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Immune responses of implanted bovine pericardium and porcine heart valves in an α1,3-galactosyltransferase knockout and wild type mouse model

알파갈 제거 및 정상 생쥐 모델에서 이식된 소심낭 및 돼지 심장 판막의 면역학적 반응

2014 년 2 월

서울대학교 대학원

의학과 박사과정

장 우 성

A thesis of the Degree of Doctor of Philosophy

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지도교수 이 정 렬

이 논문을 의학박사 학위논문으로 제출함
2013 년 10 월

서울대학교 대학원
의학과 흉부외과학 전공
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장우성의 의학박사 학위논문을 인준함
2014 년 01 월

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Immune responses of implanted bovine pericardium and porcine heart valves in an α1,3-galactosyltransferase knockout and wild type mouse model

by Woo Sung Jang

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Major in Thoracic and Cardiovascular surgery) at Seoul National University College of Medicine

January 2014

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논문 제목: Immune responses of implanted bovine pericardium and porcine heart valves in an α1,3-galactosyltransferase knockout and wild type mouse model

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ABSTRACT

Introduction: Degenerative calcification is an important mechanism of bioprosthetic failure. α-Gal epitope-induced immune responses have been reported as a potential cause of degenerative calcification in bioprostheses. The objective of this study was to evaluate the α-Gal immune reaction and anti-calcification effect of decellularization with or without α-galactosidase in glutaraldehyde-fixed bovine pericardium and porcine heart valves, using an α-Gal knockout (KO) & wild type mouse subcutaneous implantation model.

Methods: To elucidate the anti-calcification effect of decellularization with or without α-galactosidase, bovine pericardium and porcine heart valve tissues were assigned to the following 3 groups according to the tissue preparation methods: glutaraldehyde fixation only (GA), decellularization + GA fixation (Decell), and decellularization + α-galactosidase + GA fixation (Decell+α-galactosidase). The mechanical properties of the bovine pericardial tissue were measured before implantation. Each prepared tissue was subcutaneously implanted into both α-Gal KO and WT mice. Macrophage and T cell infiltration were examined via immunohistochemistry staining in the explanted tissues. Anti-α-Gal immunoglobulin (Ig) G and IgM antibodies were measured prior to implantation and at 4 weeks, 8 weeks, and 12 weeks after implantation using an enzyme-linked immunosorbent assay. The calcium contents in the explanted tissues were measured at 12 weeks after implantation.

Results: The Decell and Decell+α-galactosidase groups showed lower tensile strengths than did the GA group ($P < 0.001$, $P = 0.002$). Increased macrophage and T cell infiltration were observed in the α-Gal KO mice in all groups relative to WT mice. The

bovine pericardium showed more macrophage infiltration than did the porcine heart valve. The α-Gal-KO mice had higher anti-α-Gal IgG antibody titers than did the WT mice over time in response to both bioprosthetic materials, regardless of the tissue preparation methods (all $P < 0.001$). There were no significant differences in the anti-α-Gal IgG antibody titers according to the type of bioprosthetic material or tissue preparation method in the α-Gal-KO mice ($P > 0.05$). The calcium content was significantly lower in the porcine heart valves than in the bovine pericardium when implanted in the α-Gal-KO mice ($P < 0.001$). The calcium contents in the bovine pericardium and porcine heart valves implanted in α-Gal-KO mice were significantly lower in the Decell and Decell+α-galactosidase groups than in the GA group (all $P < 0.05$). However, there was no significant difference in calcium content between the Decell and Decell+α-galactosidase groups (all $P > 0.05$).

Conclusions: The porcine heart valve induced lower levels of macrophage/T cell infiltration and calcium deposition than did the bovine pericardium, although the anti-α-Gal IgG antibody titers did not significantly differ between the bioprosthetic tissues. Decellularization with or without α-galactosidase had significant anti-calcification effects in both the bovine pericardium and porcine heart valves implanted in the α-Gal KO mice, although there was no significant difference in the anti-α-Gal IgG antibody titers among tissue preparation methods. The discrepancies in the calcium deposition and anti-α-Gal IgG antibody titers observed in our study suggested that bioprosthetic calcium deposition might occur through a multifactorial mechanism.

Keywords: Glutaraldehyde · calcification · knockout mice · porcine heart valves
Student number: 2012-30520

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

GA: Glutaraldehyde

SDS: sodium dodecyl sulfate

α-Gal: Galα1,3-Galβ1,4GlcNAc-R

α-Gal KO: the *α*1,3-galactosyltransferase knockout

GAG: glycosaminoglycan

WT: wild type

PBS: phosphate-buffered saline

Decell: Decellularization

H & E: hematoxylin-eosin

IHC: immunohistochemistry

RT: room temperature

HRP: horseradish peroxidase

ELISA: enzyme-linked immunosorbent assay

BSA: bovine serum albumin

ANOVA: one-way analysis of variance

RM-ANOVA: repeated-measures analysis of variance

CMAH: cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase

INTRODUCTION

Glutaraldehyde (GA)-treated bioprostheses are commonly used as reconstructive biomaterials during cardiovascular surgeries. GA increases the resistance of these biomaterials to enzymatic degradation by forming surface polymers and cross-linking with collagen in the bioprosthetic tissues [1, 2]. However, some drawbacks have been observed with these GA-treated bioprostheses, including biological matrix deterioration and tissue degeneration-associated calcification during long-term implantation [3]. During the GA fixation process, cross-linking reactions between the GA aldehyde groups and collagen amine groups produce residual free aldehydes. These free aldehydes are well known to induce cell necrosis and immune responses and to eventually lead to degenerative calcification and early graft failure [2, 4-7]. Therefore, preoccupation of the free aldehydes with elements such as magnesium has been used as a method for preventing degenerative calcification [8]. In addition to free aldehydes, various factors contribute to degenerative calcification, including mechanical stress, protein and circulating cell deposition, cavity formation after proteoglycan removal, and immune response activation.

The immune response induced by the strong xenoreactive antigen Galα1,3-Galβ1,4GlcNAc-R (α-Gal) is known as the main cause of hyperacute rejection, and this mechanism has also been reported in the context of bioprosthetic degenerative calcification and failure [9, 10]. Lee et al. reported that the implantation of bovine pericardium into α1,3-galactosyltransferase knockout (α-Gal KO) mice caused significant increases in anti-α-Gal Immunoglobulin (Ig) G antibody levels and that these transplanted tissues featured some histologic evidence of chronic rejection and revealed a tendency

toward increased calcification [11]. Other reports also supported the hypothesis that an immunologic antibody-mediated reaction to the α-Gal antigen was the main cause of bioprosthetic degenerative calcification [12-16]. Furthermore, α-Gal epitopes have reportedly remained present in commercial GA-treated bioprosthetic heart valves [17].

Bioprosthesis decellularization reduces immune responses and suppresses early calcium nucleation by eliminating tissue components, while supporting the physical properties of the tissue and preserving the suitable extracellular stroma for recellularization. Common detergents used during chemical decellularization include sodium dodecyl sulfate (SDS), Triton X-100, Tween-20, MEGA 10, and deoxycholate. SDS is commonly used because of its ability to stably eliminate bioprosthetic cells [18-20]. However, several reports have indicated that decellularization is not sufficient for α-Gal epitope removal [21, 22]. Therefore, the use of α-galactosidase or the removal of α-1,3 galactosyl transferase, which synthesizes the α-Gal antigen epitope, in an α-Gal KO model can be used to remove the α-Gal antigenic epitope [23, 24] and would be expected to decrease the α-Gal antigen-antibody-mediated immune response.

