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Original Article

Establishment of optimal decellularization conditions using porcine placenta

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ABSTRACT Organ transplantation is currently the most fundamental treatment for organ failure, but there is a shortage of organ supply compared to those in need. Regenerative medicine has recently developed a decellularization technique that overcomes the limitations of conventional organ transplantation and attempts to reconstruct damaged tissues or organs to their normal state. Several decellularization methods have been suggested. In this experiment, the decellularization methods were used to find effective decellularization methods for humanlike porcine placenta. The optimal conditions for decellular support are low DNA content and high glycos amino glycans (GAGs) and collagen content. In order to satisfy this condition, SDS and Triton X-100 and SDS + Triton X-100 were used as the detergent used for decellularization in this experiment. The contents were compared according to the decellularization time (0, 12, 24, 48 and 72 hours), and the concentrations of SDS (0.2, 0.5, 0.7 and 1.0%) were mixed in 1.0% Triton X-100 to analyze the contents. When decellularized using SDS and Triton X-100, respectively, it was confirmed that the contents of DNA and GAGs were opposite to each other. And decellularization treatment for 24 hours at 0.5% SDS was able to obtain an effective decellular support. If decellularization studies of various detergents can be obtained an effective decellular support, and furthermore, cell culture experiments can confirm the effect on the cells.

Keywords: decellularization, DNA, extracellular matrix, glycos amino glycans, porcine placenta

INTRODUCTION

Organ transplantation is the most fundamental treatment method for late organ insufficiency at present. However, organ transplants are in short supply of organs compared to those in need, so many patients continue their lives with conservative treatment. Regenerative medicine is a fundamental treatment that overcomes the limitations of conventional organ transplantation and seeks to reconstruct damaged tissues or organ to their normal state. Recently, organ cell removal techniques have been developed to produce scaffolds with micro-vessels similar to living bodies. It has been reported that this may be an alternative to solve existing support issues (Chun et al., 2015). The placenta is an organ that supplies nutrients to the fetus while maintaining close contact between the

fetus and maternal tissue and contains various nutrients and hormones necessary for growth (Lee et al., 2009). The placenta is an organ rich in extracellular matrix (ECM) and an important source of stem cells. The placenta is composed of fetal and maternal parts and is closer to the fetus and uterus, respectively, and can be classified according to distinct parameters such as overall anatomical structure, fetal-maternal border type, and fetal maternal interactions. ECM of the decellularized placenta has been clinically applied for soft tissue reconstruction, such as in the treatment of patients with tumor resection. It has also been demonstrated that decellularized placenta has matrix components similar to those found in skin, growth factors involved in skin wound healing, and bioactive molecules, placental ECM. In addition, the placenta can be used as a xenograft, allogeneic and even autologous implant and has a wide range of applications (Leonel et al., 2018).

Decellularization is a new method of producing artificial scaffolds by removing cells from whole organs while maintaining their original structure (Maghsoudlou et al., 2013). Biocompatible materials based on decellularized tissues are increasingly attracting attention as functional replacements for other materials. Because, these decellularized substances are naturally derived and inherently support a variety of cellular functions. For example, the arrangement of proteins and tissue fibers in the ECM supports cell migration and proliferation, including gene expression and intercellular interactions (Schneider et al., 2016). ECM of decellularized tissues and/or organs has been widely used as a biological biomaterial for tissue engineering purposes (Leonel et al., 2018). ECM confirmed low interspecies immunogenicity and potential biocompatibility of decellularized tissues (Pan et al., 2014). The long-term goal of decellularization is to focus on functional regeneration of organ functions and ultimately on clinical transplantation of regenerated bioartificial organs, and to achieve transmission of tissues and organs across species (Guyette et al., 2014; Pan et al., 2014). In order to achieve decellularization satisfactorily, the cellular components of the tissue must be completely removed and the ECM used as the tissue scaffold must be preserved as completely as possible while maintaining the physical properties of the tissue. In addition, various cleaning agents or chemicals used in the process should be completely removed (Park et al., 2008). The tissues, organ types, decellularization methods, and terminal sterilization methods for these biological scaffolds from which ECMs are recovered vary widely (Gilbert et al., 2006). Optimal decellularized organ support tissue-specific factors, such as cell density, matrix density, and geometric considerations, including tissue thickness and shape, vary from tissue to organ (Keane et al., 2015).

