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In vitro study in the endothelial cell compatibility and endothelialization of genipin-crosslinked biological tissues for tissue-engineered vascular scaffolds

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Abstract To overcome the cytotoxicity of the chemical reagents used to fix bioprostheses, genipin, a naturally occurring crosslinking agent, was used to fix biological tissues in present study. We prepared the biological vascular scaffolds through cell extraction and fixing the porcine thoracic arteries with 1% (by w/v) genipin solution for 3 days, and then examined their mechanical properties and microstructures; glutaraldehyde- and epoxy-fixed counterparts were used as controls. HUVECs were seeded on the type I collagen-coated surface of different modified acellular vascular tissues (fixed with different crosslinking agents), and the growths of HUVECs on the specimens were demonstrated by means of MTT test, the secretion of PGI_2 and vWF by HUVECs on the various specimens was also measured. Finally, HUVECs were seeded on the luminal surface of acellular biological vascular scaffolds (<6 mm internal diameter) which were, respectively, treated in the same manner described above, and then cultured for 9 days. On the ninth day, the HUVECs on the luminal surface of these vascular scaffolds were examined morphologically and by immunohistochemistry. Genipin-fixation can markedly diminish antigenicity of the vascular tissues through partially getting rid of cell or reducing the level of free amino groups in the vascular tissues. Genipin-fixed acellular vascular tissues mimicked the natural vessels due to the maintenance of the integrity of total structure and the large preservation of the microstructures of collagen fibers and elastic fibers; therefore, it appeared suitable to fabricate vascular scaffolds in mechanical properties. Compared to controls, the genipin-fixed acellular vascular tissues were characterized by low cytotoxicity and good cytocompatibility. The HUVECs can not only proliferate well on the genipin-fixed acellular vascular tissues, but also preserve the activities and function of endothelial cells, and easily make it endothelialized in vitro. The results showed that the genipin-fixed acellular porcine vascular scaffolds should be promising materials for fabricating vascular grafts or the scaffolds of tissue-engineered blood vessels.

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1 Introduction

The number of vascular surgical operations increases because of advances in vascular surgery accompanied with an increasing prevalence of atherosclerotic arterial disease in an ageing society [1]. Therefore, there is an increasing demand for blood vessel substitute in clinic. However, the traditional blood vessel substitutes such as synthetic grafts, autografts, allografts and xenografts can not meet clinic demand due to mismatch compliance, lack of sources and antigenicity etc., especially in small-diameter (<6 mm internal diameter) vascular graft. In an attempt to address these disadvantages vascular tissue engineering which is a new multidisciplinary approach to create completely autologous, living blood vessel substitute is applied to this field. The prefabrication of vascular scaffolds is very important in vascular tissue engineered because the vascular scaffolds can provide the sites for adherence and proliferation of seed cells. Various natural and synthetic biomaterials have been used to prefabricate blood vessel substitute or tissue-engineered vascular scaffolds, especially in small-diameter (<6 mm internal diameter) vascular graft [2, 3]. Compared the synthetic materials, natural

biological tissues are composed of extracellular matrix proteins that are conserved among different species and that can serve as scaffolds for cell attachment, migration, and proliferation. Therefore, the natural biological tissues can offer better constructions for adhesion and growth of cells over synthetic materials [4]. However, naturally derived biological tissues have to be fixed with a crosslinking agent and subsequently sterilized before they can be applied as vascular grafts in vivo. The fixation of biological tissue can partially reduce antigenicity and immunogenicity and prevent enzymatic degradation [5]. After fixation, the biological tissue can also be preserved for a long time.

Various crosslinking agents have been used in the chemical modification of biological tissues. Although various synthetic crosslinking agents such as formaldehyde [5], glutaraldehyde [5] and epoxy compound [6] have been widely used in the pre-treatment of natural biomaterials for the purposes mentioned above, its application in clinics was limited by the side-effects of the synthetic crosslinking agents' treatment to natural biological tissues such as high cytotoxicity, mismatched mechanical properties and calcification. It is therefore desirable to provide a crosslinking agent suitable for used in biomedical applications that is of low cytotoxicity and that forms stable and biocompatible crosslinked vascular graft.

Recently, genipin, a naturally occurring crosslinking agent, was used to fix biological tissues in some researches. Genipin can be obtained from its parent compound geniposide, which may be isolated from the fruits of *Gardenia jasminoides* ELLIS [7, 8]. The treatment of genipin to natural biomaterials represents an effort to overcome some of the drawbacks that are typically encountered with the synthetic crosslinking agents. Although there are some investigations on the stability [9], crosslinking characteristics and mechanical properties [10] of biological tissue fixed with genipin, the data related to effects of naturally derived vascular scaffold materials fixed with genipin on the human umbilical vein endothelial cells (HUVECs) are scarce; it was not reported by other studies up to now. The research on interaction between genipin-fixed biological vascular scaffolds and HUVECs is very important because HUVEC is the most commonly used endothelial type in constructing living blood vessel substitute. In this paper, we prepared the biological vascular scaffolds by pretreatmenting porcine thoracic arteries with genipin, and glutaraldehyde- and epoxy-fixed counterparts were used as controls. The ultrastructures, mechanical properties and HUVECs compatibility (including HUVECs proliferation and functional parameters) of the biological vascular scaffolds fixed with genipin were investigated. Further, the genipin-fixed biological vascular scaffolds were successfully endothelialized in vitro.

