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Original Article

Comparison of various reagents for preparing a decellularized porcine cartilage scaffold

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Abstract: Cartilage lesion repair is difficult due to the limited self-repair capability of cartilage and its lack of vascularization. Our previous study established a sandwich model for engineering cartilage with acellular cartilage sheets (ACSs) and chondrocytes. However, there is still debate over which agent achieves the optimal decellularization of cartilage sheets. In addition, changes in the extracellular matrix after decellularization are worth studying. We aimed to determine the optimal decellularization reagents and decellularization time for preparing cartilage sheets. This study compared the effects of 2 extraction chemicals [t-octylphenoxypolyethoxyethanol (Triton X-100) and sodium dodecyl sulfate (SDS)] on cartilage sheets. The sheets were soaked in various concentrations (0.1-2%) of the extraction solutions for various time periods (24-72 h). After the decellularization process with the various treatments, we examined the cell removal and preservation of the matrix components and microstructure to determine which method was the most efficient while inducing minimal damage to the perichondrium. Both protocols achieved decellularization within an acceptable time. DNA analysis showed that the reagent removed nearly all of the DNA from the cartilage sheets. The growth factor contents in the Triton X-100 samples were higher than those in the SDS samples, quantified by enzyme-linked immunosorbent assay (ELISA). Furthermore, Triton X-100 decreased the glycosaminoglycan (GAG) and increased the chondromodulin-I contents compared with SDS. The results of a Cell Counting Kit-8 (CCK-8) assay revealed that the ACSs were not cytotoxic. In conclusion, our results demonstrate that cartilage sheets decellularized by 1% SDS for 24 h or by 2% Triton X-100 for 48 h may be suitable candidate scaffolds for cartilage tissue engineering.

Keywords: Cartilage, decellularization, detergent, acellular scaffolds, tissue engineering

Introduction

Cartilage lesions, generally caused by congenital abnormalities, trauma, or tumors, have long been common clinical problems [1]. Unlike most types of tissue, cartilage lacks access to abundant nutrients and circulating progenitor cells and possesses an avascular structure. These features contribute to the limited self-healing capacity of cartilage, making cartilage lesions highly challenging for clinicians to manage [2, 3]. Through the use of biodegradable scaffolds and cell replacement therapies, tissue engineering has become a promising approach for cartilage regeneration [4-6]. However, as cartilage is a compact tissue, the removal of native cells and the migration of seed cells into the scaffolds are difficult [7]. Thus, in our previous studies, we established a sandwich model for effectively engineering cartilage in vitro and in vivo. Cartilage from the ears of adult pigs was cut into cylinders with a diameter of approximately 6 mm and freeze-sectioned at a thickness of 10 μm, and the sheets were then decellularized and lyophilized. The chondrocytes isolated from newborn pig

Table 1. Chemical treatments for decellularization

Group	Con	S1	S2	S3	T1	T2	T3
Concentrations of SDS (%)		0.1	0.5	1			
Concentrations of Triton X-100 (%)					0.1	0.5	2
Time (h)		72	72	24	72	72	48

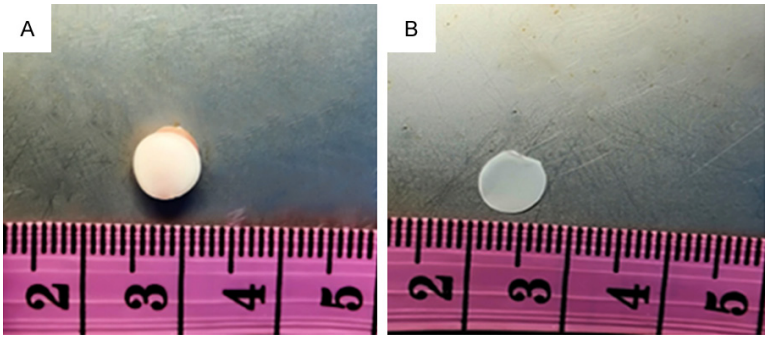

Figure 1. Characterization of pig ear cartilage. A. Macroscopic view of pre-shaped cartilage before sectioning; B. Cartilage sheets were achieved by freeze-sectioning the shaped cartilage at 10 mm thicknesses.

ears were expanded for 2 passages, and the acellular sheets and chondrocytes were then stacked layer-by-layer in a sandwich model and cultured in dishes. This model overcomes the decellularization and cell-seeding problems of using acellular cartilage sheets (ACSs) for cartilage engineering [2]. In addition, we demonstrated that ACSs possess chondrogenic induction activity, which promotes bone marrow stromal cell (BMSC) differentiation, and that the ACSs possess anti-angiogenic activity that can stabilize the engineered cartilage in vivo. Compared to synthetic materials, ACSs may be better scaffolds for cartilage engineering [8]. By cutting donor cartilage into sheets, the original chondrocytes may be able to be removed from the sheets by treating with 1% sodium dodecyl sulfate (SDS) for 24 h, as in our previous study [2].

