

Crosslinking effect of Nordihydroguaiaretic acid (NDGA) on decellularized heart valve scaffold for tissue engineering

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Abstract Decellularized heart valve scaffolds possess many desirable properties in valvular tissue engineering. However, their current applications were limited by short durability, easily structural dysfunction and immunological competence. Although crosslinking with chemical reagents, such as glutaraldehyde (GA), will enhance the mechanical properties, the low long-term stability and cytotoxicity of the scaffolds remains potential problem. Nordihydroguaiaretic acid (NDGA) is a bioactive natural product which is able to crosslink collagen and was proven to be effective in preparation of scaffold for tendon tissue engineering. In this paper, NDGA crosslinked decellularized heart valve scaffolds demonstrated higher tensile strength, enzymatic hydrolysis resistance and store stability than the non-crosslinked ones. Its mechanical properties and cytocompatibility were superior to that of GA-crosslinked heart valve matrix. Below the concentration of 10 $\mu\text{g/ml}$, NDGA has no visible cytotoxic effect on both endothelial cells (EC) and valvular interstitial cells (VIC) and its cytotoxicity is much

less than that of GA. The LC50 (50% lethal concentration) of NDGA on ECs and VICs are 32.6 $\mu\text{g/ml}$ and 47.5 $\mu\text{g/ml}$, respectively, while those of GA are almost 30 times higher than NDGA ($P < 0.05$). ECs can attach to and maintain normal morphology on the surface of NDGA-crosslinked valvular scaffolds but not GA-crosslinked ones. This study demonstrated that NDGA-crosslinking of decellularized valvular matrix is a promising approach for preparation of heart valve tissue engineering scaffolds.

1 Introduction

The use of synthetic polymer materials as a scaffold of tissue engineering heart valve (TEHV) led to many limitations such as lacking of the regulation of cell attachment and tissue remodeling [1, 2]. Therefore, extracellular matrix (ECM) scaffolds comprising natural collagen, elastin and proteoglycans, the critical elements for the structural integrity, biomechanical properties and potential adhesion/migration sites for cell in-growth [3], remains the most prevalence way for heart valve replacement. For example, decellularized porcine heart valves are ideal due to its structural similarity with human hearts [2, 3]. Furthermore, the decellularized scaffolds can significantly reduce antigenicity, ideally facilitate adhering of recipient autologous cells and easily create a living tissue [4]. However, scaffold from non-crosslinked fresh porcine valvular matrix has its own limitations such as short durability, easily structural dysfunction and immunological competence. Attempts by crosslinking chemical reagents, such as glutaraldehyde (GA), to porcine valvular matrix increased the mechanical properties and reduced immunogenicity, but the long-term stability is still low. Moreover, the cytotoxicity of GA to valvular cells prevented

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them from adhesion, proliferation and infiltration [5]. Therefore, novel crosslinking reagents which endow the decellularized valve scaffold with high mechanical properties, long durability and low cytotoxicity are under investigation.

Nordihydroguaiaretic acid (NDGA), extracted from chaparral (*Larrea tridentate Coville*), is a natural product with two functional ortho-catechols. It has many beneficial bioactivities such as antioxidant, free radical scavenger [6], anti-inflammatory and cardiovascular protection therefore being used as a therapeutic agent to treat heart disease, cancer [7], Alzheimer's disease [8], and inflammatory-immune injuries/autoimmune diseases [9]. Recently, it had been demonstrated that NDGA can crosslink collagen materials for preparation of tissue engineered tendon scaffold with high cytocompatibility and ideal mechanical properties comparable to native tendon [10–14]. The objective of this study is to assess the crosslinking effects of NDGA on decellularized heart valve matrix including the mechanical properties, anti-enzymatic degradation, NDGA release process, stability in store solution and in vitro biocompatibility.