The final goal of decellularized organ support is low DNA content and high protein and sugar content such as collagen and glycos amino glycans (GAGs). If a large amount of DNA remains in the support, it causes an immune response when transplanted into the patient. Lowering DNA content is effective, and proteins such as collagen and elastin determine the physical properties of tissues, and glycoproteins act as adhesives to attach cells and extracellular matrix, protein polysaccharides maintain tissue shape and volume (Min et al., 2011). In addition, GAGs contain sulfuric acid groups, and when charged with negative charges, they act as lubricants for joint tissues, showing moisturizing effect and viscosity. This biochemical property protects the tissue by absorbing or dispersing the pressure applied to the tissue (Ahn et al., 2003). Therefore, in order to make decellularized organ scaffolds, a low DNA content and a high content of collagen and GAGs are optimal decellularized organ scaffolds (Chae et al., 2014). There are various methods such as physical and chemical decellularization, and chemical methods are preferred in term of decellularization effects. The most commonly used solutions are Triton X-100 and sodium dodecyl sulfate (SDS). Since these two solutions are reported differently depending on the application organs, it is necessary to select a decellular solution suitable for making a scaffold.

Therefore, in this experiment, we applied existing decellularization methods advanced decellularization method is more convenient and minimize tissue damage, and find a decellularization method that can increase collagen and GAGs by minimizing DNA content, based on this, an attempt was made to establish conditions for obtaining an effective decellularized organ support.

MATERIALS AND METHODS

Porcine placenta preparation

For this experiment, the porcine placenta was recovered in the slaughter process in cooperation with Samse

slaughterhouse in Yeongcheon and immediately stored frozen at -20℃ and moved to the laboratory. Porcine placenta collected in the laboratory was removed blood using a rotary shaker (Hanna Instruments, USA) for 1 hour using 100 mL heparin (50 IU/mL). After blood removal, the remaining blood and solvent were washed with phosphate buffered saline (PBS) for 10 minutes. 1.0% antibiotics (penicillin/streptomycin) were added to 0.9% saline to remove the odor for 30 minutes and washed. Then, decellularization of the porcine placenta was performed, and the unused porcine placenta was stored in 4℃ in PBS containing 1.0% antibiotics.

Experimental design

This study was conducted to find more effective decellularization conditions of porcine placenta using porcine placenta. The experiment was conducted by designing three conditions as follows.

1) Changes in placenta shape, total DNA and DNA staining with different decellularization times, 2) GAGs and collagen contents of porcine placenta according to decellularization detergent, 3) DNA content and GAGs content of porcine placenta decellularized by mixing 1.0% Triton X-100 in each concentration of SDS

Antibiotics were not used during the decellularization process, and the porcine placenta (0 hour) without decellularization was used as a control, comparative experiments were performed using porcine placenta (12, 24, 48 and 72 hours) decellularized with detergent. After decellularization, the placenta were washed two more times for 10 minutes in 100 mL of PBS to minimize residual solvents or detergents.

Decellularization of porcine placenta

The detergents used in the experiment were all dissolved in PBS and used at the proper concentration. Porcine placenta prepared for decellularization of porcine placenta was cut into 10-11 g and used for the experiment. Then, 100 mL of the cleanser was added to the placenta of porcine and decellularized by 4℃, speed 50-60 using a rotary shaker. After decellularization treatment, the placenta were washed in PBS for 10 minutes, and when the washing was completed, the placenta were placed in PBS containing 1.0% antibiotic and stored at 4℃.

Freeze-drying of decellularized porcine placenta

Freeze-drying of the decellularized porcine placenta was transferred to the placenta stored at 4℃ to dish and then removed PBS as much as possible, and freeze-dried for 2 to 3 days at -50℃ using a freeze dryer (Kang et al., 2014). The freeze-dried placenta was pulverized into powder form using liquid nitrogen, and the extracellular matrix was used for component analysis. The remaining placenta was placed in an eppendorf tube and stored frozen at -20℃.

Histological analysis of decellularized porcine placenta

After decellularization, the placenta were washed in PBS and fixed in 10% formaldehyde for one day to perform histological and other necessary tests. Decellularization and ECM preservation were monitored for the presence and concentration of nucleic acid residues by hematoxylin-eosin (H&E) staining. Porcine placenta fixed in 10% formaldehyde was washed with PBS for 12 hours and treated with tissues, and then paraffin tissue blocks were prepared. After that, using a cutting machine at first cut to about 10 μm and then cut out to 4-6 μm if all the sides come out well. After dipping the cut tissue in 50% ethanol using a slide to expand the wrinkles, and dissolved paraffin for 1-2 hours in a dry oven at 60℃ and then stained with xylene, hematoxylin, eosin.