To evaluate the chronic immune responses associated with the bioprosthetic calcification, this study used a genetically manipulated animal model, the α-Gal KO mouse. α-Gal KO mice are an option in which to evaluate immune responses in human-like immunologic environments because similar to humans, these mice lack α-Gal epitopes. Therefore, bioprostheses from which xenoreactive α-Gal epitopes have been removed via decellularization with or without α-galactosidase could be used to reduce calcification and improve bioprosthetic durability.

The various bioprostheses used during cardiovascular surgery each have their own

characteristics and limitations. Among these materials, bovine pericardium and porcine heart valve leaflets are representative tissues that are commonly used during cardiovascular surgery. Histological and chemical differences in the compositions, characteristics, and post-implantation durabilities of these two materials have been reported [25-28]. Porcine heart valve leaflets comprise three layers of connective tissues, as follows: collagen fibers in the fibrosa layer, a glycosaminoglycan (GAG) matrix in the spongiosa layer, and elastin sheets in the ventricularis layer. The collagen exists to support the tensile load and provide some stiffness and strength to the valve. The GAGs maintain hydration and the intrinsic viscoelasticity of the tissue. Finally, the elastin matrix exists to restore the collagen structures to their resting states between loading cycles [29-33]. In contrast to porcine heart valves, bovine pericardium is almost entirely composed of type I collagen, arranged hierarchically at different levels of organization and in various structures with non-linear and anisotropic mechanical behaviors. Bovine pericardium comprises a network of collagen and elastic fibers that are embedded in an amorphous matrix composed mainly of free GAG and proteoglycans [34-36].
Previous reports have stated that bovine pericardium [11] and porcine heart valves [15, 21, 22], when implanted into α-Gal KO mice, significantly increased the levels of anti-α-Gal antibodies and exhibited histological evidence of chronic rejection. However, to our knowledge, the immune responses of these α-Gal KO mice and the effects of anticalcification treatments on bovine pericardium and porcine heart valves have not been compared. Therefore, the objective of this study was to evaluate the effects of decellularization with or without α-galactosidase on immune responses and anti-calcification in glutaraldehyde fixed bovine pericardium and porcine heart valves, using

an α-Gal knockout (KO) mouse subcutaneous implantation model.

MATERIALS AND METHODS

All animals in this study were treated in compliance with the "Guide for the Care and Use of Laboratory Animals", published by the National Institute of Health (NIH Publication No. 86-23, revised 1985). This study was approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute, Seoul National University Hospital (IACUC No. 12-0219). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. α-Gal KO (n = 72; C57BL/6 background; 6–7 weeks old; average weight, 29.8 ± 3.0 g) mice and WT mice (n = 72; average weight, 29.4 ± 2.5 g) were used as the experimental animals. α-Gal KO and WT mice were obtained from the Center for Animal Resource Development, Seoul National University College of Medicine.

Tissue preparation

Fresh bovine pericardium and porcine heart valve tissues were washed with normal saline and placed into phosphate-buffered saline (PBS; 0.1 M, pH 7.4). All tissues treated according to the tissue preparation methods were cut into round disc shapes and stored in a 2% propylene oxide solution before use. Tissues were then implanted into the subcutaneous layers of α-Gal KO and WT mice for a 12-week period.

Experimental design

The bovine pericardial and porcine heart valve tissues were assigned to the following 3 groups according to the tissue preparation methods:

Group 1: Simple GA fixation treatment (GA group)

Group 2: Decellularization + GA fixation treatment (Decell group)

Group 3: Decellularization + alpha-galactosidase + GA fixation treatment (Decell+α-galactosidase group)

Aortic and pulmonary heart valves exhibit similar structural elements containing mostly fibroblasts and comprise glycoproteins, elastic fibers, fibrillar collagens, and an amorphous substance composed of proteoglycans with similar functions but different loading pressures [37, 38]. Therefore, porcine aortic and pulmonary valves were used as interchangeable materials in this study. A schematic experimental design diagram for a given number of mice is shown in Fig. 1.

Tissue preparation methods

GA fixation treatment methods

Tissue materials were fixed in 0.5% buffered GA for 3 days at room temperature, and then in 0.25% buffered GA for 7 days at room temperature.

Decellularization methods

All tissues were disinfected in 0.1% peracetic acid, 4% (v/v) ethanol in distilled water for 2 hours at room temperature. The decellularization processes were different for the pericardium and heart valve tissues due to their natural differences in tissue composition and mechanical properties. The porcine heart valves were subsequently washed for 30 minutes in distilled water, and a hypotonic 1.0% SDS solution was added to the tissues for 24 hours at 4°C, after which a hypotonic 1.0% Triton X-100 solution was added for 24 hours at 4°C; this step was repeated for 5–6 times. The tissues were then washed in PBS

for 24 hours at 4°C. The bovine pericardium tissues were subsequently washed for 30 minutes in distilled water, and a hypotonic 0.5% SDS solution was then added for 24 hours at 4°C, followed by a hypotonic 0.5% Triton X-100 solution for 24 hours at 4°C. The tissues were then washed in PBS for 24 hours at 4°C.

α *-galactosidase treatment methods*

α-galactosidase in PBS was added to the tissues for 24 hours at 4°C, after which the tissues were washed in PBS for 24 hours at 4°C.

Mouse subcutaneous implantation protocol

Intraperitoneal injections of zolazepam + tiletamine (0.2 cc of Zoletil; Virbac, France) and xylazine (0.2 cc of Rumpun; Bayer, Germany) were used to anesthetize the mice. Four subcutaneous pouches were created in the dorsal areas of the mice after shaving and wound dressing with a povidone-iodine (Betadine®) solution. Next, a round tissue disc (6 mm in diameter) was implanted into each of the 4 pouches, and the wounds were closed with 4-0 monofilament sutures. Antibiotics, including cefazoline (20 mg/kg, Yuhan, Korea), were administered for 3 days after tissue implantation. The mice were sacrificed by CO_2 gas inhalation at 12 weeks post-implantation without any issues, and the tissue samples were then harvested.

Analysis assessments

Tensile strength and elasticity

Tensile strength and elasticity measurements were performed to identify and compare

the mechanical strengths after treatment preparation methods. These tests were performed only on pre-implantation bovine pericardium due to the limited availability of the porcine heart valve leaflets. Tensile strength was measured with the uniaxial test, using prepared 5 mm x 50 mm strips of bovine pericardium that were measured at a 30-degree angle on a tensile strength machine (K-ML-1000N, M-TECH, Seoul, Korea). The measurements were recorded with a digital force gauge (Model 5FGN, Japan Tech & Manufacture IMADA; Mitutoyo, Tokyo, Japan) at a load speed of 100 mm/min and in units of MPa (Kgf /5 mm width). The elongation percentage was measured at the breaking point while under bidirectional tension during the elasticity test.

Thermal stability test

To measure the thermal stabilization according to the tissue preparation method, a thermal stability test was performed. As in the tensile strength and elasticity tests, the thermal stability test was performed only on pre-implantation bovine pericardium due to the limited availability of porcine heart valve leaflets. The tissue shrinkage temperatures were measured using a hydrothermal method to assess the thermal stabilities of the tissues. The 8 x 30 mm tissue strips were loaded to a weight of 95 g and held at a constant extension along their long axes in a 55°C water bath. The water temperature was increased at a rate of 2.5°C/min, as measured by a waterproof digital thermometer (Alla France, France), and the width of each tissue strip was measured with a microscope. The sharp deflection point at which the tissue strips abruptly contracted was recorded, and the shrinkage temperatures were analyzed in a plotted graph.