2 Materials and methods

2.1 TEHV scaffolds preparation and crosslinking

Fresh porcine aortic heart valves were harvested from a local slaughter house and brought to the laboratory in sterile phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} . To prepare the decellularized heart valve scaffolds, heart valve were treated by trypsin solution (0.25%) at 37°C for 4 h, then wash with sterile PBS, followed by treatment in decellularization solution (0.5% triton X-100, 0.5% sodium deoxycholate and 0.02% EDTA) and agitate for 48 h at 37°C. Then, the heart valves were further treated in RNase (20 µg/ml, Sigma, America) and DNase (0.2 mg/ml, Sigma, America) for 2 h with agitating. Finally, the scaffolds were extensively washed again with PBS to get rid of the residual cell debris, and stored in 4°C for further use [15]. The decellularized heart valve scaffolds were crosslinked with NDGA by the following procedures. Fifty milligram NDGA (Sigma, America) was dissolved in 1 ml 0.1 N NaOH and then 9 ml PBS was added to get a NDGA solution of 5 mg/ml final concentration. To assure complete solubilization, additional 10 µl NaOH (10 N) should be added to the above solution [13, 16]. Then the solution can be diluted to serial concentrations of 0.5, 1.5 and 2.5 mg/ml. The scaffolds were crosslinked with NDGA solutions under continuous shaking at 120 rpm (revolutions per minute) at 37°C for 48 h. The decellularized valve scaffolds crosslinked by

6.25 mg/ml (0.625%) GA under the same conditions was served as the control [17].

2.2 Valve scaffold morphological observation

The decellularized heart valve scaffolds were photographed using an optical camera (Olympus E300, Japan). To observe the structure and collagen integrity of the heart valve matrix, the decellularized scaffolds were fixed by immersion in 2.5% GA for 4 h, dehydrated in an ascending series concentration of ethanol solution for 10 min each, and then dried in 100% hexamethyldisilazane (Sinopharm Chemical Reagent Co. Ltd., China). Finally, the specimens were coated with gold-palladium and examined by the scanning electron microscopy (SEM, JSM-6700F, JEOL, Tokyo, Japan) [18]. To validate that the scaffolds are free from cell debris, hematoxylin and eosine staining of valve samples was performed according to standard protocol [19]. Briefly, samples were fixed by immersion in 10% neutral buffered formalin for 24 h, embedded in paraffin, serially sectioned and stained with hematoxylin eosin (Sinopharm Chemical Reagent Co., Ltd., China). The sections were observed under a light microscope (Leica DM LM, Germany).

2.3 Mechanical properties determination

The mechanical determination were performed based on our previous described methods [20]. Briefly, decellularized heart valve scaffolds were trimmed as a test strap of 4 mm × 25 mm along collagen fiber. Each group was composed of six straps and was kept moist throughout testing procedure by immersed in PBS overnight and spraying PBS during the process. Tensile strength tests were performed on a TRAPEZIUM material testing instrument (Shimadzu, Japan) at a distance of 7.8 mm between the grips and at an extension rate of 2 mm/min. The ultimate tensile strength was taken as the highest load attained before failure and normalized to cross-sectional area. The elastic modulus was calculated by the linear portion of the stress/strain curve.

2.4 In vitro enzymatic degradation

NDGA-crosslinked decellularized valve scaffolds along with GA-crosslinked and non-crosslinked decellularized valve scaffolds were digested with 1000 U/ml type II collagenase (Sigma, America)/PBS (pH 7.4) at 37°C for selected duration under continuous vibration at 120 rpm. To assess the resistant of the scaffolds to enzymatic digestion, non-, GA- and NDGA-crosslinked valve scaffolds were weighted before and after enzymatic degradation. The residuals were taken out after filtering through a

0.45- μm cellulose filter to separate insolubilized matrix from solubilized collagen at predetermined intervals (1 h, 2 h, 3 h, and 24 h). The retaining weight percentage ($\Delta\text{W}\%$) was then calculated using the equation

$$\Delta\text{W}\% = \text{W}_r/\text{W}_0 \times 100,$$

where W_0 represents the original weight of each sample and W_r represents the retaining weight of the same sample after enzymatic degradation [21].

2.5 Crosslinking stability in PBS

Heart valve scaffolds crosslinked by 2.5 mg/ml NDGA were soaked in 20 ml PBS (five leaflets per group) at 37°C with continuous shaking (180 rpm). The ultimate tensile strength and released NDGA were measured at selected time points. For measurement of released NDGA, the soaking solution was sampled and changed with fresh D-Hanks solution at selected time point. The concentration of NDGA solution was determined by dissolving samples in 1% ammonium molybdate solution, and optical density (OD) was measured using a UV–Vis 8500 spectrophotometer (Techcomp Ltd., Hong Kong) at 500 nm wavelength [22]. The standard OD curve was prepared based on the OD value of serial concentrations of NDGA.

