

Changes of the Structural and Biomechanical Properties of the Bovine Pericardium after the Removal of α -Gal Epitopes by Decellularization and α -Galactosidase Treatment

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Background: Bovine pericardium is one of the most widely used materials in bioprosthetic heart valves. Immunologic responses have been implicated as potential causes of limited durability of xenogenic valves. This study aimed to determine the effectiveness of decellularization and α -galactosidase (α -gal) to remove major xenoreactive antigens from xenogenic tissues. **Materials and Methods:** Recombinant *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) α -gal or decellularization, or both were used to remove α -gal from bovine pericardium. It was confirmed by α -gal-bovine serum albumin-based enzyme-linked immunosorbent assay (ELISA), high-performance anion exchange chromatography, flow cytometry, 3,3'-diaminobenzidine-staining, and lectin-based ELISA. The mechanical properties of bovine pericardium after decellularization or α -gal treatment were investigated by tests of tensile-strength, permeability, and compliance. Collagen fiber rearrangement was also evaluated by a 20,000 \times transmission electron microscope (TEM). **Results:** Recombinant *B. thetaiotaomicron* α -gal could effectively remove α -gal from bovine pericardium *B. thetaiotaomicron* (0.1 U/mL, pH 7.2) while recombinant human α -gal removed it recombinant human α -gal (10 U/mL, pH 5.0). There was no difference in the mechanical properties of fresh and recombinant α -gal-treated bovine pericardium. Furthermore, the TEM findings demonstrated that recombinant α -gal made no difference in the arrangement of collagen fiber bundles with decellularization. **Conclusion:** Recombinant *B. thetaiotaomicron* α -gal effectively removed α -gal from bovine pericardium with a small amount under physiological conditions compared to human recombinant α -gal, which may alleviate the harmful xenoreactive immunologic responses of α -gal. Recombinant α -gal treatment had no adverse effects on the mechanical properties of bovine pericardium.

Key words: 1. Pericardium
2. Tissue engineering
3. Bioprosthesis

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INTRODUCTION

Biological heart valves have been widely used in treatments for patients with valvular heart diseases. The durability of the valves is limited by structural degeneration and calcification. Recent studies have demonstrated the possible role of immunologic responses of xenoreactive antigens as a potential cause of the limited durability of xenograft heart valves. The mechanism of failure following a heart valve implantation is not catastrophic hyperacute rejection but rather subacute or chronic bioprosthetic valve dysfunction causing the eventual xenograft leaflet calcification [1]. The immune mechanism, a major barrier of galactose- α -1,3-galactose (α -gal) in xenotransplantation, is triggered by pre-existing human xenoreactive natural antibodies, which recognize the disaccharide α -gal present on the surface of pig cells and matrices. The antigen-antibody reaction causes complement activation and membrane attack complex deposition followed by massive tissue destruction [1-5] in the xenotransplantation. There are several reports that the cause of degeneration of the xenograft heart valves may be mediated by immune responses [6-10] partly due to α -gal xenoreactive antigens expressed on the cells and connective tissues of the valves [7,11]. To eliminate the harmful effects of xenoreactive major antigens, various tissue treatment techniques including dye oxidation methods, crosslinking, and decellularization have been attempted, but the majority of antigenicity remained even after glutaraldehyde treatment [6,12]. We have previously reported that α -gal epitopes on the porcine valves could be effectively removed using recombinant human α -galactosidase A (α -galactosidase A), but significant tissue damage was also noticed during the process [13]. In this study, we investigated the effectiveness of human recombinant α -galactosidase on synthetic α -gal and porcine aortic endothelial cells (PAECs) using various methods. We also evaluated the effectiveness of *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) recombinant α -galactosidase on the bovine pericardium. Furthermore, the mechanical properties of bovine pericardium, which was treated with decellularization or α -galactosidase and fixed with glutaraldehyde, were investigated. The degree of collagen fiber rearrangement of the bovine pericardium treated with decellularization or α -galactosidase was de-

termined using a transmission electron microscope (TEM).

MATERIALS AND METHODS

1) Methods of tissue treatment (study groups)

The bovine pericardial tissues were divided into six groups according to the treatment method. (1) Group 1: fresh pericardial tissue (control group). (2) Group 2: the pericardial tissue was treated with recombinant *B. thetaiotaomicron* α -galactosidase in 50 mM tris buffer for 24 hours (pH 7.2, 4°C). (3) Group 3: the pericardial tissue was decellularized with 0.25% sodium dodecyl sulfate (SDS) and 0.5% Tritonx-100 solution. (4) Group 4: the pericardial tissue was fixed with a 0.5% glutaraldehyde (GA) solution for 3 days, and then fixed with a 0.25% GA solution for 7 days (pH 7.4, at room temperature). (5) Group 5: the pericardial tissue was treated with recombinant *B. thetaiotaomicron* α -galactosidase in 50 mM tris buffer for 24 hours (pH 7.2, 4°C) and fixed with a 0.5% GA solution for 3 days, and then it was fixed with a 0.25% GA solution for 7 days (pH 7.4, at room temperature). (6) Group 6: the pericardial tissue was decellularized with 0.25% SDS and a 0.5% Tritonx-100 solution, fixed with a 0.5% GA solution for 3 days, and then fixed with a 0.25% GA solution for 7 days (pH 7.4, at room temperature). The bovine pericardial tissues for each analysis were obtained from the same animal (there were no differences between the experimental groups) to avoid a clustering effect.

