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Histologic Characteristics and Mechanical Properties of Bovine Pericardium Treated with Decellularization and α-Galactosidase: A Comparative Study

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Background: Bioprostheses for cardiovascular surgery have limitations in their use following as calicification. α-galactosidase epitope is known as a stimulant of immune response and then shows a progressing calcification. The objective of this study was to evaluate histologic characteristics and mechanical properties of decellularization and treated with α-galactosidase. ***Materials and Methods:*** Bovine pericardial tissues were allocated into three groups: fixation only with glutaraldehyde, decellularization with sodium dodesyl sulfate and decellularization plus treatment with α-galactosidase. We confirmed immunohistological characteristics and mechanical properties as fatigue test, permeability test, compliance test, tensile strength (strain) test and thermal stability test. ***Results:*** Decellularization and elimination of α-gal were confirmed through immunohistologic findings. Decellularization had decreased mechanical properties compared to fixation only group in permeability (before fatigue test p=0.02, after fatigue test p=0.034), compliance (after fatigue test p=0.041), and tensile strength test (p=0.00). The group of decellularization plus treatment with α-galactosidase had less desirable mechanical properties than the group of decellularization in concerns of permeability (before fatigue test p=0.043) and strain test (p=0.001). ***Conclusion:*** Favorable decellularization and elimination of α-gal were obtained in this study through immunohistologic findings. However, those treatment including decellularization and elimination of α-gal implied the decreased mechanical properties in specific ways. We need more study to complete appropriate bioprosthesis with decellularization and elimination of α-gal including favorable mechanical properties too.

Key words: 1. Bioprosthesis
2. Decellularization
3. Glutaraldehyde
4. α-Gal

INTRODUCTION

Clinically, prosthetic valves are the most widely used treatment for heart valve disease, and they can substitute for human valves in patients who need heart valve transplantation. Bioprosthetic valves have notable advantages in terms of anti-coagulant therapy and blood dynamic properties, and their use is thus continuously increasing. Nevertheless, the durability of bioprosthetic valves is not yet satisfactory, and this is the essential factor to be overcome [1]. The possible rea-

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sons for damage to bioprosthetic valves are mechanical fatigue, which leads to physical changes; bioprosthetic valve calcification by valve manufacturing solvent; and inflammation and immune responses to the valves [2,3].

A recent study has shown that the α-galactosidase epitope (a typical heteroantigen presenting on the animal tissue surface) is present on bioprosthetic valves in clinical use. The study reported that the patients who received bioprosthetic valve transplantation showed an increase in plasma antibody levels of the α-galactosidase epitope. Thus, damage to bioprosthetic valves was closely linked with an immune response, especially with the animal immune response [4]. The α-galactosidase epitope is the carbohydrate ring present on the surface of the animal plasma membrane, and they are expressed in all mammals apart from primates [5,6]. There is a difference in the levels of α-galactosidase epitope expression depending on the species, but the α-galactosidase epitope is well known for stimulating a strong immune response in primates [7]. Thus identifying methods of manufacturing clinically suitable bioprosthetic valves while minimizing both calcification caused by tissue fatigue and also the use of toxic fixation and heteroantigens is the focus of this study.

This study focused, more specifically, on identifying the optimal pericardium. Transfection of bacterial genetic recombinants of α-galactosidase into decellurized bovine pericardium was performed to remove the α-galactosidase epitope. Remaining heteroantigen expression and histological properties were examined by immunohistochemistry staining and mechanical examination investigated the physio-physical characterstics in great detail.

MATERIALS AND METHODS

1) Xenograft pericardium harvest

Under control of a veterinarian at the abattoir, a healthy bovine was killed and fresh pericardium was extracted. The pericardium was then put into cold phosphate buffered saline (PBS) solution (0.1 M, pH 7.4, 4°C), then transported to the laboratory in a cold ice box.

2) Decellularization and α-galactosidase treatment

(1) General procedure: As soon as the pericardium arrived at the laboratory, the surrounding tissue was removed and washed in distilled water that had been pretreated with 0.1% phosphate buffered saline and 4% ethanol for 1 hour. The pericardium was then washed with fresh distilled water for 3 hours. Decellularization of the right pericardium was performed in a rotating shaker.

First, the pericardium was placed in a 4°C hypotonic solution (Tris, 10 mmol/L; ethylendiamine tetra acetic acid [EDTA], 0.05%; aprotinin, 5 KIU/mL; Neomycin trisulfate 50 mg/L; pH 8) for 16 hours then placed in 0.25% sodium dodesyl sulfate (SDS)-treated hypotonic solution for 24 hours. After that, the pericardium was treated with hypertonic solution (Tris, 50 mmol/L; NaCl, 0.15 mol/L; EDTA, 0.05%; aprotinin, 5 KIU/mL; Neomycin trisulfate 50 mg/L; pH 8) at 4°C for 8 hours. Thereafter, the pericardium was stored in a 4°C of isotonic solution for 16 hours, and this was the period in which α-galactosidase (0.1 U/mL) was added to certain designated pericardium fragments. Finally, the pericardium was washed with PBS solution for 1 hour at room temperature.