2 Materials and methods

2.1 Preparation of genipin-fixed biological vascular scaffold materials

2.1.1 *Fixation process*

Fresh porcine thoracic arteries procured from Longchang pigs aged 6 months, from a slaughterhouse, were used as raw materials. The arteries were 5–10 mm in inside lumen diameters. Warm ischemic time was no more than 6 h from the time of tissue extraction to processing. Fresh porcine thoracic arteries first were immersed in PBS containing 0.1% trypsin/0.02% EDTA for 24 h at 37°C with constant stirring. Subsequently, they were immersed in a 1% solution of Triton X-100 (octylphenoxypolyethoxyethanol, Sigma Chemical Co., St. Louis, MO, USA) in tris-buffered salt solution for 72 h at 37°C with constant stirring. Samples then were thoroughly rinsed in Hank's physiological solution and digested with DNase and RNase at 37°C for 1 h. This was followed by a further 24 h extraction with Triton X-100 in tris-buffer. Finally, all samples were washed for 48 h in Hanks' solution. A 1% (by w/v) genipin (Wako Pure Chemical Industries, Ltd., Osako, Japan) solution was employed to fix these acellular porcine arteries, and this genipin solution was buffered with phosphate-buffered saline (PBS) (0.01 M, pH 7.4). Meanwhile, the samples fixed with 0.625% glutaraldehyde (GA) solution (buffered with phosphate-buffered saline, 0.01 M, pH 7.4) and 4% ethylene glycol diglycidyl ether (EGDE) solution (buffered with sodium carbonate/sodium bicarbonate, 0.21:0.02 M, pH 10.5) were used as a control. The samples of each group were all fixed for 72 h.

2.1.2 *Mechanical examination*

The mechanical testing was conducted in a Plexiglas tank containing saline at 37°C on an Instron material testing machine (Mini 44, Canton, MA, USA). A segment of vessel (nature, genipin treated, EGDE treated or glutaraldehyde treated) was longitudinally opened and cut into 40 mm × 10 mm rectangular slab specimens with the long axis aligned along the circumferential axes of the vessel. The thickness of the sample was obtained using a micrometer. The tested tissue strip was mounted between two screw-tightened brass grips lined with sandpaper to prevent slippage. The upper grip was attached to the fixed load cell, while the lower grip was secured to the bottom of the Plexiglas tank. Prior to each test, samples were preconditioned with three linear loading cycles to ~33% of the force at failure to remove stress history. The tested tissue strip was then extended at 10 mm/min from 0 g load

until the tissue strip ruptured. Fracture was taken to occur when the first decrease in load was detected during extension. The ultimate tensile strength was taken as the force at which fracture occurred divided by the initial cross-sectional area. The linear tensile modulus was defined by the slope of the region extending from 25 to 75% of the peak failure stress.

2.1.3 Histology

The nature samples and the acellular samples cross-linked with genipin were examined histologically by Masson staining for collagen fibers and Verhoeff iron hematoxylin staining for elastic fibers.

2.2 The HUVECs compatibility and endothelialization of genipin-fixed biological vascular scaffold materials in vitro

2.2.1 Endothelial cell harvest, culture and characterization

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins as previously described [11]. Briefly, human umbilical veins was incubated for 20 min at 37°C, 5%CO_2 in PBS containing 0.05% trypsin/0.02% EDTA to obtain endothelial cells. Subsequently, the cell suspension was centrifuged for 7 min at 200 g, after which the endothelial cells were cultured to the third passage in 75 cm^2 tissue culture polystyrene (TCPS) flasks (Costar, Cambridge, MA) coated with 2 mg ml^{-1} fibronectin (Sigma). HUVECs were cultured at 37°C in humidified 95% air/5% CO_2 in culture medium (M199, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 20 mM HEPES, all from Gibco) containing 20% pooled new born calf serum. Before testing cellular compatibility, the media was aspirated from TCPS flasks and the cells were washed briefly with Hanks' balanced salt solution. Cell were detached from TCPS by incubation with trypsin (Gibco, 0.05% trypsin with 0.02% EDTA in PBS) for 1 min, transferred to M199 media containing 20% fetal calf serum, and centrifuged (800 g for 5 min at 4°C). The resultant pellet was resuspended in the M199 growth medium (containing 15% new born calf serum, 5% foetal calf serum, Endothelial cell growth factor 20 μg ml^{-1}, heparin 100 μg ml^{-1}, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 20 mM HEPES) to a concentration of 2×10^4 cell/ml. Before seeding, Cell identity was confirmed by in situ von Willebrand/Factor VIII staining (Sigma-Aldrich, Steinheim, Germany) using the EnVision™ AP detection system (Dako, Hamburg, Germany).