2) Construction and preparation of recombinant α -galactosidase

(1) Human α -galactosidase A: The full-length cDNA of human α -galactosidase A (gene ID 2717) was cloned from the human fibroblast cDNA library using polymerase chain reaction (PCR). The insert was then ligated into the pMSG vector (Amersham, Uppsala, Sweden). Standard methods were used for subcloning and PCR to produce mammalian expression vectors encoding α -galactosidase A (Isu abxis, Seoul, Korea).

(2) *Bacteroides thetaiotaomicron* 1,3 galactosidase: The gene for the α -galactosidase from *B. thetaiotaomicron* (designated BtGal110B) was amplified from the corresponding genomic DNA by PCR and was introduced into the pET28a vec-

tor to express His₆-tagged proteins using appropriate restriction sites for protein expression in *Escherichia coli* (*E. coli*) Rosetta2 (DE3) (Novagen, Madison, WI, USA). *E. coli* was grown in a Luria-Bertani medium supplemented with 34 $\mu\text{g/mL}$ chloramphenicol and 30 $\mu\text{g/mL}$ kanamycin, and then induced at an A₆₀₀ nm of ca. 0.6 with 1 mM of isopropyl-1-thio- β -D-galactopyranoside. The harvested cell pellet was lysed in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole) using an ultrasonicator (Misonix Inc., Farmingdale, NY, USA). The crude lysates were centrifuged for 20 minutes at 13,000 rpm at 4°C, and the expressed proteins were purified using nickel-NTA agarose column (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction.

3) Decellularization

The bovine pericardial tissues were initially washed with 0.1% peracetic acid and distilled water with 4% ethanol for 2 hours to reduce the bioburden. These tissues were treated with a hypotonic buffered solution with 0.25% SDS for 24 hours at 4°C, and washed with distilled water for 1 hour afterward. Then they were treated with hypotonic solution with 0.5% TritonX-100 for 24 hours at 4°C, and then washed with distilled water for 12 hours at 4°C. Subsequently, they were treated with isotonic solution for 24 hours at 4°C. These tissues were finally treated with a hypertonic buffered solution (II) for 6 hours at 4°C, followed by washing thoroughly in phosphate buffered saline.

4) Enzyme-linked immunosorbent assay

Bovine serum albumin (BSA) conjugating synthesized α -gal-BSA was used as a solid phase antigen and was plated with 1 $\mu\text{g/mL}$ in phosphated-buffered saline (PBS) on the microtiter wells for 1 hour at 37°C. The plates were then blocked with 5% (w/v) BSA with PBS. Various concentrations of recombinant α -galactosidase A in Hepes buffer at various pH conditions were added to each well and were incubated for 1 day under room temperature. After being washed three times with PBS containing 0.05% (v/v) Tween 20, the wells were treated with anti α -gal specific monoclonal antibodies in PBS containing 0.01% (v/v) Tween 20 and 3% (w/v) BSA for 1 hour at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G

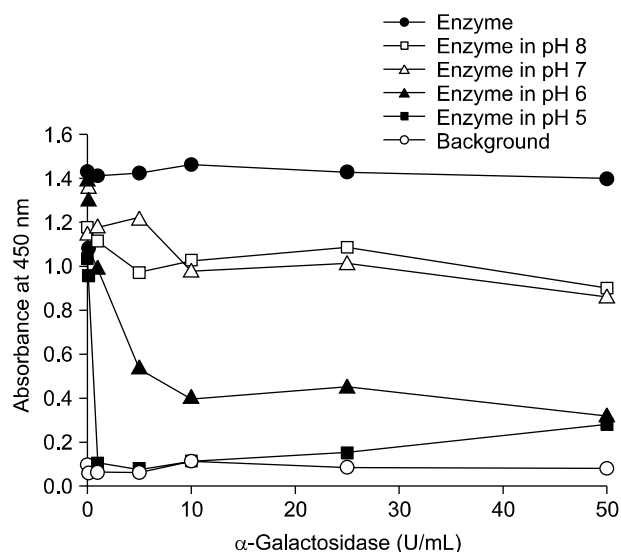


Fig. 1. Measurement of recombinant human α -galactosidase A activity by enzyme-linked immunosorbent assay. The mouse anti α -gal antibody determined the degree of degrading α -gal-Bovine serum albumin according to the efficiency of recombinant human α -galactosidase A under various pH conditions. White circle, background; black circle, no enzyme; white square, enzyme in pH 8.0; white triangle, enzyme in pH 7.0; black triangle, enzyme in pH 6.0; black square, enzyme in pH 5.0.