(2) α-Galactosidase treatment: In this experiment, DNA recombinant α-galactosidase was provided by Seoul National University Microbiology Laboratory and the following steps show the methodology of obtaining α-galactosidase: from *Bacteroides thetaiotamicron* (*B. thetaiotamicron*) genomic DNA, the α-galactosidase gene (BtGal 110B) was amplified and separated using polymerase chain reaction. Then the separted α-galactosidase gene was applied to the C-terminal of the pET28a vector, which would express histidine at the C-terminal. Afterwards, the vector was transfected into *Escherichia coli (E. coli)* Rosetta 2 (DE3) (Novagen, Madison, WI, USA). In order to confirm transfecton, DNA sequencing with the 377 ABI Prism instrument (Applied Biosystems, Foster City, CA, USA) was used.

E. coli was incubated in pretreated Luria Bertani media with 34 μg/ mL chloramphenicol and 30 μg/mL kanamycin at 37°C, in 200 rpm up to (A 600 nm of 0.6 to 0.8) in exponential steps. Then 1 mM isopropyl-1-thio-β-D galactopyranoside was added and it was incubated for another 4 hours. The incubated *E. coli* was centrifuged for 15 minutes, then resuspended with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole), and the *E. coli* cell wall was

gradually segmented and destroyed by using an ultrasonicator (Misonix Inc., Farmingdale, NY, USA). Afterwards, it was centrifuged for 20 minutes at 13,000 rpm to remove cell debris. Then α-galactosidase protein was isolated with a nickel-nitrilotriacetic acid agarose column (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

3) Fixation

Control pericardium (cellurized but not treated), decellurized pericardium, and α-galactosidase treated on decellurized pericardium were fixed using the same procedure. Fixation was performed at room temperature with 0.5% gultaraldehyde (GA) for 3 days then treated with 0.25% GA at room temperature for 7 days and stored at 4°C.

4) Staining of α-galactosidase antigen determinant epitope

Each of the valves and pericardium tissue samples were cut into 5×5 mm pieces and washed 3 times with PBS buffer solution for 5 minutes each. Then the tissues were placed in 30% sucrose solution until they sink down at the bottom. The tissues were left as frozen sections and placed on slides. Cold acetone was added to fix them for 10 minutes. Afterwards, the slides were washed with PBS buffer solution 3 times for 5 minutes each.

Biotin-attached *Griffonia simplicifolia* I isolectin B4 (GS-IB4; Invitrogen, Carlsbad, CA, USA) was diluted in PBS solution then dropped at a volume of 0.1 mL onto the slides to incubate for 1 hour at 37°C. Horseradish peroxidase bound to avidin with a protein concentration of 5 μg/mL was diluted in PBS buffer solution, which was then dropped in 0.1 mL quantities onto the slides. Aftterwards, the slide was incubated at 37°C for 1 hour. Thereafter, 0.1 mL of 3.3'-diaminobenidine (DAB) was placed on the slide and washed three times with distilled water after 1 minute, hematoxylin treatment for 1 minute, and a series of HCl, ammonia, ethanol, and neo-clear process was performed. Then the mounting medium was dropped onto the slides and covered with a coverglass. The extent of α-galactosidase antigen staining was then observed.

5) Optical microscope examination

After completing the treatment of the pericardium, they were fixed with a 10% formalin solution. Paraffin embedding tissues were made and sliced into 2 to 4 μm sections. They were then stained with hematoxylin-eosin and Masson's trichrome to be observed under the microscope.

6) Electron miscroscope examination

Electron microscope examination required slicing the tissue into 1 mm thicknesses and fixing them with 4% GA-PBS solution (0.1 mol/L phosphate buffer, pH 7.4). Afterwards another fixation was performed with 1% OsO_4 (0.1 mol/L phosphate buffer, pH 7.4) then embedded into Epon LX 112 fixative.

7) Fatigue test

A circular device 25 mm in diameter was designed and constructed in order to carry out the experiment. It was used to apply a pulsating pressure to one side of the pericardium fragment. This setting allowed reproduction of the physiological environment in which pericardium is transplanted for use as valve. Hence, the device could reproduce pulsating pressure from 0 to 180 mmHg. In this experiment 240 rpm, which is 4 Hz and human physiological internal pressure of 80 mmHg were used for 49 days to carry out the fatigue test.

8) Permeability test

When the processes ofdecellurization, fixation, and storage were completed for the pericardium, 100 mmHg of pressure was applied for 1 hour on one side of the pericardium in physiological saline. Then the volume of saline that had penetrated through the pericardium was recorded in units of mL/hr · cm^2. The difference in permeability could be interpreted as the association between colloidal transformation within tissue (permanent stretching of collagen fiber and the potential appearance of a gap).