Pronase test

To detect changes in protein digestibility, a pronase test was performed. In this test, 10 x 10 mm tissue strips were weighed after being dried at 80°C. Next, 0.5 mg/mL of pronase in 0.01 M HEPES (pH 7.4), 0.1 M glycine, and 0.01 M CaCl2 was added to the strips, which were then incubated with shaking at 50°C for 24 hours. The tissues were dried again at 70°C after removing the soaking solution. Finally, the ratios of the tissue weights before and after the pronase treatment were determined.

Microscopic examinations

Post-implantation tissue samples from each group were examined by light microscopy. Tissue samples were fixed in 10% formalin and embedded in paraffin wax, and 4-μm-thick sections were cut. Post-implantation tissue sections were stained with hematoxylin-eosin (H & E) to identify structural changes, including collagen fibers and inflammatory cellular reactions, and according to the von Kossa method to detect calcification.

Immunohistochemistry (IHC)

Harvested tissue samples were stained for murine macrophages and T cells. Antibodies against the F4/80 antigen, a mouse macrophage marker (1:300 dilution; eBioscience, San Diego, CA, USA), and murine CD4, a T cell marker (1:300; eBioscience) were used.

Preparation of frozen tissue sections

Freshly dissected tissue blocks were placed onto a pre-labeled tissue disposable base

mold (Simport, QC, Canada; 15 x 15 x 5mm) that had been covered with cryo-embedding media (optimal cutting temperature compound; Leica Microsystems GmbH, Wetzlar, Germany), and stored at -80ºC until ready for sectioning. Prior to sectioning, the frozen tissue blocks were transferred to a cryotome cryostat at -20ºC, and cut into a 4-µm-thick slices with the cryotome.

Immunostaining of frozen tissue sections

To ensure proper fixation, fresh tissue blocks were immersed in acetone that had been pre-cooled to -20ºC for 10 min. The solution was then discarded, and residual acetone was allowed to evaporate from the tissue sections for more than 20 min at room temperature (RT). After the tissue sections were rinsed twice with 300 ml of 10 mM phosphate buffered saline (PBS) at a neutral pH for 5 min per wash, they were incubated in a 0.3% H_2O_2 solution in PBS at RT for 10 min to block endogenous peroxidase activity. The tissue sections were again rinsed with 300 ml PBS in 3-5 min washes, and a protein blocking buffer (DaKo North America Inc., California, USA) was added to cover the specimens in order to reduce the background. The sections were incubated for 20 min at RT and rinsed in 300 ml PBS 3 times for 5 min each. After that, two drops (50 µl/drop) of Avidin and d-Biotin (Invitrogen Corporation, Camarillo, CA, USA) were applied to the tissues, followed by 10-min incubation at RT. After rinsing, 100 µl of an appropriately diluted primary antibody (1:300) was applied to each section, followed by incubation overnight at 4ºC. On the following day, 100 µl of an appropriately diluted horseradish peroxidase (HRP)-conjugated donkey anti-rat IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA) secondary antibody was added to the sections on the slides,

followed by one hour incubation at RT. Next, 100 μl of a diaminobenzidine (Lab Vision Corporation, Fremont, CA, USA) substrate solution was applied to the sections on the slides to reveal the antibody staining color after rinsing. The slides were counterstained by immersion in Hematoxylin (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 min. The tissue slides were dehydrated in 6 graduated alcohol solutions (70%, 80%, 90%, 95%, 100%, and 100%) for 5 min each. After clearing the tissue slides in 3 changes of xylene for 5 min and attaching coverslips with a mounting solution, the antibody staining was observed via microscopy.

Calcium content analysis

Tissue samples harvested from the bovine pericardium and porcine heart valves were washed in normal saline, dried at 70°C for 24 hours, and weighed. These tissue samples were hydrolyzed in 5.0 N HCl and dried again at 75°C for 24 hours. Next, the calcium contents were measured by colorimetry according to the O-cresolphthalein complexone method, using an automatic chemistry analyzer (Hitachi 7070; Hitachi High Technologies Corporation, Japan). The calcium and phosphorus contents were expressed in units of μg/mg dry weight.

Enzyme-linked immunosorbent assay (ELISA) for anti-α-Gal antibody measurements

To measure the activities of serum IgM and IgG antibodies against anti-α-Gal in the α-Gal KO (n = 70) and WT mice (n = 70), an ELISA was used, and results were obtained at pre-implantation and at 4, 8, and 12 weeks post-implantation. Approximately 0.2–0.4 ml of blood were obtained via infraorbital venous-plexus sampling. Synthetic α-Gal

epitopes, which were linked to bovine serum albumin (α-Gal-BSA; Dextra, Reading, UK) as a solid-phase antigen, were used as the primary antigen. Microtiter plates were coated with 100 μl per well of 1 μg/mL α-Gal-BSA in PBS, pH 7.4, for both the IgM and IgG isotypes, and the plates were incubated for 1 hour at 37ºC. Mouse sera were added to the immobilized α-Gal-BSA-coated wells in three serial dilutions (1:20, 1:40, and 1:80) in BSA-Tween 20 (PBS, pH 7.4 plus 3% BSA (GIBCO^{TM}, Invitrogen Corporation, New Zealand) and 0.01% Tween 20), and the plates were incubated for 1 hour at 37ºC. The secondary antibodies were horseradish peroxidase-conjugated rabbit anti-mouse IgM (Jackson ImmunoResearch) and goat anti-mouse IgG (Jackson ImmunoResearch), used at 1:10000 dilutions in 3% BSA/PBS-Tween 20, for IgM and IgG, respectively. The color reactions were developed with a tetramethylbenzidine solution (BD Biosciences, San Diego, CA, USA), and the absorbance at 450 nm was measured on an ELISA reader. The Thermo Electron-Lab System (Labsystems) was used to measure the titers of anti-α-Gal antibodies at 450 nm and determine the optical densities.

Statistical analysis

Continuous variables were expressed as means ± standard deviations. Student's *t*-test was used for normally distributed continuous variables. A one-way ANOVA was used for comparisons among more than 3 groups. The Bonferroni test with equal variances and the Tamhane test with unequal variances were used as post hoc methods. Changes in the parameters over time between two groups were compared with a repeated-measures analysis of variance (RM-ANOVA). A p-value < 0.05 was considered statistically significant. These analyses were performed with the SPSS statistical software package

(SPSS version 19.0, SPSS Inc., Chicago, IL, USA).