2.6 Cell culture and proliferation analysis

The effect of NDGA on cell proliferation was evaluated using human umbilical vein endothelial cell (ECs) and porcine valve interstitial cells (VICs). Human umbilical vein ECs were purchased from Invitrogen Gibco Cell Culture Co. Ltd. (Catalogue Number: C-003-5C) and maintained in DMEM medium (Gibco, America) supplemented with 20% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C, 95% humidified in 5% CO_2 atmosphere. VICs were isolated from bovine aortic heart valves. The endothelial cells were removed by scraping from the harvested aortic valve leaflets. The leaflets were then minced to small pieces about 1–2 mm^3 , and digested enzymatically in test tubes containing DMEM medium (Gibco) supplemented with 2 mg/ml type II collagenase (Sigma-Aldrich, 386 U/mg solid) for 1 h at 37°C under continuous shaking. The released cells were washed and cultured in the same condition as ECs. Cultures at 2–6 passages were used in the cell proliferation experiments.

NDGA was dissolved in DMSO and diluted by DMEM medium containing 10% FBS to the desired final concentrations (1, 10, 25, 50 and 100 $\mu\text{g}/\text{ml}$). The DMSO concentration was held constant in NDGA solution with different concentrations (no more than 10%), and a 10% DMSO in DMEM solution was used in all experiments as a control. Cells were inoculated in 96-well plates at the

density of 10^3 cells/well in 100 μl complete medium. After 1 day, the medium was replaced with medium supplemented with NDGA, or GA in serial concentrations. Cells were incubated for 6 days and analyzed using MTT assay. The optical densities were measured at 490 nm [23, 24]. The relative cell viability (%) was calculated according to the following equation [25]

$$\text{Relative cell viability} = \text{OD value of test group} / \text{OD value of control group} \times 100.$$

The LC50 (50% Lethal Concentration) of NDGA and GA on ECs and VICs are determined as the dose of 50% cell viability according to the viability curve.

2.7 In vitro cytocompatibility of crosslinked scaffolds

Non-, NDGA- and GA-crosslinked heart valve scaffolds (10 mm \times 10 mm) were put in a 6-well plate and rinsed with 5 ml cell culture medium for 24 h and then seeded with ECs at a density of 3×10^6 cell/ cm^2 . The scaffolds were incubated at 37°C for 2 h to allow the cells to adhere to the scaffolds and then additional 5 ml cell culture medium with 10% FBS was added to each well. After cultured for 24 h, the seeded scaffolds were collected for SEM observation as the methods described in 2.2 [26].

2.8 Statistical analysis

All data are presented as mean \pm standard deviation. Four samples were represented for each data point. Statistical analysis for determination of differences in the measured properties between groups was accomplished using two-tailed analysis of variance, performed with a computer statistical program (Student's *t*-test), and *P* values < 0.05 were considered statistically significant [20].

3 Results

3.1 Morphology of decellularized heart valve

As shown in Fig. 1, there was no cell residual substance or cellular components left in the valvular matrix. The decellularization procedure yielded a natural porous network. The morphology of the heart valve matrices crosslinked by different concentrations of NDGA all demonstrated excellent porous micro architecture (Fig. 1). NDGA-crosslinking valve leaflets carried the brown color from NDGA and almost as soft and stretched as the fresh natural valves, while GA-crosslinked heart valve leaflets were a little stiff and crimped (Fig. 2). The result showed that NDGA-crosslinked valves are morphologically closer

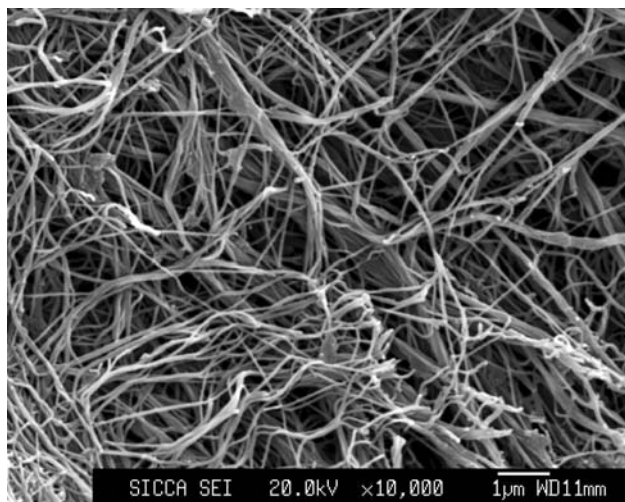


Fig. 1 SEM photo of the decellularized porcine aortic heart valve. An excellent porous micro architecture was observed. There were no cellular components left in the matrix



Fig. 2 Digital image of non-crosslinked and crosslinked heart valve scaffolds. From left to right: 1: non-crosslinked; 2: 6.25 mg/ml GA; 3: 1.5 mg/ml NDGA; 4: 2.5 mg/ml NDGA; 5: 5 mg/ml NDGA

to natural valve than GA-crosslinked ones. Histological analysis revealed that NDGA-crosslinked valvular ECM resulted in porous scaffolds without apparent disruptions of histoarchitecture (Fig. 3).