9) Compliance test

As soon as the pericardium decellurization, fixation and storage processes were complete, different pressures were applied to the pericardium in physiological saline. The 120,

140, 160, 180, and 200 mmHg of hydrostatic pressure were exerted on one side and the modified pericardium volume was recorded.

When the pressure increased from 100 to 200 mmHg, it was defined as the ($\Delta V/\Delta P$) change in volume per change in pressure. If the surface area of the precardium is different, a comparison can be made by dividing by the unit area, yielding the unit of $\mu L/mmHg \cdot cm^2$. A possible link between the change in pressure and collagen fiber could be evaluated with by the degeneration and relaxation of, and damage to the fiber.

10) Tensile strength and strain tests

The collagen fibers of the bovine pericardium are not arranged running in the same direction, so they sometimes cross over each other or bend. Hence, collagen fibers are not equally distributed in the bovine pericardium. Thus, a random slice does not represent the tensile strength of a specific portion of pericardium.

This limitation was overcome by spreading out the treated pericardium and twisting it by intervals of 30°, and taking a slice in each orientation until slices from 6 different orienations were obtained. In each orientation, a 5×50 mm rectangular section was obtained and tensile strength was measured across the 5 mm direction. The mean recorded values were set to be equal to the representative values of pericardium tensile strength.

Recording was done with a digital force gauge automated materials testing system (Model 5FGN Japan Tech & Manufacture IMADA; Mitutoyo, Tokyo, Japan), at a load speed of 100 mm/min in units of MPa (=kgf/5 mm width). In order to observe the correlation between the thickness and tension, calipers (Mitutoyo Thickness Gauge, Digimatic 543-122-15; Mitutoyo) were used to measure specimen and sample thicknesses repeateadly. The percentage of elongation was measured at the point in which the pericardium was torn by bidirectional tension.

11) Thermal stability examination

A pericardium fragment was cut to 8×30 mm and 95 g of tension was constantly applied. Afterwards, the fragement was put in 55°C distilled water and the water temperature was increased at a rate of 2.5°C/min. The temperature at which the heterotransplanted pericardium fragment contracted rapidly was noted for each group and comparisons were made.

12) Statistics

A Kruskal-Wallis test was used to examine the differences in each interval, and statistical significance was evaluated using the Mann-Whitney U-test. Pre- and post-fatigue results within the group were compared using a Wilcoxon signed rank test. The confidence interval was set at 95%.

RESULTS

1) Microscope observation

Hematoxylin and eosin (H&E) stained slides of the pre-fatigue test results were as follows (Fig. 1): the decellurized slides showed no nucleus in the cells. However, some loss in the surrounding colloid could be noted in the decellurized and α-galactosidase-treated slides. Notably, the α-galactosidase-treated slides showed a greater loss in surrounding colloid. H&E stained slides of the post-fatigue test results are shown in Fig. 1. It can be seen that the post-fatigue results resembled those shown in Fig. 2. There was also no change in colloidal alteration in the pre- and post-fatigue tests. It should be noted that the post-fatigue test showed consistency in structure compared to the pre-fatigue test. Fig. 3 shows a slide stained with Masson's trichrome before the fatigue test. The result is similar to that obtained from H&E staining, showing the lack of a histological difference between the two results. An increase in the loss of colloid is shown from the decellurized to the α-galactosidase-treated samples. Nevertheless, the structure of the two treated samples does not differ greatly compared to the samples that only have undergone fixation. The Masson's trichrome stained slides of the post-fatigue test results are shown in Fig. 4. In the post-fatigue test samples, the loss in colloid was not shown compared to the pre-fatigue test samples. However, there was a greater adjustment of the structure and lowering of the density in the decellurized and α-galactosidase-treated samples, in that order.

(1) Results of electron microscope observation: The result of the pre-fatigue test is shown in Fig. 5. Little differ-

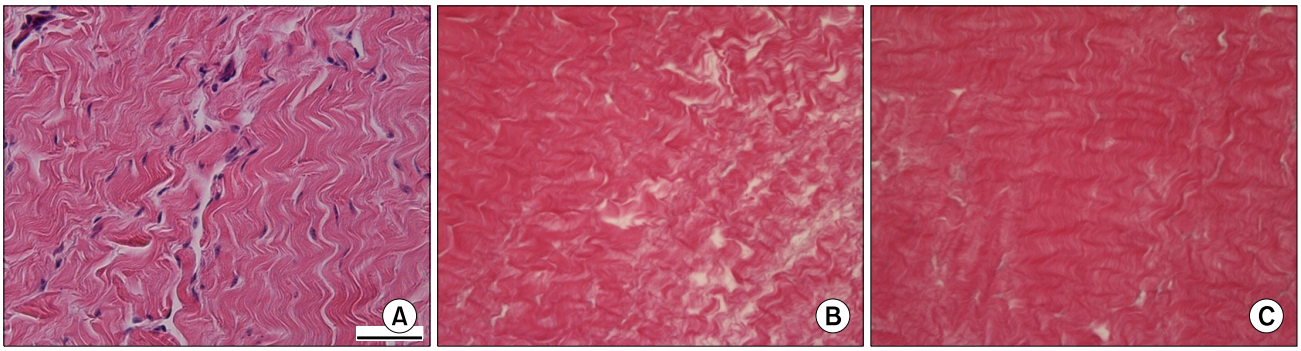

Fig. 1. H&E staining (before the fatigue test). (A) Glutaraldehyde fixation only; (B) glutaraldehyde fixation+decellularization; (C) glutaraldehyde fixation+decellularization+ α-galactosidase. B and C had no visible cells. The interstitial matrix of B and C was less dense than that of A (H&E, ×400).