Analysis of extracellular matrix (ECM) components in decellularized porcine placenta

ECM includes collagen, elastin, and complex polysaccharides, which affect cell survival, adhesion, differentiation, chemotaxis and induce long-term reconstruction. In this experiment, the degree of decellularization was measured by measuring DNA content, glycos amino glycans (GAGs), and collagen in lyophilized decellularized placental scaffolds. DNA content was measured using a nanodrop kit (Thermo Scientific, USA) and GAGs were absorbed at 525 nm using a dimethylemethylene blue assay kit (Bio-protocol, USA). Collagen content was measured by absorbance using hydroxyproline assay kit (Cell Biolabs, USA) using 540-560 nm as the basic wavelength.

Statistical analysis

In order to eliminate the subjective view of the experiment, placement and observation were carried out in a complete batch method. The standard deviation (± SD)

and significance test for discrete variables were tested using the SAS (statistics analytical system, wersion 9.4, USA) program. The significance between each treatment group was tested at the 5% level using Duncan's multiple test.

RESULTS

Changes in placenta shape, total DNA and DNA staining with different decellularization times

Decellularization was treated for 0, 12, 24, 48 and 72 hours to determine DNA content changes in porcine placenta. Table 1 shows the DNA contents of porcine placenta according to decellularization time of porcine placenta. After decellularization, placenta decellularized in sodium dodecyl sulfate (SDS) for 12 and 24 hours (10.24 and 9.36 ng/μg) showed significantly lower DNA content than Triton X-100 (30.65 and 30.10 ng/μg) and SDS + Triton X-100 (14.18 and 16.02 ng/μg, $p < 0.05$). As a result, it was confirmed that when decellularized with SDS, a large amount of DNA could be removed. This is consistent with the report that SDS tends to dissolve cytoplasm and nuclear membranes and to denature proteins, thus destroying the structure of native tissues (Seong et al., 2010). After decellularization of porcine placenta, there was no significant difference between SDS and SDS + Triton X -100 at 0, 48 and 72 hours. However, Triton X-100 (24.45, 28.42 and 38.44 ng/μg) showed significantly higher DNA content than SDS (22.17, 9.75 and 11.70 ng/μg) and SDS + Triton X-100 (22.03, 13.26 and 13.36 ng/μg, $p < 0.05$). This is consistent with the finding that the nonionic Triton X-100 does not damage the binding between proteins (Rieder et al., 2004; Gilbert et al., 2006). Decellularization was treated for 0, 12, 24, 48 and 72 hours to determine the appearance change of porcine placenta. Fig. 1 shows the changes in the appearance of porcine placenta with decellularization time. As shown in Fig. 1, no significant differences were found in the appearance of the porcine placenta after treatment 0, 12, 24, 48 and 72 hours.

Decellularization was treated for 0, 12, 24, 48 and 72 hours for DNA staining of porcine placenta. The results of DNA staining of porcine placenta with decellularization time are shown in Fig. 2. Nuclei were observed at 0 hours, but almost no nuclei were observed in DNA staining after 12, 24, 48 and 72 hours treatment. Consistent with reports that SDS or Triton X-100 are effective at removing nuclei in cells (Khan et al., 2015).

As a result, decellularization of porcine placenta using SDS for 12 hours or 24 hours is considered more effective for DNA removal.

Table 1. The content of DNA of decellularized placenta with time of each detergent

Treatment (ng/μg)	Decellularization time (h)				
	0	12	24	48	72
SDS[1)]	22.17 ± 0.03^{Ab}	10.24 ± 0.01^{Cc}	9.36 ± 0.04^{Ec}	9.75 ± 0.08^{Db}	11.70 ± 0.08^{Bb}
Triton X-100	24.45 ± 0.25^{Ea}	30.65 ± 0.21^{Ba}	30.10 ± 0.16^{Ca}	28.42 ± 2.54^{Da}	38.44 ± 0.25^{Aa}
SDS + Triton X-100	22.03 ± 0.00^{Ac}	14.18 ± 0.12^{Cb}	16.02 ± 0.17^{Bb}	13.26 ± 3.99^{Bb}	13.36 ± 4.00^{Db}

[1)]Sodium dodecyl sulfate.