2.2.2 The proliferation of HUVECs cultured on the genipin-fixed biological vascular scaffold materials

Porcine thoracic arteries were fixed according to previous description in chapter "Fixation process". A segment of fixed vessel with 10 mm in inside diameters was longitudinally opened, and the vessel wall was cut into 0.3 cm × 0.3 cm foursquare slab specimens. These specimens were divided into four groups as follows:

1. Fixed with glutaraldehyde for 72 h and coated with type I collagen (Sigma). (Group 2, $n = 5$)
2. Fixed with EGDE for 72 h and coated with type I collagen. (Group 3, $n = 5$)
3. Fixed with genipin for 72 h and coated with type I collagen. (Group 4, $n = 5$)
4. As a control experiment for comparison, the polystyrene 96-well flat-bottom culture plate (Corning Costar, Cambridge, MA, USA) coated with type I collagen was also used as a test specimen. (Group 1, $n = 5$)

The proliferation of HUVECs cultured on these specimens fixed with these three crosslinking agents was determined as previously described [6]. Briefly, the M199 growth medium (containing 15% new born calf serum, 5% foetal calf serum, endothelial cell growth factor 20 μg ml^{-1}, heparin 100 μg ml^{-1}, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 20 mM HEPES, all from Gibco) was distributed onto each specimen placed in the bottom of well in a 96-well plate. After 24 h culture in a incubator, the M199 growth medium in each well was sucked off, and 200 μl M199 growth medium suspension of HUVECs at 2×10^4 cell/ml was evenly distributed onto each well. The cell culture was maintained at 37°C in humidified 95% air/5% CO_2. Using the MTT assay, the viable cells cultured on each test specimen were determined at 1, 3, 5, 7 and 9 days after cell seeding. During the cell-culture period, M199 growth medium was changed daily.

2.2.3 The vWF and PGI_2 secretion of HUVECs cultured on the various test specimens

Cell cultures were washed with a solution of bovine serum albumin (BSA, Sigma A6003, 5 mg/ml) in M199 growth medium without serum (M199/1% BSA, 37°C), followed by 30 min incubation in M199/1% BSA. Subsequently, supernatant medium was collected, centrifuged (10 min, 400 g, 4°C) and stored at −20°C until assayed. vWF was determined using an ELISA [12] (Gradipore, North Ryde, Australia). PGI_2 concentrations were determined using a competitive EIA for the stable hydrolysis product of PGI_2, 6-keto-prostaglandin F1a (Amersham, England) [13].

2.2.4 Endothelialization experiments

The endothelialization experiments were carried out on fixed acellular biological vascular scaffold according to a protocol described in the literature [6]. Three 10 cm length of <6 mm internal diameters porcine thoracic arteries were, respectively, treated in the same manner described in chapter “Fixation process”, and then coated with type I collagen, finally washed with PBS. After culturing with the M199 growth medium (prepared in the same manner described above) in the incubator for 24 h, each vascular construction was clamped at one end, and HUVECs suspension (5×10^5 cell/ml) was injected into the lumen of each conduit several times with the use of a 5-ml disposable syringe with a 26-gauge needle, finally the other end of each conduit was also clamped. The conduits were put into sterile cylinders, and placed on a modified blood roller, and rotated at 16 revolutions per hour for 1 h at 37°C. After seeding, the conduits were placed in M199 growth media and incubated at 37°C in a 5% CO_2 atmosphere for 9 days. During the cell-culture period, M199 growth medium was changed daily.

At the ninth day postseeding, segments (2.5 cm) were cut from the end of each conduit. These segments were processed through a series of dehydrating EtOH–H_2O rinses and embedded in paraffin. Following removal of the paraffin through a series of rehydrating rinses with xylene substitute and EtOH–H_2O; the sections were stained with Hematoxylin and Eosin Y (H&E) to color the cell nuclei blue, and observed with a light microscope. The samples also were examined by standard immunohistochemistry for Factor VIII staining.

Samples for SEM examination were fixed in 2% glutaraldehyde solution, and gradient dehydrated at critical point followed by AuPd sputtering. Samples were observed in JSM-255 scanning microscope (JEOL, Japan).

2.2.5 Statistics

Result data were expressed as mean ± standard deviation of the mean. Comparisons between groups were performed by ANOVA test. Statistical significance was set at $P < 0.05$.