3.2 Mechanical properties

NDGA crosslinking significantly increased the mechanical strength of decellularized heart valve scaffolds (Fig. 4a). The tensile strength increased with the increase of the NDGA concentration and reached the maximum of 17.2 ± 1.7 MPa at 2.5 mg/ml NDGA crosslinking. This maximum tensile strength was about twice higher than that of non-crosslinked control 8.7 ± 1.0 MPa and even

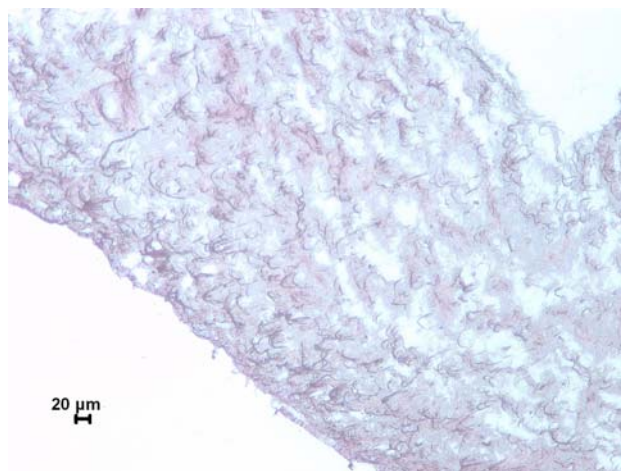


Fig. 3 Histological micrograph of HE-stained NDGA-crosslinked decellularized heart valve ECM

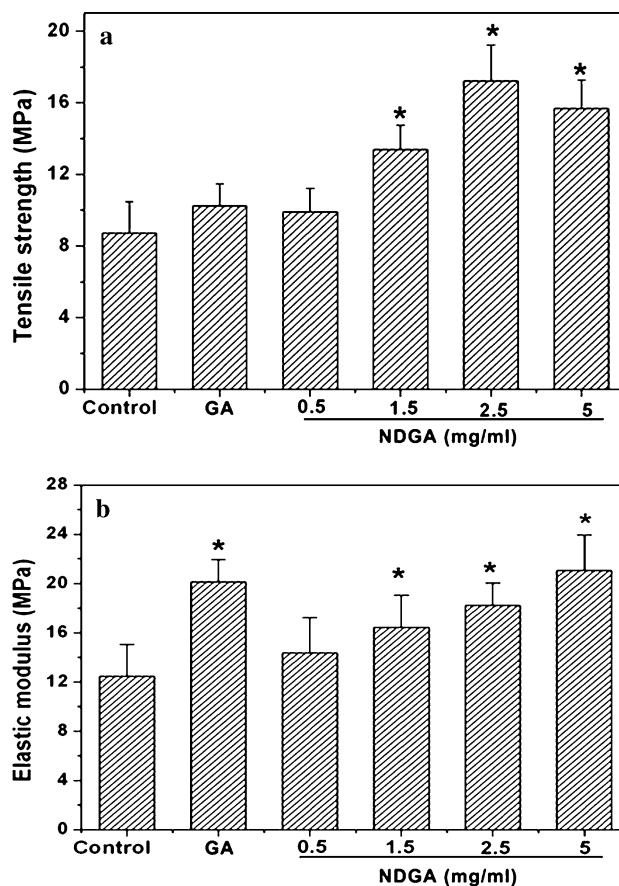


Fig. 4 Mechanical properties of heart valve scaffolds crosslinked by NDGA. **a** ultimate tensile strength; **b** elastic modulus. Control: decellularized heart valve matrix without crosslinking. GA: 6.25 mg/ml GA-crosslinked heart valve scaffolds. NDGA: Decellularized heart valve scaffolds crosslinked by different concentrations of NDGA. * $P < 0.05$

surpassed the GA-crosslinked heart valve scaffolds 11.2 ± 2.0 MPa. The increase of the elastic modulus was correlated with the NDGA concentration. The highest was

21.1 ± 2.6 MPa (at 5 mg/ml NDGA crosslinking), which is almost the same as that of GA-crosslinked (Fig. 4b).