When used in the decellularization process, SDS results in the denaturation of protein structures [9], which may also destroy the protein function. Therefore, decellularization techniques that are able to preserve functional proteins are worth investigating, and the development of other chemical protocols is clearly needed. Chemical decellularization is a method that primarily relies on chemicals to lyse and remove cells and their components from the surrounding extracellular matrix (ECM). Chemicals frequently used for decellularization include SDS and t-octylphenoxypolyethoxyethanol (Triton X-100) [10, 11]. In addition, various formulations of trypsin and tri-n-butyl phosphate (TnBP) are also commonly used to remove nucleic acids from the material [12, 13]. However, no standard treatment method is available for the decellularization of cartilage sheets. Here, we investigated the decellularization effect of chemical detergents. Cartilage from adult pig ears was harvested, cut into sheets, and decellularized by treatment with various concentrations of SDS and Triton X-100 for various times. The decellularization efficiency was investigated by histology and the preservation of matrix components was examined by an enzyme-linked immunosorbent assay (ELISA). Taken together, the purpose of this study is to thoroughly characterize the effects of SDS and Triton X-100 removal protocols on the decellularization of a porcine osteochondral scaffold.

Materials and methods

Cartilage sheet preparation

Porcine ear samples were obtained from a local abattoir in Wenzhou, China. Cartilage was harvested from adult pig ears, and all surrounding tissues were carefully removed using a scalpel. The cartilage was then cut into cylinders with a diameter of 7 mm, and cartilage sheets were then acquired by freeze-sectioning at a thickness of 10 μm. All the experimental protocols were performed in strict accordance with the Animal Care and Use Committee of Wenzhou Medical University.

Decellularization methods

We examined different concentrations of 2 chemical detergents for different treatment times (**Table 1**). After SDS (Amresco, Solon, OH) and Triton X-100 (Solarbio) treatment, the sheets were rinsed 5 times with sterile water and lyophilized in a vacuum freeze-dryer (Virtis