3 Results

3.1 Microstructures of genipin-fixed biological vascular scaffold materials

After fixation, the genipin-fixed acellular tissues were stiffer than the EGDE-fixed acellular tissues and the fresh tissues, but were similar to the glutaraldehyde-fixed acellular tissues. Histological examination of the acellular vascular tissues after the fixation process showed intact total framework. The microcosmic structure of collagen fibers (Fig. 1) and elastic fibers (Fig. 2) preserved well after fixation with genipin. In addition, Masson staining did

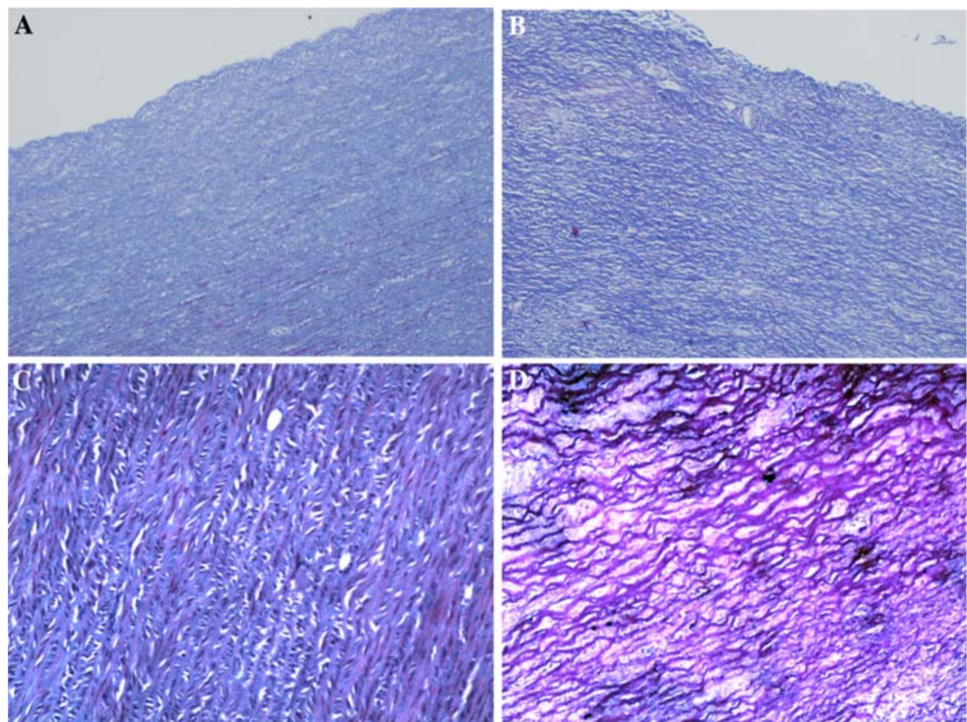

Fig. 1 Photomicrographs of: **a** natural porcine vascular tissues before decellularization process, **b** genipin-fixed acellular porcine vascular tissues (Masson staining, 150× magnification); **c** natural porcine vascular tissues before decellularization process, **d** genipin-fixed acellular porcine vascular tissues (Masson staining, 300× magnification)

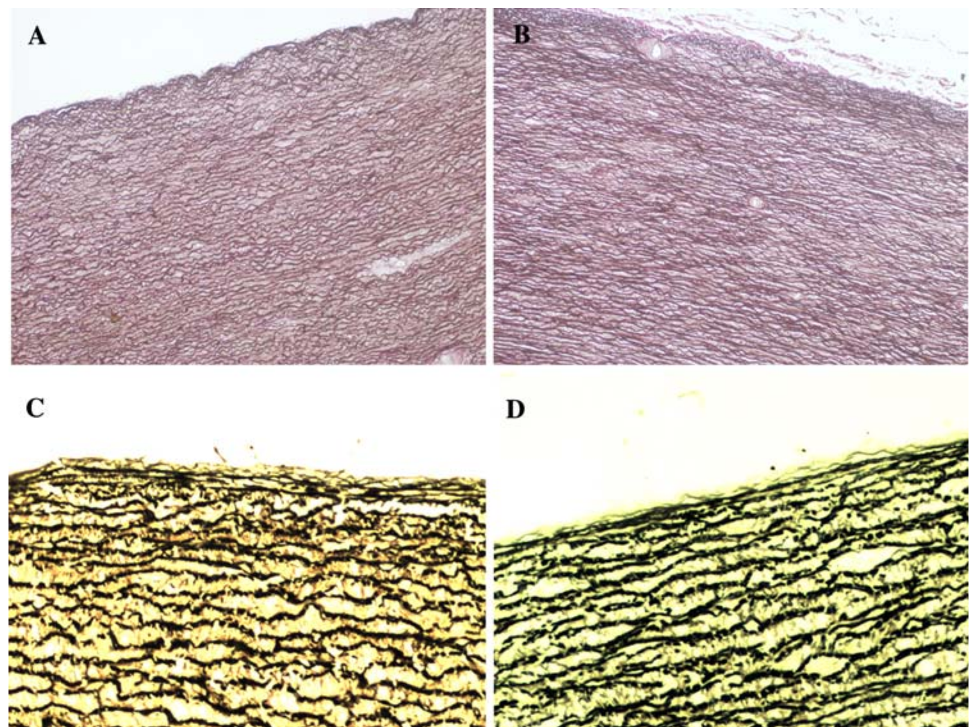

Fig. 2 Photomicrographs of: **a** natural porcine vascular tissues before decellularization process, **b** genipin-fixed acellular porcine vascular tissues (Verhoeff iron hematoxylin staining, 150× magnification); **c** natural porcine vascular tissues before decellularization process, **d** genipin-fixed acellular porcine vascular tissues (Verhoeff iron hematoxylin staining, 300× magnification)

not show the signs of remaining nuclear material in the vessel walls, indicating successful decellularization through the thickness of the vessels.

