VSMCs were seeded onto 96 well-plates at a density of 5000 cells/ well (n=10). 100 μl above mentioned extracts solution or positive control solution were added into each well and cells were incubated for 1, 3, 5, 7 and 9 days. The extraction solution was replaced every other day. After adding the CCK-8 assay solution, OD450 nm was determined to calculate the relative cell growth rate (RGR) as $RGR= A_{exp}-A_{blank}/ A_{neg.}-A_{blank}$. The toxicity of extract medium from decellularized rabbit aortas were evaluated according to 6 levels of RGR value: ≥100%, 75-99%, 50-74%,30-49%, 15-29%, 0-14% as level 0, 1, 2, 3, 4, 5, respectively.

Cell adhesion assay

Lyophilized de-cellularized rabbit aortas with a length of 5 cm was cut longitudinally. Rat ECs and VSMCs cultured in complete DMEM medium were seeded into the inner or outer surface of sterile lyophilized decellularized rabbit aortas at a density of 10^6 cells per vessel, respectively (n=6). After incubation at 37 °C for 4 h, unattached cells were counted according to a previously reported method (17). Cell adhesion rates were calculated as a percentage of attached cells/ seeded cells (10^6) ×100%.

Hydrophilicity of decellularized rabbit aorta

Aortic specimens (n=5) of various weights were soaked in 100 ml of PBS (pH7.4, 37 °C). After 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24 h, the surface water were wiped out with filter paper and the weight of each aortic specimen were determined. The water hydrophilicity of the aortic specimen at different time points was calculated as $(W_{wet}-W_{dry})/W_{dry}\times100$.

Animal surgery

Animal surgery was performed as previously described (3,18). Briefly, rat ECs or VSMCs were seeded onto the inner and outer layer of decellularized rabbit aortas. 3 weeks later, the aortic scaffold was cut off longitudinally and a small piece of engineered tissue (around 0.5 × 0.5 cm) was engrafted underneath the back skin of BALB/c nude mice after anesthesiaed (2% Pentobarbital Sodium, i.p. injection with a dosage of 40 μg/g). The outer surfaces of decellularized aortic sections were in close contact with the back skin tissue. Mice were sacrificed at 2, 4, 6 and 8 weeks after surgery (n=4). Organs were fixed in formalin, embedded in paraffin and cross sections were measured by H&E staining method.

Canine greater saphenous vein ECs and VSMCs were seeded onto the inner and outer layer of rabbit aortic scaffolds as mention above and cultured for 3 weeks before transplantation. Four dogs weighing 20 to 30 kg (aged at 2 to 3 years) were used in transplantation experiments. Anesthesia was induced by intramuscular injection of atropine sulfate (0.05 mg/kg), xylazine (5 mg/kg), and ketamine hydrochloride (12 mg/kg) and maintained with pentobarbital sodium (8 to 10 mg/kg). The femoral artery was exposed through a longitudinal incision. After given heparin (1 mg/kg) by intravenously, the proximal and distal portions of the femoral artery were clamped and 2 cm of the femoral artery was transected. To substitute the 2 cm-length common femoral artery, the cell-seeded graft for the cell-donated dog were transplanted. All vascular graft was sutured in an end-to-end fashion using 8.0 Prolene suture. After awaking, animals were recovered and monitored carefully, and no immuno-suppression was used post-operation. Prophylactic antibiotics were given just after the operation. The recipient dogs were harvested with the adjacent 2 cm of proximal and distal femoral artery for histological evaluation with light microscopy after staining with H&E at 2 months after transplantation.

Statistical analysis

Data are expressed as mean±S.D. and statistically analyzed with software SPSS 16.0. A 2-tailed Student's t-test was used to compare individual groups, while multiple groups in a cytotoxicity assay were compared with one-way ANOVA. A level of $P<0.05$ was considered significant.