(1:10,000) was added to each well for 1 hour at 37°C. Following several washes with PBS containing 0.05% (v/v) Tween 20, the reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) solution (Pierce, Rockford, IL, USA). Optical density was measured at 450 nm using the Thermo Electron-Lab Systems (Labsystems, Vienna, VA, USA).

5) High-performance anion-exchange chromatography with pulsed amperometric detection

A previously published protocol was followed [14]. Briefly, 12 U/mL of human α -galactosidase A in 100 mM Hepes buffer at pH 5.0 was incubated with 0.5 mM synthetic type I α -gal (Genechem, Seoul, Korea) at 37°C. The reaction was discontinued for 0 hour, 2 hours, and 6 hours as indicated in Fig. 1. Hydrolysis of the α -gal type I epitopes (Gala1, 3Galb1, and 4Glc) by α -galactosidase A was monitored using high-performance anion-exchange chromatography with pulsed amperometric detection with a CarboPac PA-100 column (4 \times 250 mm, i.d. 4 μm). The eluates were eluted by a NaOH gradient elution at a flow rate of 1 mL/min (Dionex,

Sunnyvale, CA, USA).

6) Flow cytometry

The previously published protocol was used after minor modifications [15]. Briefly, 3×10^5 of PAECs with 10 U/mL human α -galactosidase A were incubated in 100 mM of Hepes buffer (pH 5.0) for 4 hours at 37°C. They were then centrifuged at 300 xg for 2 minutes and were washed with PBS twice. Afterwards, they were finally incubated in 1 ug/mL of Griffonia simplicifolia 1-B4 (GSIB4)-fluorescein isothiocyanate (FITC) at 37°C for 1 hour. A flow cytometry was performed using a flow cytometer (Coulter Epics XL; Beckman Coulter, Fullerton, CA, USA).

7) Immunohistochemistry

Frozen blocks were sectioned into 4-um thicknesses. The sectioned samples were incubated in 1 ug/mL biotinylated GSIB4 lectin (Vector Lab, Burlingame, CA, USA). They were washed with PBS and treated with 5 ug/mL avidin-HRP (Molecular Probes, Eugene, OR, USA), and α -gal epitopes on the valves were visualized using 3,3'-diaminobenzidine (DAB; Vector, Burlingame, CA, USA) as a substrate. The DAB staining intensity of the valve tissues was captured under light microscopy.

8) Lectin-based enzyme-linked immunosorbent assay for α -gal epitopes on tissues

The punched pieces of the tissues were homogenized using a pulverizing machine cooled by liquid nitrogen. They were then incubated overnight in 0.5 mL of diluted isolectin-biotin of 10 ug/mL at 37°C with 120 rpm agitation. After that, an unbound lectin solution was collected by centrifugation at 1,500 rpm. From this, 1:1, 1:10, and 1:100 diluted solutions were obtained, and 5 ug/mL α -gal-BSA was added to each of these three solutions and the standard curve sample (IB4-biotin, 5-500 ng/mL in 3% BSA/PBS blocking buffer). These samples were immobilized on the microtiter well plate for 1 hour at 37°C, which blocked non-specific binding with bovine serum albumin. After washing, 0.1 ug/mL of the streptavidin-conjugated HRP was incubated in each sample well for 1 hour at 37°C. Following several washes with distilled water, the reaction was developed with a TMB solution (Pierce, Rockford, IL, USA) and ceased with a 2M H₂SO₄

solution. The absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Labsystems, Vienna, VA, USA) at 450 nm.

9) Permeability and compliance testing

Permeability variations were evaluated by measuring the leaked volume of saline solution and applying constant pressure of 100 mmHg on the porcine pericardial tissues (1 cm², n=12) for 1 hour. This test is performed to evaluate the differences of penetration, elongation, and the degrees of the gap that have appeared in the collagen fiber bundles. Compliance was evaluated by measuring the transformed volume of saline solution during the pressure increases from 100 mmHg to 200 mmHg on one side of the pericardial tissues (n=12). The unit is set as $\mu\text{L}/\text{mmHg} \cdot \text{cm}^2$ by dividing the unit area. Changes of compliance indicate the stiffening of the collagen fibers in the high-strain working region, where the elastic behavior is dominated by the fibers themselves [16].

