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The study of a new detergent (octyl-glucopyranoside) for decellularizing porcine pericardium as tissue engineering scaffold

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ABSTRACT

Background: The use of extracellular matrix (ECM) derived from decellularized tissue is increasingly frequent in regenerative medicine and tissue engineering. However, it is recognized that currently used decellularization procedures have negative effects on ECM integrity. The objective of this study was to investigate the impact of a decellularization protocol with a new detergent on the ECM integrity of porcine pericardium (PP) compared with other traditional detergents.
Materials and methods: Fresh PP were decellularized by sodium deoxycholate in combination with Triton X-100 (SDT), sodium dodecyl sulfate (SDS), and octyl-glucopyranoside (OGP), respectively. Histologic analysis and scanning electron microscopy were performed to confirm the removal of cells and to examine the structure of ECM. DNA content was examined by the method of DNA extraction. Mechanical properties and biochemical compositions of ECM were also studied.
Results: Histologic analysis and DNA determination demonstrated that SDS and OGP completely removed the cells, and the major ECM structure was preserved well for PP treated with 1% (wt/vol) OGP but disrupted for PP treated with SDS; whereas treatment with SDT was insufficient to remove cells from PP. Uniaxial tensile tests showed that PP decellularized by OGP had similar mechanical properties to native PP, whereas the mechanical properties of PP decellularized by SDS and SDT decreased. The biochemical compositions of PP decellularized by OGP were also well conserved, except that glycosaminoglycans markedly decreased. Moreover, the results obtained in the MTT study further indicated that the cytotoxicity of PP decellularized by OGP was significantly lower than that decellularized by SDS and SDT.
Conclusion: It is suggested that the environmentally friendly and nontoxic OGP can be used as a decellularizing agent. The OGP method could achieve both complete removal of cells from native PP and preservation of the matrix structure; thus, it might be a suitable approach to preparation of tissue engineering heart valve scaffold.
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1. Introduction

Cardiovascular diseases represent a major worldwide health care issue. Over 225,000 heart valve replacement operations are performed worldwide annually [1]. The number of patients requiring heart valve replacement is estimated to triple from approximately 290,000 in 2003 to over 850,000 by 2050 [2]. Worldwide heart valve allograft scarcity urges international research to generate biologic substitutes for heart valve transplantation. The use of biologic scaffold materials composed of extracellular matrix (ECM) is increasingly frequent in regenerative medicine and tissue engineering. ECM-based biomaterials have many inherent advantages over synthetic polymer materials for surgical and regenerative medicine applications. These protein-based materials have significant mechanical strength and retained biological activity and can promote regeneration of tissue [3]. In recent years it has been convincingly demonstrated that the ECM is involved in cell–cell signaling, the regulation of cell proliferation, cell differentiation, and cell migration [4–8]. Decellularized tissues for these applications began with the development of decellularized porcine small intestine submucosa as a novel material for surgical repair [9].

When a natural biomaterial is to be used as the scaffold for tissue engineering heart valve, the cell component must be removed completely, because this is the main factor of immune response after implantation [10,11]. Decellularization techniques have been developed in an attempt to reduce the antigenicity of xenogeneic biomaterials, a critical barrier in their use as tissue engineering scaffolds. The ultimate goal of any decellularization protocol is to remove all cellular material without adversely affecting the composition integrity, mechanical property, and eventual biological activity of the remaining ECM [6]. Commonly used methods of decellularization include physical treatments and chemical and biologic agent methods [12]. Sonication, agitation, and freezing and thawing [12] are commonly used methods to disrupt cell membranes, release cell contents, and facilitate the subsequent rinsing and removal of cell remnants from the ECM. Various detergents or enzymatic agents have been used to remove cells and cellular debris in xenogeneic biomaterials. Commonly used decellularization methods are a nonionic detergent, tert-octylphenylpolyoxyethylen (Triton X-100) [11,13–16]; an anionic detergent, sodium deoxycholate [11,14,16]; sodium dodecyl sulfate (SDS) [10,11,13,15,16]; and an enzymatic agent, trypsin [11,13–17]. However, numerous studies have demonstrated inadequate removal or some damage to ECM ultrastructure [10,12,14,15,17,18].

The ultrastructure of ECM scaffolds can be largely preserved throughout processing steps required for decellularization of the tissue if care is taken to avoid harsh chaotropic agents [5]. The reason for the choice of octyl-glucopyranoside (OGP) was mainly that it is a new nonionic surfactant with highly biodegradable, low-irritation, and low-toxicity properties [19,20]. Research shows that OGP can destroy bacteria membrane structure, owning broad-spectrum antibacterial property, such as against gram-negative bacteria, positive bacteria, etc, and regards OGP as the ideal mild nonionic surfactant [21]. The traditional detergents have certain toxicity [22,23], and the detergents remaining in the scaffold after decellularization could also be toxic to cells. Cytotoxicity is possible even at reduced agent concentrations and will inhibit or completely negate the beneficial properties of a cell-free ECM scaffold [22]. Hence, we hypothesized that the OGP method, which reduces cytotoxicity by a large margin, will remove cells from porcine pericardium (PP) while maintaining biomaterial functional properties. However, few investigations were performed to report about the decellularization effect of OGP.

In this study, the efficiency of decellularization using different concentrations of OGP was investigated to optimize the appropriate decellularization concentration of OGP, and then different detergents were used to remove cells of the pericardium tissues. The best detergent was selected by histologic analysis, measuring the mechanical properties and biological properties of the decellularized PP.

2. Materials and methods

2.1. *Reagents and sample preparation*

Antibiotic solution (AS) and L-4-hydroxyproline were obtained from Gibco Co (New York). Deoxyribonuclease I, ribonuclease, p-dimethylaminobenzaldehyde, and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich (St. Louis, MO). Quant-iT PicoGreen dsDNA assay kit was obtained from Invitrogen Co (New York). Fastin Elastin assay kit was obtained from Biocolor Ltd (Newtownabbey, UK). OGP, ethylenediamine tetraacetic acid (EDTA), and other reagents (analytical grade or equivalent grade) were obtained from Aladdin Co (Shanghai, China).

Preparation of PP has been previously described [14]. Briefly, 45 pieces of PP with size 50×60 mm from 45 pigs aged about 4–6 mo were extracted directly under clean conditions at a slaughterhouse, blood was washed out with 0.9% NaCl solution, and fat was dissected. Warm ischemic time was less than 2 h. The extracted pericardium was stored in sterile phosphate-buffered saline (PBS) containing 1% (vol/vol) AS and was transported to the laboratory in an ice box.