Each value is mean ± standard deviation.

$^{a-c}$Means with the different superscripts in the same column are significantly different at $p < 0.05$ by Duncan's multiple range test.

$^{A-E}$Means with the different superscripts in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test.

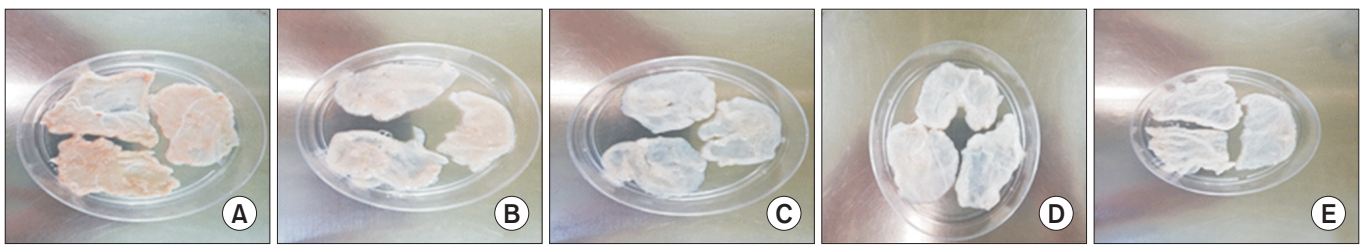

Fig. 1. Decellularized porcine placenta over time. (A) None-decellularized placenta. (B) Placenta decellularized for 12 hours. (C) Placenta decellularized for 24 hours. (D) Placenta decellularized for 48 hours. (E) Placenta decellularized for 72 hours.

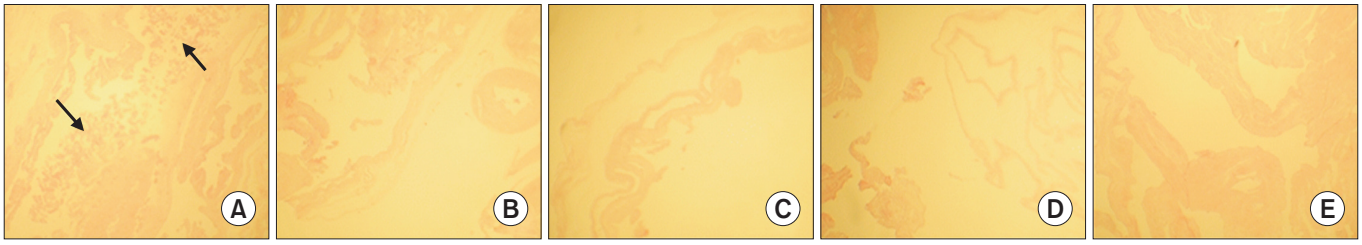

Fig. 2. Hematoxylin and eosin staining of decellularized placenta over time (X100). (A) None-decellularized placenta. Arrow points to the nucleus of the cell. (B) Placenta decellularized for 12 hours. (C) Placenta decellularized for 24 hours. (D) Placenta decellularized for 48 hours. (E) Placenta decellularized for 72 hours.

Table 2. The content of glyco samino glycans (GAGs) of placenta decellularized with time of each detergent

Treatment (ng/μg)	Decellularization time (h)				
	0	12	24	48	72
SDS[1)]	0.64 ± 0.02^{Aa}	0.29 ± 0.01^{Bc}	0.25 ± 0.01^{Cc}	0.24 ± 0.00^{Cc}	0.20 ± 0.01^{Dc}
Triton X-100	0.56 ± 0.01^{Ac}	0.50 ± 0.02^{Ba}	0.51 ± 0.02^{Ba}	0.52 ± 0.02^{ABa}	0.53 ± 0.03^{ABa}
SDS + Triton X-100	0.59 ± 0.00^{Ab}	0.35 ± 0.01^{Bb}	0.31 ± 0.00^{Cb}	0.35 ± 0.01^{Bb}	0.32 ± 0.00^{Cb}

[1)]Sodium dodecyl sulfate.

Each value is mean ± standard deviation.

[a–c]Means with the different superscripts in the same column are significantly different at $p < 0.05$ by Duncan's multiple range test.

[A–D]Means with the different superscripts in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test.