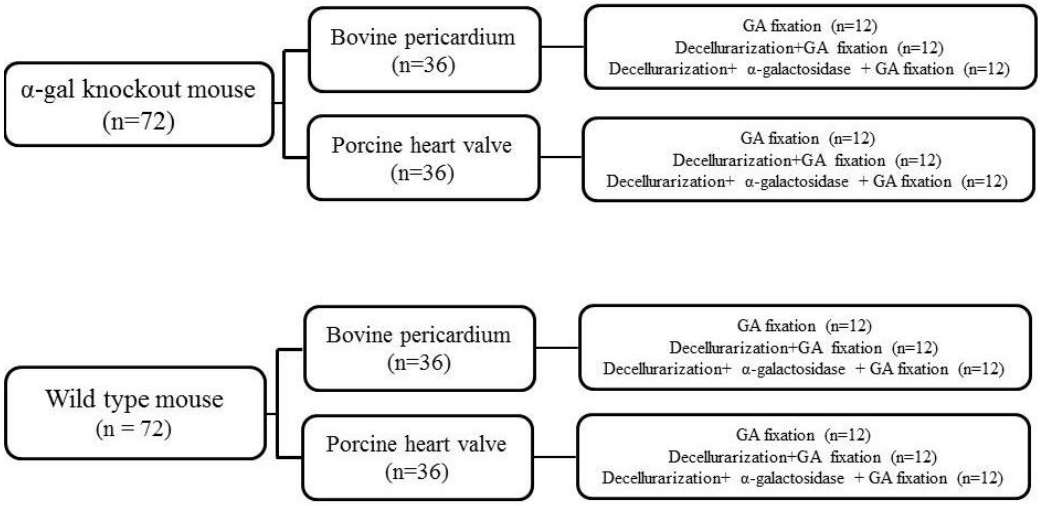

Figure 1. Schematic diagram of the experimental design for a given number of mice

RESULTS

Bovine pericardium tensile strength and elasticity tests

Tensile strength was significantly lower in the Decell (n = 12; 9.9 ± 1.1 MPa) and Decell+α-galactosidase groups (n = 12; 12.6 ± 5.1 MPa) than in the GA group (n = 12; 17.2 ± 2.3 MPa; $P < 0.001$ and $P = 0.002$, respectively). The elasticity was also significantly lower in the Decell (n = 12; 74.9 ± 9.0%) and Decell+α-galactosidase groups (n = 12; 65.7 ± 10.3%) than in the GA group (n = 12; 90.8 ± 11.0%; $P < 0.001$ and $P < 0.001$, respectively).

Thermal stability and pronase test

The thermal stabilities were similar among the three groups (GA group: 84.1 ± 0.7, n = 5; Decell group: 83.6 ± 0.7, n = 10; and Decell+α-galactosidase group: 83.6 ± 0.6, n = 10; $P = 0.252$). No differences were observed in the bovine pericardium pronase stability with respect to the tissue preparation methods ($p = 0.210$). However, the porcine heart valve pronase stability was significantly lower in the Decell (n = 10; 68.8 ± 13.7) and Decell+α-galactosidase groups (n = 10; 68.9 ± 11.3) than in the GA group (n = 10; 83.5 ± 8.7; $P = 0.003$ and $P = 0.003$, respectively).

Microscopic examination

Inflammatory cell infiltration was identified by H & E staining. Large amounts of inflammatory cell infiltrates were observed in the GA group. However, in the α-Gal KO mice, lower levels of inflammatory cell infiltration were observed in the Decell and Decell+α-galactosidase groups than in the GA group. Von Kossa staining revealed that in

the α-Gal KO mice, calcium deposition was also reduced in the Decell and Decell-α-galactosidase groups compared with the GA group, whereas in WT mice, no gross changes in calcium deposition were observed with respect to the tissue preparation method (Fig. 2).

IHC

F4/80 staining revealed significant macrophage infiltration in both the α-Gal KO and WT mice. However, the α-Gal KO mice exhibited higher levels of macrophage infiltration than did the WT mice. Notably, higher levels of macrophage infiltration were observed in the bovine pericardium than in the porcine heart valves. No difference was observed in the degree of macrophage infiltration with respect to the tissue preparation method (Fig. 3).

CD4 staining revealed a high degree of T cell infiltration in the α-Gal KO mice, whereas little or no T cell infiltration was observed in the WT mice. No difference was observed in the degree of T cell infiltration with respect to the type of prosthetic tissue used (bovine pericardium vs. porcine heart valves) or the tissue preparation method (Fig. 4).

Anti-α-Gal antibodies

The absolute serum IgG and IgM anti-α-Gal antibody titer values increased over time, irrespective of the mouse type. The IgG anti-α-Gal antibody titers demonstrated a statistically significant increase over time in the α-Gal-KO mice (all $P < 0.05$), except in the GA with bovine pericardium group (Table 2). Furthermore, the IgG titer displayed a

tendency to increase over time in the α-Gal-KO mice with a peak at 8 weeks, followed by a decrease.

Comparison between bovine pericardium and porcine heart valves in α-Gal KO and WT mice

The anti-α-Gal IgG antibody titers were consistently higher in the α-Gal-KO mice than in the WT mice for all tissue preparation methods (all $P < 0.001$). However, the anti-α-Gal IgM antibody titers did not differ significantly with respect to the tissue preparation method between the α-Gal-KO and WT mice (all $P = 1.000$). No differences were observed in the anti-α-Gal IgG and IgM antibody titers with respect to the use of bovine pericardium versus porcine heart valves (all $P > 0.05$).

Comparison according to the tissue preparation methods

The anti-α-Gal IgG antibody titers were not significantly reduced in the Decell ($P = 0.997$) and Decell+α-galactosidase groups ($P = 0.527$) compared with the GA group, in the *α*-Gal KO mouse. Additionally, no significant differences were observed in the anti-α-Gal IgG antibody titers between the Decell and Decell+α-galactosidase groups following bovine pericardium ($P = 0.865$) and porcine heart valve ($P = 0.536$) implantation in *α*-Gal KO mice.

No significant differences were observed in the anti-α-Gal IgM antibody titers with respect to the tissue preparation method and the type of bioprosthetic material in *α*-Gal KO mice (all $P = 1.000$). There were also no significant differences in the anti-α-Gal IgM antibody titers between the Decell and Decell+α-galactosidase group with respect to the

use of bovine pericardium ($P = 0.984$) and porcine heart valves ($P = 1.000$) in α-Gal KO mice (Tables 2, 3; Fig. 5, 6).

Calcium content

Comparison between α-Gal KO and WT mice

For the bovine pericardium, the calcium contents in tissues from the GA, Decell, and Decell+α-galactosidase groups were significantly higher in α-Gal KO mice than in WT mice (all $P < 0.001$). For the porcine heart valves, the calcium contents in tissues from the GA, Decell, and Decell+α-galactosidase- groups did not significantly differ between the α-Gal KO and WT mice ($P = 0.221$, $P = 0.253$, and $P = 0.589$, respectively).

Comparison between the bovine pericardium and porcine heart valves

In the α-Gal KO mice, the calcium contents were significantly lower in the porcine heart valves than in the bovine pericardium in the GA, Decell, and Decell+α-galactosidase groups (all $P < 0.01$). In the WT mice, the calcium content was lower in the porcine heart valves than in the bovine pericardium in the GA group ($P < 0.001$).

Comparison according to the tissue preparation methods

In the α-Gal KO mice, the bovine pericardium calcium contents were significantly lower in the Decell (13.4 ± 2.3, n = 23; $P = 0.003$) and Decell+α-galactosidase groups (13.7 ± 3.6, n = 22; $P = 0.009$) than in the GA group (16.4 ± 2.8, n = 24). Similarly, the calcium contents in the porcine heart valves were significantly lower in the Decell-GA (6.3 ± 1.2, n = 18; $P = 0.013$) and Decell+α-galactosidase groups (5.5 ± 2.1, n = 17; P

<0.001) than in the GA group (8.4 ± 3.7, n = 19). There were no significant differences in the calcium contents between the Decell and Decell+α-galactosidase groups in either the bovine pericardium or the porcine heart valves ($P = 0.854$ and $P = 0.325$, respectively; Fig. 7).

