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Preparation of decellularized and crosslinked artery patch for vascular tissue-engineering application

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Abstract There is an urgent clinical need of tissue-engineering (TE) vascular grafts, so this study was for developing a fast and simple way of producing TE vascular scaffold. The TE vascular scaffold was prepared with pepsin, DNase and RNase enzymatic decellularization and crosslinked with 0.1, 1, 5% glutaraldehyde (GA), respectively. The samples were underwent analyses of burst pressure; suture strength; cytotoxicity; enzymatic degradation in vitro; degradation in vivo; rehydration; biocompatibilities detected with hematoxylin and eosin (H&E), scan electron microscope, immunohistochemistry both in vivo and in vitro; macrophage infiltration and calcification using Von Kossa staining. After being decellularized the scaffold had a complete removal of cellular components, an intact collagen structure. The burst pressure and suture strength were similar to native artery. 0.1% GA crosslinked scaffold showed less cytotoxicity than 1 and 5% GA groups ($P < 0.05$) and was resistance to enzymatic degradation in vitro. Once being implanted, 0.1% GA group was resistant to degradation and formed endothelium, smooth muscle and adventitia with few macrophages infiltration. However, there appeared calcification in implants compared with that in native artery. This study demonstrated that DVPs producing methods by enzymatic decellularizing and crosslinking with 0.1% GA could be used for clinical TE vascular graft manufacture.

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1 Introduction

Synthetic polymer vascular patches are often used in cardiovascular surgery for pericardial substitute, intracardiac defect repair, stenosis enlargement [1]. However prosthetic and bioprosthetic materials currently in use are lack of growth potential and easily infected [2, 3]. Tissue engineering is a new discipline that offers the potential for creating replacement structures from autologous cells and biodegradable scaffolds [4]. Natural scaffolds are composed of extracellular matrix proteins that are conserved among different species and that can serve as scaffolds for cell attachment, migration, and proliferation [5]. In addition to inherent cell compatibility, biological tissues possess the desired shape and the strength of the tissues from which the materials are derived [5].

Several decellularizing methods including detergents, enzymes and sonication are being explored to produce a complete acellular tissue matrix by specifically targeting the

removal of cellular components [5, 6]. Decellularization can remove nonstructural protein to reduce the immunity after implantation [6]. Crosslinking methods aim to stabilize collagen-based biomaterials to prevent tissue degradation in vivo, to minimize the immune response, and sterilize the tissue before implantation [7]. The tissue once being decellularized is subjected to be crosslinked by using varied chemicals. Glutaraldehyde (GA) is the most commonly used crosslinker in natural tissue treatment. GA crosslinking accomplishes the task of reducing immunogenicity of the graft, but several problems such as cytotoxicity are encountered, so it is important to check their toxic limit [8] to find the suitable GA crosslinking concentration without toxicity.

Many studies successfully acquired suitable scaffold by decellularization and crosslinking, however few researches explored the degradation situation and biocompatibility. In this study, we investigated the processing method by enzymatic decellularizing sheep aortic arterial patch and crosslinking with GA. Base on previous studies concerning glutaraldehyde in tissue crosslinking we focused on exploring the toxic limit of glutaraldehyde concentration in development of decellularized vascular patch, and further explore the burst pressure, suture strength test; cytotoxicity; resistant enzymatic degradation in vitro; degradation in vivo; rehydration; biocompatibilities both in vivo and in vitro using H&E, electron microscope, immunohistochemistry; further tracing macrophage infiltration and calcium formation after implantation.

2 Materials and methods

2.1 MSCs isolation and characterization

2.1.1 MSCs isolation

As described in previous report [9], briefly, bone marrow was centrifuged together with a Ficoll density gradient for 15 min at 3,000 rpm. Bone marrow mononuclear cells were isolated from the layer between the Ficoll reagent and blood plasma, the isolated mononuclear cells were incubated at 37°C, 90% humidity, and 5% CO_2 incubator with Dulbecco's modified eagle medium (DMEM Invitrogen) supplemented with 15% fetal bovine serum (FBS), 1% Penicillin–Streptomycin, the medium was changed at every 3 days. Dense cell clones were trypsinized with 0.25% trypsin and the cells were plated in 75 cm^2 flasks until confluence. The second passage was used.

2.1.2 Proliferation curve

The second passage MSCs were seeded in a 96-well microplate at a density of 1.0×10^4 cells per well containing 200 μl culture medium at 37°C in a 5% CO_2 atmosphere. Every day, the cells were detached with EDTA and the cell number was counted using a hemocytometer. Cell population doubling time (*DT*) was calculated based on the formula $DT = t \times \lg 2/(\lg Nt - \lg N0)$. *DT* means cell doubling time, *t* is the cell culture time, *Nt* represent cell number after *t* hours culture and *N*0 is the cell number when cell inoculation.

2.1.3 MSCs osteogenic differentiation

MSCs cells were seeded into 24-well plates (Costar, IL, USA) at 5×10^4 cell/well and after the cells reached 80% confluence, the medium was changed with that containing in DMEM supplemented with 10% FBS, 5 mM beta-glycerophosphate, and 100 μg/ml ascorbic acid, 10^{-8} mol/l dexamethasone. After 4 weeks, the cells were fixed with 4% paraformaldehyde for 30 min, washed with distilled water, incubated in 5% $AgNO_3$ for 30 min, washed again and treated with 2.5% sodium thiosulfate for 5 min.

2.2 Producing decellularized and crosslinked vascular patches (DVPs)

Decellularized vascular patch was produced according to the report with modifications [9] Aorta from 6-month-old sheep were obtained from a local abattoir. Artery segments were immediately frozen at −80°C, thawed and then placed in a 500 ml bottle containing 75% ethanol on an orbital shaker for 72 h. The tissue was then placed in distilled water and transferred into 0.125% pepsin and DNase and RNase for 4 h. After rinsed in distilled water, the processed tissue was frozen dried and crosslinked in 0.1, 1, 5%, aqueous GA solution, respectively at 37°for 24 h. The aqueous GA was buffered with phosphate-buffered saline. After rinsed in distilled water for 96 h, the processed tissue was frozen dried.