GAGs and collagen contents of porcine placenta according to decellularization detergent

Decellularization was treated with 0, 12, 24, 48 and 72 hours using SDS, Triton X-100 and SDS + Triton X-100 to determine the degree of decellularization of porcine placenta. After decellularization of porcine placenta by detergent, the results of glycos amino glycans (GAGs) contents are shown in Table 2. As shown in Table 2, the content of GAGs was significantly higher in Triton X-100 (0.50, 0.51, 0.52 and 0.53 μg/mg) than in SDS (0.29, 0.25, 0.24 and 0.20 μg/mg) and SDS + Triton X-100 (0.35, 0.31, 0.35 and 0.32 μg/mg) after 12, 24, 48 and 72 hours of decellularization ($p < 0.05$). This is because SDS, an ionic detergent, dissolves the cytoplasm and nuclear membranes, tends to denature proteins and destroy tissue structure and remove GAGs. And Triton X-100, a nonionic detergent, breaks the bond between lipid and protein, but does not damage the bond between proteins (Seong et al., 2010; Min et al., 2011).

After decellularization of porcine placenta by detergents at 0, 12, 24, 48 and 72 hours, the result of collagen content are shown in Fig. 3. As shown in Fig. 3, there was no significant difference between SDS, Triton X-100 and SDS + Triton X-100 in terms of collagen content. However, at 0 hours, collagen content of SDS group was higher than that of other groups ($p < 0.05$). Thereafter, at 12, 24, 48 and 72 hours there was no significant difference in SDS + Triton X-100 but higher collagen content.

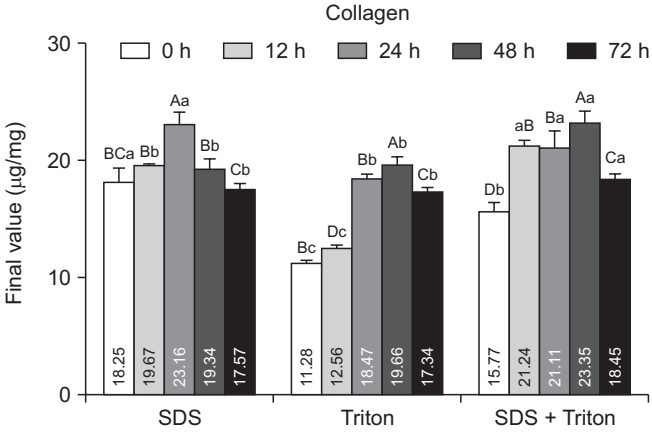

Fig. 3. Collagen content of placenta after decellularized with time of each detergent. [a–c]Means with the different treatment in the same decellularization time are significantly different at $p < 0.05$ by Duncan's multiple range test. [A–E]Means with the different decellularization in the same treatment are significantly different at $p < 0.05$ by Duncan's multiple range test.

In case of decellularization of human placenta using SDS and SDS + Triton X-100, the nuclei are well removed

and high levels of GAGs and collagen content can be used to make decellularization scaffold (Flynn et al., 2006). However, this study showed other results. It is thought that different results may have been obtained because the concentration of detergent used in this study was high.

DNA content and GAGs content of porcine placenta decellularized by mixing 1.0% Triton X-100 in each concentration of SDS

In order to measure the degree of decellularization of porcine placenta, experiments were performed by mixing SDS concentrations at 0.2, 0.5, 0.7 and 1.0%, respectively, and mixing them with 1.0% Triton X-100. The DNA contents of the placenta decellularized by mixing different concentrations of SDS in Triton X-100 are shown in Table 3. As shown in Table 3, the DNA content was significantly higher at SDS concentration of 0.2% ($p < 0.05$). No significant differences were found at other concentrations, but significant differences were observed for each concentration of SDS at 24 hours ($p < 0.05$). And when the concentration of SDS is 0.5%, it was confirmed that the DNA content is the lowest. These results were in agreement with the findings that optimal decellularization conditions exist when the concentration of SDS is between 0.3 and 0.5% (Chun et al., 2015).

Table 4 shows the contents of GAGs in placenta decellularized by mixing different concentrations of SDS in Triton X-100. As shown in Table 4, the content of GAGs was significantly higher at 0.2% SDS ($p < 0.05$). The content of GAGs was high at 0.5% SDS, but the placenta decellularized for 24 hours at 0.7% SDS (35.30 ng/μg) was not significantly different from 0.2% SDS (33.93 ng/μg, $p < 0.05$). These results reported that the higher the concentration of SDS, the lower the content of GAGs (Sullivan et al., 2012). In this study, however, the higher the concentration of SDS, in part, the higher the content of GAGs. This may be because the detergent did not properly diffuse into the placenta during decellularization.