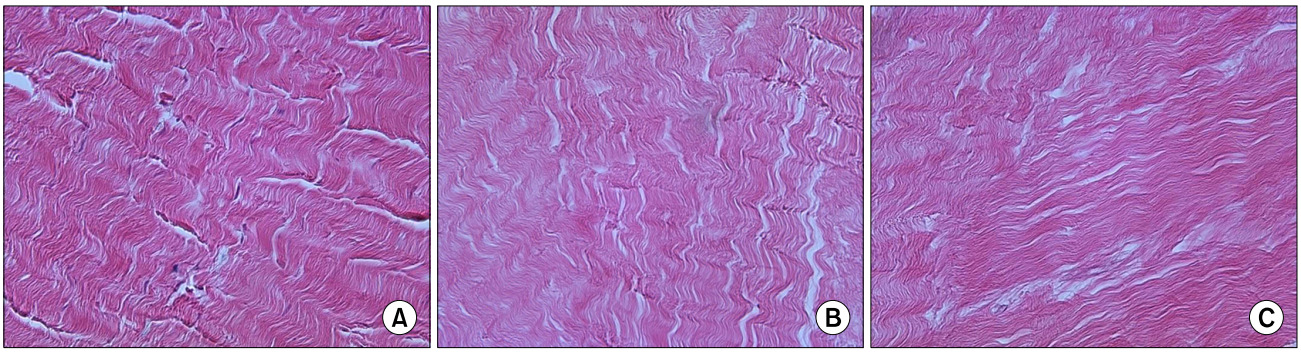

Fig. 2. H&E staining (after the fatigue test). (A) Glutaraldehyde fixation only; (B) glutaraldehyde fixation+decellularization; (C) glutaraldehyde fixation+decellularization+ α-galactosidase. A, B, and C had less dense interstitial matrix than pre fatigue test ones after the fatigue test than before. B and C showed relative derangement (H&E, ×400).

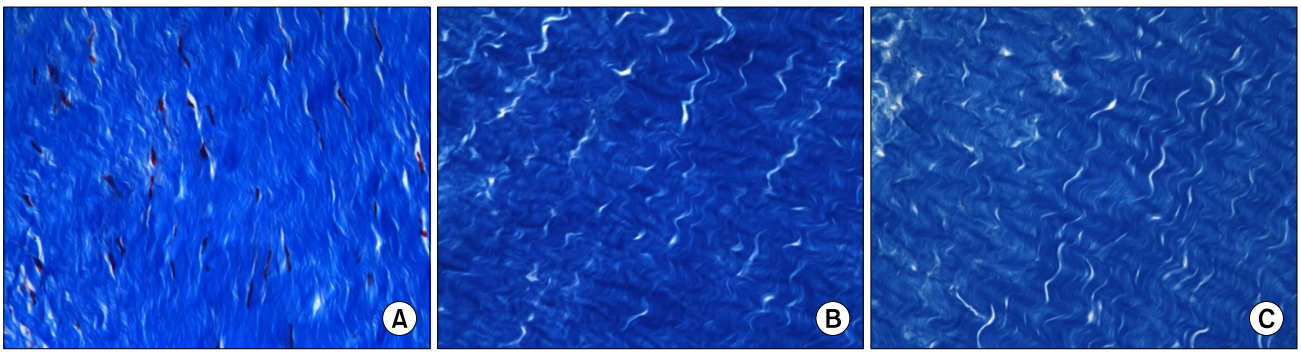

Fig. 3. Masson's trichrome staining (before the fatigue test). (A) Glutaraldehyde fixation only; (B) glutaraldehyde fixation+decellularization; (C) glutaraldehyde fixation+decellularization+ α-galactosidase. B and C had no visible cells. There was no derangement of the interstitium in B and C (×400).

ence between electron micropy results was visible to the naked eye. However, the density of the structures were shown to be lowered in the decellurized and α-galactosidase-treated samples compared the ones that were only fixed. The electron microscope results of the post-fatigue test is shown in Fig. 6. Generally, collidal density was lower in the decellurized and