3.2 Mechanical properties

Biomechanical analysis was performed on the fresh, the EGDE-fixed, the genipin-fixed, and the glutaraldehyde-fixed vascular tissues. The results are showed in Table 1.

As shown in the table, the values of "Tensile stress at maximum load" and "E-Modulus" for the glutaraldehyde-fixed and genipin-fixed vascular tissues were greater than that of the fresh and EGDE-fixed vascular tissues ($P < 0.05$), while there is no significant difference in same test items between the glutaraldehyde-fixed and genipin-fixed vascular tissues. The values of "Tensile strain at maximum load" for the fresh vascular tissues were greater than that of the EGDE-fixed, GA-fixed or genipin-fixed vascular tissues ($P < 0.05$). Although the values of "Tensile strain at maximum load" for the genipin-fixed were greater than that of the EGDE-fixed and glutaraldehyde-fixed, there were no statistical differences among them.

3.3 Proliferation of HUVECs cultured on the fixed biological vascular tissues

Figure 3 illustrates the optical density readings obtained in the MTT assay for the HUVECs cultured on the surfaces of type I collagen coated-polystyrene culture plate and each group of the acellular vascular tissues treated with three different crosslinking agents.

As shown in the Fig. 3, the optical density reading for the cells cultured on the surface of the tissues of Group 2 declined continuously. On the other hand, the optical density readings for the cells cultured on the surfaces of the tissues of Group1, Group3 and Group4 increased with

Table 1 Mechanical properties of porcine vascular tissues ($\bar{X} \pm S$, $n = 4$)

Treated arteries	Tensile strain at maximum load (%)	Tensile stress at maximum load (Mpa)	E-Modulus (Mpa)
Fresh vessel	227.44 ± 16.8**	1.08 ± 0.097	0.942 ± 0.0169
EGDE-vessel	98 ± 14.3	0.95 ± 0.22	1.19 ± 0.107
GA-vessel	108 ± 20	1.99 ± 0.47*	1.602 ± 0.90*
Genipin-vessel	136.97 ± 14.27	2.068 ± 0.463*	1.596 ± 0.428*

* $P < 0.05$ compared with fresh and EGDE-fixed vessel tissues; ** $P < 0.05$ compared with EGDE-fixed, GA-fixed and genipin-fixed vessel tissues

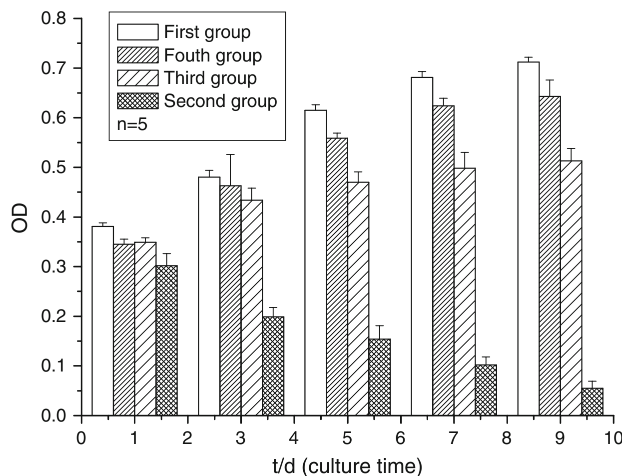

Fig. 3 Proliferation of the HUVEC cultured on various substrates. First group: the polystyrene 96-well flat-bottom culture plate; Second group: GA-fixed acellular vessel tissues; Third group: EGDE-fixed acellular vessel tissues; Fourth group: genipin-fixed acellular vessel tissues

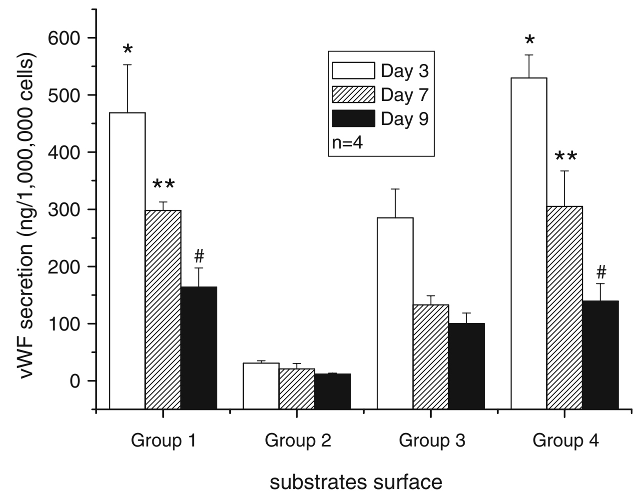

Fig. 4 vWF secretion by HUVECs cultured on various substrates. * $P < 0.05$ compared with GA-fixed (Group 2) and EGDE-fixed (Group 3) acellular vessel tissues (Day 3); ** $P < 0.05$ compared with GA-fixed, EGDE-fixed acellular vessel tissues (Day 7); # $P < 0.05$ compared with GA-fixed acellular vessel tissues (Day 9); Group 1: the polystyrene 96-well flat-bottom culture plate; Group 4: genipin-fixed acellular vessel tissues