3.3 Crosslinking stability in PBS

In order to assess the stability of the NDGA-crosslinked scaffold, the valve scaffolds were immersed in PBS for up to 120 days. No significant change in the ultimate tensile strength was observed (Fig. 5). This result implied that NDGA-crosslinked scaffolds maintained stability in PBS over a long period without losing mechanical properties. During the short soaking period, the excess NDGA released rapidly from NDGA-crosslinked scaffolds and reached the maximum in 3 days as shown in Fig. 6. After 10 days, NDGA could not be detected any longer in the solution.

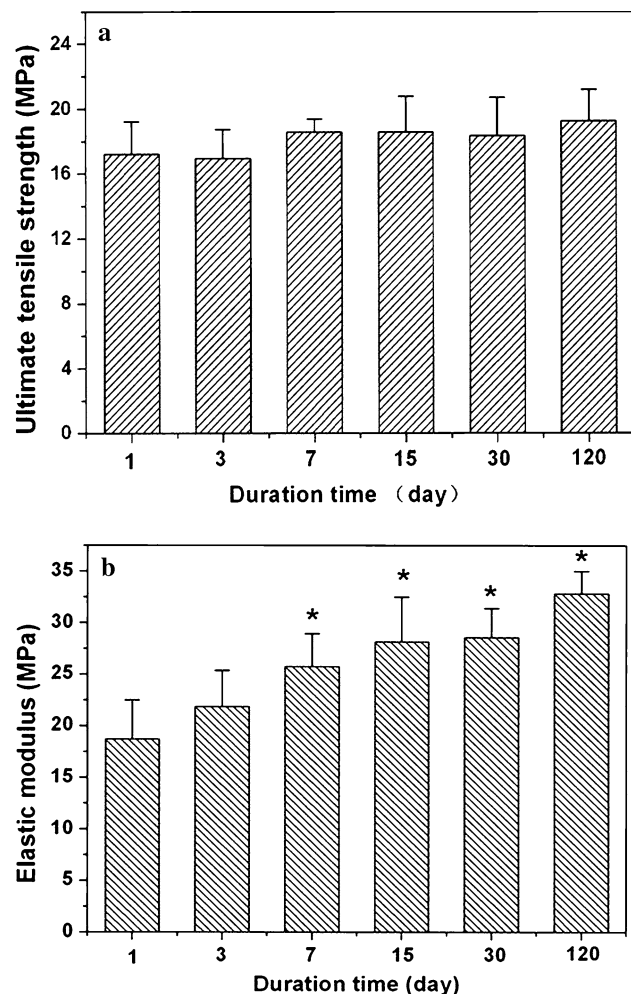


Fig. 5 a Ultimate tensile strength and b elastic modulus of 2.5 mg/ml NDGA-crosslinked heart valve matrices soaked in PBS

3.4 In vitro enzymatic degradation

Figure 7 illustrated the capacity of enzymatic hydrolytic resistance of non-, GA- and NDGA-crosslinked valve scaffolds at different time points. At the first hour, the non-crosslinked decellularized heart valve left only 16.3 ± 0.3% of the original weight while GA-crosslinked and NDGA-crosslinked heart valve scaffolds remained almost intact. The weight of the valvular scaffolds decreased while the hydrolytic time proceeded. Twenty-four hours later, the scaffolds crosslinked with NDGA of concentration

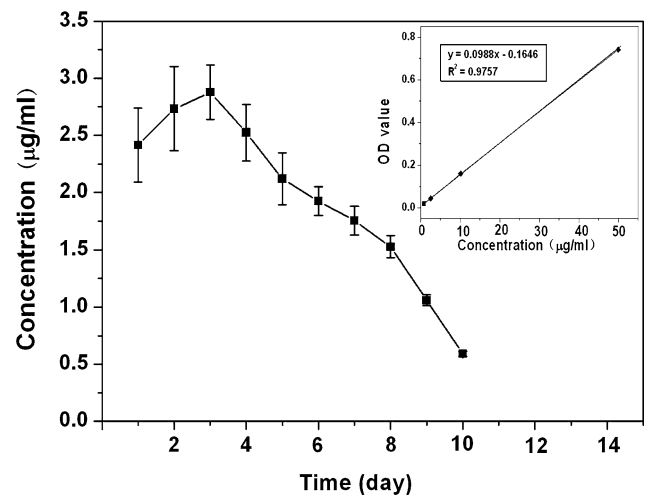


Fig. 6 NDGA releasing. The NDGA released from 2.5 mg/ml NDGA-crosslinked heart valve scaffolds. This result showed that, after 10 days soaking, there was almost no NDGA release from the NDGA-crosslinked scaffold. The upright insert is the standard optical-to-concentration curve of NDGA