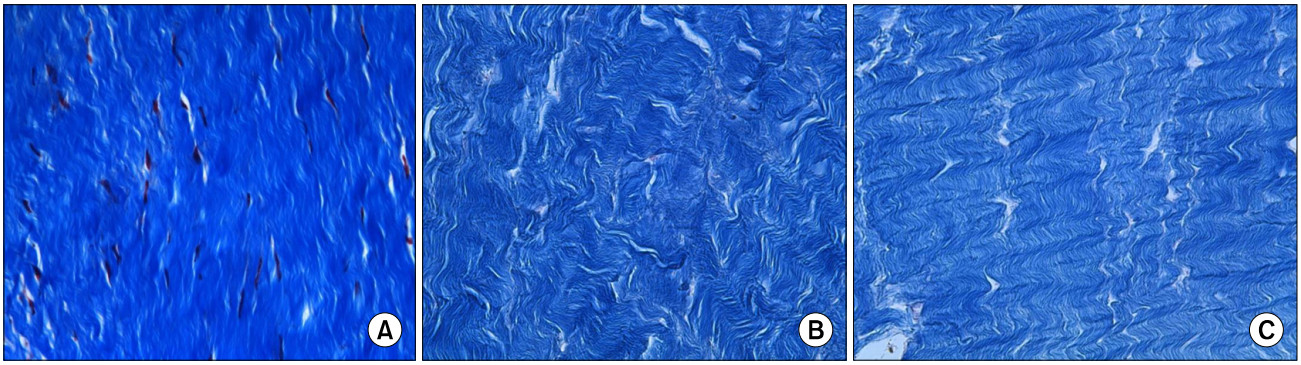

Fig. 4. Masson's trichrome staining (after the fatigue test). (A) Glutaraldehyde fixation only; (B) glutaraldehyde fixation+decellularization; (C) glutaraldehyde fixation+decellularization+ α-galactosidase. Derangement of the interstium in B and C (×400).

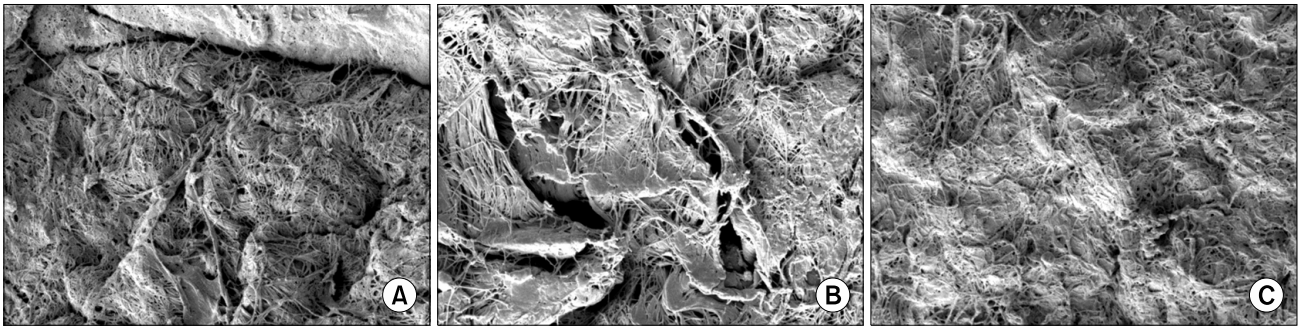

Fig. 5. Electron miscroscopic findings (before the fatigue test). (A) Glutaraldehyde fixation only; (B) glutaraldehyde fixation+decellularization; (C) glutaraldehyde fixation+decellularization+ α-galactosidase. There was no difference in the interstitial derangement among A, B and C (×3,000).

Fig. 6. Electron microscopic findings (after the fatigue test). (A) Glutaraldehyde fixation only; (B) glutaraldehyde fixation+decellularization; (C) glutaraldehyde fixation+decellularization+ α-galactosidase. Interstitial derrangement was observed in B and C. C had more deranged interstitium than B (×3,000).

α-galactosidase-treated samples compared to the samples that were only fixed. Within the two treated samples, the α-galactosidase-treated ones had a lower collidal density. The DAB stained slides are shown in Fig. 7. Decellurized and fixed sample slides were detected by a darker brown α-galactosidase signal compared to the background ones. Nonetheless, the α-galactosidase-treated samples had a similar signal strength to the background signal, which suggests that the