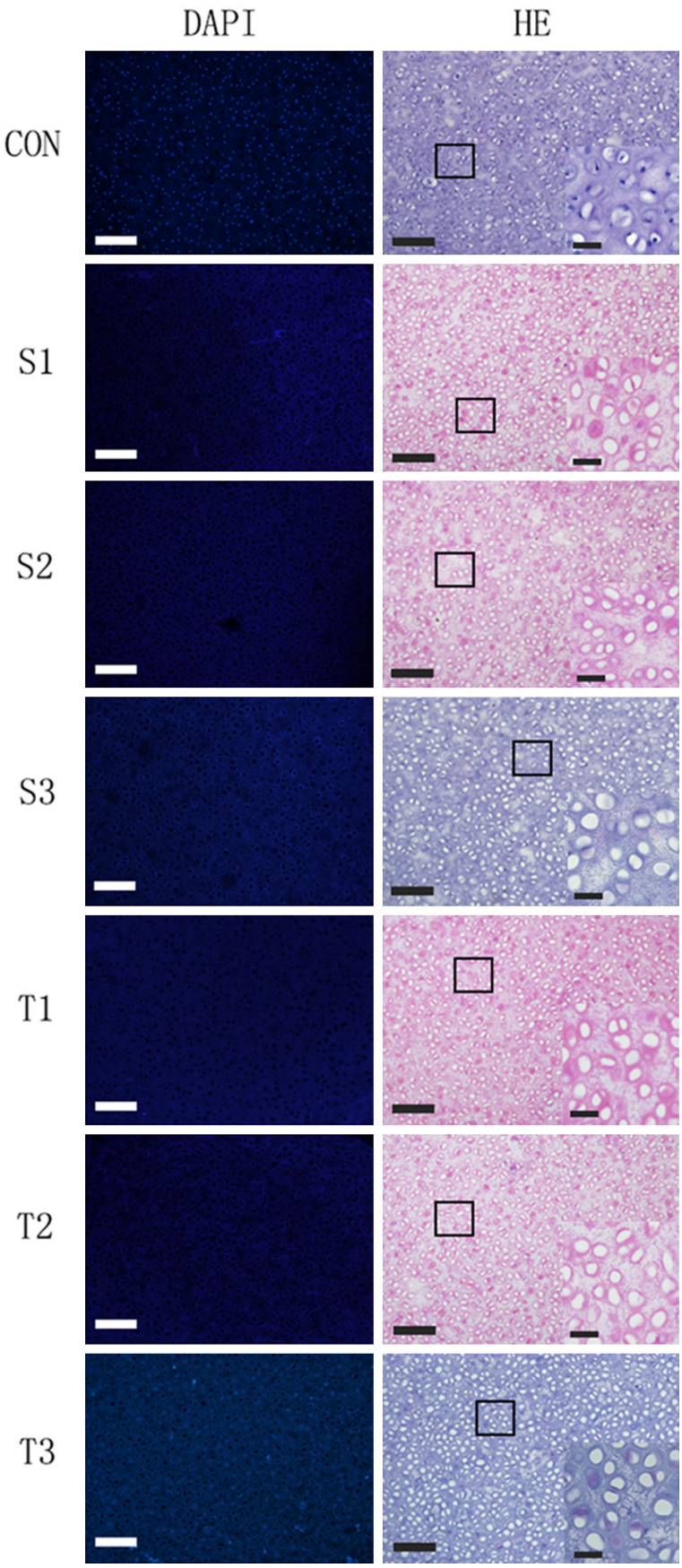

Figure 2. DAPI and H&E staining of the cartilage sheets. The cartilage sheets were treated with different groups (con, S1, S2, S3, T1, T2, T3). No DAPI-positive cell nuclei were observed after treatment. The H&E staining results showed that the cell components were present in the control group, while they were completely removed after decellularization. Scale bars in the low-magnification pictures: 400 μm. Scale bars in the high-magnification pictures: 50 μm.

Benchtop 6.6, SP Industries, Gardiner, NY).

Histology

Decellularized and undecellularized tissue specimens (n = 20) were mounted on glass slides. Hematoxylin and eosin (H&E) staining was used to evaluate the cellular content and general structure of the cartilage sheets. Cell nucleus staining was performed by staining sheets with 40,6-diamidino-2-phenylindole (DAPI, Biomol, Plymouth Meeting, PA). The cartilage cells were visualized by toluidine blue staining, and the sheets were stained with safranin O to visualize the glycosaminoglycan (GAG) deposits.

Quantification of DNA

To determine the DNA content in the cartilage sheets as previously described [14], the DNA was first extracted from fresh or decellularized cartilage sheets by using a DNA isolation kit for tissues (Roche Applied Sciences, Indianapolis, IN, USA). Then, the DNA content was quantified by measuring the absorbance at 260/280 nm using a Nanodrop spectrophotometer (Labtech, Ringmer, UK).