2.3 Burst testing of the decellularized aorta

A 50 mm long decellularized aorta was immersed in the PBS and used for burst pressure test. Two side of this tubular scaffold were clamped. A catheter was placed in the lumen, connected to a 50 ml syringe and monitored by using a manometer (testo926, Testo, Germany) which was connected via a control valve. The scaffold was progressively pressurized until vessel failure when air bubbles from the water can be observed.

2.4 Suture strength testing of the DVPs

A 30×20 mm vascular patch was used for suture strength test with Instron 4456 (Norwood, MA, USA).

The 5-0 polypropylene suture (Ethilon USA) was used for stitching two side of the patch. A continuous elongation (5 mm/min) was applied along the longitudinal axis of the scaffold until the sutures were pulled through the vessel edges.

2.5 MSCs seeding analysis

The uncrosslinked and crosslinked DVPs were cut into sections approximately 20 × 5 mm. MSCs suspensions were seeded (2×10^5 cells/ml) onto the surface of the DVPs. The constructions were cultured for 5 days and were performed H&E stain. The seeding cells on the scaffold were stained with PKH26 (sigma) and observed with a florescent microscope.

2.6 Cytotoxicity of DVPs

MSCs were first seeded in a 96-well microplate at a density of 1.0×10^4 cells per well containing 200 μl DMEM supplemented with 15% fetal bovine serum (FBS), 1% Penicillin–Streptomycin at 37°C, in 5% CO_2 atmosphere. After 24 h of culture, the culture medium was replaced with fresh medium containing 200 μg/ml uncrosslinked and crosslinked DVPs (cut into small pieces) and the medium was replaced in every 2 days and alone DMEM as a control. At the intervals, the cell number was counted using a hemocytometer.

2.7 In vitro enzymatic degradation

The uncrosslinked DVPs and crosslinked DVPs with 0.1, 1, 5% GA were tested enzymatic degradation using collagenase type II digestion (Sigma-Alderich). The uncrosslinked and crosslinked DVPs were cut into to a mean weight of 5 ± 0.1 g, specimens were immersed in 20 ml collagenase/D-Hanks solution (pH 7.4) with a concentration of 1,000 U/ml, incubated at 37°C for 12 h under continuous shaking, then all residual specimens were weighed again. After freeze-drying the degradation ratios of test samples were calculated from the following expression: $(Wd-We)/Wd$. The *Wd* is the weight of the original test samples and the *We* is the weight of the digestive treated sample.

2.8 Degradation analysis in vivo

After cytotoxicity, cell seeding and enzymatic degradation experiments, the 0.1% GA crosslinked DVPs was selected for further study. The uncrosslinked and 0.1% GA crosslinked DVPs samples (50 × 50 mm) were implanted subcutaneously for degradation analysis in vitro. C57BL/6 mice (20 ± 2 g) ($n = 5$) were used for subcutaneously implantation. At weeks 2, 4, 6 and 8, the samples were harvested for histological examination. Animal care was in compliance with regulations of Xiamen University on experimental animals and animal experiments were approved by the Animal Care Committee of the University of Xiamen. All the surgical procedures were performed in a sterile animal operation room in Zhongshan Hospital Xiamen University.

2.9 Rehydration analysis

The uncrosslinked and crosslinked DVPs were cut into to a mean weight of 5 ± 0.1 g and were determined by immersing dried samples in PBS. After 24 h, samples were removed from the solution. Subsequently, samples were blotted with a piece of tissue to absorb excess water on surface. The swelling ratios *Rs* (%) of test samples were calculated from the following expression: $(Ws-Wd)/Wd$. The *Ws* is the weight of the swollen test sample and the *Wd* is the weight of the dried test sample.

2.10 Surgical techniques

Animal care was in compliance with regulations of Xiamen University on experimental animals and animal experiments were approved by the Animal Care Committee of the University of Xiamen. All the surgical procedures were performed in a sterile animal operation room in Zhongshan Hospital Xiamen University. The sheep that donated the bone marrow ($n = 3$) were anesthetized with intramuscular injection of ketamine and intravenous injection of pentobarbital and ventilated with a mixture of O_2, N_2 and isoflurane during the operation. The aorta was exposed. After heparin (100 unit/kg) being given intravenously, a 15 × 5 mm of aorta wall was cut and implanted with MSCs seeded 0.1% GA crosslinked DVPs using a 5-0 polypropylene suture (Ethicon, USA). No anti-coagulants or anti-platelets were administered post-operatively.

2.11 Histology analysis

The DVPs and the implant harvested 2 months after surgery were fixed in 10% buffered formaldehyde solution, dehydrated with a graded ethanol series, and embedded in paraffin. The specimens were cut into 4 μm-thick sections and stained with H&E. The elastin and collagen in the samples were stained with van Gieson's method and Masson's trichrome method. The calcium formation was detected with Von Kossa staining and counterstained with hematoxylin. The implanted specimens were also stained immunohistochemically for vWF, SM α-actin for detection

of endothelium, smooth muscle cell and stained for CD163 and CD68 for detection of macrophage infiltration. Labeled MSCs were examined using a fluorescence microscope.

2.12 Electron microscopy

The DVPs and the implant harvested 2 months after surgery were analyzed with scanning electron microscopy (SEM). Specimens were fixed in 1% buffered GA and 0.1% buffered formaldehyde, respectively, dehydrated with a graded ethanol series and dried. The dried samples were mounted on an aluminum stub, coated with gold using a Sputter Coater and examined with a VEGAII LMU scanning electron microscope (Tescan, Czech Republic).