DISCUSSION

The purpose of this study was to find a method to facilitate the previous decellularization method and to minimize tissue damage using the existing decellularization method using the porcine placenta, and to find the conditions to effectively obtain decellularized organ support.

Table 3. The content of DNA of decelluarized placenta depending on each sodium dodecyl sulfate (SDS) level with 1.0% Triton X-100

Concentration of SDS (%)	Decellularization time (h)			
	0	12	24	48
0.2	20.46 ± 1.23^{Aa}	9.86 ± 0.36^{Da}	12.70 ± 0.50^{Ca}	14.57 ± 0.14^{Ba}
0.5	20.46 ± 1.23^{Aa}	7.70 ± 0.47^{Bb}	8.10 ± 0.30^{Bc}	6.85 ± 0.16^{Cc}
0.7	20.46 ± 1.23^{Aa}	8.17 ± 0.05^{Db}	10.47 ± 0.29^{Bb}	8.81 ± 0.15^{Cb}
1.0	20.46 ± 1.23^{Aa}	8.18 ± 0.02^{Bb}	7.25 ± 0.07^{Cd}	6.73 ± 0.10^{Dc}

Each value is mean ± standard deviation.

$^{a-d}$Means with the different superscripts in the same column are significantly different at $p < 0.05$ by Duncan's multiple range test.

$^{A-D}$Means with the different superscripts in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test.

Table 4. The content of glyco samino glycans (GAGs) of decelluarized placenta depending on each sodium dodecyl sulfate (SDS) level with 1.0% Triton X-100

Concentration of SDS (%)	Decellularization time (h)			
	0	12	24	48
0.2	69.20 ± 1.85^{Aa}	42.90 ± 0.20^{Ba}	33.93 ± 0.76^{Da}	37.23 ± 1.08^{Ca}
0.5	69.20 ± 1.85^{Aa}	16.13 ± 0.06^{Cb}	20.87 ± 0.70^{Bb}	14.10 ± 0.61^{Db}
0.7	69.20 ± 1.85^{Aa}	13.93 ± 1.15^{Cc}	35.30 ± 0.96^{Ba}	8.13 ± 1.79^{Dc}
1.0	69.20 ± 1.85^{Aa}	12.87 ± 0.68^{Bc}	3.67 ± 0.95^{Dc}	9.57 ± 0.74^{Cc}

Each value is mean ± standard deviation.

$^{a-c}$Means with the different superscripts in the same column are significantly different at $p < 0.05$ by Duncan's multiple range test.

$^{A-D}$Means with the different superscripts in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test.

When decellularization was performed using various detergents, the best support was obtained from SDS and Triton X-100 (Chun et al., 2015). In this experiment, SDS and Triton X-100 were used, and SDS and Triton X-100 were mixed to use SDS + Triton X-100. SDS showed the most removal of DNA, but Triton X-100 did not remove much DNA. However, GAGs were higher in Triton X-100 than other groups and significantly lower in SDS, which is consistent with previous studies (Seong et al., 2010; Min et al., 2011). In addition, it was reported that the content of DNA and GAGs is different as the concentration of SDS changes (Sullivan et al., 2012; Yang, 2014). In this study, as the concentration of SDS increases, the contents of DNA and GAGs decreased. And when the decellularization was performed for 24 hours at 0.5% concentration of SDS, it was confirmed that the content of GAGs is high and the DNA content is low.

Therefore, the results of this study showed that the DNA content and GAGs content were opposite when decellularized using SDS and Triton X-100. Sullivan et al. (2012) reported that DNA and GAGs differed by the difference in SDS concentration. The low DNA content and high content of GAGs did not yield satisfactory results. A combination of SDS and Triton X-100 is considered more effective to meet both DNA and GAGs conditions. However, it is thought that an effective decellularized organ support can be obtained by treating the decellularization for 24 hours with the concentration of 0.5% of the ionic detergent SDS rather than mixing the two detergents at 1.0% concentration. In addition, various methods of decellularization studies using various detergents may yield more effective decellularized organ support. And the decellularized organ scaffold obtained by the effective decellularization method can be used to determine the effect on the cells through co-culture experiments using adipose stem cells or C_2C_{12} cells.

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