2.2. *OGP optimization*

To optimize the OGP concentration, porcine pericardial tissues were decellularized at different OGP concentrations ($n = 5$ per group). Briefly, PP was placed in mixed solution: 10 mM Tris–HCl (pH 7.5), 0.02% EDTA, 1% AS, and 1 mM PMSF containing 0.5%, 1%, 2%, and 5% (wt/vol) OGP, respectively, at 4°C for 24 h for cells lysis. PP was then removed and incubated with a nuclease digestion solution (NDS) (2.5 Kunitz units [KU]/mL deoxyribonuclease I, 7.5 KU/mL ribonuclease, 0.15 M NaCl, 2 mM $MgCl_2(H_2O)_6$, 1% (vol/vol) AS in 50 mM Tris–HCl [pH 7.6]) at 37°C for 24 h for nucleic acid degradation [10]. Then all samples were washed in PBS at 4°C for 24 h. Histologic analysis (only hematoxylin-eosin [HE] stain and Weigert stain) was conducted to assess the effect of decellularization at different OGP concentrations ($n = 5$ per group).

2.3. *Decellularization procedure*

Porcine pericardial tissues were divided into four groups randomly, each group having five tissue segments.

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Unprocessed, native PP served as control (Group A) and was stored in sterile PBS containing 1% AS. In Group B, the PP was placed in mixed solution: 10 mM Tris–HCl (pH 7.5), 0.02% EDTA, 1% AS, and 1 mM PMSF containing 1% (wt/vol) OGP at 4°C for 24 h. PP was then removed and incubated with NDS at 37°C for 24 h. In Group C, PP was treated with 0.5% SDS in PBS (pH 7.4) containing 0.02% EDTA, 1% AS, and 1 mM PMSF at 37°C for 24 h [11]. Afterward, PP was agitated in NDS at 37°C for 24 h. In Group D, PP was treated with 1% sodium deoxycholate in combination with 1% Triton X-100 (SDT) in PBS (pH 7.4) containing 0.02% EDTA,1% AS, and 1 mM PMSF at 37°C for 24 h [14]; then PP was agitated in NDS at 37°C for 24 h. In all treated groups, an additional 24 h washing in PBS at 4°C was performed under continuous shaking to remove cellular remnants.

2.4. *Histologic assessment of decellularized PP*

Decellularized PP by different detergents were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections ($n = 5$ per group) were cut at 5 mm thickness and stained with HE stain, Weigert stain, and Movat pentachrome stain to identify the presence of any residual intact nuclei within the samples and assess the structure of collagen fibers and elastin fibers.

2.5. *Quantification of DNA content*

DNA content of tissues was quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen), according to the manufacturer's instructions. Briefly, PP was lyophilized, weighed, incubated vortically in lysis buffer at 55°C overnight, and centrifuged at 13,000 rpm for 5 min, and supernatant was collected, diluted in TE buffer, and mixed with the Quant-iT PicoGreen reagent. Fluorescence was measured at 535 nm with excitation at 485 nm, and DNA content was quantified based on standard curve.

2.6. *Scanning electron microscopy*

The morphology and structural integrity of native and decellularized PP were observed by scanning electron microscopy (SEM) (Hillsboro, America FEI, TET 0625). For this, wet samples were freeze-dried and then examined by SEM ($n = 5$ per group). Samples were mounted on aluminum stubs, sputter-coated with gold, and imaged at 14 kV.

2.7. *Uniaxial tensile testing*

Tensile properties of the native and decellularized PP were measured using a universal testing machine (Model 5543; Boston, Instron) at a constant speed of 10 mm/min. Strips of PP (50×4 mm) were cut from each PP along the collagen fiber direction. Initial gauge length, width, and thickness were determined using digital calipers. For each PP, the elastic modulus, ultimate tensile strength, and strain at fracture were determined by strain-stress curve. During testing, the PP strips were kept in a water bath at 37°C.

2.8. *Quantitative biochemical assays*

The wet weights of native and decellularized PP were measured before they were frozen at −80°C for 1 h and lyophilized for 24 h; after that, the dry weight was obtained. The water content of PP was calculated as (wet weight − dry weight)/dry weight [10].

Collagen content was calculated from the detected hydroxyproline content with a colorimetric assay [24]. Briefly, lyophilized PP were incubated in 6N HCl at 106°C for 24 h, neutralized, oxidized with chloramine-T, and reacted with p-dimethylaminobenzaldehyde. Absorbance was measured at a wavelength of 550 nm, and values were compared with an L-4-hydroxyproline standard. A 1:11.1 (wt/wt) ratio of hydroxyproline to collagen was used to calculate the collagen content of the PP.

The elastin of lyophilized PP was extracted following described methods [25]. Samples were incubated with 0.25 M oxalic acid at 100°C for 1 h; after cooling to room temperature, PP samples were centrifuged at 10,000*g* for 10 min; and supernatant containing soluble elastin was collected. Oxalic acid extraction was repeated three times. The supernatant from three extractions was pooled. Elastin content was analyzed using the Fastin Elastin kit (Biocolor Ltd) according to the manufacturer's instructions.

Sulfated glycosaminoglycans (GAG) were quantified using the colorimetric method. Native and decellularized PP samples were lyophilized, minced, and weighed. Then, approximately 20 mg samples were placed in 1 mL papain solution (0.125 mg/mL in 20 mM sodium phosphate, 5 mM L-cysteine, pH 6.2) and digested at 60°C for 3 h. The sulfated GAG content of digested samples was assessed per triplicate spectrometrically at 540 nm with the 1,9-dimethyl-methylene blue assay using chondroitin sulfate (from shark cartilage) standard curves and expressed as micrograms per milligram of dry tissue [26].

2.9. *In vitro cytotoxicity study*

The cytotoxicity of the decellularized PP was evaluated *in vitro* using a mouse-derived established cell line of L929 fibroblasts [27]. The native and decellularized PP samples were placed in the saline solution for 24 h at 37°C in 5% CO_2 for extraction of liquid. The cells were trypsinized and seeded into 96-well plates at a density of 1×10^3 cells/well, then topped up with 100 μL Dulbecco's modified Eagle medium. Meanwhile, 100 μL extraction liquid of pericardial tissues was added into each well. The cell culture was performed at 37°C in a humidified atmosphere of 5% CO_2. Using the MTT assay, the cell viability in each well was determined 3 d after cell seeding. Photomicrographs were taken and relative growth rate of the L929 fibroblasts was also calculated.