Table 1. Mechanical test in the bovine pericardium

Group	Tensile strength		Elasticity
	Thickness (mm)	Ultimate strength (Mpa)	Elongation at ultimate strength (%)
GA (n=12)	0.4 ± 0.03	17.2 ± 2.3	90.8 ± 11.0
Decell (n=12)	0.5 ± 0.02	9.9 ± 1.1	74.9 ± 9.0
Decell+α-Gal (n=12)	0.4 ± 0.08	12.6 ± 5.1	65.7 ± 10.3

GA: Glutaraldehyde; Decell: Decellurarization; Decell+α-Gal: decellurarization-α-galactosidase

Table 2. Changes in the anti-α-Gal IgG titers over time according to tissue treatment

Type	Tissue material	Tissue treatment	Pre. (n)	Post. 4wks(n)	Post. 8wks (n)	Post. 12wks(n)	*P* value
KO	Bovine pericardium	GA	1.4±0.3(12)	1.6±0.3(11)	1.8±0.3(10)	1.5±0.1(9)	0.066
		Decell	1.7±0.2(12)	1.7±0.0(10)	2.0±0.2(9)	1.5±0.1(7)	0.004*
		De-Gal	1.7±0.3(12)	1.7±0.1(11)	2.0±0.3(11)	1.5±0.0(8)	<0.001*
	Porcine valve	GA	1.6±0.4(12)	1.7±0.0(9)	2.0±0.3(9)	1.5±0.2(9)	0.005*
		Decell	1.7±0.3(12)	1.7±0.0(11)	1.9±0.3(9)	1.5±0.0(8)	<0.001*
		De-Gal	1.6±0.3(10)	1.7±0.0(8)	1.9±0.3(8)	1.5±0.0(8)	<0.001*
WT	Bovine pericardium	GA	0.5±0.1(11)	0.5±0.1(10)	0.5±0.1(9)	0.5±0.1(9)	0.543
		Decell	0.5±0.1(12)	0.5±0.1(12)	0.5±0.1(12)	0.5±0.1(11)	0.987
		De-Gal	0.5±0.0(11)	0.5±0.1(11)	0.5±0.2(10)	0.5±0.1(10)	0.948
	Porcine valve	GA	0.6±0.1(12)	0.6±0.1(12)	0.6±0.2(12)	0.6±0.1(11)	0.971
		Decell	0.5±0.0(12)	0.5±0.1(12)	0.6±0.2(12)	0.6±0.1(11)	0.753
		De-Gal	0.6±0.3(12)	0.6±0.1(8)	0.6±0.1(8)	0.6±0.1(8)	0.205

IgG:immunoglobulin G; Pre:pre-implantation; Post:post-implantation; KO:α-Gal knockout mouse; WT:wild-type mouse; GA:glutaraldehyde group; Decell:Decellurarization group; De-Gal:Decellurarzation-α-galactosidase group

Table 3. Changes in the anti-α-Gal IgM titers over time according to tissue treatment

Type	Tissue material	Tissue treatment	Pre (n)	Post 4wks(n)	Post 8wks (n)	Post 12wks(n)	*P* value
KO	Bovine	GA	0.5±0.1(12)	0.6±0.1(11)	0.5±0.1(10)	0.5±0.1(9)	0.001*
		Decell	0.6±0.1(12)	0.6±0.1(10)	0.5±0.1(9)	0.5±0.1(7)	0.246
		De-Gal	0.6±0.1(12)	0.6±0.1(11)	0.5±0.1(11)	0.6±0.1(8)	0.452
	Porcine	GA	0.6±0.1(12)	0.6±0.1(9)	0.5±0.1(9)	0.6±0.1(7)	0.280
		Decell	0.6±0.1(12)	0.6±0.1(11)	0.6±0.2(9)	0.6±0.1(8)	0.072
		De-Gal	0.6±0.2(10)	0.6±0.1(9)	0.5±0.1(9)	0.6±0.2(8)	0.197
WT	Bovine	GA	0.6±0.1(11)	0.6±0.2(10)	0.5±0.1(9)	0.5±0.1(9)	0.274
		Decell	0.6±0.1(12)	0.6±0.1(12)	0.6±0.2(12)	0.6±0.1(11)	0.164
		De-Gal	0.6±0.1(11)	0.6±0.1(11)	0.5±0.1(10)	0.6±0.1(10)	0.352
	Porcine	GA	0.6±0.1(12)	0.6±0.1(12)	0.5±0.1(12)	0.5±0.0(11)	0.072
		Decell	0.6±0.1(12)	0.6±0.1(12)	0.5±0.1(12)	0.5±0.1(11)	0.464
		De-Gal	0.6±0.1(12)	0.6±0.1(8)	0.5±0.1(8)	0.5±0.1(8)	0.112

IgM:immunoglobulin M; Pre:pre-implantation; Post:post-implantation; KO:α-Gal knockout mouse; WT:wild-type mouse; GA:glutaraldehyde group; Decell:Decellurarization group; De-Gal:Decellurarzation-α-galactosidase group