Results

Morphological characterization of decellularized rabbit aorta scaffold

Decellularized rabbit aortas were conducted in PBS solution containing Triton X-100, sodium deoxycholate and nuclease for 48 h. Decellularized rabbit aortas exhibited a loose structure and a uniform decellular texture, which show a white colored, intact tubular structure. Both inner and outer membranes are smooth and there

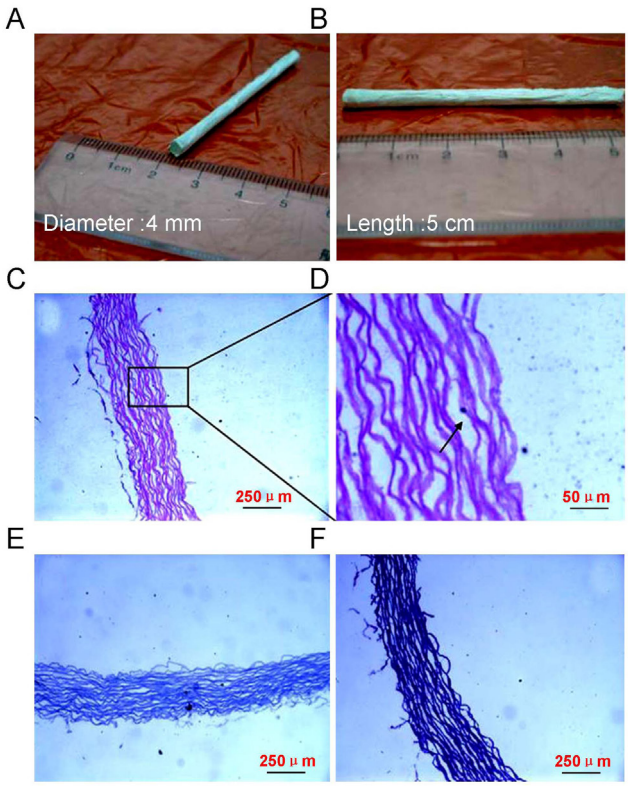

Figure 1. Physical characteristics of decellularized rabbit aortas. Vessel diameter and Longitude length are shown in A. Histological structure of decellularized rabbit aortas as examined by Immunostaining of H&E (B, zoomed in C), Masson's (D) and Fibers assay staining (E, F).

are no structural difference and comparing with fresh unprocessed rabbit aortas. The diameter and length our decellularized rabbit aortas are of 4 mm and 5 cm, respectively. A representative vessel is shown in Fig. 1A.

To determine decellularization efficiency, cell contents were firstly examined by H&E staining. As shown in Fig. 1B&C, ECs in decellularized rabbit aortas were almost completely removed as there were no nucleus can be observed in the intimal area of the aorta. In addition, there were also almost no VSMCs can be observed (frequency <0.5 % throughout the whole aorta) in the deeper area of medium between elastic lamina. Moreover, there were also no adhered immune cells or adventitia cells can be found in vascular wall, indicating that the decellularization process was successful. Importantly, as shown in Fig. 1D by Masson's Trichrome staining, structures of multiple layers of elastic lamina were largely preserved without destruction. The structure is loose with many empty extra-cellular matrix interspaces, used to be occupied by VSMCs. Thick collagen fibers and multiple layers of elastic lamina were intertwined into each other with small sized collagen in between. Lastly, Masson's Trichrome staining. VVG staining (Fig. 1E, F) also indicates that there was a loose purple structure with lots of intact collagen fibers after decellularization.

Culture of rat vascular cells on decellularized rabbit aorta scaffold

We next seeded rat VSMCs in the external surface of decellularized rabbit aortas and cultured the complex. The results showed that rat VSMCs could adhere, infiltrate and proliferate well in the external surface of decellularized aorta scaffolds. As shown in Fig. 2A, 2B by H&E staining assay, after 4 weeks, multiple, 2 to 4 cell-layers of VSMCs can be observed in the adventitia area of the scaffold. Moreover, VSMCs were also confirmed by immuno-staining with Asma (Fig. 2C). Similarly, rat ECs were seeded and allowed to grow in the inner surface of decellularized rabbit aorta scaffolds. As shown in Fig. 2D&2E, 3 weeks later, a single layer of scattered ECs can be found in the inner surface of the scaffold. Rat ECs were also verified by immuno-staining of EC marker Factor VIII (Fig. 2F).