2.10. *Statistical analysis*

Results are expressed as mean ± standard deviation. The significance of the differences between native and decellularized samples was determined by 1-way analysis of variance. Differences are considered statistically significant when $P < 0.05$.

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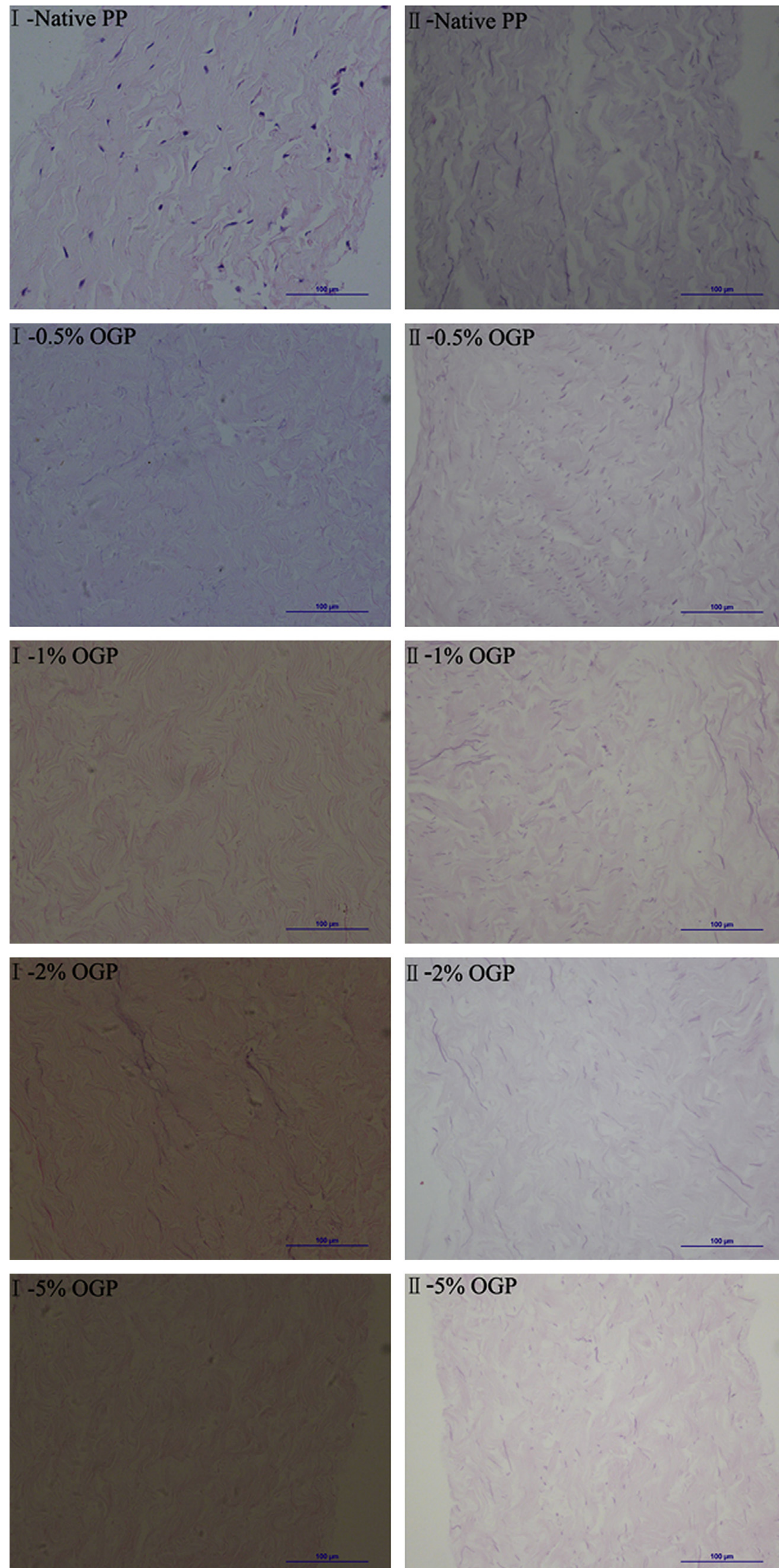

Fig. 1 – HE (column 1) and Weigert (column 2) staining of PP treated with 0.5% (wt/vol), 1% (wt/vol), 2% (wt/vol), and 5% (wt/vol) OGP, respectively, and native PP. In PP processed with 0.5% (wt/vol) OGP, cell debris were still observed by HE stain (column 1, 0.5% OGP). Compared with native PP, they were completely removed when the concentration of OGP was 1% (wt/vol), but it

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3. Results

3.1. OGP optimization

Representative images of HE and Weigert stain are shown to evaluate the effect of decellularization at different OGP concentrations (Fig. 1). Histology of decellularized PP revealed that treatment with 1% (wt/vol) OGP for 24 h followed by agitation in NDS and 24 h washing with PBS converted native PP into an almost cell-free scaffold, with no apparent disruptions of the pericardium histoarchitecture.

3.2. Decellularization efficiency

Decellularization efficiency was examined histologically and by DNA quantification. Representative images showed gross tissue morphology, presence of residual nuclei, and collagen and elastin structure of PP (Fig. 2). For native PP, the results suggested that the histoarchitecture of ECM in native PP was integrated. The results indicated that cells in PP can be removed adequately and the ultrastructure and components of PP are well preserved by the OGP decellularization method compared with the other two decellularization methods.

The decreased DNA content means cells are removed from tissue. Thus, the determination of DNA content in PP can be used for the evaluation of cell residual in the PP. Figure 3 shows that there was a significant difference in the DNA content between all three experimental groups and the control group. In addition, it showed a relatively lower level in the PP treated with OGP and SDS. This is in agreement with the results of HE staining and implies that the vast majority of DNA can be removed by the OGP decellularization method.

3.3. Scanning electron microscopy

Figure 4 shows the surface morphology of native and decellularized PP. It can be seen that PP decellularized by OGP (Fig. 4B) showed a similar structure to native PP (Fig. 4A). No clearly distorted or disrupted collagen and elastin fibers were encountered. However, fibers of PP treated with SDS were irregular and lost wavelike structure on the tissue surface (Fig. 4C), and cell remnants were observed on the surface of PP treated with SDT (Fig. 4D). In agreement with histologic analysis, the results of scanning electron microscopy (Fig. 4B) implied that the morphology and structure of fibers were well preserved in the PP treated with OGP.