2.13 Statistical analysis

Experiment of burst pressure test, suture retention test and cell proliferation were repeated at least three times, and the data were expressed as means ± SD. Student t test was used to evaluate comparative values and determine statistical significance. A value of $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 MSCs isolation and characterization

As we know that MSCs were absent specific markers [10, 11], so we demonstrated uniform appearance (Fig. 1a), proliferation curve (Fig. 1b), double population time and potential differentiation (Fig. 1c) to show MSCs stem cell characteristics. The first adherent cells isolated from rat bone marrow grew as spindle- or stellate-shaped cells, and developed into visible colonies 3–5 days after the initial plating. They remained dormant for 3–5 days, then began to multiply rapidly, and reached confluence on the 8th to the 10th day. The floating cells were removed from the medium and subsequently passaged. After the second passage, the adherent cells appeared to have a more uniform appearance (Fig. 1a).

3.1.1 *Proliferation curve*

The proliferation curve (Fig. 1b) was an inverted "S" curve, the number of cells slightly decreased at the 1st day; there was a massive increasing at the 4th day; at the 5th day the cells increased significantly, the proliferation was stable at the 8th day and reached a plateau period. Cell population doubling time was 32 h.

3.1.2 *Osteogenic differentiation ability*

Van kossa staining was carried out to determine whether MSCs could form mineralized nodules after induction. The data showed that mineralized nodules stained positively 28 days after osteogenic induction (Fig. 1c).

3.2 Characteristics of DVP

After being decellularized and frozen-dry, the decellularized artery showed a tubular scaffold (Fig. 2a). The cellular components were removed completely from fresh arteries (Fig. 2b). The acellular scaffolds were left with only collagen (Fig. 2c). Scanning electron microscopic examination revealed that no endothelial cells on the luminal side (Fig. 2d).

3.3 Burst testing

The uncrosslinked matrix can resist an average of 1,706 mbar of burst pressure, the fresh artery can resist 2,156 mbar; It was 1,762 mbar in 0.1% group, 1,886 mbar in 1% group, 1,898 mbar in 5% group, respectively. However there is no significant difference between the crosslinked scaffold, no significant difference between fresh artery and decellularized scaffold, no significant

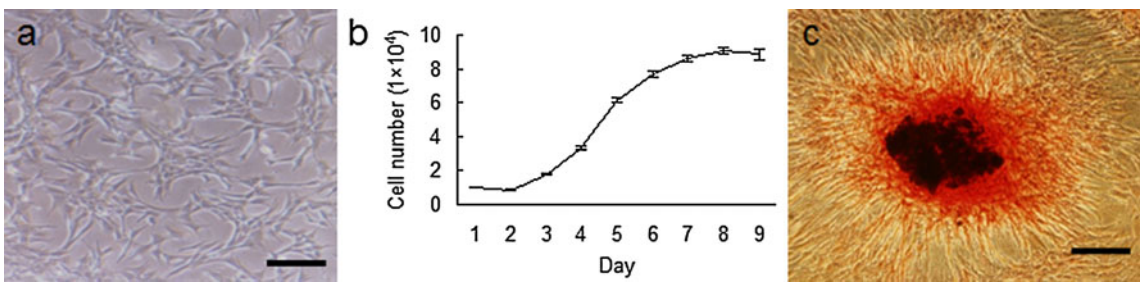

Fig. 1 Characteristics of MSCs. **a** The spindle-shaped, uniform appearance of MSCs (*Bar* = 50 μm); **b** proliferation curve of MSCs; **c** Von Kossa staining indicated calcium formation after osteogenic induction (*Bar* 100 μm).

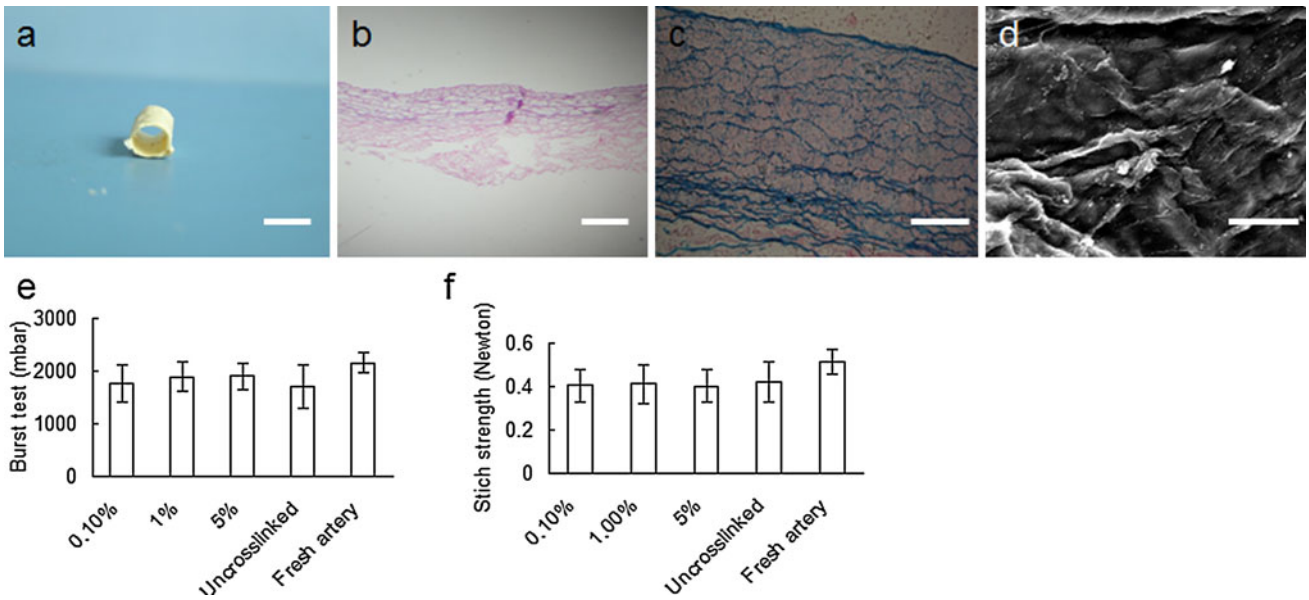

Fig. 2 Characteristics of DVPs. **a** Gross view of decellularized aorta (*Bar* = 5 mm); **b** H&E staining showed that the cellular components were removed from fresh aorta (*Bar* = 200 μm); **c** Masson's staining showed that the acellular scaffolds were left with collagen (*Bar* = 100 μm); **d** scanning electron microscopic examination revealed that no endothelial cells on the luminal side (*Bar* = 50 μm); **e** There is no significant difference of burst strength between crosslinked and uncrosslinked DVPs ($n = 5$); **f** There is no significant difference of suture strength between crosslinked and uncrosslinked DVPs ($n = 5$)