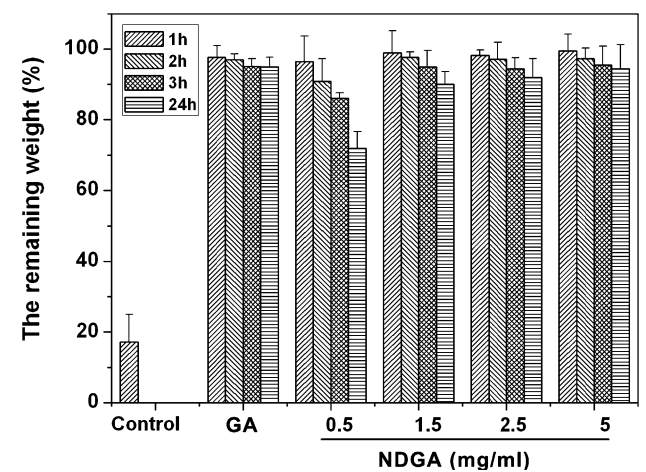


Fig. 7 The relative remaining weight of non-, GA- and NDGA-crosslinked valvular scaffolds. Control: non-crosslinked decellularized heart valve scaffolds. GA: 6.25 mg/ml GA-crosslinked heart valve scaffolds. NDGA: Decellularized heart valve scaffolds cross-linked by different concentrations of NDGA

2.5 mg/ml and above still maintained $91.9 \pm 1.5\%$ of the initial weight, which is close to that of GA-crosslinked (6.25 mg/ml). This result suggests that the enzymatic stability of the NDGA-crosslinked heart valve scaffolds is comparable to that of GA-crosslinked, and both are superior to natural heart valve scaffolds.

3.5 Effects of NDGA on cell proliferation

After 6 days of culture, the cell proliferation in the presence of NDGA was determined by MTT assay and LC50 was calculated to quantitate the level of cytotoxicity. The results showed that, GA exhibited an obvious inhibition effect on cell proliferation and the LC50 is as low as 0.9 $\mu\text{g/ml}$ on EC and 2.3 $\mu\text{g/ml}$ on VIC. In contrast, NDGA had little inhibitory effect on cell proliferation below the concentration 10 $\mu\text{g/ml}$ (Fig. 8), the LC50 of NDGA is about 32.6 $\mu\text{g/ml}$ on EC and 47.5 $\mu\text{g/ml}$ to VIC. In contrast, the lowest concentration of GA, at which no

cytotoxicity was observed, was 0.1 $\mu\text{g/ml}$, is 100 times lower than that of NDGA.

3.6 Cell attachment on NDGA-crosslinked scaffolds

After 24 h culture, the human umbilical vein ECs could not adhere to the GA-crosslinked scaffolds (Fig. 9a, b). In contrast, ECs could attach to and spread on the NDGA-crosslinked scaffolds (Fig. 9c, d). ECs exhibited cobblestone morphology with small microvilli on the scaffolds is similar to ECs on native heart valve.

4 Discussion

Heart valve ECM is a complex mixture of both structural and functional proteins, glycoproteins and proteoglycans arranged in a unique, tissue specific three dimensional ultra-structure [27, 28]. Among them the collagen is the main component which can support high mechanical properties [29]. It serves as structural support, attachment sites for cell surface receptors, and as a reservoir for signaling factors, which modulate biological processes such as cell attachment, migration and proliferation. Therefore, the decellularized heart valve ECM is a biologically based tissue engineering scaffold for heart valve replacement. In the present study, the decellularized porcine heart valve ECM was crosslinked with NDGA to yield an artificially modified scaffold. It maintained excellent porous network suitable for tissue engineering (Fig. 1) and exhibited increased tensile strength and maintain softness as compared with GA-crosslinked matrix. The present result showed that NDGA is superior to GA in maintaining the valves natural configuration (Fig. 2), high ultimate tensile strength and relative low elastic modulus (Fig. 4).