Hydrophilicity and cell adhesion of decellularized rabbit aortas

In addition, we also determined the hydrophilicity of decellularized rabbit aortas. As shown in Fig. 3A, after 15 min, the weight of lyophilized decellularized rabbit aortas sharply increased in PBS solution with a water hydrophilicity of 227±21.2%. The hydrophilicity reached a plateau at 12 h with 519±23%, and maintained these levels for another 36 h. In addition, de-cellularized rabbit aorta constitutes a property for supporting of cell adhesion and growth. As shown in Fig. 3B, the cell adhesion rates of rat VSMCs and ECs to the outer and inner layer of decellularized rabbit aorta were 64.32±2.03% and 52.77±1.19%, respectively.

Mechanical properties of decellularized rabbit aortas.

We next assessed the mechanical properties of decellulatized aortas. After 4 weeks co-culture with seeded rat VSMCs, the mechanical properties of decellularized scaffolds were measured. As shown in Figure 3C&3D,

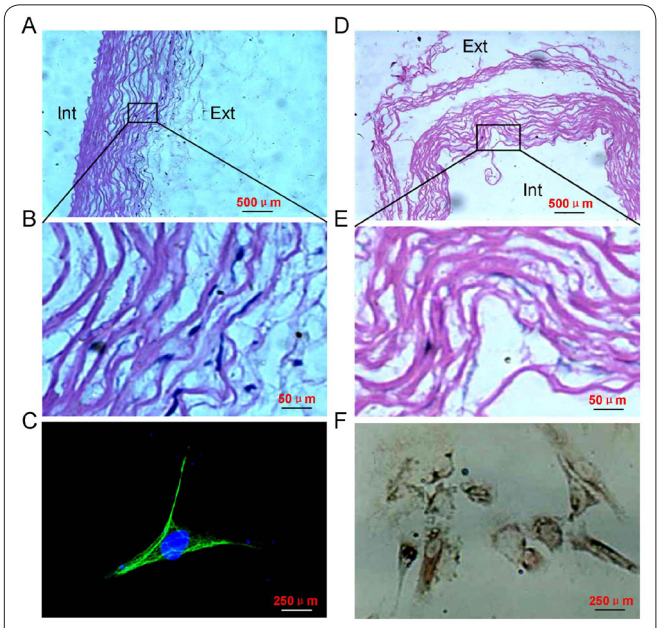

Figure 2. Rat VSMCs were cultured in external surface of decellularized rabbit aortas. 4 weeks later aortic sections Incorporating cells were examined by H&E staining (A, zoomed in B) . Cultured rat VSMC was confirmed by immunostaining with αSMA (green), nucleus was shown by DAPI counterstaining (blue) in overlay image. (C). Rat ECs were cultured in internal surface of decellularized rabbit aortas. 3 weeks later, aortic sections Incorporating cells were examined by H&E staining (D, zoomed in E) . Cultured rat ECs were confirmed by immunostaining of EC marker Factor VIII (F).

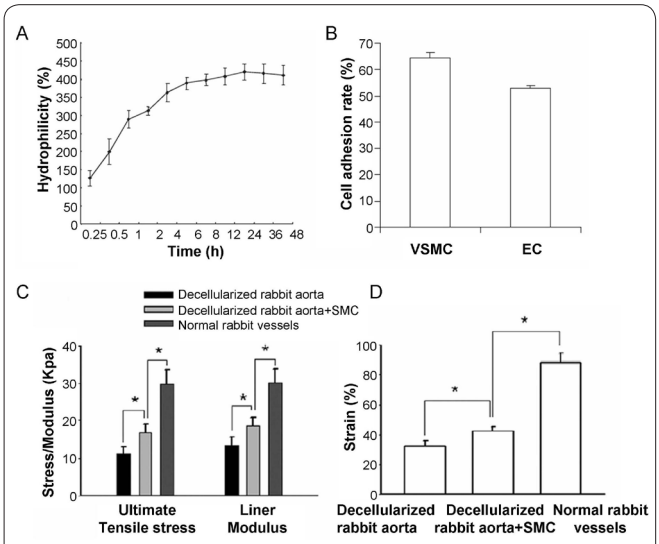

Figure 3. Curves of hydrophilicity changing with the time (A) and cells adhesion (B) of VSMCs to decellularized rabbit aortas. The mechanical properties of the scaffolds and normal rabbit vessels were measured using biaxial tensile testing of ring sections of the constructs. Ultimate tensile stress and strain at failure are shown in (C) and (D). Bars are mean ± S.D. from five independent experiments. *P<0.05.