difference between uncrosslinked and crosslinked DVPs (Fig. 2e) ($P > 0.05$).

3.4 Suture retention analysis

The highest suture strength was fresh artery, 0.51 (Newton). The suture strength in fresh artery was little higher than those of decellularized matrix, however there was no significant differences between them ($P > 0.05$). The suture strength of uncrosslinked patches is 0.42 (Newton), while that of 0.1, 1, 5% GA crosslinked matrix were 0.41, 0.41 and 0.4 (Newton), respectively. There was no significant difference between uncrosslinked matrix and crosslinked matrix ($P > 0.05$). There was no significant differences in different GA concentration treated matrix ($P > 0.05$) (Fig. 2f).

3.5 MSC seeding analysis

Seeding and culturing MSCs on the abluminal surface of 5 and 1% GA crosslinked DVPs displayed a poor adhesion that could be observed by histological analysis (Fig. 3a, b), SEM analysis (Fig. 3e, f) and fluorescent detection (Fig. 3i, j); MSCs seeded on the luminal surface of uncrosslinked and 0.1% GA crosslinked DVP displayed positive adhesion over the 5-day culture period that could be observed by histological (Fig. 4c, d) and SEM analysis (Fig. 4g, h) and fluorescent detection (Fig. 3k, l) showing near confluent layers of cells.

3.6 Cytotoxicity of DVP

As shown in Fig. 3m, the proliferation rate of the MSCs cultured with medium containing uncrosslinked DVPs was little higher than that of control medium and 0.1% GA crosslinked DVPs. It showed a obvious suppression of cell proliferation in 1 and 5% GA crosslinked group, especially in 5% GA group, which revealed that crosslinked DVPs with 1 and 5% GA affected cell growth and proliferation. This indicated that the effect of toxicity of crosslinker can be related to the concentration.

3.7 Enzymatic degradation analysis

After being digested in vitro, the weight loss of all crosslinked tissue was significantly less than that of uncrosslinked, as shown in Fig. 4e. After enzymatic digestion, the relative weight loss of uncrosslinked was 43.3%, the crosslinked groups was 10.8% in 0.1% group, 7.8% in 1% group and 5.9% in 5% group respectively. H&E analysis showed that main structure protein of scaffold was digested and became irregular fiber in uncrosslinked group (Fig. 4a), while in crosslinked group (Fig. 4b, c, d), it showed a well preserved structure, especially in 5% crosslinked group (Fig. 4d).