10) Tensile strength testing

A uniaxial test was used to compare the mechanical properties of the differently treated tissues. Tissue strips (5×50 mm, 10 strips for each group, 60 strips for fresh or fixed bovine pericardium) were cut in different directions to overcome material anisotropy. Tissue thickness was measured at 3 points using a thickness gauge (Quick-Mini 700-117; Mitutoyo, Kawasaki, Japan). Tensile properties were evaluated using a tensile testing machine (K-ML-1000N; M-TECH, Seoul, Korea) equipped with a digital force gauge (DS2-50N; IMADA, Tokyo, Japan) operating at an extension rate of 100 mm/min. Ultimate strength and strain at the fracture were evaluated from the recorded stress-strain curves.

11) Transmission electron microscopy

The bovine pericardial tissues were fixed with 2.5% of formaldehyde and 2.5% of glutaraldehyde in 0.1 mol/L of phosphate buffer at pH 7.2, then stained with 2% OsO₄ in the same buffer. They were then dehydrated with graded ethanol and embedded in Araldite/Epon. Thin sections were contrasted with uranyl acetate and lead citrate. Observations and photographic records were obtained with a TEM

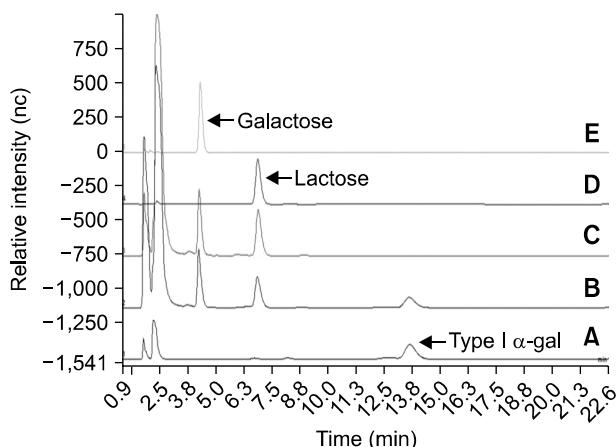


Fig. 2. Measurement of recombinant human α -galactosidase A activity using ion exchange column chromatography. (A-C) The α -gal synthetic type I was treated with recombinant human α -galactosidase A for 0 hour, 2 hours, and 6 hours. The output productions of monosaccharide which was cut by enzyme, were analyzed by high-pressure anion-exchange column chromatography. Galactose (E) and lactose (D) were used as controls.

(JEM-1400; JEOL, Tokyo, Japan) at 80 kV.

12) Statistical analysis

Statistical analyses were performed using one-way analyses of variance Sigma Stat ver. 3.0 (SPSS Inc., Chicago, IL, USA). Statistical differences were compared using either the Student's t-test or a paired t-test. Probability values of <0.05 were considered statistically significant.

RESULTS

1) Recombinant human α -galactosidase A activity using ELISA, ion exchange column chromatography, and removal of α -gal epitopes on PAECs

To investigate the optimal condition of recombinant human α -galactosidase A activity, we established an α -galactosidase A activity assay using an ELISA (Fig. 1) and ion exchange column chromatography (Fig. 2). Recombinant human α -galactosidase A effectively removed α -gal under acidic conditions, but not under physiological conditions. Recombinant human α -galactosidase A was most effective under pH 5.0 (pH 5.0 $>$ 6.0 $>$ 7.0 $>$ 8.0) at 37°C in a concentration dependent manner (Fig. 1). Treatment of α -gal synthetic type I

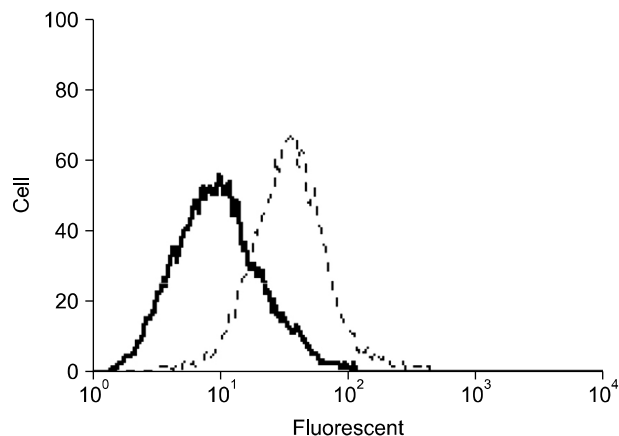


Fig. 3. Removal of α -gal epitope from porcine aortic endothelial cells (PAECs) by recombinant human α -galactosidase A. PAECs were treated with 10 U/mL recombinant human α -galactosidase A (dotted line) or mock treated (solid line). Griffonia simplicifolia 1-B4-fluorescein isothiocyanate was used to detect the presence of α -gal on the surface of PAECs using flow cytometry.

with recombinant human α -galactosidase A was performed for 0 hour, 2 hours, and 6 hours. High-pressure anion-exchange column chromatography demonstrated that the α -gal synthetic type I disappeared and output products such as galactose and lactose appeared in a time-dependent manner (Fig. 2). The α -gal levels were examined using GSIB4-FITC and flow cytometry. α -Galactosidase A significantly removed α -gal epitopes expressed on the surface of PAECs (Fig. 3).