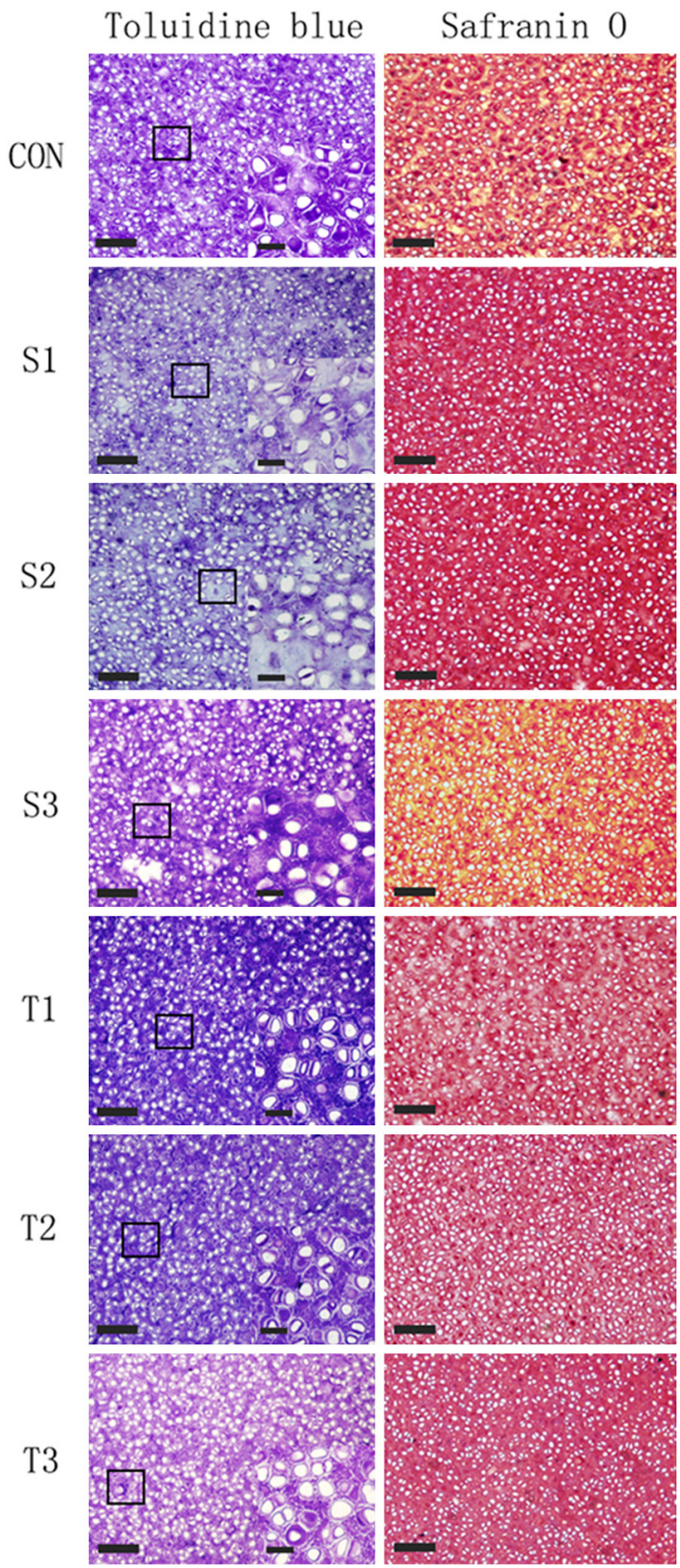

Figure 3. Toluidine blue and safranin O staining of the cartilage sheets. The cartilage sheets were treated with different groups (con, S1, S2, S3, T1, T2, T3). The toluidine blue and safranin O staining results showed that in the decellularized groups, some cell matrix could be observed. Scale bars in the low-magnification pictures: 400 μm. Scale bars in the high-magnification pictures: 50 μm.

Growth factor, GAG, collagen II and chondromodulin-I ELISA

To extract growth factors from decellularized cartilage sheets, 50 sheet pieces were suspended in 1 mL of extraction buffer (0.5 M acetic acid, 50 mM Tris-HCl (pH 7.4) and 0.1x protease inhibitors), followed by stirring at 4°C for 3 days. To extract GAG, collagen II and chondromodulin-I, a suitable amount of sample tissue was mixed with 500 μL of phosphate-buffered saline (PBS) and homogenized. The mixture was then centrifuged at 12,000 rpm/min for 30 min at 4°C, and the supernatant was collected for protein quantification and ELISA. The amounts of transforming growth factor-β1 (TGF-β1), insulin-like growth factor-1 (IGF-1) and bone morphogenic protein-2 (BMP-2) in the extraction solution were measured by ELISA kits purchased from R&D Systems. The GAG, collagen II and chondromodulin-I contents were measured by ELISA kits (Shanghai Boyun Biotech Co., LTD, China) following the same protocol.

Cell isolation and culture

Primary chondrocytes were isolated from the knee cartilage of mice as previously described [15]. Briefly, cartilage pieces were collected under aseptic conditions by a

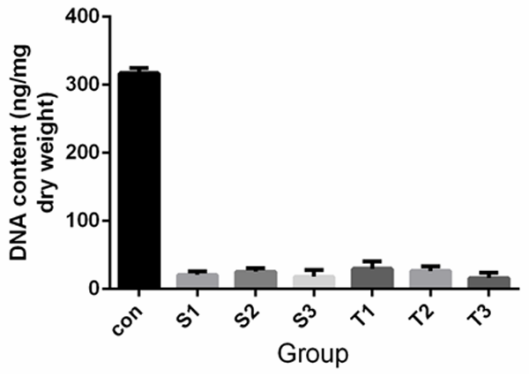

Figure 4. Content of DNA in cartilage sheets. The cartilage sheets were treated with different groups (con, S1, S2, S3, T1, T2, T3). The results showed the near-complete removal of the DNA from the decellularized cartilage sheets. There was a significant ($P<0.01$) reduction in DNA in all decellularized samples compared to fresh cartilage sheets.

dissecting microscope, and digested with 0.1% collagenase type II for 4 h at 37°C. Then the solution was centrifuged at 1000 rpm for 5 min and the supernatant was discarded. Finally, the solution was suspended and seeded onto culture dishes in DMEM with 10% fetal bovine serum (FBS, HyClone) in a humidified atmosphere of 5% CO_2 at 37°C. Cells at passage 3 were used for further experiments.

Scanning electron microscopy (SEM)

Decellularized or control cartilage sheets were loaded onto copper plates, coated with gold, and then examined under a field emission scanning electron microscope (S-3000N, HITACHI, Japan). The morphological changes were compared among the groups.

Cytotoxicity assay

The cytotoxicity was evaluated by Cell Counting Kit-8 (CCK-8; Sigma) assays following the instructions of the supplier. Briefly, decellularized or control cartilage sheets (treated with double distilled H_2O_2) were placed on the bottoms of 96-well plates, and chondrocytes were seeded directly onto the sheets at a density of 5×10^3 cells/well, with each well containing 200 μL of medium at 37°C in a 5% CO_2 atmosphere. After 24 h, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was added to each well, and the plate was incubated for 1 h at 37°C with 5% CO_2. The optical density (OD) absorbance was read using a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00, (GraphPad Software, CA, USA). Significant differences between two groups were determined by multiple t-tests corrected for multiple comparisons using the Holm-Sidak method. $P<0.05$ was considered statistically significant.