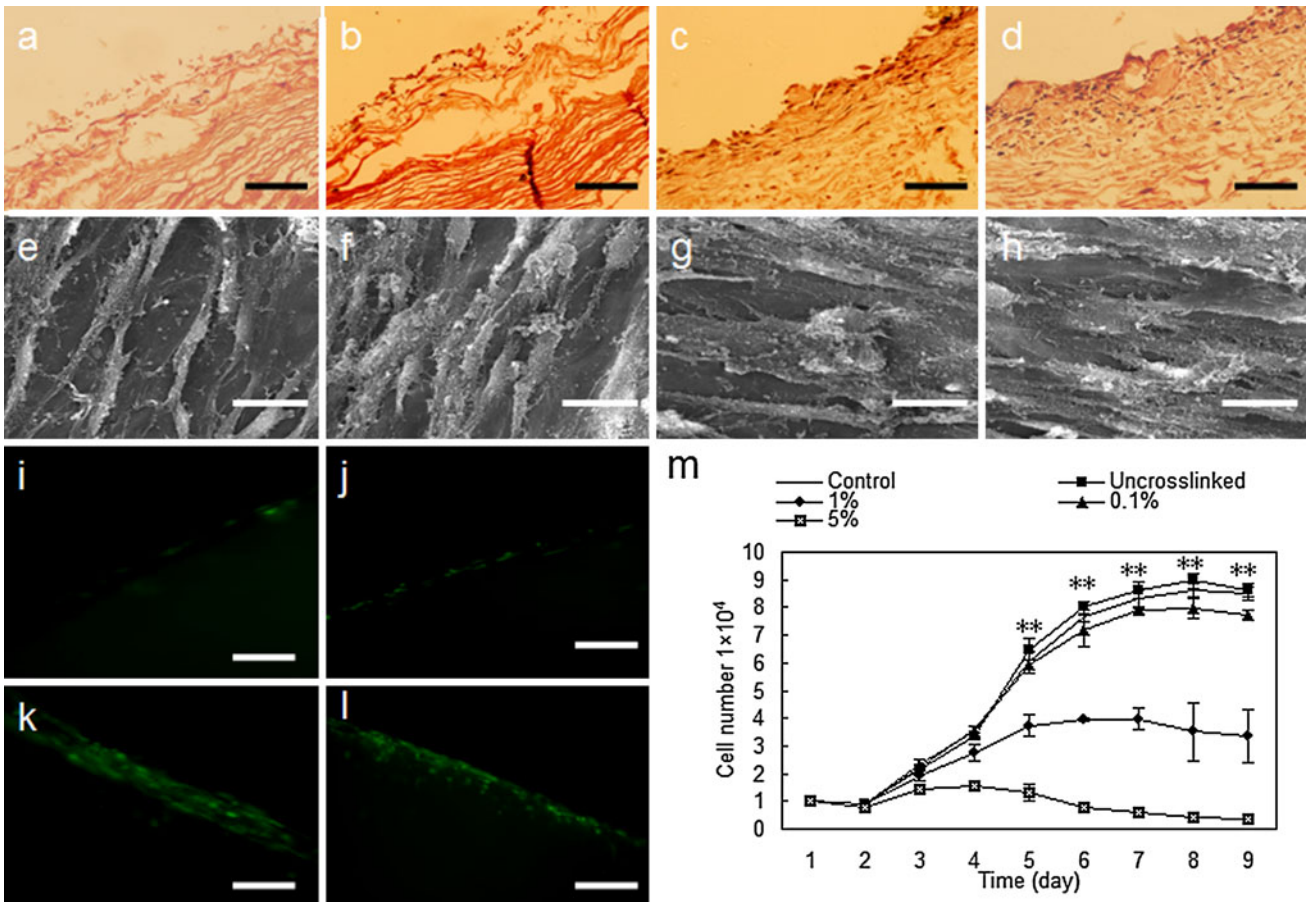

Fig. 3 Cytotoxic analysis of DVPs. **a** Uncrosslinked; **b** 0.1%; **c** 1%; **d** 5% (the upper panel was tested with H&E staining (200 μm)); **e** uncrosslinked; **f** 0.1%; **g** 1%; **h** 5%, the middle panel was tested with SEM. (*Bar* = 50 μm). **i** uncrosslinked; **j** 0.1%; **k** 1%; **l** 5%, the lower panel was fluorescently tested. (*Bar* = 200 μm); **m** cell proliferation assays were performed for checking cytotoxicity of uncrosslinked and crosslinked DVPs, there was no significant different between 0.1%, control and uncrosslinked group. ($P > 0.05$), 0.1% group showed a significant higher cell proliferation than 1% crosslinked group. **Statistically different ($P < 0.01$). Data for the growth curves represent means ± SD of two groups ($n = 5$)

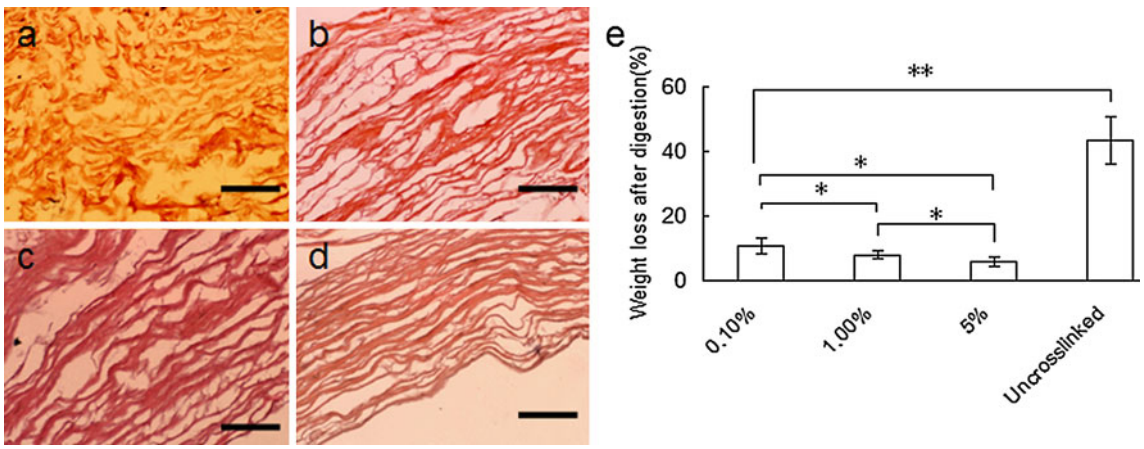

Fig. 4 Enzymatic degradation analysis. **a** Uncrosslinked (*Bar* = 100 μm); **b** 0.1% (*Bar* = 100 μm); **c** 1% (*Bar* = 100 μm); **d** 5% (*Bar* = 100 μm); **e** The weight loss of uncrosslinked and crosslinked DVP after enzymatic digestion. *Statistically different ($P < 0.05$). **Statistically different ($P < 0.01$) ($n = 5$)