3.4. Tensile test

A successful decellularization process must remove cells completely, while maintaining mechanical properties of the tissues. Data on ultimate tensile strength, strain at fracture, and elastic modulus of native and decellularized PP are shown in the Table and Figure 5. The ultimate tensile strength and strain at fracture of PP treated with OGP were not significantly different from native PP (Table, Fig. 5A and B); however, the elastic modulus of PP treated with OGP was lower than native PP (Table, Fig. 5C). However, the PP treated with SDS and SDT had a much lower ultimate tensile strength, strain at fracture, and elastic modulus than native PP (Table, Fig. 5). There was a significant difference in the tensile properties of PP decellularized by SDS and SDT compared with native PP.

3.5. Quantitative biochemical assays

The effect of decellularization may be reflected indirectly by water content. The water content of PP treated with OGP, SDS, and SDT, respectively, was increased slightly from that of native PP (Fig. 6). It was used to characterize hydrophilic properties of decellularized tissues. With the removal of cells, hydrophobic lipid, and soluble proteins, their original positions would be replaced by water, and thus the water content may increase. Consequently, the water content of PP treated with OGP and SDS had increased slightly.

The collagen content showed no significant difference between the native PP and decellularized PP (Fig. 7). This result implies that the collagen content was not reduced during the decellularization process. The collagen fiber structure of PP decellularized by SDS and SDT was damaged, whereas the collagen fiber structure of PP decellularized by OGP was conserved (Fig. 2). Collagen fibers provide the tissue with stability and tensile strength. This is in agreement with the lack of significant difference in ultimate tensile stress between native PP and OGP-treated PP, which demonstrated that both the collagen structure and composition of PP decellularized by OGP were well preserved.

The elastin content in the PP treated with OGP, SDS, and SDT, respectively, was lower than that in native PP (Fig. 8). Furthermore, elastin in PP decellularized by SDS was significantly reduced compared with that in the PP treated with OGP and SDT. This is in agreement with the results of the elastic modulus of the uniaxial tensile test (shown in Fig. 5C).

The GAG content of PP decellularized by OGP (0.02 ± 0.022 μg/mg dry weight) was significantly different from that in native PP (0.31 ± 0.026 μg/mg dry weight) (Fig. 9) ($P < 0.05$, $n = 5$). Total GAG content of PP decellularized by SDS and SDT was 0.29 ± 0.059 μg/mg dry weight and 0.13 ± 0.055 μg/mg dry weight, respectively (Fig. 9). The OGP or SDT decellularization protocol did have a noticeable effect on the GAG content of the pericardium samples, whereas samples treated with SDS did not show a significant difference, with regard to GAG content, from native PP. This difference is because quantitation of glycosaminoglycans is affected by residual SDS [10,28].

seemed that collagen fibers of PP treated with 2% (wt/vol) OGP and 5% (wt/vol) OGP were sparse and deranged, especially for 5% (wt/vol) OGP decellularization (column 1). Collagen fibers of PP treated with 1% (wt/vol) OGP remained wavelike (column 1, 1% OGP). The results were also in agreement with Weigert stain (column 2). It was obvious that collagen fibers and elastin fibers of PP treated with 0.5% (wt/vol) and 1% (wt/vol) OGP were preserved. All scale bars represent 100 μm. (Color version of figure is available online.)

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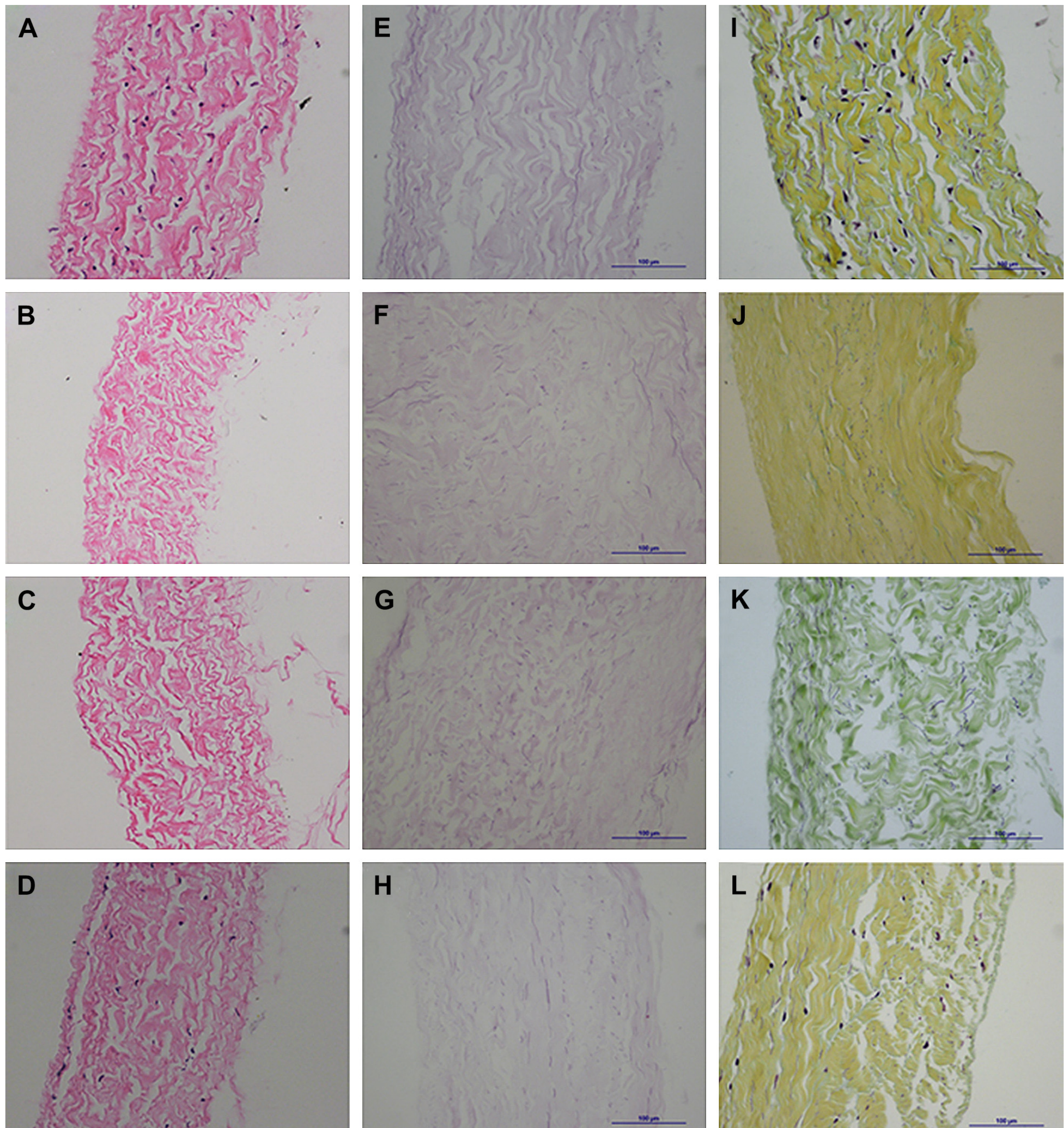