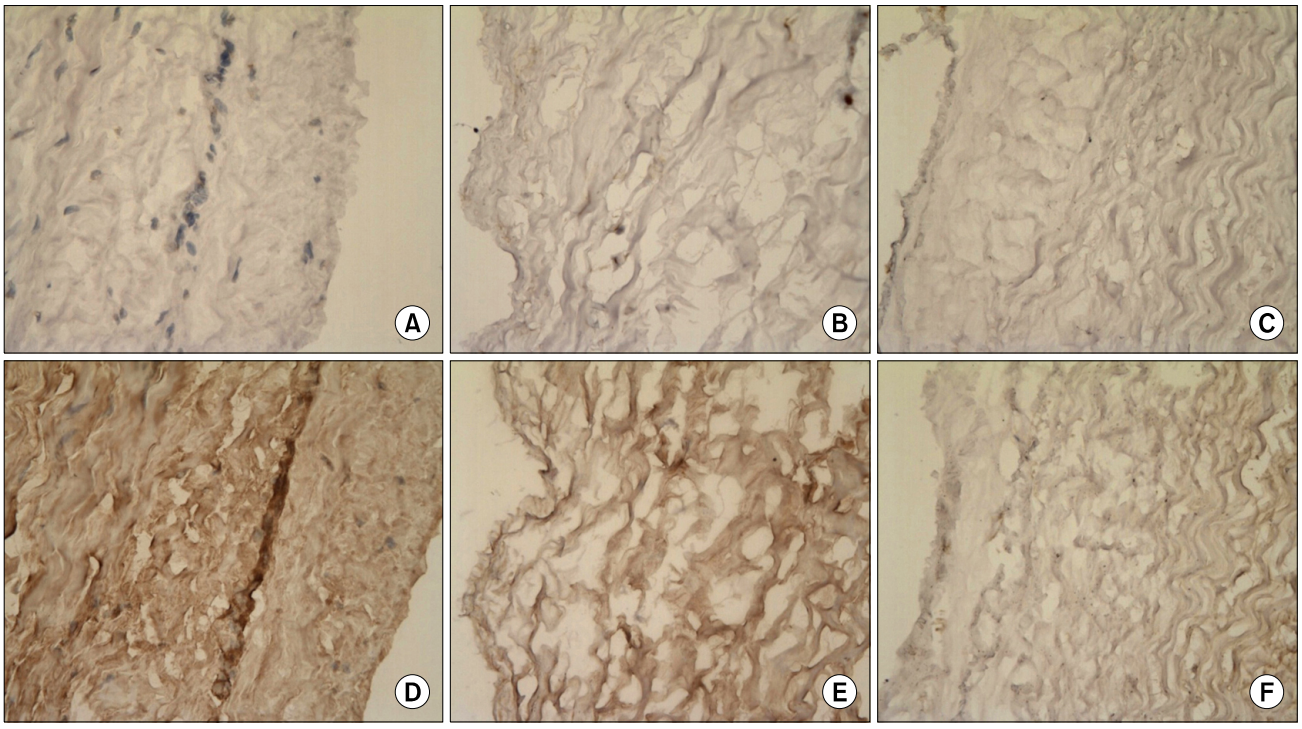

Fig. 7. 3.3'-diaminobenidine (DAB) staining. (A, D) Glutaraldehyde fixation only; (B, E) glutaraldehyde fixation+decellularization; (C, F) glutaraldehyde fixation+decellularization+ α-galactosidase. (A－C) Background image; (D－F) α-gal signal. D, E, and F were α-gal signal images. F had no α-gal signal (×400).

α-galactosidase signal had almost disappeared.

2) Mechanical test results

(1) Permeability test: The pre- and post-fatigue test results are shown in Table 1, respectively. Each permeability group showed a significant difference in both pre- and post-fatigue tests (pre-fatigue test p=0.01; post-fatigue test p=0.024). Specifically, the permeability of the fixed and decellurized groups in the pre-fatigue test showed a significant difference (p=0.02) and that of the decellurized and α-galactosidase-treated groups was significantly different as well (p=0.043). The permeability after the fatigue test showed a significant difference betweeen the fixed and decellurized groups (p=0.034), but no significant difference was observed between the decellurized and α-galactosidase-treated groups (p=0.289).

(2) Compliance test: The compliance test results before and after the fatigue test are shown in Table 2, respectively. Before the fatigue test, there was no significant difference in compliance between the groups (p=0.092). However, compliance after the fatigue test among the three groups showed a significant difference (p=0.029). Notably, the fixed and decellurized groups after the fatigue test showed a significant difference in compliance but no difference was observed between the decellurized and galactosidase treated groups (p=0.372).

(3) Tension and elasticity examination: Each group of tension and elasticity test results are shown in Table 3. Recording of the elasticity and tension after the fatigue test could not be performed due to the size of the pericardium fragment. The tension test showed a significant difference in each of the three groups (p=0.00). In particular, there was a significant difference between the decellurized and fixed group but no significant difference was observed between the decellurized and α-galactosidase-treated groups (p=0.705). The elasticity test also showed a significant difference in each of the three groups (p=0.00). Unlike the previous test, there was a significant difference between the decellurized and

Table 1. Permeability test

Group	Before the fatigue test			After the fatigue test		
	Sample no.	Permiability at 1 hr (mL)	Mean±SD	Sample no.	Permiability at 1 hr (mL)	Mean±SD
GA fix only			0.32±0.15			0.53±0.26
	1	0.39		1	0.75	
	2	0.20		2	0.22	
	3	0.20		3	0.73	
	4	0.51		4	0.42	
D+GA fix			1.38±0.59			2.39±0.24
	1	2.24		1	2.52	
	2	0.95		2	2.12	
	3	1.26		3	2.54	
	4	1.07				
D+α-galactosidase+GA fix			2.14±0.54			3.04±0.72
	1	1.63		1	3.27	
	2	2.14		2	1.98	
	3	1.91		3	3.36	
	4	2.88		4	3.57	

SD, standard deviation; GA fix, glutaraldehyde fixation; D, decellularization.