increasing the culture duration. The results suggest that the cellular compatibilities of the EGDE-fixed acellular tissues and the genipin-fixed acellular tissues were significantly superior to their glutaraldehyde-fixed counterpart. Compared with the EGDE-fixed acellular tissues, the cellular compatibilities of the genipin-fixed acellular tissues were better.

3.4 The vWF and PGI_2 secretion of HUVECs cultured on the various test substrates

As shown in the Fig. 4, vWF secretion by HUVECs cultured for 3 days and 7 days on control substrates (group 1) and genipin-fixed acellular vessel (group 4) was significantly higher when compared to HUVECs cultured on GA-fixed (group 2) and EGDE-fixed (group 3) acellular vessel tissues. After 9 days of proliferation, vWF secretion by HUVECs cultured on control substrates and genipin-fixed acellular vessel was still significantly higher than that cultured on GA-fixed acellular vessel, but no significant difference compared to HUVECs cultured on EGDE-fixed acellular vessel. In addition, vWF secretion by HUVECs cultured on various substrates gradually reduced with the elapsing of culture time.

PGI_2 secretion by HUVECs cultured for 3, 7 and 10 days on both control substrates (group 1) and genipin-fixed acellular vessel (group 4) was significantly higher compared to cells cultured on GA-fixed (group 2) and EGDE-fixed (group 3) acellular vessel tissues (Fig. 5). The Phenomenon similar to vWF secretion was also observed that PGI_2 secretion by HUVECs cultured on various substrates gradually reduced with the elapsing of culture time.

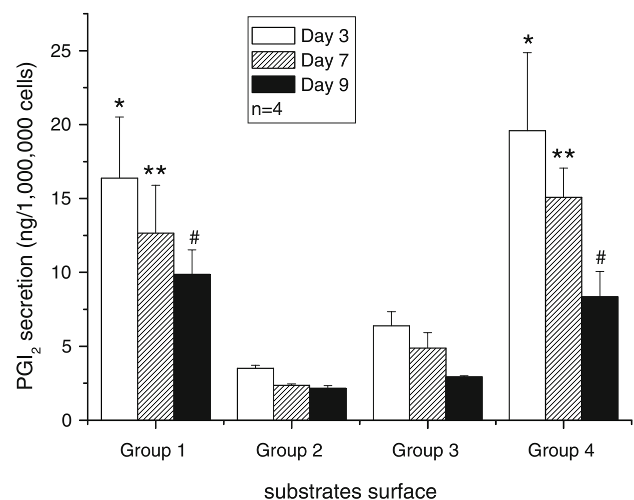

Fig. 5 PGI_2 secretion by HUVECs cultured on various substrates. * $P < 0.05$ compared with GA-fixed (Group 2) and EGDE-fixed (Group 3) acellular vessel tissues (Day 3); ** $P < 0.05$ compared with GA-fixed, EGDE-fixed acellular vessel tissues (Day 7); # $P < 0.05$ compared with GA-fixed and EGDE-fixed acellular vessel tissues (Day 9); Group 1: the polystyrene 96-well flat-bottom culture plate; Group 4: genipin-fixed acellular vessel tissues

3.5 Endothelialization experiments

After coating with type I collagen, several fixed biological vascular scaffolds were seeded with HUVECs and examined histologically after 9 days. As shown in Fig. 6, cells seeded and cultured on the luminal surface of the biological vascular scaffolds treated with genipin were unable to

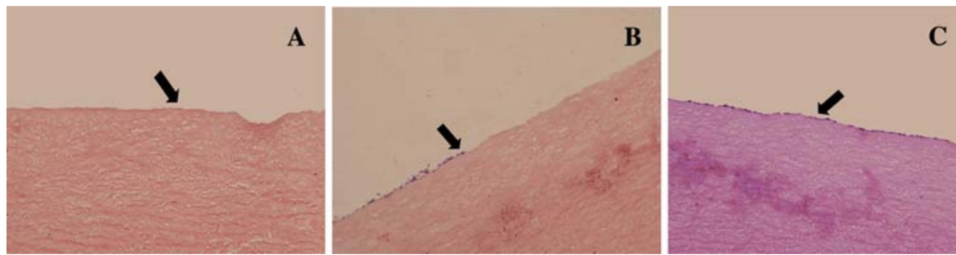

Fig. 6 HUVECs grown (9 days) on the luminal surface tubular biological vascular scaffolds after fixing; note the acellularity of the fixed materials. The cells (C) formed a monolayer (*arrowheads*) that was unable to invade below the tubular biological vascular scaffolds (150× magnification, H&E stain). (**a** glutaraldehyde-fixed; **b** EGDE-fixed; **c** genipin-fixed)