Fig. 2 – HE staining of PP treated with OGP (B), SDS (C), and SDT (D), and native PP (A) (magnification ×200); Weigert staining of PP treated with OGP (F), SDS (G), and SDT (H), and native PP (E); and Movat pentachrome stain of PP treated with OGP (J), SDS (K), and SDT (L), and native PP (I). For native PP, there were a great number of cells remaining in the PP on the histologic section of HE staining (A) and Movat pentachrome stain (I). The collagen fibers of the section were wavelike and compact, whereas the elastin fibers of the section were clear and continuous, as shown by Weigert staining (E) and Movat pentachrome stain (I). In the OGP treatment group, no cells remained in PP and the collagen fibers of the section remained wavelike (B, J), and the elastin fibers of the section were clear and continuous (F, J). In the SDS treatment group, no cells remained in PP and the collagen fibers of the section were broken and deranged (C, K). The elastin fibers in the PP were in malalignment (G, K). In the SDT treatment group, HE-stained (D) and Movat pentachrome–stained sections (L) of the tissues revealed an incomplete cell removal from the matrix. Black/blue-stained nuclei were still found in the tissues. Weigert stain (H) and Movat pentachrome stain (L) showed partly distorted elastic fiber in comparison with native tissue. All scale bars represent 100 μm. (Color version of figure is available online.)

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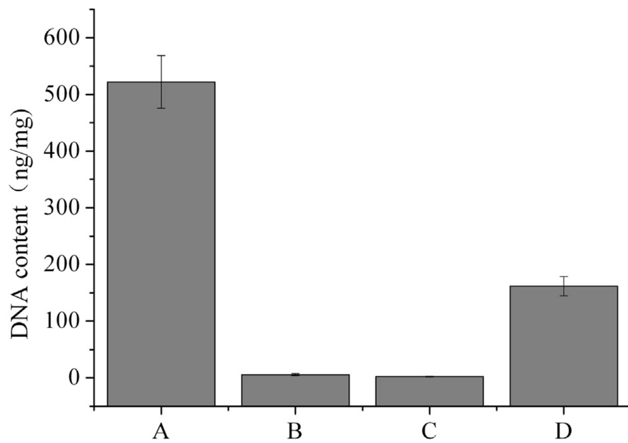

Fig. 3 – DNA extraction tests show that there was very little DNA content in PP decellularized by the OGP (B) and SDS (C) methods, and a little DNA remained by SDT (D) methods, compared with the native PP (A) without decellularization. This means that the DNA, directly correlated to adverse host reactions, can also be removed effectively by the OGP decellularization method. Data are mean ± standard deviation ($P < 0.05$, $n = 5$).

3.6. *Cytotoxicity of decellularized PP*

Figure 10 shows an optical micrograph of an L929 fibroblast when cultured with the extraction liquid from the native PP (A) and PP decellularized by OGP (B), SDS (C), and SDT (D) for 3 d. The cell morphology remained normal for all four groups. The cell density for the OGP group was similar to that of native PP, which means OGP had lower cytotoxicity for PP decellularization, whereas the cell density for the SDS and SDT (D) groups were much lower than that of native PP, so SDS and SDT had higher cytotoxicity than OGP. This result was also confirmed by Cebotari *et al.* [22] and Caamano *et al.* [23]. Consequently, the extraction liquid of PP treated with OGP was significantly superior to all other counterparts.

The relative growth rate of the L929 fibroblasts was also calculated among the four groups using tissue culture plate as control in Figure 11. It can be seen from Figure 11 that the cell relative growth rate for the OGP group (B) (97.03% ± 3.45%) was similar to the native PP (A) (97.23% ± 4.5%), whereas the cell relative growth rates for the SDS (C) and SDT (D) (61.7% ± 3.82% and 62.9% ± 6.65% [$P < 0.05$, $n = 4$], respectively) were much lower than that of native PP. That means that OGP is not cytotoxic but SDS and SDT are cytotoxic as decellularization agents. This result was coincident with the cell number observation in Figure 10.

4. Discussion

Xenogeneic and allogeneic cellular antigens are recognized as foreign by the host and therefore induce an inflammatory response or an immune-mediated rejection of the tissue [11–14,18,26,27]. The goal of a decellularization protocol is to efficiently remove all cellular and nuclear material while conserving the composition and biological activity, and

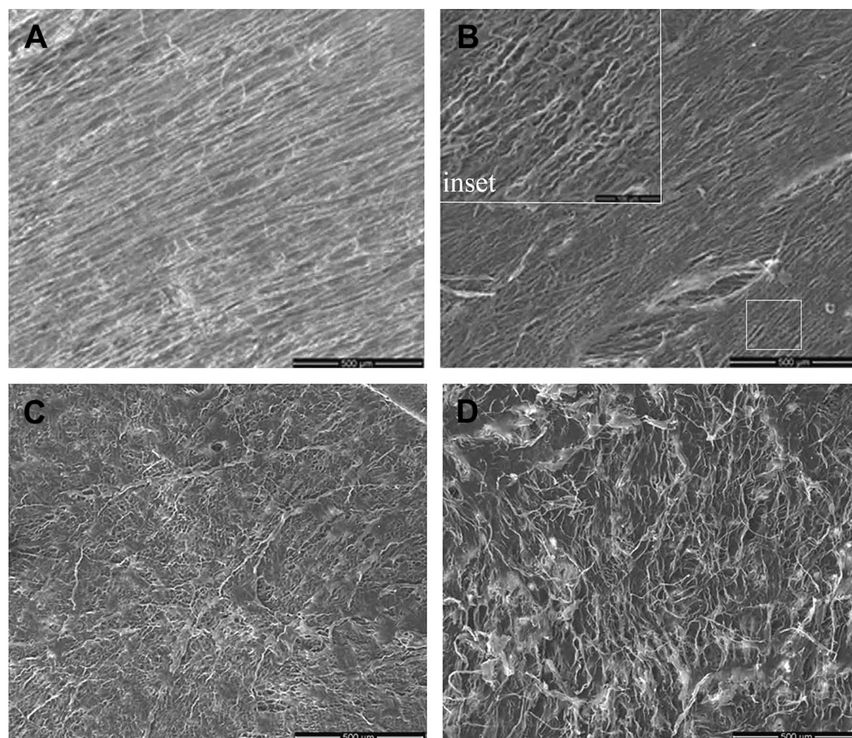

Fig. 4 – Scanning electron micrographs of native PP (A) and PP decellularized by OGP (B), SDS (C), and SDT (D). Surface morphology of PP treated with OGP was similar to native PP, and that of PP treated with SDS and SDT was distorted. Scale bar represents 500 μm. Inset: wavelike collagen fibrils of OGP-treated PP (scale bar equal 100 μm).