the ultimate tensile stress of linear modulus, strain at failure of normal rabbit aortas were significantly higher than that of decellularized aorta with seeded VSMCs. The mechanical properties of tissue engineered scaffolds were slightly better than that of decellularized aortas without cell reconstitution. After decellularization, the biomechanics of rabbit aortas were greatly impaired, which might be due to the damage to the ECM. After co-culture with VSMCs, mechanical properties of decellularized aortic scaffolds were dramatically improved. Index of Ultimate tensile stress and strain at failure are significantly higher than that of tissues without seeded cells. Both engrafted VSMCs and newly secreted ECM remodeling could contribute to the improvement of the engineered tissues.

Scanning electron micrograph (SEM) and transmission electron microscope (TEM) of cells decellularized rabbit aorta complex. The decellularized rabbit aortas were further characterized by SEM and TEM. Rat VSMCs were seeded on the external surface of a lyophilized aorta scaffold. After 4 weeks, the ultra-structure of the cell-scaffold complex were analyzed by SEM microscopy. As shown in Fig. 4A&4B, SEM microscopy demonstrated a tight structure of extra-cellular matrix with embedded cells. Seeded cells were growing nicely to form a connective layer that fills up the porous surface of the scaffold which makes it hard to distinguish individual cells. Similar results were observed for rat ECs grown on the internal surface of a lyophilized aorta scaffold (Fig. 4C, 4D). Transmission electron microscopy results showed that decellularized rabbit aortas co-cultured with rat VSMCs (Fig. 4E) or rat ECs (Fig. 4F) formed typical organelle properties of VSMC, such as actin filaments and dense patches in the external surface as well as W-P bodies of ECs in the internal surface of the scaffold (as shown in arrows).

Cytotoxicity and biocompatibility of decellularized rabbit aorta scaffold

We then tested the cytotoxicity of decellularized rabbit aortas by using the CCK-8 method. Rat ECs and VSMCs were cultured in various concentrations of aortic extraction solution for 1, 3, 5, 7 and 9 days. The effects of extraction solution on rat ECs or VSMCs growth are shown in Fig. 5A and 5B. No significant toxic effect of decellularized aorta extract solution was found. Moreover, there were also no any toxicity throughout the time span of culturing. The relative cell growth rates for rat ECs or VSMCs were similar to that of negative controls. The extract solution of decellularized rabbit aorta scaffold showed low cell toxicity to rat vascular cells. The values of relative cell growth rate were assessed as toxicity level 0-1.

Finally, we assessed the biocompatibility of the cells-decellularized rabbit aortas complex *in vivo*. Biocompatibility refers to specific properties of a material to test whether it elicits an immune response, or is able to integrate with a particular cell type or tissues. As decellularized vessels have the advantage of reduced immune

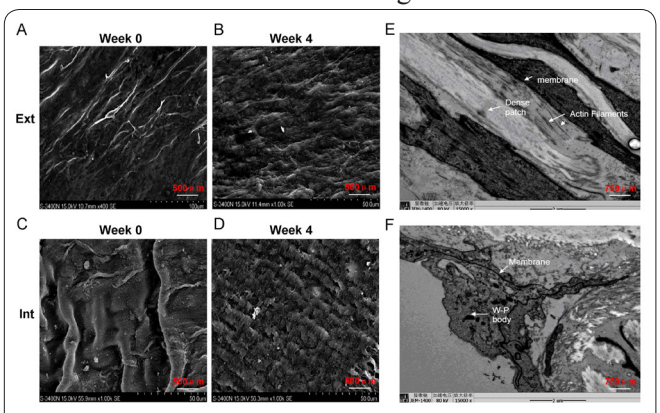

Figure 4. Representative scanning electron microscopic (SEM) images on external surface of decellularized rabbit aortas at week 0 (A) and 4 weeks after culturing with rat VSMCs (B). Representative images of scanning electron microscopic data on internal surface of decellularized rabbit aortas at week 0 (C) and 4 weeks after culturing with rat ECs (D). Representative images of transmission electron microscopy in decellularized rabbit aortas co-cultured with rat VSMCs (E) or rat ECs (F).