Table 2. Compliance test

Group	Before the fatigue test			After the fatigue test		
	Sample no.	Compliance (⊿V/⊿P)	Mean±SD	Sample no.	Compliance (⊿V/⊿P)	Mean±SD
GA fix only			0.8±0.26			0.6±0.19
	1	0.7		1	0.9	
	2	0.9		2	0.5	
	3	0.5		3	0.6	
	4	1.1		4	0.5	
D+GA fix			1.4±0.44			1.1±0.25
	1	1.9		1	0.8	
	2	1.1		2	1.4	
	3	1.2		3	1.0	
				4	1.0	
D+α-galactosidase+GA fix			1.2±0.36			1.2±0.13
	1	1.0		1	1.2	
	2	0.9		2	1.0	
	3	1.3		3	1.2	
	4	1.7		4	1.3	

⊿V/⊿P, diffrence ratio of volume and pressure; SD, standard deviation; GA fix, glutaraldehyde fixation; D, decellularization.

α-galactosidase-treated groups (p=0.01), but no significant difference was noted between the fixed and decellurized groups.

(4) Thermal stability examination: The thermal stability results of only fixed, decellurized then fixed, and decellurized then α-galactosidase-treated samples were 86.25°C, 83.75°C, and 83.75°C, respectively. There was no significant difference among each test group.

Table 3. Tensile strength and strain test

Group (n=10)	Thickness (mm)	Strength (MPa)[a)]	Strain (%)[b)]
GA fix only	0.31±0.04	18.96±5.14	20.67±5.16
D+GA fix	0.40±0.04	7.29±1.64	22.50±4.32
D+α-galactosidase +GA fix	0.41±0.05	8.55±2.65	39.50±10.86

GA fix, glutaraldehyde fixation; D, decellularization.
[a)]Tensile stress at break (kgf/5 mm width).
[b)]Tensile strain at break (%).

DISCUSSION

Recently, the main problem of tissue valve durability has been caused primarily by tissue imperfections, and such imperfections are reported to arise from inflammation and the immune response to transplanted tissue. The aim of this study was to find a way to minimize the transplanted tissue immune response and identify any physical changes in the tissue when it was put through various processes. As mentioned in the introduction, if bioprosthetic valves used clinically could last longer and provide more effective transplantation experiences to patients, it would reduce the burden to patients significantly. In Korea, no development of clinical bioprosthetic valves has been performed and relatively little research in this area is in progress at the moment. Thus, more research on the development of bioprosthetic valves would allow for the advancement of Korean bioprosthetic valve manufacturing.

Generally, the methodology of fixing and preserving prosthetic tissue in GA is widely used. This allows the tissue's collagenous fibres to maintain stable cross-links with GA and to produce GA polymer at the surface. The polymer reduces the immune response and enzyme degradation of the prosthetic tissue [8]. There is a change in transplanted tissue properties according to the GA concentration used for fixation [9]. When it has been fixed by GA, tissue calcification and toxifcation present some problems [10]. Thus, there have been several studies attempting to reduce tissue toxification and calcification. At the same time, it was reported that the celluar components in the tissue are closely realated to the immune response, so the importance of decellurization emerged as a solution to the problem [11].

Furthermore, homologous or heterologous tissues have been decellurized and used as tissue scaffold so that patients' cells could grow on a scaffold *in vivo* or *ex vivo*. This allows cells to continuously produce cellular matrix to extend the tissue's durability. There is the hope that these techniques could induce the growth of juvenile tissues through tissue enginnering techniques. The aim of decellurization is, firstly, to minimize the immune resoponse by completly removing cellular components. Secondly, it aims to stabilize the physical characteristics of the matrix and to preserve appropriate extra-cellular matrix for recellurization.

Furthermore α-galactosidase antigen determinant epitope, which showed peracute rejection in animal organ transplantation, is present in animal valves or pericardium tissue. The presence of anti-α-gal antibody in the tissue valve reacts in the early stage of transplantation and causes an immune and inflammation response. Thus, the hypothesis of these reasons for causing valve calcification and consequential damage have been very persuasive. Hence, removing α-galactosidase antigen determinant epitope from within the tissue valve should contribute to increasing the immune-durability of the valve. In the present study, 0.25% SDS detergent was used in combination with the osmotic methodology of hypo- and hypertonic solution for decellurization. This was based on research by Sung et al. [12] who showed the effectiveness of decellurization.

There are several methods for removing α-galactosidase antigen determinant epitope, which is present extensively on the surface of the cells. α-Galactosidase can be used to eliminate cell surface presenting α-galactosidase antigen determinant epitope or alpha-1,3 galactosyl transferase which synthesizes α-galactosidase antigen determinant epitope can be removed by α-galactosidase knockout [13,14]. Using an α-galactosidase knockout pig would mimize peracute rejection in the organ as well as valve tissue; therefore, the organ could be transplanted to the patient safely. However, this technique is not economical.