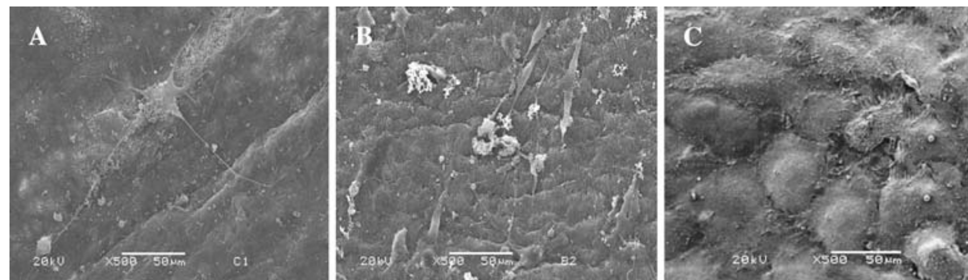

Fig. 7 Representative SEM-images of HUVECs on the luminal surface of biological vascular scaffolds treated with glutaraldehyde (**a**), treated with EGDE (**b**), and treated with genipin (**c**), cultured for 9 days

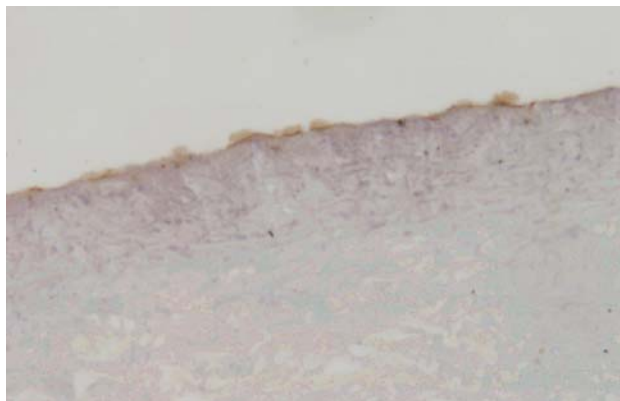

Fig. 8 Factor VIII related antigen is positive in HUVECs cultured on the luminal surface of tubular biological scaffolds fixed with genipin (150× magnification)

invade the scaffold and formed confluent monolayer, while no cell was observed on the luminal surface of the biological vascular scaffold fixed with glutaraldehyde. Compared with cells cultured on the surface of the biological vascular scaffolds treated with genipin, the counterpart of the biological vascular scaffold treated with EGDE did not reach a confluent monolayer.

As shown in Fig. 7, scanning electron microscopy showed the results similar to the light microscopy.

On the other hand, after incubating in incubators for 9 days, Factor VIII positive cells that formed confluent monolayer were detectable on the luminal surfaces of the biological vascular scaffolds treated with genipin (Fig. 8). This result indicated that the cells cultured on the luminal surfaces of the biological vascular scaffolds treated with genipin preserved the activities of endothelial cells.

4 Discussion

Vascular tissue engineering is a new multidisciplinary approach to create completely autologous, living small-diameter blood vessel substitute and alleviate the disadvantages of synthetic grafts, autografts, allografts and xenografts. The prefabrication of vascular scaffolds and their endothelialization are very important in tissue-engineered vascular grafts. Ideally, the vascular scaffolds should mimic the natural vessels; they should contain the well organized extracellular matrices, which confer the bulk of the mechanical properties, and contain collagen and elastin in quantities that are similar to native vessels [14]. On the other hand, thrombosis can occur when blood comes in contact with a surface other than the endothelium, therefore a confluent, functional and antithrombogenic endothelial layer is essential for small diameter arterial graft success. To date, HUVEC is the most commonly used endothelial type in vascular tissue engineering and is routinely used to investigate in vitro the adhesion and proliferation of endothelial cells on matrices or vascular scaffolds.

In this study, the genipin was employed to pre-treatment (crosslink) the biological vascular scaffold. Some researchers reveal that genipin can react with the free amino groups of lysine, hydroxylysine, or arginine residues within biological tissues to form intramolecular and intermolecular crosslinks with cyclic structure within

collagen fibrils in biological tissues [15, 16]. After fixation, it was noted that the genipin-fixed acellular tissues were stiffer than the EGDE-fixed acellular tissues and the fresh tissues, but were similar to the glutaraldehyde-fixed acellular tissues. This may be due to their different cross-linking bridge structure. After cross-linking, a network crosslinking structure can be created intramolecularly and intermolecularly within collagen fibrils in glutaraldehyde-fixed biological tissues [17], as well as a heterocyclic crosslinking structure in genipin-fixed biological tissues. Meanwhile, the cross-linking bridge structure within EGDE-fixed biological tissues presents the linearity [18, 19]. Therefore, the extents of tissue shrinkage for the glutaraldehyde-fixed tissues and the genipin-fixed tissues are larger than that for the EGDE-fixed tissues. The shrinkage of tissues reduces the free volume in tissues and thus expels some water molecules out of the fixed tissues. Consequently, the genipin-fixed and glutaraldehyde-fixed tissues were stiffer than the EGDE-fixed tissues and the fresh tissues.