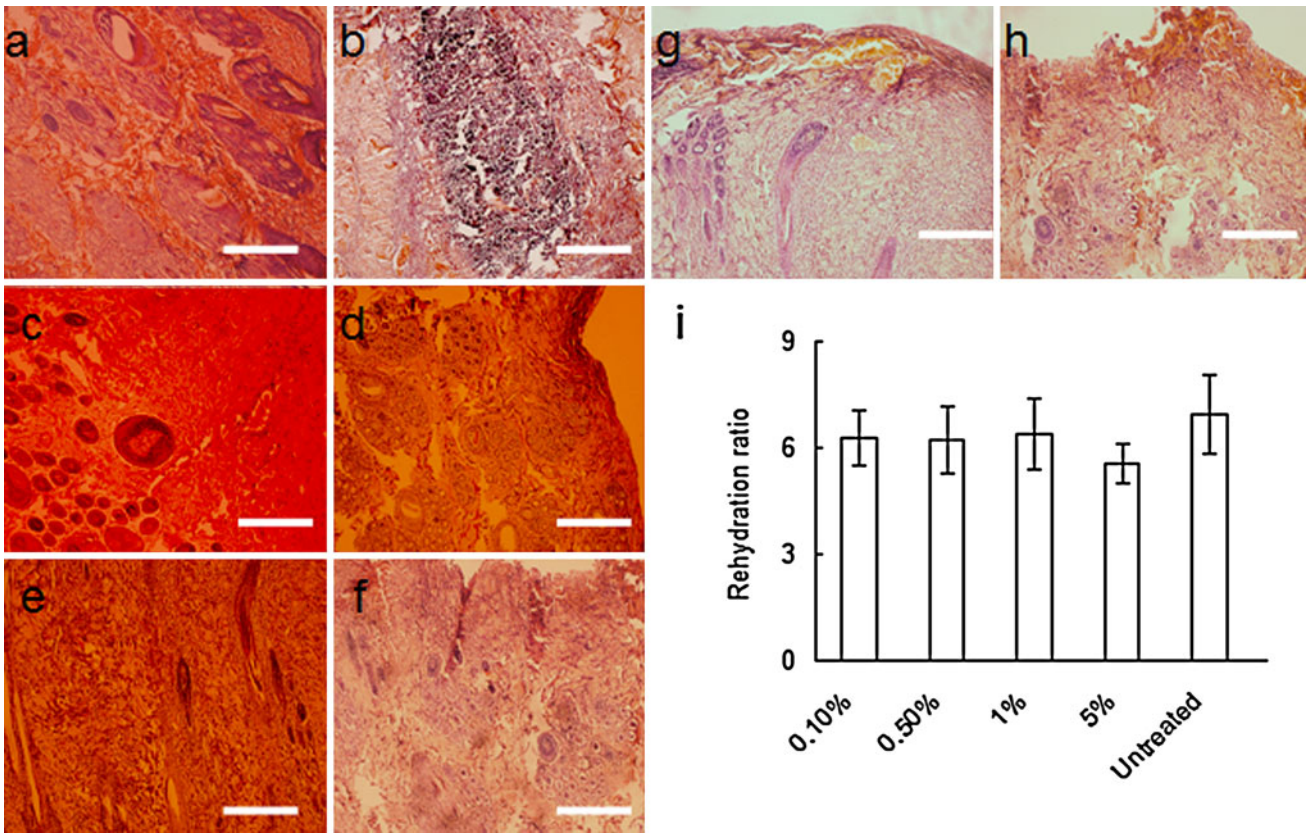

Fig. 5 Degradation of DVPs in vivo and rehydration test. **a** 2 weeks (0.1% GA); **b** 2 weeks (uncrosslinked); **c** 4 weeks (0.1% GA); **d** 4 weeks (uncrosslinked); **e** 6 weeks (0.1% GA); **f** 6 weeks (uncrosslinked); **g** 8 weeks (0.1% GA); **h** 8 weeks (uncrosslinked) (*Bar* = 100 μm); **i** After rehydration, there is no significant difference between uncrosslinked and crosslinked DVPs ($P > 0.01$) ($n = 5$)

3.8 Degradation in vivo

After being implanted subcutaneously, the uncrosslinked DVPs gradually degraded into pieces, especially at 8 week (Fig. 5b, d, f, h); while the 0.1% GA crosslinked DVPs kept intact to a great degree with little signs of degradation at 8 week (Fig. 5a, c, e, f).

3.9 Rehydration analysis

The decellularized matrix showed a high capacity of absorbing water. The swelling ratios of dried DVPs were about 6.3 ± 0.8 (0.1% crosslinked), 6.2 ± 0.9 (1% crosslinked), 6.4 ± 1 (5% crosslinked) and 6.9 ± 1.1 (uncrosslinked). There is no significant difference between uncrosslinked and crosslinked scaffolds (Fig. 5i).

3.10 Histology of the implanted vascular patch

The MSCs seeded DVP was implanted (Fig. 6). After 2 months, similar to native aortic histological analysis

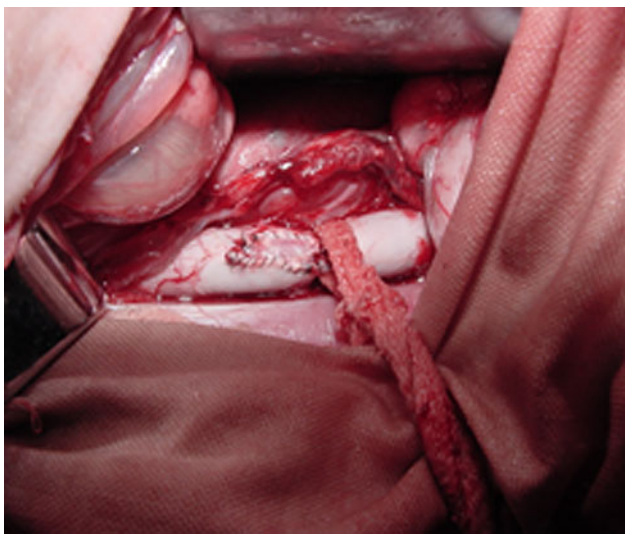

Fig. 6 Surgical procedure implantation of the DVPs seeded with MSCs to the aorta of sheep who donated the MSCs

(Fig. 7a) the vascular patch showed well regeneration of vascular structure which had endothelium, media and adventitia (Fig. 7b). Masson's trichrome (Fig. 7c) and van