Results

Preparation and characterization of ACSs

Cartilage sheets were achieved by freeze-sectioning the shaped cartilage at 10-mm thicknesses (**Figure 1**).

Upon DAPI staining, DAPI-positive cell nuclei were obtained in the control group. H&E staining revealed many cells scattered among the cartilage spaces in the cartilage sheets. The cell components were completely removed in both types of sheets after decellularization with different concentrations of the 2 chemical detergents for different treatment times (**Figure 2**). Some cell matrix could be observed in the decellularized groups, following toluidine blue and safranin O staining (**Figure 3**).

Quantification of DNA

The results showed the near-complete removal of DNA from the decellularized cartilage sheets. There was a significant ($P<0.01$) reduction in the DNA content in all decellularized samples compared to fresh tissue (**Figure 4**). Meanwhile, there was no significant ($P>0.05$) difference between the DNA content in the cartilage from the SDS-treated and Triton X-100-treated groups.

SEM

SEM was used to analyze the morphological changes before and after treatment. Empty lacunar structures were observed in all cartilage sheets, and the surface of the cartilage sheets appeared to remain intact after decellularization (**Figure 5**).

Cytotoxicity assay

No effects on cellular proliferation were found in the S3 and T3 groups (**Table 1**), as there

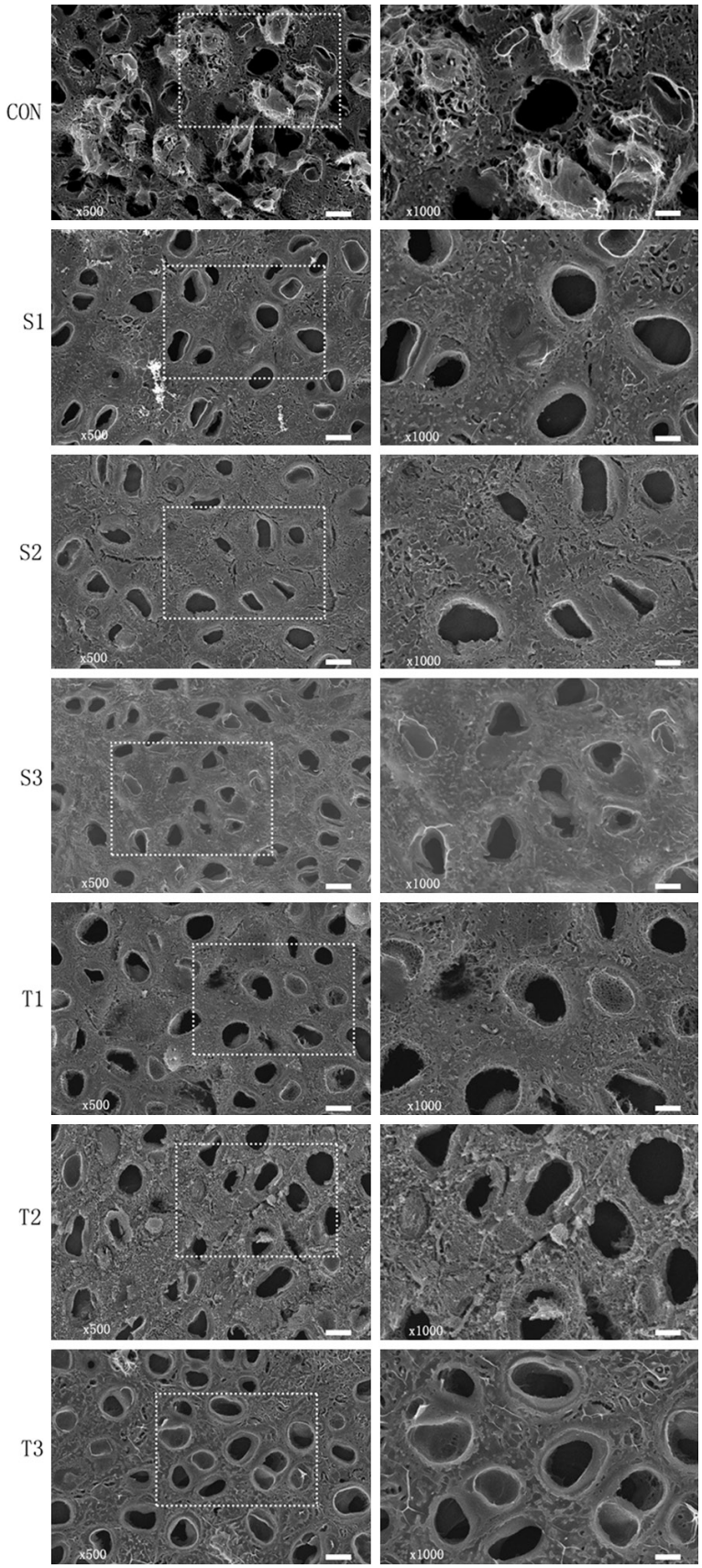

Figure 5. Scanning electron micrographs of the cartilage sheets. The cartilage sheets were treated with different groups (con, S1, S2, S3, T1, T2, T3). In the control samples, abundant matrix could be observed on the sheets, while empty lacunar structures were observed in all cartilage sheets, and that the surface of the cartilage sheets appeared to remain intact after decellularization. Scale bars in the low-magnification pictures: 200 μm. Scale bars in the high-magnification pictures: 100 μm.