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Table – Tensile properties of PP treated with OGP, SDS, SDT, and native PP.

Different PP	Ultimate tensile strength (MPa)	Strain at fracture (%)	Elastic modulus (MPa)
Native PP	14.36 ± 0.82	87.63 ± 2.49	76.59 ± 10.34
Treated with OGP	13.46 ± 0.55	88.64 ± 3.46	58.49 ± 3.66*
Treated with SDS	8.41 ± 1.37*	64.66 ± 10.07*	37.16 ± 5.49*
Treated with SDT	8.02 ± 1.78*	80.07 ± 12.13	50.95 ± 7.51*

Data are presented as mean ± standard deviation.
* $P < 0.05$ ($n = 5$ per group) compared with the native PP.

maintaining the mechanical properties of the ECM scaffold [6]. However, alterations both in the structural composition and in the mechanical properties of the remaining ECM can be induced during the decellularization protocols [12]. Moreover, residual cytotoxic reagents will cause cytotoxicity during the decellularization process, resulting in prevention of cell proliferation. The present study was focused on finding the most effective nontoxic and ECM-preserving method to produce decellularized scaffolds.

The efficiency of cell removal, as well as preservation of the matrix integrity, is highly dependent on the decellularization method used. Cell remnant removal is necessary, because the presence of dead cells or cell remnant potentially lead to inflammation and/or calcification *in vivo* [29]. In the present study, we chose to decellularize PP with OGP, and different concentrations were investigated to optimize the appropriate decellularizing concentration of OGP and then compared with SDS and SDT decellularization methods. OGP, as a glucose-based surfactant and nonionic detergent, has a range of beneficial physical and performance properties, including very rapid biodegradability, low human and animal toxicity, and surface interactions, which synthetized by low cost (based on natural starting materials), renewable saccharide components and appropriate renewable (natural fatty acids) hydrophobic pendant groups. Various studies showed that nonionic detergents are mild and relatively nondenaturing, as they break lipid–lipid interactions and lipid–protein interactions rather than protein–protein interactions [30,31].

DNA content below a specified level and/or lack of visible nuclear material indicates that cells have been effectively removed. Thus, it should ensure that the tissue is essentially devoid of immunogenic active molecules [32]. It is considered that the potential adverse immune response can be elicited by xenogeneic DNA [18,32,33]. Keane *et al.* [18] have shown that the presence of xenogeneic DNA within ECM has been suggested as a possible cause of an "inflammatory response" in patients. Indeed, many commercial biologic scaffolds contain varying amounts of residual DNA [33]. Based on the adverse cell and host responses that have been avoided *in vivo*, the acceptance criteria of residual DNA can satisfy intended decellularization. Crapo *et al.* [12] pointed out that the DNA content should be lower than 50 ng dsDNA per mg ECM dry weight. In our study, the DNA content of PP decellularized by OGP and SDS is lower than 50 ng dsDNA per mg ECM dry weight. These data suggested that the OGP method might achieve an efficient decellularization.

The collagen fiber and elastin fiber architecture of an ECM scaffold plays an important role in determining its mechanical properties [8,24]. It is commonly believed that all methods of decellularization damage the architecture of ECM and cause loss of composition [12]. Because the collagen structure and elastin fiber of PP treated with SDS and SDT may be disrupted (Fig. 2C, G, K and Fig. 2D, H, L, respectively), the biomechanical properties of PP treated with SDS and SDT decreased markedly (Fig. 5). Although the elastic modulus of PP decellularized by OGP was lower than native PP, histology assessment, SEM observation, and tensile test showed that the OGP method did not alter the architecture of ECM by a large margin.

At present, there is no consensus on the effects of any individual decellularization agent upon mechanical properties. Zou and Zhang [15] reported that 0.1% SDS appeared to maintain critical mechanical and structural properties. However, Mendoza-Novelo *et al.* [26] reported that there is a reduction of almost 50% on tensile strength of bovine pericardium decellularized by SDS when compared with native bovine pericardium. In addition, Yang *et al.* [14] used 0.5% Triton X-100 combined with 0.5% sodium deoxycholate to decellularize bovine pericardium and found that the

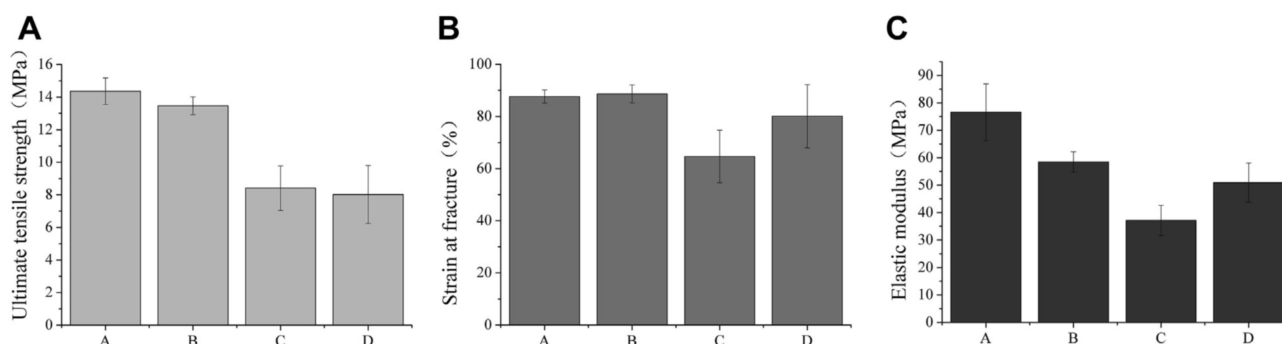

Fig. 5 – Tensile properties of PP decellularized by OGP (B), SDS (C), and SDT (D), and native PP (A). Ultimate tensile strength, strain at fracture, and elastic modulus of native PP and decellularized PP are presented. Data are presented as mean ± standard deviation, *$P < 0.05$ ($n = 5$ per group) compared with control (A).