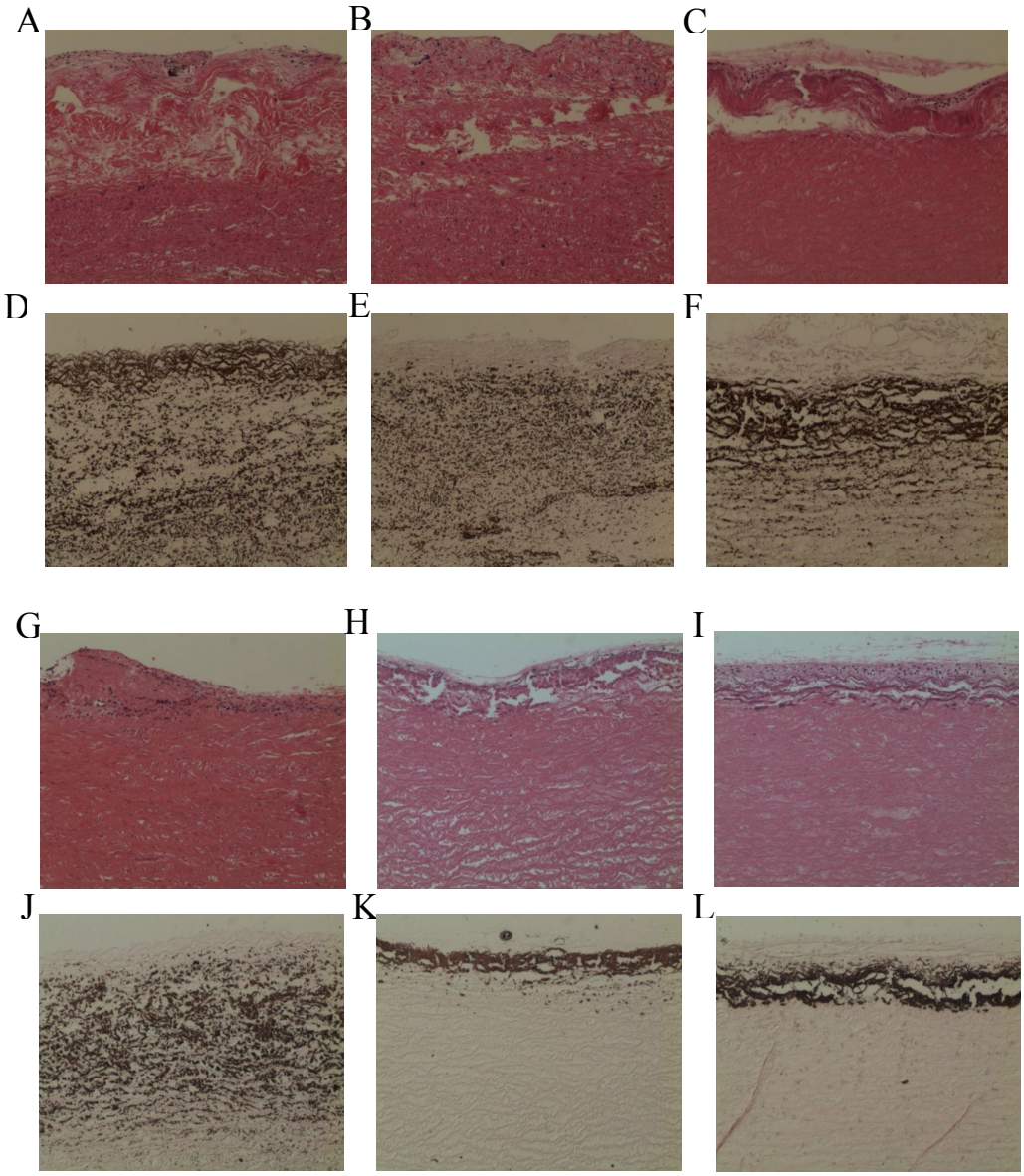

Figure 2. Representative microscopic examination of porcine heart valves.

Large numbers of inflammatory cells were observed in the GA group. However, among the α-Gal KO mice, a lower degree of inflammatory cell infiltration was observed in the Decell and Decell+α-galactosidase groups. Additionally, in the α-Gal KO mice, calcium

deposition was reduced in the Decell and Decell+α-galactosidase groups compared with that in the GA group.

A-C, G-I: H&E staining; D-F, J-L: Von Kossa staining.

A, D, G, and H: GA fixation; B, E, H, and K: decellularization-GA fixation; C, F, I, and L: decellularization-α-galactosidase-GA treatment.

A-F: wild type mice; G-L: α-Gal KO mice.

Decell:decellularization; GA:glutaraldehyde; H&E:Hematoxylin-eosin; KO:knockout.

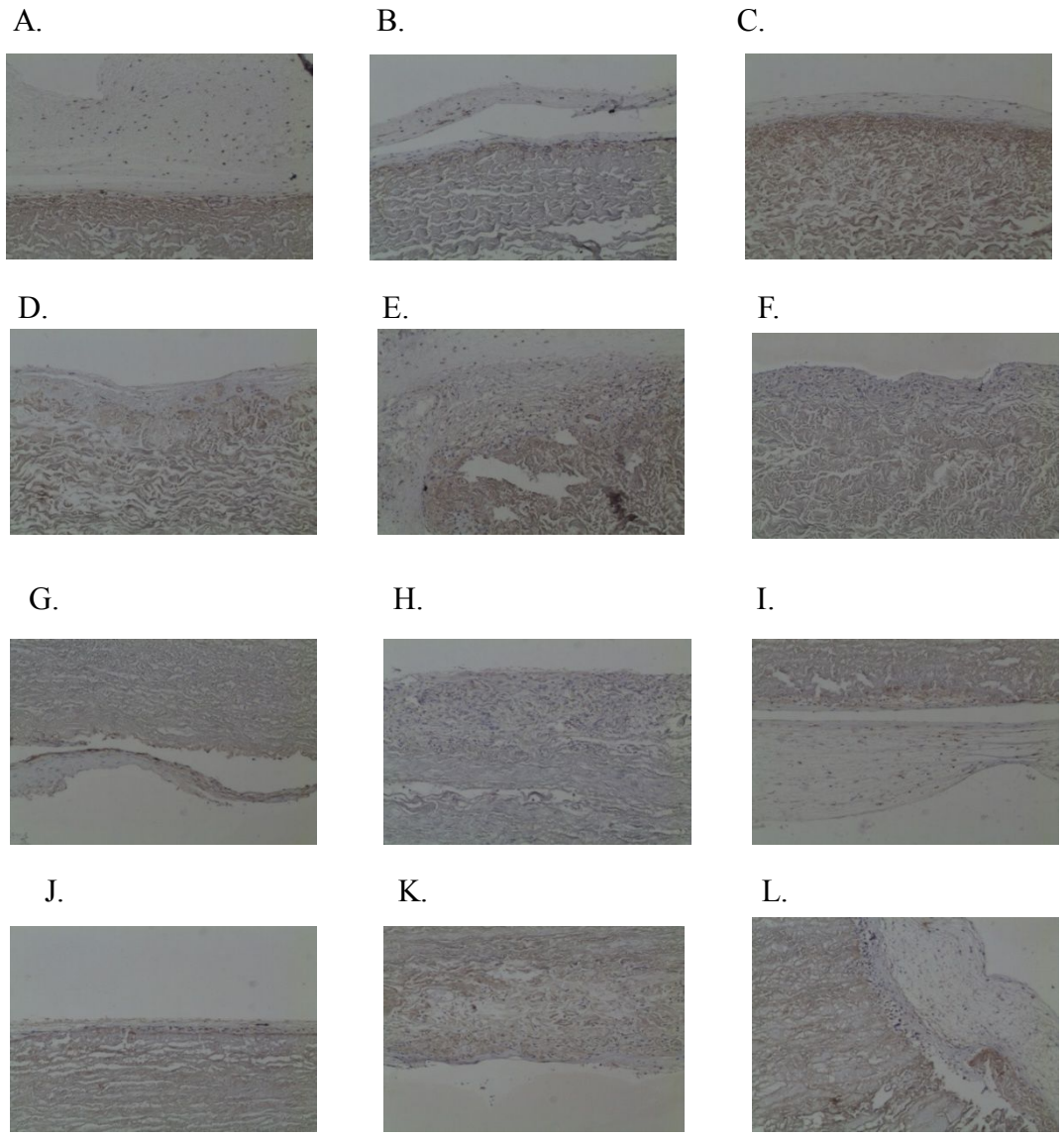

Figure 3. Representative immunohistochemistry of harvested bovine pericardium and porcine heart valves (F4/80 macrophage staining) from WT and α-Gal KO mice (original magnification, 100x). α-Gal KO mice showed higher levels of macrophage infiltration than did WT mice. Notably, a higher level of macrophage infiltration was observed in the bovine pericardium than in the porcine heart valves.

A-F: bovine pericardium; G-L: porcine heart valves.

A, G: GA group in WT mice; B, H: GA group in α-Gal KO mice; C, I: Decell group in WT mice; D, J: Decell group in α-Gal KO mice; E, K: Decell+α-galactosidase group in WT mice; F, L: Decell+α-galactosidase group in α-Gal KO mice.

Decell:decellularization; GA:glutaraldehyde; KO:knockout; WT:wild type.