were no differences in the OD values among the 3 groups at each time. Thus, the decellularized cartilage sheets were not cytotoxic (**Figure 6**).

Quantification of growth factors

To detect whether growth factors remained in the cartilage sheets after decellularization extracts from decellularized cartilage sheets and unprocessed sheets were analyzed for TGF-β1, IGF-1 and BMP-2 by ELISA. The amounts of growth factors in the decellularized group decreased with a longer decellularization time. The IFG-1 and BMP-2 contents in the Triton X-100 samples were higher than those in the SDS samples ($P<0.01$) (**Figure 7**).

Quantification of GAG, collagen II and ChM-I

The GAG, collagen II and ChM-I contents exhibited greater losses over 72 h of decellularization (**Figure 8**). The GAG content was lower in the Triton X-100 samples than in the SDS samples (**Figure 8A**). In contrast, the ChM-I content was higher in the Triton X-100 samples than in the SDS sam-

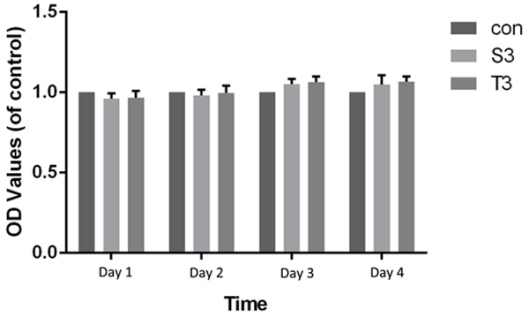

Figure 6. The cells were cultured with different groups (con, S1, S2, S3, T1, T2, T3) for several days (1, 2, 3 or 4 days). The cytotoxicity was determined by a CCK-8 assay. The values are mean ± SD. * = P<0.05, ** = P<0.01 compared with the control group.

ples (**Figure 8C**). The data are presented as the means ± SD. * = P<0.05 ** and *** = P<0.01 for comparisons between groups).

Discussion

Scaffolds provide a 3-D structure for the growth of cells in tissueengineering. Theoretically, acellular matrices derived from the same type of tissue would make ideal scaffolds for engineering new tissue. Many acellular matrices have been used as scaffolds for tissue engineering [10, 16-18]. Furthermore, in compact tissues, the removal of native cells and migration of seed cells into the scaffolds are difficult. By cutting the tissue into pieces, we could easily remove the cells with a very gentle treatment while maximally preserving the natural components, including the activity of some important factors, in the matrix. Several methods of decellularization, including physical [19], chemical [20] and enzymatic [12, 17] treatments, have been reported in the literature. Studies have shown that the efficiency of a particular decellularization protocol is dependent on the tissue of interest [21]. The present investigation analyzed the effects of 2 common chemical agents on the cartilage. Detergents such as SDS and Triton X-100 are commonly used to decellularize tissues [10, 22]. In previous reports of decellularization, SDS and Triton X-100 were reported to remove cells well from tracheal [23] and nasal septal cartilage [24]. However, how the extracellular matrix components, such as growth factors and ChM-I, change after decellularization has not been further studied. In this study, we explored the use