2) Removal of α -gal epitopes from the PAECs and the bovine pericardium using recombinant *Bacteroides thetaiotaomicron* α -galactosidase A

The bovine pericardium was decellularized. The α -gal epitopes on the bovine pericardium were sequentially stained with biotinylated lectin and avidin-HRP. GSIB4 is known to bind to the α -gal epitopes [17-19]. There were residual α -gal epitopes obtained on the surface of the decellularized bovine pericardium. To investigate the additional effect of recombinant *B. thetaiotaomicron* α -galactosidase on decellularized bovine pericardium, it was incubated in the presence of recombinant *B. thetaiotaomicron* α -galactosidase after decellularization (pH 7.2, 24 hours, 4°C) and stained, as described above, to observe how residual α -gal epitopes exit. Compared to the only decellularized bovine pericardium, the

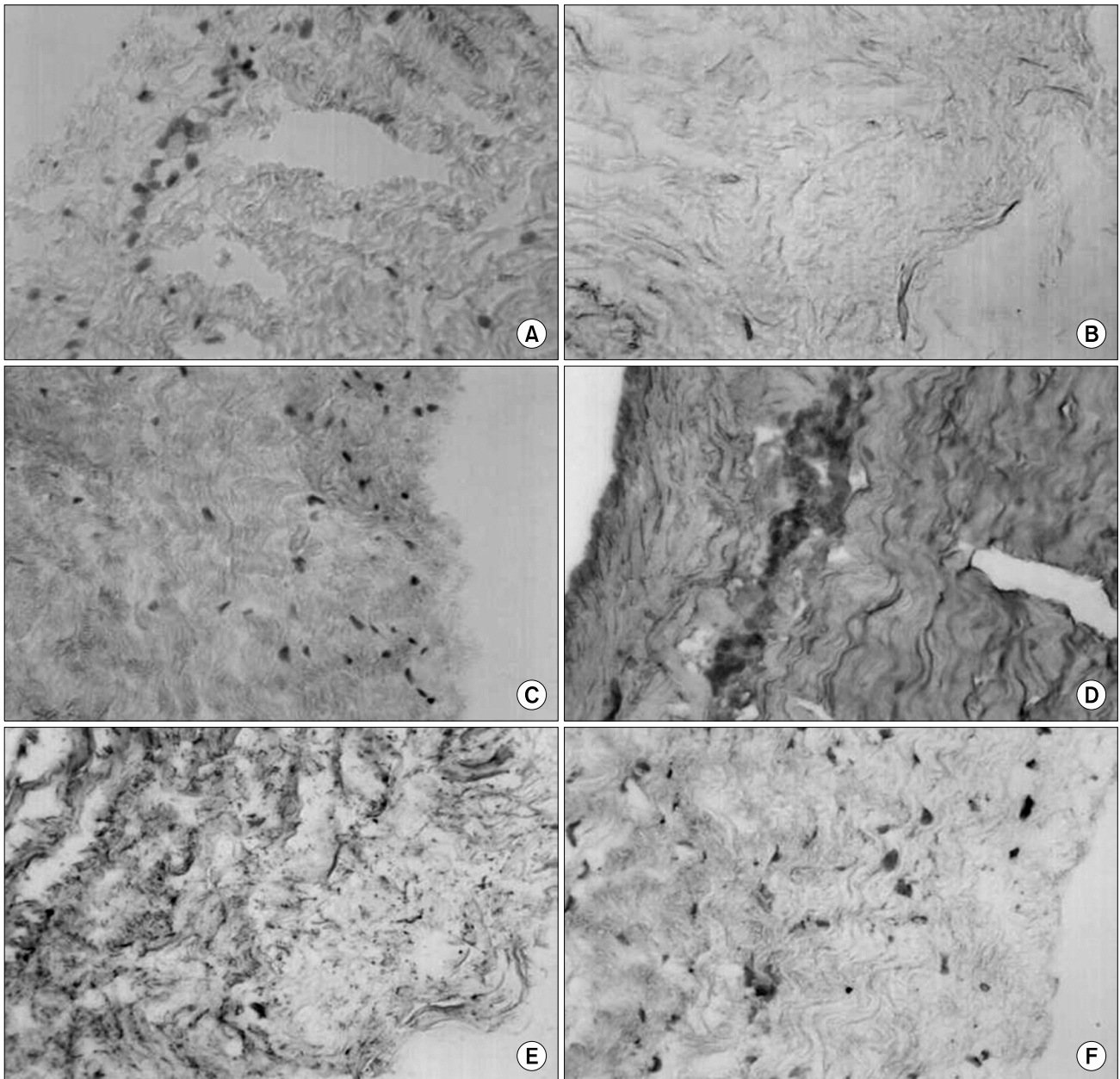


Fig. 4. Removal of α -gal epitopes on the bovine pericardium by recombinant *Bacteroides thetaiotaomicron* α -galactosidase using lectin histochemistry. Fresh bovine pericardium (D). Bovine pericardium treated with decellularization (E). Bovine pericardium treated with decellularization and α -galactosidase (F). (A), (B), and (C) were negative control of (D), (E), and (F), respectively. The frozen tissue samples were stained with biotinylated lectin and avidin-peroxidase to detect α -gal epitope. 3,3'-Diaminobenzidine staining intensity of tissues was captured by light microscopy ($\times 400$).

α -gal epitopes on the surface of the bovine pericardium were nearly completely removed (Fig. 4).

3) Quantitative evaluation of α -gal on the surface of the bovine pericardium

The amounts of isoelectin from the fresh bovine pericardium, the bovine pericardium treated with decellularization,

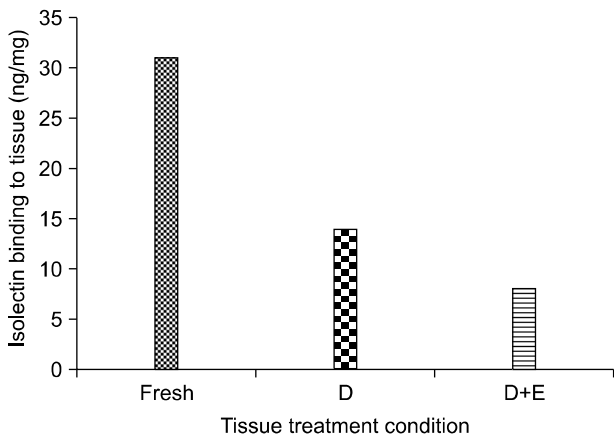


Fig. 5. Quantitative evaluation of α -gal on the surface of bovine pericardium. The isolectin binding to fresh bovine pericardium (fresh), bovine pericardium treated with decellularization (D), and bovine pericardium treated with decellularization (D) and recombinant *Bacteroides thetaiotaomicron* α -galactosidase (E), (D+E).