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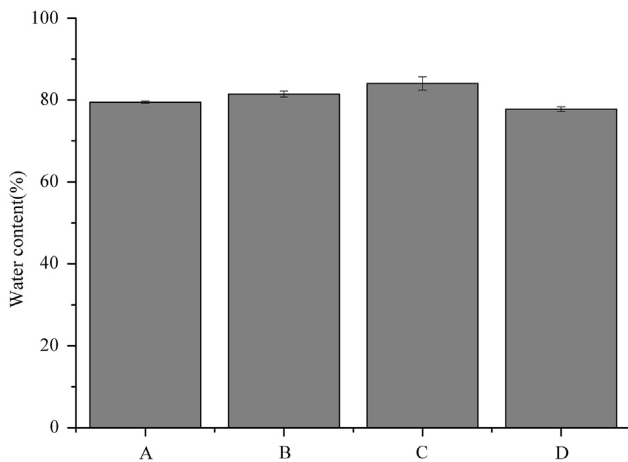

Fig. 6 – Water content per dry weight in PP treated with OGP (B), SDS (C), and SDT (D), and native PP (A) ($n = 5$ per group).

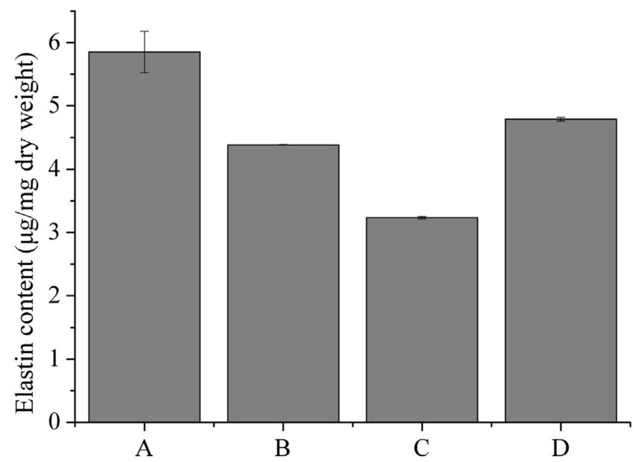

Fig. 8 – Elastin content per dry weight in PP treated with OGP (B), SDS (C), and SDT (D), and native PP (A) ($n = 5$ per group).

method changed the mechanical properties, with a lower elastic modulus, maximal stress, and maximal strain. Moreover, Wilczek [34] also demonstrated that a prolonged treatment with SDS leads to a denaturation of the collagen fibers. Differences in incubation time and SDS concentration used in the decellularization process will cause different properties of tissues. Liao *et al.* [13] performed 0.1% SDS on aortic valves for 48 h, and Zhou *et al.* [11] used 1% SDS on porcine hearts and bovine pericardium for 24 h. Fitzpatrick *et al.* [35] investigated the mechanical behavior of decellularized porcine aorta using uniaxial tensile tests as well. They used three decellularization protocols, Triton X-100, SDS, and a combination of Triton X-100 and sodium deoxycholate. Their results showed a decreased stiffness in all the decellularized samples with different decellularization protocols. There have been reports that SDS may have adverse effects when used to decellularize tissue [36]. Initial reports on the adverse effects of SDS (1% wt/vol) on the integrity of tissue matrices limited the use of this detergent for decellularization [37]. Thus, the specific mechanical testing that should be performed is dependent on the intended clinical application [6].

To determine whether the decellularization methods change ECM composition, biochemical assays were performed. Collagens are an indispensible component of ECM. Collagens, which constitute the main structural element of the ECM, provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development [4–8]. Collagen content of decellularized PP was not observed to be significantly different from that in native PP. The elasticity of tissue is achieved by a random network of elastic fibers embedded in the ECM. Elastin is highly flexible but has very low stiffness, surrounding the collagen fiber bundles [5,8]. Many researchers investigated the impact of different decellularization methods on elastin fibers. For example, Petersen *et al.* [25] reported that elastin was partially depleted from matrices decellularized by 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate *via* quantitative assay. Wong *et al.* [10] also reported that elastin content was significantly decreased in the presence of 0.1% (wt/vol) SDS. Similarly,

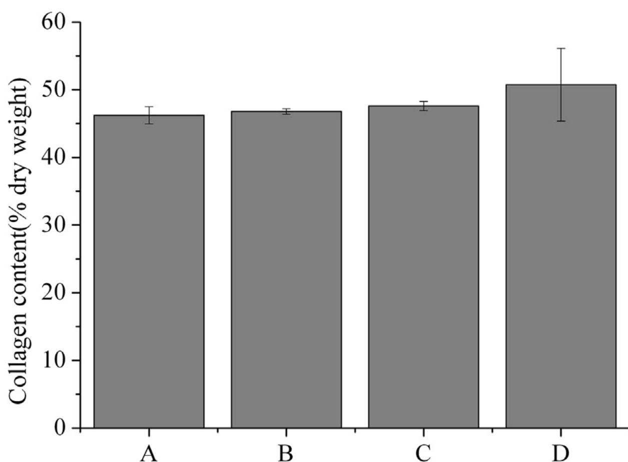

Fig. 7 – Collagen content per dry weight in PP treated with OGP (B), SDS (C), and SDT (D), and native PP (A) ($n = 5$ per group).

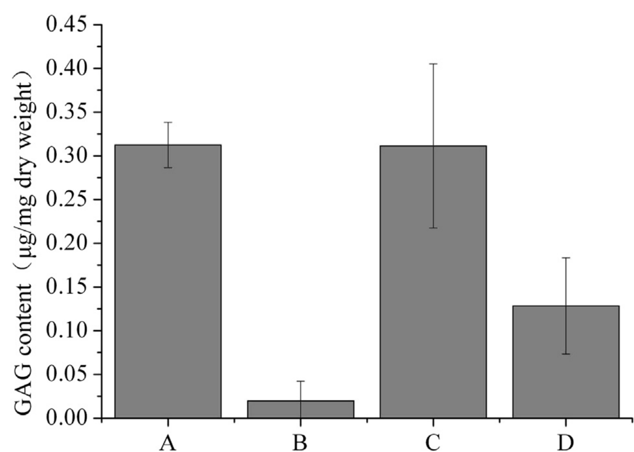

Fig. 9 – GAG content per dry weight in PP treated with OGP (B), SDS (C), and SDT (D), and native PP (A) ($n = 5$ per group).