NDGA-crosslinked valvular scaffolds could maintain the good mechanical property for as long as 120 days without significantly changes the elastic modulus (Fig. 5). The crosslinking of NDGA to the native decellularized heart valve is fairly stable. There is rarely excess NDGA being released from the crosslinked TEHV scaffolds (Fig. 6) after 10-day soaking in PBS. Furthermore, NDGA-crosslinked valvular scaffolds were resistant to collagenase digestion (Fig. 7). Their well-performed stability is critical because TEHV scaffolds have to withstand strong biomechanical stress in vivo after implantation [30]. Taking these factors into account, the steady crosslinking structures could be kept for long time to meet the requirements of clinical applications.

GA forms adduct with free primary amines of amino acid residues in proteins, particularly lysine side chains. The constituent aldehydes reaction of GA can result in the formation of chemical bonds between neighboring

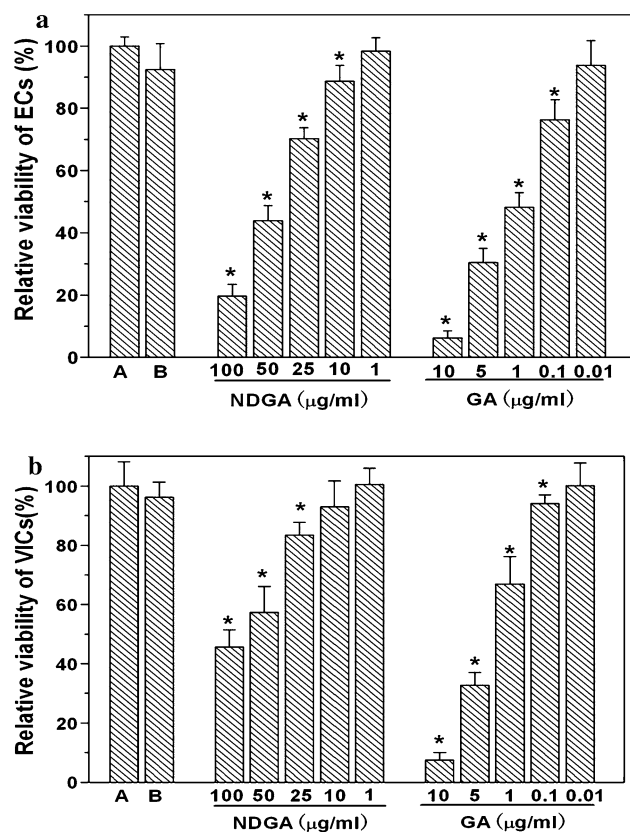
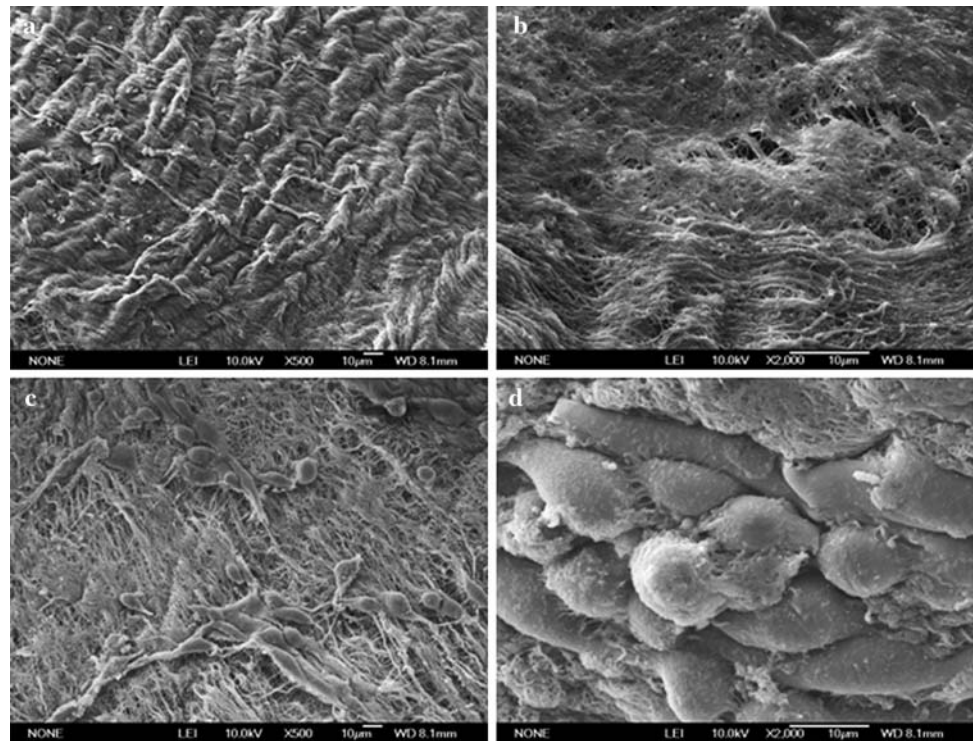