and the bovine pericardium treated with decellularization and α -galactosidase were 30.91, 13.69, and 7.91 ng/mg (n=1), respectively. The total amounts of bound isolectin were determined by subtracting the remaining amounts of isolectin from the initial total amounts after tissue incubation. Each isolectin of the decellularized tissues, and the decellularized tissues treated with α -galactosidase was lowered by 55.72% and 74.41%, respectively, compared to fresh bovine pericardium (Fig. 5). After decellularization and α -galactosidase processing, α -gal epitopes on the surface of the bovine pericardium were removed to a noticeable extent.

4) Permeability and compliance testing

The effects of treatment with α -galactosidase and decellularization of the tissues were monitored by measuring the leakage volume and compliances. The leakage volume of the tissue treated with α -galactosidase (group 2) and the decellularized tissue (group 3) was not significantly different from the fresh tissue (group 1) (group 1 vs. group 2, p=0.454; group 1 vs. group 3, p=0.580), and their compliance was also not different (group 1 vs. group 2, p=0.315; group 1 vs. group 3, p=0.365). In the GA-fixed groups, the leakage volume of the tissue treated with α -galactosidase (group 5) and the decellularized tissue (group 6) was not different from the simple GA-fixed tissue (group 4) (group 4 vs. group 5,

Table 1. Permeability and compliance of variously treated bovine pericardium (n=12)

Groups	Permeability (mL/hr · cm ²)	Compliance: 100 to 200 mmHg (μL/mmHg · cm ²)
Group 1	0.63±0.40	1.46±0.35
Group 2	0.76±0.628	1.60±0.55
Group 3	0.53±0.62	1.9±0.79
Group 4	3.26±1.04	4.71±0.90
Group 5	3.76±1.33	5.23±1.21
Group 6	4.57±2.43	6.44±1.75

Values are presented as mean±standard deviation.

p=0.517; group 4 vs. group 6, p=0.098). However, there were significant differences in permeability and compliance between the unfixed groups (group 1, 2, and 3) and the fixed groups (group 4, 5, and 6) (p<0.001) (Table 1).