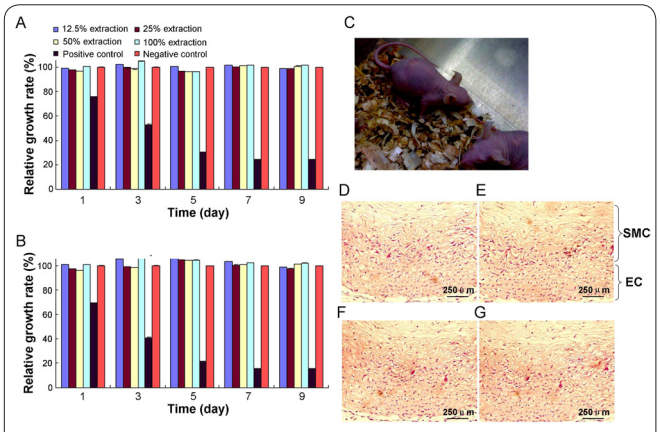

Figure 5. Cytotoxicity effects of extraction solution of decellularized rabbit aorta on rat VSMCs (A) and ECs (B) growth. Decellulatized rabbit aorta, as a source of rat vascular cells was subcutaneously implanted in the back of nude BALB/c mice(C). images of mice one week post surgery. Cross-sections of implanted cell-aorta complex at 2(D), 4(E), 6(F) and 8(G) weeks post surgery, respectively.

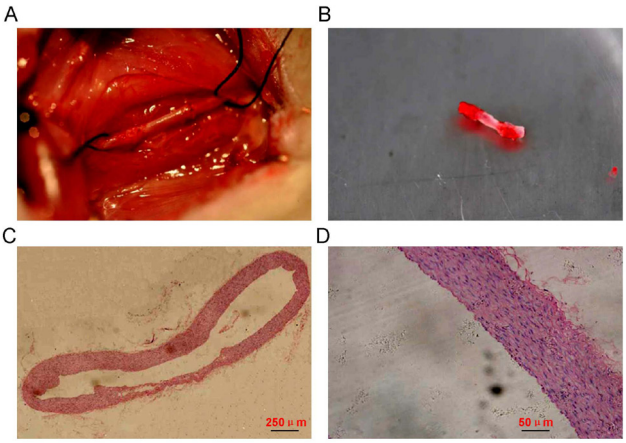

Figure 6. In vivo transplantation of decellularized rabbit aorta into a canine femoral artery. A, representative image of microsurgery transplantation of reconstituted decellularized rabbit aorta with autologous vascular cells at 2 month past surgery. B. representative image of dissected vessel segment at 2 months past transplantation. H&E staining of cross sections of transplanted aortic scaffold segments were shown in C and D.

resistance, we tested the integration of a cells-decellularized rabbit aorta complex in a tissue transplantation model with a BABL/c mouse. This avoids effects of immune resistance between rat cells and the host. Rat ECs or VSMCs grew on the decellularized scaffold for 4 weeks and then a small piece (an area of ~0.25 cm^2) of tissue was implanted subcutaneously underneath the back skin of the nude mice (Fig. 5C). From 2 weeks after transplant, sustained cell proliferation and nearby cell migration can be observed, which indicate that the decellularized aorta incorporates well with the local tissue from the host (Fig. 5D). For up to 8 weeks, the seeded cells were still viable and proliferated well. No clear impairment of vessel structures were observed (Fig. 5E, 5F, 5G).