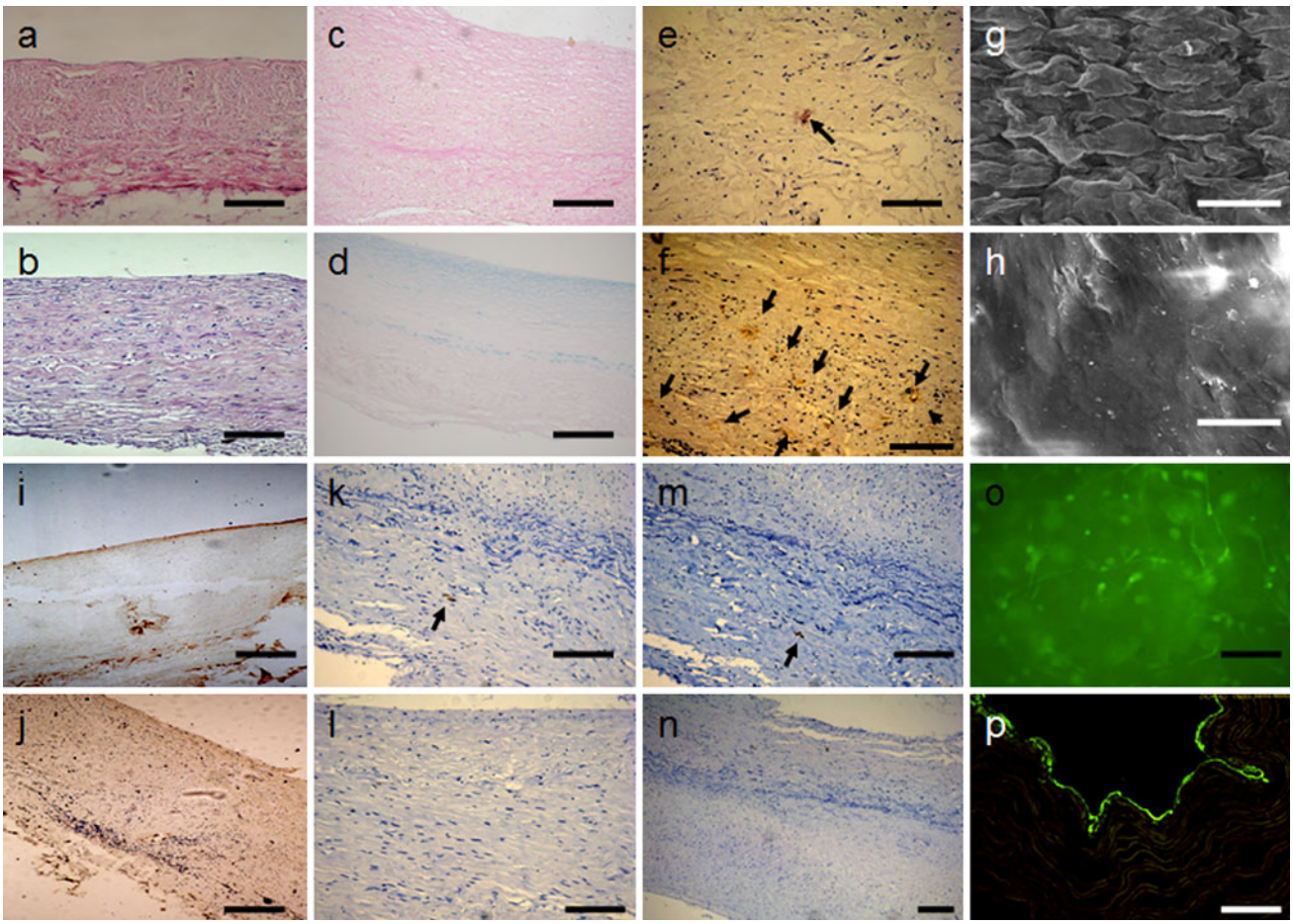

Fig. 7 Postoperative analysis of implants. **a** H&E staining of the normal aorta; **b** H&E staining of the implant showed the formation of endothelium, media, and adventitia; **c** Van Gieson collagen staining showed regenerating collagen; **d** Masson trichrome staining indicated collagen formation; **e** Von Kossa staining showed one calcium formation in native artery (*Bar* = 100 μm); **f** Von Kossa staining showed much calcium formation in implant (*Bar* = 100 μm); SEM showed the endothelium in the normal artery **g** and endothelium formation of the implant (**h**); **i** vWF immunohistochemical staining presented the endothelium of the implant; **j** α-SM immunohistochemical staining indicated SMCs in medial layers of implant (*Bar* = 200 μm); **k** CD163 immunohistochemical staining indicated few macrophage infiltration in implant and **l** native artery as control (*Bar* = 100 μm); **m** CD68 immunohistochemical staining indicated few macrophage infiltration in implant and **n** native artery as control (*Bar* = 100 μm); the labeled MSCs were seeded on DVPs **o** (*Bar* = 50 μm); **p** the labeled MSCs were detected on endothelium layers of the implants. (*Bar* = 100 μm)

Gieson's (Fig. 7d) staining showed the presence of a certain amount of collagen and elastin. However, there appeared much calcium formation in 0.1% GA crosslinked vascular patches (Fig. 7f) than native tissue (Fig. 7e). Immunohistochemistry presented that luminal sides of the vascular patch implant was stained positively for vWF (Fig. 7i) and SM α-actin (Fig. 7j), indicating endothelial and smooth muscle regeneration. There appeared few macrophage infiltration as indicator of inflammation, but there was no significant difference between implants (Fig. 7k, m) and native tissue (Fig. 7l, n) ($P > 0.05$).

3.11 SEM analysis of the implants

SEM analysis showed a regeneration of endothelium 2 months after transplantation (Fig. 7g). However compared it with native endothelium (Fig. 7h), the endothelium formation on the grafts showed an irregular endothelium and underdeveloped appearance.

3.12 Detection of labeled cells

The MSCs were labeled and seeded on the 0.1% GA crosslinked DVP (Fig. 7o). Two months after implantation,

the labeled MSCs were detected in the graft patch (Fig. 7p) and indicated that the MSCs survived and contributed the vascular regeneration. Fluorescence intensity diminished due to the dilution of the cell membrane incorporated dye with cell division.

4 Discussion

In the study, the enzymatic cell extraction process was used to remove the cellular components of sheep aorta and produced an extracellular scaffold. Light microscopy of the decellularized scaffold revealed an intact connective tissue matrix with no components of cells and left open spaces. Scan electron microscopy indicated that all cellular components were removed from the sheep aortic artery. Additionally, the special staining analyses confirmed that the DVPs was consisted primarily of insoluble collagen, elastin. After being crosslinked, the mechanical property of DVPs remained unaltered, capability against enzymatic degradation was strengthened both in vitro and in vivo. 0.1% GA crosslinked DVPs had a better biocompatibility than 1 and 5% GA crosslinked DVPs. The DVPs crosslinked with 0.1% GA and seeded with MSCs were implanted in vivo. The implants were remodeled and formed three layers of vascular wall.