5) Tensile strength testing

The tensile strengths of the tissue treated with α -galactosidase (group 2) and the decellularized tissue (group 3) were not significantly different from the fresh tissue (group 1 vs. group 2, p=0.692; group 1 vs. group 3, p=0.348). In the GA-fixed groups, the tissue treated with α -galactosidase (group 5) was not different from the simple GA-fixed tissue (group 4; p=0.722). The decellularized tissue (group 6) was also not different from the simple GA-fixed tissues (group 4; p=0.800) (Table 2). However, there was a significant difference between the unfixed groups and the GA-fixed groups (p<0.001). The tissues in the GA-fixed groups tended to be thicker than the unfixed groups, but the tensile strength of the tissues in GA-fixed groups was lower. However, strain at fracture was higher in the GA-fixed groups than in the unfixed groups.

6) Transmission electron microscopy

The gaps between the collagen fiber bundles of the decellularized bovine pericardium (Fig. 6B) and the decellularized bovine pericardium treated with α -galactosidase (Fig. 6C) displayed similar collagen patterns. The general measurement of the gaps from the collagen bundles was obtained to compare the shapes and sizes of the collagens. To analyze the tendency toward the gap formation, two areas from the

Table 2. Tensile strength of variously treated bovine pericardium

Groups	Sample size	Thickness (mm)	Ultimate strength (MPa)	Strain at fracture (%)
Group 1	n=40	0.29±0.05 (0.21–0.42)	17.15±2.86 (11.59–23.03)	42.42±7.70 (30.00–60.00)
Group 2	n=40	0.31±0.07 (0.21–0.45)	16.82±3.61 (10.87–23.14)	43.58±8.72 (30.00–63.33)
Group 3	n=40	0.36±0.06 (0.23–0.50)	16.37±4.49 (10.36–24.44)	40.42±8.17 (30.00–66.67)
Group 4	n=40	0.36±0.46 (0.24–0.45)	15.18±2.63 (11.37–19.70)	50.33±8.96 (33.33–66.67)
Group 5	n=40	0.39±0.06 (0.30–0.545)	14.94±3.38 (9.30–21.65)	55.00±10.78 (40.00–73.33)
Group 6	n=40	0.44±0.06 (0.34–0.58)	15.35±3.02 (10.95–21.35)	64.00±6.37 (50.00–73.33)

Values are presented as mean±standard deviation.

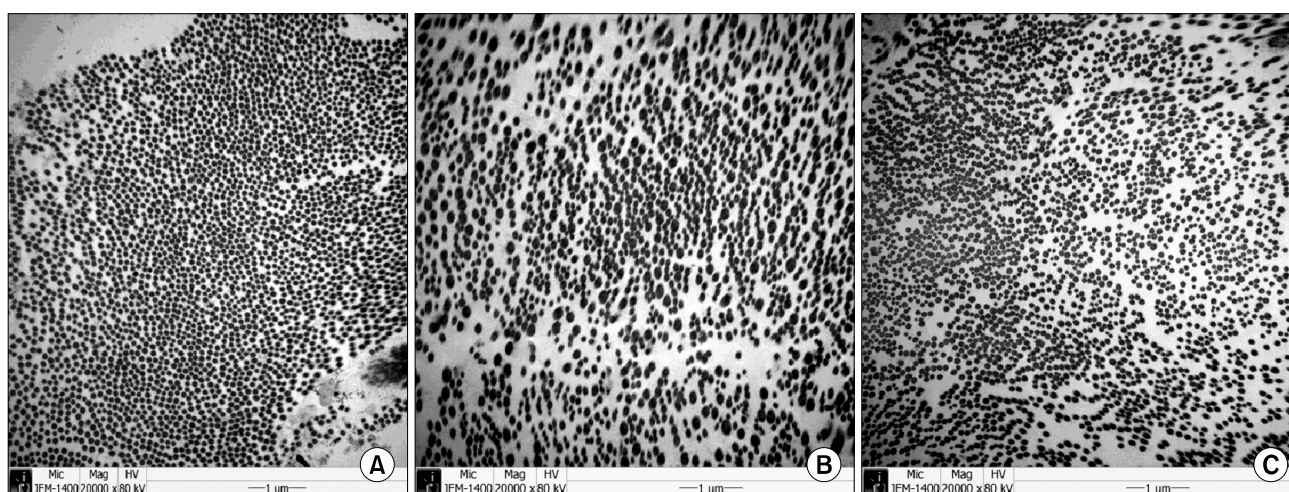


Fig. 6. Transmission electron microscope (TEM) assessment of bovine pericardium ($\times 400$). TEM results for the fresh bovine pericardium (A), bovine pericardium treated with decellularization (B), and bovine pericardium treated with decellularization and recombinant *Bacteroides thetaiotaomicron* α -galactosidase (C).

same part of the pericardium with the same distribution of collagen bundles were taken as specimens after enzymatic processes.

DISCUSSION

An early failure of xenogeneic heart valves, especially in children, has been a major drawback in clinical situations. Furthermore, the progressive degeneration of implanted bio-prosthetic heart valves caused by their limited durability is still a major obstacle to overcome. Among many factors, the presence of α -gal epitopes on cells or tissues may play a key role in inducing early xenoimmune inflammatory responses on an implanted xenograft heart valve [17].