As autologous cells are expected to be seeded onto aortic scaffolds in the clinical use, the immunogenicity of transplantation will be resulted largely from the scaffolding materials. To assess the immunogenicity of rabbit decellularized aortic scaffold in an immune competent condition *in vivo*, we also examined the immuno-genicity of rabbit aortic scaffold in an *in vivo* transplantation experiments, using rabbit aortic scaffolds reconstituted with autologous vascular cells to replace a segment of femoral artery from dogs. The main reason using canine animal model is due to the relative large caliber of the decellularized rabbit aortic scaffolds. The illustrated surgical process and aortic scaffold segments dissected 2 months after transplantation were shown in Figure 6 (A,B). No clear occlusion of reconstitute aortic scaffolds was observed. This is confirmed by H&E staining in Figure 6 (C,D), which demonstrates that transplanted aortic scaffold incorporates well with the local tissue of the host with no occlusion, no thrombosis formation, no overt rejection or impaired cell proliferation. Although long term effects of immune responses after transplantation needs to be carefully examined, our experiments clearly demonstrate that decellularized rabbit aorta constitutes a promising candidate in vascular tissue engineering for auto-transplantation.

Discussion

In typical tissue engineering approaches, scaffold materials are utilized as templates to support live cells growth *in vitro*, providing a three dimensional space for seeded cells to adhere, growth and metabolize. The basic requirements for appropriate vascular ECM are optimal biocompatibility, functional nutrition delivery and easy for cells to adhere and growth. Therefore, the selection, preparation and evaluation of scaffold materials are the crucial issues in tissue engineering. After removing cellular components, decellularized vascular scaffolds should ideally possess favorable properties such as minimized immunogenicity, an intact extra-cellular matrix, adequate mechanistic characteristics, low cytotoxicity and facilitated cell infiltration and repopulation, making them ideal for artificial blood vessel materials (7,19).

Currently, the most commonly used methods for decellularized process are enzyme digestion, detergent methods or a combination of both. As early as the 1990s, Wilson *et al* had applied a combination method using both enzyme and detergents to prepare acellular matrix allograft small caliber vascular prostheses (14). Triton X-100 is a nonionic surfactant. Its hydrophilic head groups allow dissolving of the cell membrane and penetration deeply into the vessel wall to prepare the decellularized aorta scaffolds. Previously, various concentrations of Triton X-100 were investigated by preparing porcine thoracic aortas acellular matrix. SEM data demonstrated that Triton X-100 could not only totally remove cellular nuclei in the aortas, but also cleared remove most of the organelles, with very few lipophilic organelle remnants (6). Aortic scaffolds obtained by this method have a maintained arrangement of collagen and elastic proteins, intact vessel structure and elastic tubular configuration. In this study, we have integrated these methods (20,21) into an improved protocol to successfully prepare small-caliber rabbit scaffolds with a combination use of Triton X-100, sodium deoxycholate, EDTA, RNase and DNase. However, future studies are needed to address whether by avoiding the use of trypsin can improve the mechanic property of the decellularized aortas.

The decellularization process used in the present study can not only significantly reduce the immonogenicity of allograft aortas, but can also enlarge the porosity of the extra-cellular matrix (22). Microscopic and histological examination showed hollow structures with large holes. Barely any cellular residues along the intimal area can be observed and only very few cells can be seen in the deeper area of media in the aorta. Decellularized aorta with a high porosity has a high surface area, which can promote cell adhesion and follow-up tissue remodeling. In addition, large pores in the aortic scaffold provide important channels for the exchange of nutritious materials, the metabolism of implanted cells, and the passing in and out of gases into deeper areas of cell-scaffold structure. Therefore, an increase in porosity potentially represents an improved nutrient transfer. It has been shown that cells seeded on the scaffold materials migrate poorly to the deeper area when culturing multilayer engineered tissue (23). With the prolonged culture condition in vitro, even those cells that have