In this study, the biological vascular scaffolds were almost completely decellularized by cell extraction and genipin-fixation (Fig. 1). The removal of original resident cells in biological vascular scaffolds reduced the antigenicity derived from cells, and thus markedly diminished the immune response elicited to these materials in vivo [20]. In addition, Sung et al. [10] reported in their study that a higher fixation index was achieved at the end of genipin-fixation. A higher fixation index often implies a lower level of free amino groups left in the fixed tissues. The reduction of free amino groups in the biological tissue also diminished its antigenicity [21]. In a word, the antigenicity of biological tissues is very low after cell extraction and complete fixation with genipin.

After fixing with genipin, the total structure of the vascular tissues remained integrity, and the microstructures of collagen fibers and elastic fibers was largely preserved (Figs. 1 and 2). This structure similar to that of fresh tissues was suitable for the adhesion and proliferation of cells, and mimicked the natural vessels to the utmost extent.

The circumferential tensile properties (circumferential tensile strength and E-Modulus) of vascular scaffolds is very important, because scaffolds should have adequate strength to resist rupture during implantation. As showed in Table 1, the circumferential tensile properties of the genipin-fixed and glutaraldehyde-fixed vascular tissues were significantly greater than that of the fresh and EGDE-fixed vascular tissues. These results means that the genipin-fixed and glutaraldehyde-fixed vascular tissues are in possession of the stronger strength to resist rupture of vascular scaffolds. This indicated that the genipin-fixed vascular tissues were suitable to serve as the scaffold of tissue-engineered blood vessel.

Although various synthetic crosslinking agents such as formaldehyde, glutaraldehyde and epoxy compound have been widely used in the pre-treatment of natural biomaterials, there is a serious drawback existing in the synthetic crosslinking agents-fixed vascular tissues, that is, cytotoxicity. Therefore, the genipin was used in this study. The HUVECs compatibility of each test tissues sample was quantitatively compared by the MTT assay in the study. The type I collagen was coated on the surfaces of each test tissues sample in order to facilitate the adhesion of cells. The MTT assay results of HUVECs proliferation for each test tissues sample is shown in Fig. 3. It is reported that the cytotoxicity of the crosslinking agents-fixed vascular tissues is attributed to the continuous leaching-out of the unreacted crosslinking agents within the tissue interstices [9]. Genipin may react with free amino groups and form a tertiary amine structure which is more stable than Schiff base formed by glutaraldehyde-fixed and linear crosslinking structure formed by EGDE-fixed [9], thus the leaching-out amount of the genipin is less. Furthermore, the genipin itself is a naturally occurring crosslinking agent; its cytotoxicity is very low. Wherefore, the HUVECs compatibility of genipin-fixed vascular tissues was significantly better than those fixed with glutaraldehyde or EGDE.

The secretion of two factors affecting platelet adhesion and aggregation (PGI_2), blood coagulation (vWF) was measured to compare the function of HUVECs on various substrates. It is reported that at low density endothelial cells secreted more of the vWF and PGI_2 than at high density [22, 23]. In this study, compared to glutaraldehyde-fixed acellular tissues, secretion of two factors was increased after 3 days of culture due to activation of HUVECs on control substrates, EGDE-fixed and genipin-fixed acellular tissues shortly after seeding. Upon prolonged culturing the secretion of these substances gradually decreased, similar to the description in the literature (Figs. 4 and 5). The secretion of two factors by HUVECs on the glutaraldehyde-fixed acellular tissues was very low and remained unchanged; this result suggested that the HUVECs on the glutaraldehyde-fixed acellular tissues basically had lost the activities of endothelial cells. The secretion of two factors by HUVECs on the genipin-fixed acellular tissues was significantly higher compared to the EGDE-fixed acellular tissues and comparable to the control substrates. This indicated that the function of the HUVECs cultured on the EGDE-fixed acellular tissues was also affected. To sum up, HUVECs cultured on the genipin-fixed acellular tissues not only proliferated well, but also preserved the activities and function of endothelial cells.

In the endothelialization experiments, HUVECs were seeded and cultured on the luminal surfaces of these fixed biological vascular scaffolds for 9 days. The results showed that the luminal surface of the biological vascular

scaffolds treated with genipin was successfully repopulated with HUVECs, and resulted in a complete coverage of HUVECs (Figs. 6c and 7c). After culturing for 9 days, the HUVECs on the luminal surface of the biological vascular scaffolds treated with genipin still preserved the activities of endothelial cells (Fig. 8). These indicated that the biological vascular scaffolds fixed by genipin had very good endothelial cell compatibility and could well be endothelialized in vitro.

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