Enzymatic extraction of cells should keep a balance between the effective removing of cellular protein and over digestion resulting in damage to the structure protein and mechanical properties [12]. It was reported that ethanol pretreatments could stabilize collagen against enzymatic damage, reduce the total water content, and minimize calcification [12, 13]. So in this study, we pretreated the samples with ethanol so that the scaffold was stabilized and allowed the enzyme to diffuse into the matrix and extract the cells with sufficient time, the ethanol-pretreated scaffold maintained gross structural integrity after enzymatic digestion.

It was reported that crosslinking can mask potentially immunogenic epitopes and render the matrix resistant to host proteolysis [9, 13]. There appeared many crosslinker such as Genipin [14], nordihydroguaiaretic acid [15], glutaraldehyde acetals [16], procyanidins [17], quercetin [18], however only glutaraldehyde (GA) was widely used in crosslinking biomaterials, some of these biomaterials have been for clinical application [8, 19]. It is known that GA reacts with amino acids of lysine residues such as methionine, tryptophan, histidine and tyrosine in proteins forming covalent bond between adjacent chains of the collagen matrix, which induces the formation of interchain crosslink and resulted in stabilization of the matrix against chemical and enzymatic degradation [17]. In this study, GA was shown to effectively crosslink the arterial tissue without adversely affecting the tissue's mechanical properties. Burst-pressure and suture strength in this study of crosslinked scaffold had no difference with uncrosslinked scaffold; clearly, both of them exceed the physiological pressure range of 80–120 mmHg. Before implantation, the suture retention strength of the scaffold was measured to determine whether the grafts could withstand forces exerted in anastomosis. The mechanical properties of crosslinked DVPs were not statistically different from that of the native arteries, suggesting that the processed vascular graft scaffold had sufficient suture retention strength to withstand anastomosis forces. Therefore, the vascular grafts could be practically sutured without rupture.

Our results also showed that GA crosslinked matrix could significantly resist the collagenase digestion and the structure protein was maintained and well preserved after enzymatic degradation analysis both in vitro and in vivo. So the crosslinked scaffold could resist the degradation, the higher concentration of GA crosslinking came up with the lower degradation rate.

Glutaraldehyde concentration is crucial in the crosslinking process. Higher GA concentration may achieve a more stable scaffold, but it also potentiates more side effects, such as cytotoxicity [20]. In this study, we tested different concentration of GA solution for crosslinking and increasing the washing time for removing GA residue from scaffold to avoid the potential cytotoxicity. Results revealed that 0.1% GA was much less cytotoxic to MSCs as compared with 1 and 5% GA crosslinking. Our in vitro assays showed that 0.1% crosslinked material has no or little adverse effect on MSCs adhesion and proliferation over the 5-day culture period under static conditions. So low GA concentration GA would reduce the side effect to a great degree and keep the mechanical and enzymatic degrading capacities at the same time. We also traced calcium deposition in vivo. Although the ethanol was used for prevention of calcification, 0.1% GA crosslinked DVPs still manifested calcium presence. These calcium formation could be reduced by multi-step anticalcification procedures [21, 22].

Bone marrow derived mesenchymal stem cells (MSCs) are a rare population of non-hematopoietic stromal cells. They have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma. In the absence of specific markers, MSCs have been defined following isolation and culture expansion, by their expression of various molecules including CD90, CD105 and CD73 and absence of markers like CD34, CD45, and CD14. MSCs can replicate as undifferentiated cells and that these cells displayed a stable phenotype and remained as a monolayer in vitro [10, 11]. MSCs were used to differentiate into endothelial cell and develop into a vascular component. Many researchers used marrow-derived MSCs to create tissue engineering blood vessels [9, 23]. Vitality and proliferation of MSCs seeded on DVPs are

essential to maintain the structure and function. We seeded the MSCs on 0.1% GA crosslinked DVP and construct a tissue engineering vascular patch. MSCs can connect to each other to establish a three-dimensional cell network in the scaffold. We implanted this crosslinked decellularized vascular patch seeded with undifferentiated MSCs to the sheep who donated the cells to check how it would remodel in vivo. This patch was exposed under the arterial blood pressure, it remodeled under normal blood flow in vivo. Major stresses applied to vascular patch inside the body are hoop stress induced by blood pressure, that is normal stress in the wall circumferential direction, wall shear stress developed by blood flow, and axial stress by elongation in the axial direction. The vascular patch was responded to changes in these stresses, and change their dimensions, structure, and properties [24]. After transplantation, this MSCs seeded vascular patch kept patent without bulging and thrombosis formation. In this study MSCs should contribute to endothelization in animal model in vivo. This tissue-engineering vascular constructs contain living cells, they may have the potential to grow, self-repair, and self-remodel. After 2 months, the blood vessel had regeneration of endothelium and smooth muscle and the presence of collagen and elastin the labeled MSCs could be detected.

5 Conclusion

This study demonstrated a fast and simple way of producing a soft vascular matrix by using enzymatic treatment and 0.1% GA crosslinking. Although studies showed the successes that acellular scaffold transplantation exhibited vascular patent and tissue regeneration [25, 26], decellularized scaffolds only allowed partial autologous recellularization [27]. Tissue-engineered heart valves created from mesenchymal stem cells, injected directly in a decellularized scaffold, exhibited satisfactory hemodynamic and histologic aspects [28]. As the seeding cells, MSCs known as its potential nature of rapid replication and potential differentiation will be an important source of clinical vascular tissue engineering. There appeared several clinical studies of vascular tissue engineering, the results showed the optimization [29–32]. The fast and simple producing method of tissue engineered blood vessels could eventually lead to a clinically and commercially successful product.

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