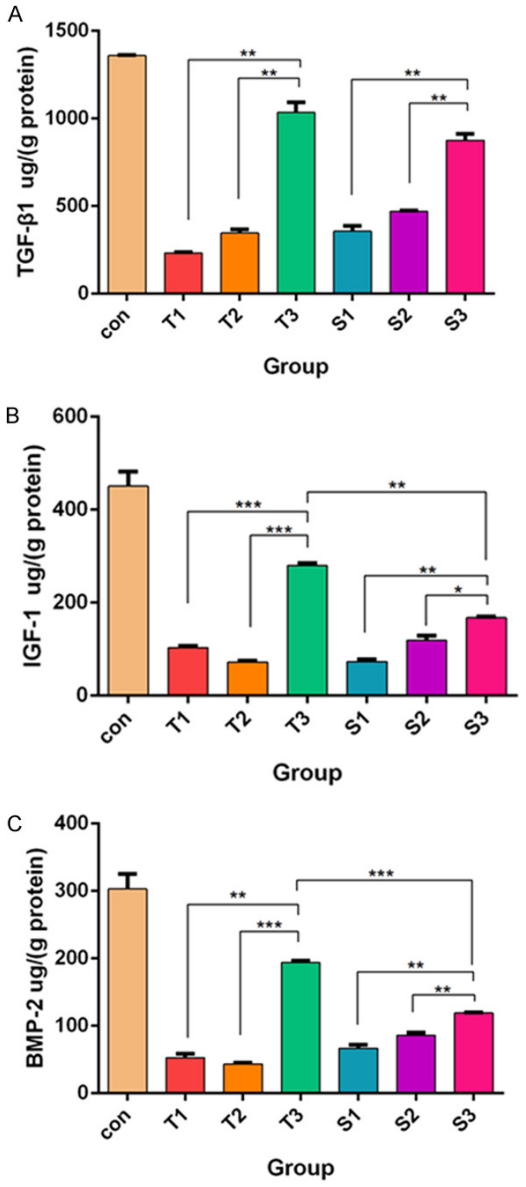

Figure 7. Content of growth factors in ACS extracts by ELISA. The cartilage sheets were treated with different groups (con, S1, S2, S3, T1, T2, T3). The ELISA results showed that the groups of T3 and S3 contained higher contents of growth factors (TGF-β1, IGF-1, BMP-2), with the T3 group exhibiting the highest growth factor content. Data are the mean ± SD. * = P<0.05 ** and *** = P<0.01 for comparisons between groups.

of an anionic detergent (SDS) and a nonionic detergent (Triton X-100) to decellularize pig ear cartilage sheets and compared the histological structure and biological properties of decellularized cartilage sheets as ideal scaffolds for cartilage tissue engineering.

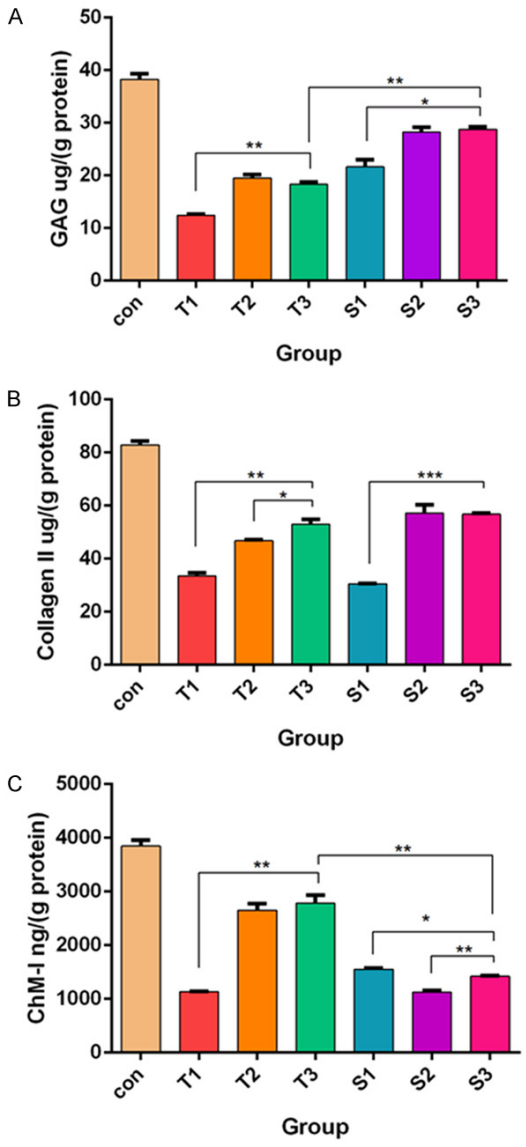

Figure 8. Quantification of GAG, collagen II and ChM-I by ELISA. The cartilage sheets were treated with different groups (con, S1, S2, S3, T1, T2, T3). The ELISA results showed that the GAG, collagen II and ChM-I content exhibited losses following 72 h of decellularization. The GAG content was lower in the Triton X-100 samples than in the SDS samples, while the ChM-I content was higher.

As cells are the main immunogenic factors in tissue, the immunogenicity of the acellular matrices must be eliminated before such matrices can be used for tissue engineering. Therefore, evaluating the decellularization efficiency of detergents on cartilage sheets is a prerequisite for further studies. We established different concentrations and time gradients of SDS and Triton X-100 and sought to determine the most suitable time period for the removal of cells from the matrix for each tested concentration. The results of H&E, DAPI and toluidine blue staining showed that almost no cells remained in the cartilage sheets in the groups treated with S1-3 and T1-3 (**Table 1**). The results of DNA quantification showed that the DNA was nearly completely removed, which was consistent with the histology assay. Moreover, for the same concentrations, the decellularization efficiency of SDS was superior to that of Triton X-100 when comparing the S3 and T2 groups. Interestingly, for concentrations of only 0.1% and 0.5%, the optimal decellularization times for SDS and Triton X-100 were approximately the same. We recommend that when the cell-free reagent concentration is low, the length of time should be the main factor influencing the decellularization. In addition, 1% SDS and 2% Triton X-100 required less time for decellularization than the other lower concentrations. Therefore, the concentrations of the detergents affect the decellularization efficiency, and achieving a suitable concentration of SDS or Triton X-100 played an important role in the decellularization. However, whether to increase the concentration of chemical agents or prolong the decellularization time remains controversial, as the structure of the cartilage sheets could be destroyed. The optimal solution is to find a balance between the concentration and time. In addition, SEM revealed well-preserved empty lacunar structures in the decellularized groups compared with the CON group.