Using enzmes to eliminate animal α-galactosidase antigen determinant epitope is relatively simple and inexpensive, but there is a re-expression of α-galactosidase antigen determinant epitope in living tissue [15]. Thus, if research is based

on fixed heart valves or pericardium rather than living organs, using an enzyme would be useful. In this experiment, genetic recombinant α-galactosidase was used, provided by the Seoul National University Microbiology Lab.

As observed under the microscope, decellurization and removal of α-galactosidase antigen determinant epitope occurred as expected. However, there are some limitations in the methodology of decellurization. Evaluating the loss of cellular nuclei is insufficient to prove the efficiency of the decellurization technique. In this study, techniques that resulted in relatively less calcification but were efficient for decellurization were used. However, during the mechanical tests, the decellurized fragments had significantly lower statistical values in their permeability, compliance, and tension tests compared to the fragments that were only fixed.

Therefore, more research on improving the efficiency of decellurization methodology and physical properties of the resulting tissue is needed. Also, SDS detergent was used for 24 hours to perform decellurization in this experiment, but more research is needed to improve on the decellurization treatment time, detergent concentration, detergent choice, and accompanying mechano-physical control.

DAB staining was used to confirm the elimination of animal α-galactosidase antigen determinant epitope. Optical microscope examination was possible, but the degree of elimination could not be evaluated accurately. Hence, quantitative examination of animal α-galactosidase antigen would probably be needed. Currently, a typical way of extracting animal α-galactosidase antigen determinant epitope is using type I lectin of B4 lectin (GS-IB4), which specifically interacts with Galα1-3Gal and is obtained from *Griffonia simplicifolia* seeds [16-18].

Kirkeby et al. [19] performed a study using α-galactosidase antigen rich neoglycoprotein, rat's laminin reacted with GS-IB4, and monoantibody M86, a free antibody present in the human plasma. They evaluated each of the reactions with enzyme-linked immunosorbent assay and showed that the GS-IB4 lectin binding site was different from the monoclonal antibody M86 and human anti-gal antibody. When α-galactosidase of neoglyprotein and rat's laminin was eliminated using α-galactosidase and reacted with either lectin or anti-gal antibody, there was a greater reduction in the reaction rate with anti-gal antibody than with lectin. Immuno-enzyme measurement is more accurate than direct immunostaining. And, although this approach with the GS-IB4 test does not express α-galactosidase antigen epitope, it still implies that there are α-galactosidase antigens remaining that could interact with human free antibodies.

This result also correlates with the work of LaVecchio et al. [20], where α-galactosidase antigen determinant epitope stimulated anti-galactosidase immunoglobulin M, and anti-galactosidase immunoglobulin G. In the present study, reduction in expression of α-galactosidase antigen determinant epitope was shown in α-galactosidase-treated bovine pericardium, and confirmed by DAB staining. This can be inferred from the fact that when the samples were transplanted into the human, the inhibition of free antibodies was not guaranteed.

Therefore, if a complete inhibition of the animal immune response must be achieved, then the removal of α-galactosidase antigen determinant epitope from the animal cellular surface should be performed. Furthermore, the specific interaction of α-galactosidase antigen determinant with GS-IB4 and either anti-gal monoclonal antibody or human anti-gal antibody interaction must be verified so that elimation from the cellular surface of the α-galactosidase antigen determinant epitope could be accurately evaluated.

Nevertheless, when the samples were decellurized and α-galactosidase antigen was removed, there was a reduction in permeability, compliance, tension, and elasticity compared to the samples that were only fixed. Also, in the permeability test, there was a greater increase in the permeability when the samples were either decellurized or decellurized along with the eliminatation of α-galactosidase before the fatigue test. There was no significant difference in the compliance test or tension test compared to the decellurized group. On the other hand, there was a significant reduction in elasticity compared to the decellurized group

It was thus found that α-galactosidase antigen determinant epitope affected the physical properties of the pericardium; in particular, the elasticity and permeability were significantly decreased. Therefore, more research must be performed to find a method for removing antigen determinant epitope from pericardium while maintaining its physical properties. Furthermore, quantitative study on improving α-galactosidase treat-

ment to increase its immunological properties is also needed. Regarding the thermal stability test, the three groups showed no significant differences and the observed results were stable.

CONCLUSION

The following conclusions could be made from this study. Decellurization with SDS is effective and this was proven by microscopic examination. The GA-fixed group showed a significant decrease in permeability, compliance (after the fatigue test), and tensile strength. After removing α-galactosidase antigen determinant epitope, treated pericardium showed a significant decrease in permeability (before the fatigue test results) and in elasticity compared to the decellularized group. Bacteria recombinant α-galactosidase was shown to be effective under light microscope examination. Thermal stability remained constant, not depending on the presence of α-galactosidase antigen determinant epitope.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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