It has been reported that porcine heart valves preserved

with glutaraldehyde alone do not have significantly longer longevities because of α -gal epitopes; the antigens of the xenograft valves [20] remained on commercially available porcine heart valves after treatment with glutaraldehyde [21,22]. The exact molecular mechanisms of degeneration have still not been proven, but, in clinical situations, α -gal epitope-induced immune responses develop rapidly in patients after implanting the xenograft heart valves [10,23,24]. Thus, eliminating α -gal epitopes on implantable xenograft heart valves may possibly improve the durability of the valves. Previously we reported the possibility that α -gal epitopes could be removed from porcine valves using recombinant human α -galactosidase A [13].

The human recombinant α -galactosidase A (10 U/mL) could remove both α -gal-BSA immobilized in microtiter

wells, and α -gal epitopes expressed on porcine aortic endothelial cells under the conditions of pH 5.0 and 37°C for 24 hours. The data derived from ion exchange column chromatography suggests that pH, rather than temperature, is important for activity of the human recombinant α -galactosidase A. As many as 10^6 to 10^7 of α -gal epitopes can be expressed on the surface of pig cells and tissues [25]. The decellularization procedure was initially introduced to diminish or attenuate the immune problems induced by the remaining α -gal epitopes on the porcine heart valves. In case where the decellularization procedure has been performed, α -gal antigens have still existed in the connective tissues; thus, the xenoreactive immune responses caused by remaining α -gal have been considered to have an important role in the initial triggering of the degeneration processes [6,26]. Therefore, α -galactosidase was introduced to cut α -gal epitopes on the surface of the matrix tissues directly. The activity of the human α -galactosidase A is optimized most under acidic pH conditions, and acidic tissue injuries can be a problem. Therefore, we evaluated the effectiveness of the treatment with recombinant *B. thalotaiomicron* α -galactosidase and the decellularization procedure in removing the α -gal epitopes on the bovine pericardial tissues under neutral pH conditions by using GSIB4 lectin-relative histochemistry. The activity of *B. thalotaiomicron* recombinant α -galactosidase (0.1 U/mL) was 100 times higher than human α -galactosidase A under the condition of pH 7.2 and 4°C after having been incubated for 24 hours. The removal of α -gal epitopes expressed on the bovine pericardial tissues was analyzed qualitatively by using a DAB staining test and quantitatively by using lectin-based ELISA assay. The amounts of isolectins of the decellularized tissues and the decellularized tissues after treating with α -galactosidase were lowered by 55.72% and 74.41%, respectively, compared to the fresh bovine pericardial tissues. Modified biomechanical properties of the pericardial tissues after the treatment with α -galactosidase and the decellularization were assessed by evaluating the permeability, compliance, and strength because a preservation of the native ultrastructures of extra cellular matrices of the tissues during the processes is desirable. In the unfixed groups, the leaked volume and compliance were similar to ($p > 0.05$) the fresh bovine pericardial tissues after the treatment with α -galacto-

sidase only or decellularization. However, there was a significant difference ($p < 0.05$) between the unfixed groups and the fixed groups. This indicated that the elongation of the collagen bundles and the extensibility of the tissues were affected by the fixation process rather than the decellularization procedure or the treatment with α -galactosidase as the fixation process can change the water contents, osmolarity, and collagen saturation of the tissues. The same pattern was also observed in the tensile strength tests. Some mechanical changes of the scaffold after treating with enzymes have been thought to be an usual finding, but in these experiments no significant differences were observed in the mechanical properties after the treatment with α -galactosidase or the decellularization process. The arrangements of the collagen fiber bundles on TEM findings changed more extensively in the tissues after the decellularization process than in the tissues after the treatment with α -galactosidase. However, incompletely removed α -gal epitopes on the scaffolds and matrices of the porcine heart valves, which are not accessible to α -galactosidase, can induce residual xenoreactive immune responses.

One limitation of this study is that the xenoreactive immune responses induced by xenograft heart valves were only confirmed in vitro. Further studies are required to prove the positive relationship between the degree of calcification of heart valves and the degree of xenospecific immune responses in the tissues implanted in non-human primates after removing the α -gal epitopes on the xenograft heart valves using α -galactosidase.

CONCLUSION

α -Gal on the xenograft heart valves might be removed completely using recombinant α -galactosidase and performing a decellularization procedure without modifying the mechanical properties of the tissues. No changes of the mechanical properties were confirmed by performing a compliance test, a permeability test, a tensile strength test, or by observing the TEM findings. The application of the decellularization process and the treatment with α -galactosidase on the xenograft tissues is probably beneficial for diminishing the xenograft immune responses; thus, it can provide a prolonged durability for xenograft tissue.

CONFLICT OF INTEREST

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

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