A functional scaffold not only can support cell growth but can also promote the formation of new tissue [25]. Collagen II and GAG are the main components of the ACS ECM and are absolutely necessary in guiding cellular survival, attachment, proliferation, differentiation, and migration [26]. The ideal decellularized ACS ECM should contain similar collagen II and GAG contents to those of natural cartilage sheets. Cartilage sheets are a proteoglycan-rich ground substance [27], and the result of safranin O staining showed that both the control cartilage sheets and decellularized cartilage sheets were rich in proteoglycans. We calculated the GAG and collagen II contents in the test samples by ELISA. The GAG exhibited varying degrees of decline after decellularization, with the most GAG losses in the T3 Triton X-100 group and the S3 SDS group; however, the S3

group contained a significantly greater GAG content than the T3 group. There was no significant difference in collagen II between the two groups. In addition, the collagen II protein was well retained in the T3 and S3 groups, which indicated that the collagen II loss during the decellularization process is related to the treatment time. Therefore, the T3 and S3 groups yielded optimal results.

Although cartilage expresses many angiogenic factors during endochondral ossification, it is an avascular tissue. The cartilage-specific noncollagenous matrix protein chondromodulin-I (ChM-I) has been shown to be a strong angioinhibitor [28], and the loss of ChM-I from the articular cartilage might be partly responsible for promoting blood vessel invasion in the cartilage during the progression of OA [29]. Therefore, ChM-I may play an essential role in determining the avascular nature of the cartilage scaffold. The results showed that ChM-I content was generally higher in the groups decellularized with Triton X-100 than in those decellularized with SDS. The reason for this may be that the chemical properties of the decellularizing reagents exert a larger influence on the ChM-I content. The scaffolds decellularized with Triton X-100 exhibited an obviously softer appearance, and the T3 group of scaffolds achieved better results.

In addition, some growth factors, such as TGF-β and IGF, are known factors produced by mature chondrocytes that have chondrogenic potential [30-32]. More importantly, our data show that ACSs contain a certain amount of growth factors, including TGF-β1, IGF-1 and BMP-2 (**Figure 7**). These matrix and growth factors are known to possess chondrogenic induction activity. Upon comparing the Triton X-100 and SDS groups, the results showed that the decellularization time had a greater influence on the cytokine levels. In addition, the T3 and S3 groups contained higher contents of growth factors, with the T3 group exhibiting the highest growth factor content.

We also tested the biocompatibility of the treated specimens, which is the most important characteristic of decellularized scaffolds for tissue engineering.

A wide variety of chemicals are used during the decellularization process. Chemicals remaining in the tissue after decellularization may be toxic to the reseeding cells when the scaffold is implanted in vivo. Therefore, we extensively washed the specimens with sterile water 5 times at the end of the decellularization procedure to clear any residual reagents and then evaluated the toxicity of the scaffolds by CCK-8 assays. The results showed that the scaffold extracts had no effect on cellular proliferation, which indicated that the scaffolds were noncytotoxic and that the residual reagents were successfully removed.

Our preliminary data revealed that the decellularization of cartilage sheets with Triton X-100 and SDS could remove almost all cells from the sliced tissue, and such decellularized cartilage matrices could be ideal for cartilage engineering. The cytokine and ChM-I activities remain to be further tested.

In conclusion, this investigation is the first to introduce the possibility of using cartilage sheets decellularized with 2 different agents as tissue-engineered cartilage scaffolds. In light of the many protocols used in the literature to remove cellular content from ex vivo tissues, our study investigated different concentrations of 2 popular chemical treatments applied for different amounts of time in an effort to determine which decellularization method was the most effective in preserving the ECM integrity of the native tissue. Overall, a 1% SDS treatment for 24 h and 2% Triton X-100 treatment for 48 h retained the majority of the ECM components after a thorough cell removal, preserved the empty lacunar structures, retained the growth factors and ChM-I, and demonstrated favorable biocompatibility. Therefore, the cartilage sheets treated via this strategy are suitable candidate scaffolds for use in cartilage tissue engineering. In vivo studies are needed to further determine the potential of this decellularization method for cartilage tissue engineering.

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Disclosure of conflict of interest

None.

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