penetrated into the deeper area of the scaffold will lose their ability for future proliferation and secretion of extra-cellular matrix. This might be due to a poor delivery of nutrient or the accumulation of metabolic toxins that eventually lead to the death of those implanted cells. Another favorable property for an ideal tissue engineered scaffold is a relatively high cell adhesion affinity, which can promote cell adhesion and healing. In this study, cell adhesion rates of rat VSMCs and ECs to the outer and inner layer of decellularized rabbit aorta were 64.32±2.03% and 52.77±1.19%, respectively. In addition, lyophilized decellularized aorta is highly hydrophilic, with a maximal hydrophilicity of 519±23%. As hydrophilicity is an important factor for cell adhesion, a high hydrophilicity normally represents a good cell adhesion affinity. In addition, hydrophilicity also plays an important role in the delivery and exchange of nutritious materials within the scaffolds. A highly hydrophilic tissue scaffold is beneficial for cell uptake of nutrition under culturing conditions (24,25).

The biocompatibility of the aortic scaffolds largely decides the feasibility and safety of their clinical application. Cytotoxicity is widely used to evaluate the biocompatibility of biological materials on which cells will grow (2). In this study, we have adopted a well established method, a CCK-8 colorimetric protocol to quantitatively evaluate the cytotoxicity of decellularized aorta extraction solution. No significant morphological changes were observed when culturing rat ECs or VSMCs in various concentrations of decellularization extraction solution. Cell proliferation rates for both cell types are higher than 75% throughout the time span during which they were cultured in a conditioned medium. The cytotoxicity of both cells was evaluated between 0-1, clearly demonstrating that our decellularized rabbit aorta scaffolds are nontoxic to rat ECs and VSMCs. The scaffold allows cells to adhere, growth and remodel afterwards as SEM resutlt showed that seeded cells had covered the inner and outer surface of the scaffold. Indeed, in addition to investigating the cytotoxicity of aortic scaffold on seeded cells, the biocompatibility of engineered tissues are also importantly, which include examination of the immune reactivity between implanted tissue and the host. In our study, to avoid the potential immune response between the host and rat vascular cells, we have adopted BALB/c nude mice which allow us to test the growth of xenografted rabbit aortic vessels.

Collectively, our study has provided several novel insights to blood vessel tissue engineering. Firstly, currently available synthetic polymeric materials have been successfully applied in large or medium-caliber vessel grafts. However, they lack the ability to self-grow or self-repair that will limit their clinical use. In addition, in small-diameter engineered tissues under condition of low shear stress of blood flow, synthetic materials can result in initial hyperplasia, thrombosis, restenosis, calcification and infection. Although decellularized aortic scaffolds from large animals such as pigs or dogs have been extensively investigated, small diameter vessels (< 5 mm) are clinically more relevant. In addition, the migration of cells into decellularized pig aorta was shown to be inadequate either *in vitro* or *in vivo*, which is possibly due to its tight matrix organization or incomplete decellularization processing.[11] We have successfully prepared small-diameter (~4 mm) decellularized rabbit aortic scaffolds on which both rat VSMC sand ECs can adhere, infiltrate and proliferate. Lyophilized scaffolds can be re-endothelized by rat ECs and repopulated by multiple layers of rat VSMCs, making it a potential engineered tissue for future clinical use. Secondly, various decellularization methodologies have been explored to de-cellularize blood vessel tissues. Enzymatic digestion by trypsin or detergents such as Triton X-100 is the most frequently used method to remove cellular components. Different methods with various concentrations of detergents or enzymes will result in distinct extents of cell clearance, adhesion and vessel wall mechanistic properties. As trypsin might potently damage the vascular matrix scaffold, we have successfully optimized a protocol which allows us not only to totally remove most of the cellular remnants but also to avoid the use of trypsin. In conclusion, we have successfully prepared small-diameter (~4 mm) decellularized rabbit aortic scaffolds, which have a porous structure with few cell remnants. Both rat aortic VSMCs and ECs cultured on decellularized aortas were adhere, proliferate well. And both rat VSMC sand ECs can adhere, infiltrate and proliferate. Importantly, no overt cytotoxicity was observed when grown in extraction solution of aortic scaffolds. Implanted scaffold structure and proliferation of seeded cells were well maintained up to 8 weeks in animals. The decellularized rabbit aortic scaffolds may represents a potential tool as a small diameter scaffold in vascular tissue engineering.

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