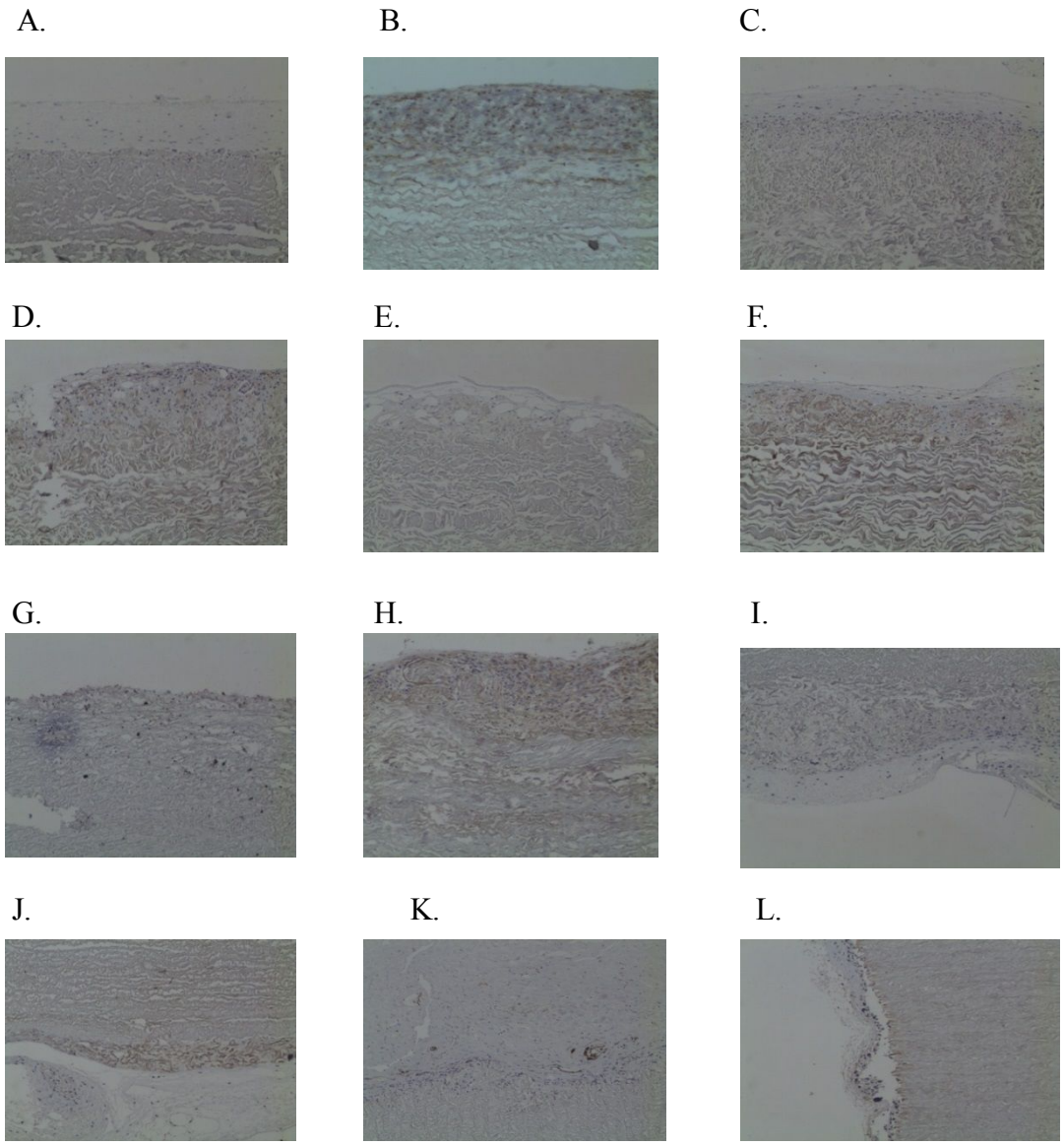

Figure 4. Representative immunohistochemistry of harvested bovine pericardium and porcine heart valves (CD4 T cell staining) in WT and α-Gal KO mice (original magnification, 100x). Large amounts of T-cell infiltration were observed in the α-Gal KO mice. A-F: bovine pericardium; G-L: porcine heart valves.

A, G: GA group in WT mice; B, H: GA group in α-Gal KO mice; C, I: Decell group in

WT mice; D, J: Decell group in α-Gal KO mice; E, K: Decell+α-galactosidase group in WT mice; F, L: Decell+α-galactosidase group in α-Gal KO mice.

GA:Glutaraldehyde; Dell:Decellularization; KO:knock out; WT:wild type.

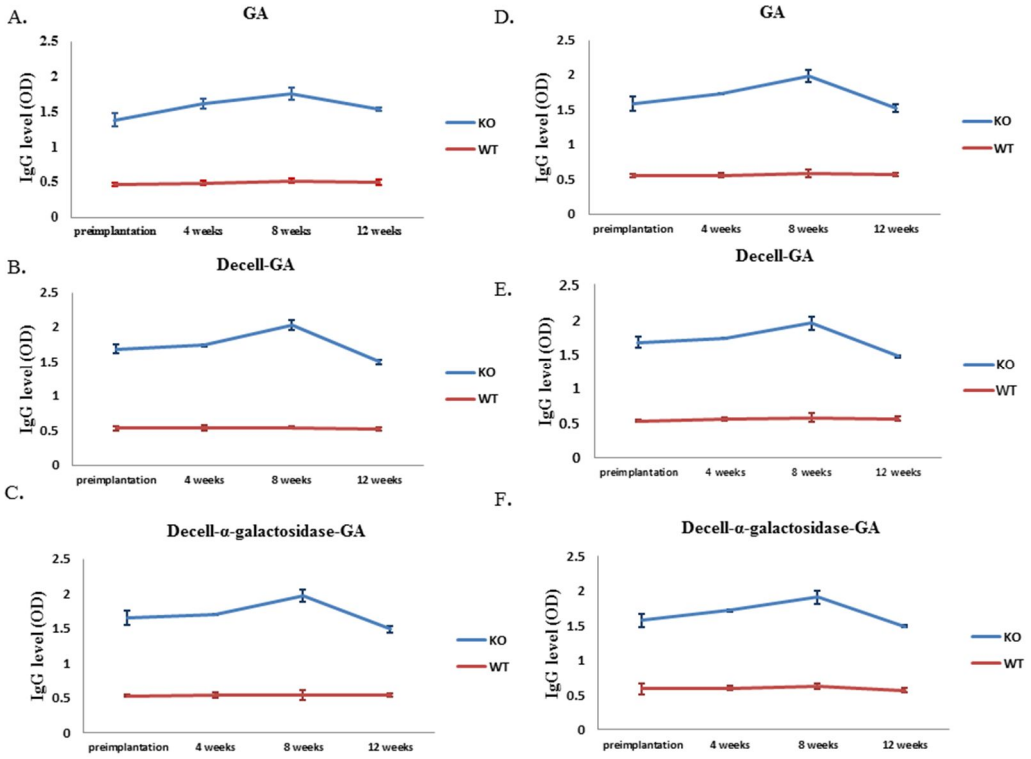

Figure 5. Changes in the anti-α-Gal IgG titers (1:40 dilution) over time, according to the mouse type and tissue preparation method. The IgG anti-α-Gal antibody titers increased significantly over time (all $P < 0.05$, except GA group in bovine pericardium) and were consistently higher in α-Gal-KO mice than in WT mice, regardless of the tissue preparation method (all $P < 0.001$).

Error bars represent the standard errors of the means. A-C: bovine pericardium; D-F: porcine heart valve.

GA:Glutaraldehyde; Decell:Decellularization; KO:α-Gal knock-out mice; OD:optical density; WT:wild type mice.