Fig. 8 Effect of NDGA and GA on cell proliferation of EC (a) and VIC (b). NDGA was dissolved in (DMSO) before dilution into the culture medium. The cells were cultured for 6 days in the absence or presence of the indicated NDGA or GA concentrations. The LC50 of NDGA is about 32.6 $\mu\text{g/ml}$ on EC and 47.5 $\mu\text{g/ml}$ to VIC. The lowest concentration of GA, at which no cytotoxicity was observed, was 0.1 $\mu\text{g/ml}$, is 100 times lower than that of NDGA. A. culture medium; B. culture medium with 1% DMSO. * $P < 0.05$

Fig. 9 SEM images of ECs seeded on the heart valve matrices after 24 h: **a** GA-crosslinked heart valve; **b** The enlarged photo of (a); **c** NDGA-crosslinked heart valve; **d** The enlarged photo of (c)



peptides, which led to the decrease of the flexibility of the ECM scaffolds. This might be the reason of the rigidity of GA-crosslinked scaffolds [23, 31]. In recent years, it was reported that genipin, another naturally occurring compound, can crosslinking biological tissues by forming covalent bond with collagen molecules, yielding good stability and biocompatibility, and it was effective to stabilize heart valve tissues [32]. In contrast, NDGA is a dicarboxylic acid with two potentially reactive o-quinone functionalities which cross-link each other at each end forming a highly cross-linked NDGA polymer, causing the collagen fibrils being embedded in and reinforced, and resulted in the increase of mechanical properties and stability either in PBS or in enzymatic hydrolysis solution [11, 13]. Our results were consistent with the mechanism proposed by Koop in their tendon crosslinking study [11].

The long-term success of TEHV scaffold depends on the ability of its living valvular components ECs and VICs to function normally and to maintain homeostasis [26]. According to our experiment, GA demonstrated significant inhibitory effect on the proliferation of ECs and VICs even at the low concentration of 0.1 µg/ml, which might be the major cause of the rapid deterioration of GA-crosslinked decellularized heart valves in vivo. In contrast, the LC50 of NDGA on EC was 32.6 µg/ml and to VIC is 47.5 µg/ml, which is 36 times and 20 times higher than that of GA, respectively. The low cytotoxicity of NDGA on both ECs and VICs indicated that NDGA was more cytocompatible

than GA. Therefore, the NDGA crosslinking provided a well cytocompatible TEHV scaffold without inhibition in cells proliferation.

The blood-contacting surfaces of native heart valves are lined by ECs which are in response to fluid shear stress and provide a normally functioning surface for preventing platelet adhesion and thrombosis [30]. Our results showed that ECs could adhere to NDGA-crosslinked scaffolds and tend to form a layer after 24 h, while no cell could be observed on the GA-crosslinked heart valve surface (Fig. 9c, d). Moreover, morphology of ECs on NDGA-crosslinked TEHV scaffolds was similar to that on native heart valve [26]. Taken together, the NDGA-crosslinked TEHV scaffold can facilitate EC attachment and proliferation, suggesting that NDGA crosslinking might be an ideal method for producing biocompatible TEHV scaffolds.

5 Conclusion

This study demonstrates that NDGA-crosslinking is an effective way to improve the mechanical properties and biocompatibility of decellularized heart valve scaffolds. The NDGA crosslinked valve scaffolds showed superior properties to those of non-crosslinked and GA-crosslinked ones. Meanwhile, it is structurally and functionally equivalent to non-crosslinked native valve scaffolds with pliable and porous network and excellent cytocompatibility to

valvular cells. These results suggest that NDGA might be an effective crosslinking reagent for preparation scaffolds for heart valves tissue engineering.

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