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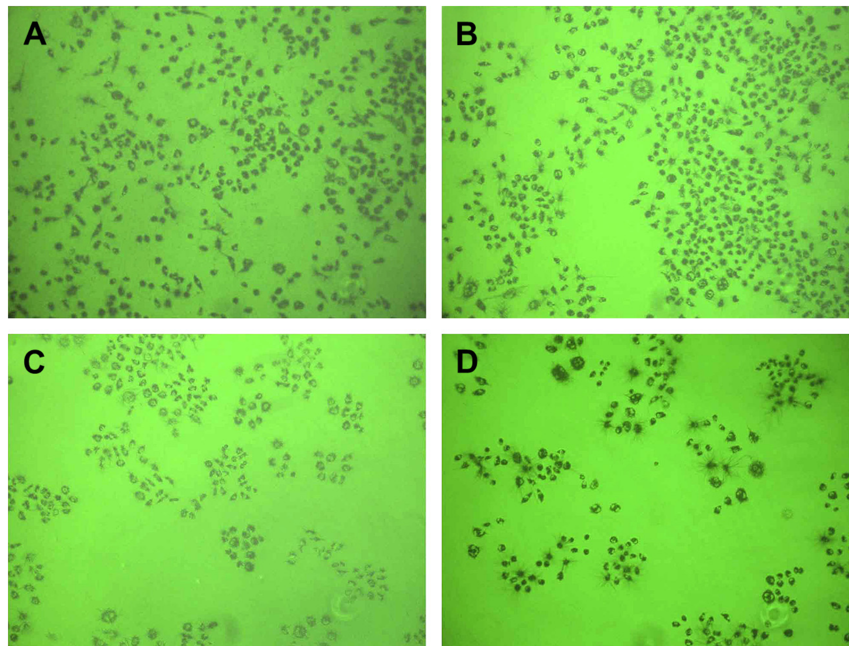

Fig. 10 – The number of living L929 fibroblasts cultured in the extraction liquid of PP decellularized by OGP (B), SDS (C), and SDT (D), and native PP (A) after adding MTT (magnification ×200). (Color version of figure is available online.)

Schenke-Layland *et al.* [38] showed a minor loss of elastin during the decellularization process. Consequently, in the present study, the decreasing elastin content of PP decellularized by OGP can be acceptable. The result of GAG content indicated that a significant amount of GAG was lost during the decellularization procedure. A similar finding was reported by Wong *et al.* [10] for bovine pericardium tissue antigen removal, Mendoza-Novelo *et al.* [26] for bovine pericardium decellularization, and Reing *et al.* [28] in porcine dermis. SDS has been reported to remove GAG from tissue [10,12,28]. The loss of GAG may benefit decellularization processing because the removal of GAG would allow increased access of the decellularization reagents to diffuse into the tissue [39].

Rieder *et al.* reported cytotoxicity of porcine aortic valves and leaflets treated with SDS (0.1% wt/vol) when tested using venous endothelial cells. The authors concluded that residual SDS in the tissue was the cause of cytotoxicity [40]. Therefore, in our study, although the cells in PP treated with SDS were removed completely, ECM scaffold had been destroyed; meanwhile, their mechanical properties remarkably decreased and residual SDS caused cytotoxicity (Fig. 10), which was in agreement with previous reports [36,37,40]. In summary, the decellularization process may affect the ultrastructure of ECM and tensile properties of tissues; thus, the purpose is to make this influence minimal. When PP samples were decellularized by 1% (wt/vol) OGP, cells were removed completely, the same structure as that of PP was maintained (Figs. 1 and 2), and the biochemical compositions of decellularized PP were well preserved (Figs. 7 and 8). Additionally, MTT tests demonstrated that OGP-treated tissues were nontoxic (Fig. 10). Furthermore, the results of uniaxial tensile tests showed that mechanical properties of PP decellularized by 1% (wt/vol) OGP were well maintained (Fig. 5), meeting the results of quantitative biochemical compositions of ECM. These results showed that the OGP method not only removed cells of PP adequately but also preserved the biomechanical properties of PP with nontoxic residual.

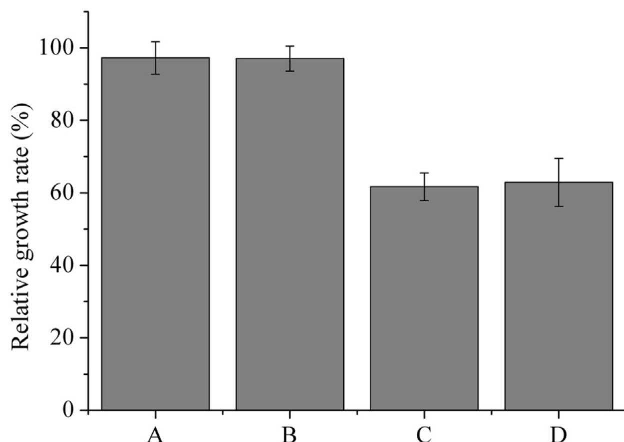

Fig. 11 – Relative growth rate of the L929 fibroblasts of PP decellularized by OGP (B), SDS (C), and SDT (D), and native PP (A) (n = 5 per group).

There were several limitations in the present study. Only tests of different concentrations of OGP were conducted, whereas processing times of the OGP procedure were not investigated. Since different effects of decellularization times are distinct [11,13,34], it is also important to study the influence on different decellularization times. Future *in vivo* implantation studies are therefore also required to demonstrate whether PP treated with OGP cause an immune response. Although there are limitations, we can say that the tissue decellularization with the novel method described in this study resulted in complete cell

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removal, the preservation of the structure, and hardly any cytotoxicity.

In conclusion, this work demonstrated the feasibility of using OGP as a novel decellularization reagent in the decellularization of biological tissues. The results suggest that the OGP method is superior to either the SDS method or the SDT method, as it achieves not only complete decellularization but also the preservation of both the mechanical function and the matrix structure of the tissues, and that it might be a suitable approach to construct tissue engineering heart valve scaffold. Moreover, the data obtained in the MTT assay imply that the cytotoxicity of PP decellularized by OGP is significantly lower than that of PP decellularized by SDS and SDT. The results obtained in this *in vitro* study demonstrate that OGP, as a new type of green nontoxic degradable detergent, can be used as a decellularization agent.

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