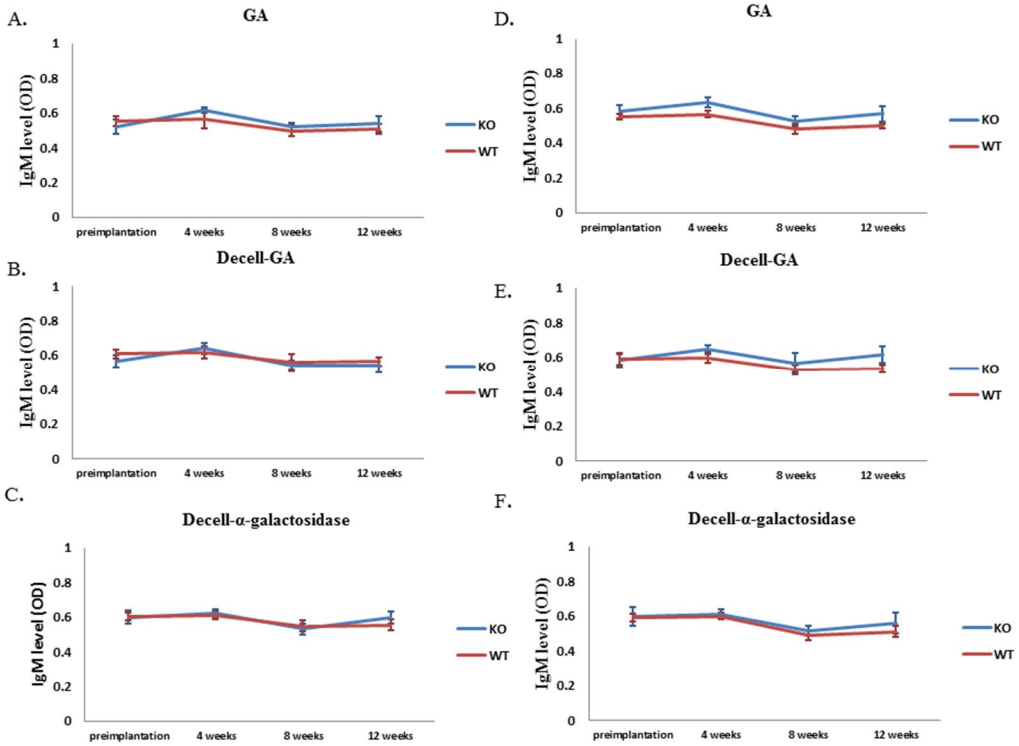

Figure 6. Changes in the anti-α-Gal IgM titers (1:40 dilution) over time, according to the mouse type and tissue preparation method. IgM titers did not differ significantly between the α-Gal-KO and WT mice over time, despite the use of different tissue treatments (all $P = 1.000$).

Error bars represent the standard errors of the means. A-C: bovine pericardium; D-F: porcine heart valve.

GA:Glutaraldehyde; Decell:Decellularization; KO:α-Gal knock-out mice; OD:optical density; WT:wild type mice.

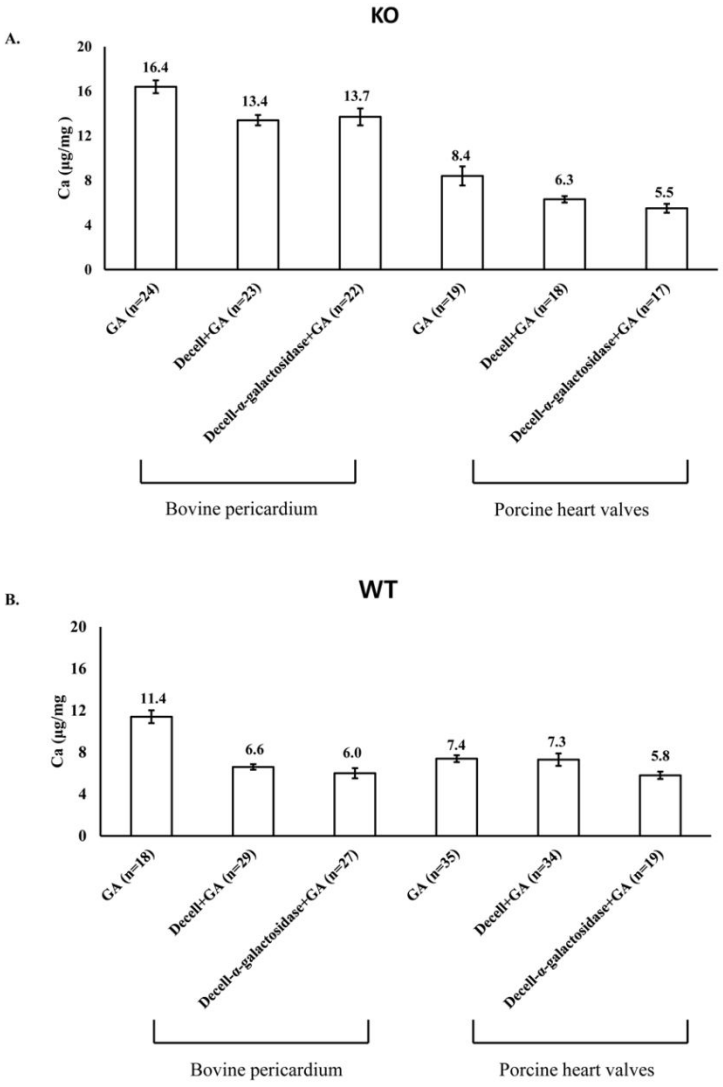

Figure 7. Calcium contents according to the tissue preparation method in wild type and α-Gal KO mice. A. Calcium contents in α-Gal KO mice. B. Calcium contents in wild type mice.

In the *α*-Gal KO mice, the calcium contents were significantly lower in the porcine heart valves than in the bovine pericardium in the GA, Decell, and Decell+α-galactosidase

groups (all $P < 0.01$). The calcium contents were also significantly lower in the Decell-GA and Decell+α-galactosidase groups than in the GA group in both the wild type and α-gal KO mice.

Error bars represent the standard errors of the means.

KO:α-Gal knockout mice; Decell:decellularization; WT:wild type mice.

DISCUSSION

Anti-α-Gal antibodies are abundant in humans, comprising an estimated 1% of circulating immunoglobulins, and are present in the serum as IgG, IgM, and IgA isotopes [39-42]. Anti-α-Gal antibodies are very highly specific for the α-Gal epitope, a carbohydrate antigen found on glycolipids and glycoproteins [43, 44]. These natural human xenoreactive anti-α-Gal antibody-α-Gal antigen interactions are an important mechanism of hyperacute rejection responses and present a major obstacle to xenotransplantation [7, 45]. However, the xenograft bioprostheses that are implanted into human bodies during cardiovascular surgery do not induce hyperacute rejection because they consist of nonviable tissue, and hyperacute rejection is therefore not a primary concern after cardiovascular surgery. Rather, chronic rejection induced by a persistent α-Gal antigen-mediated response is an important mechanism that is responsible for the degenerative calcification of the bioprosthetics used in cardiovascular surgery. Several reports have demonstrated a significant increase in anti-α-Gal IgG antibody levels after bioprosthetic valve replacement, leading to degenerative calcification [9, 15, 17, 26, 28]. Therefore, an immunologic approach to reducing this degenerative calcification might improve the duration and function of implanted bioprostheses.

Generally, in non-primate mammal models [46], implantation of bovine pericardium or porcine heart valve tissues will not provoke anti-α-Gal immune responses because, unlike in humans, both the donor and recipient express the α-Gal epitope. Therefore, α-Gal KO mice, which lack the α-Gal antigen due to genetic modification and thereby possess an immunological environment similar to that in humans, were used in this study. For this reason, xenoreactive immune responses to the α-Gal antigen and antibody

interactions were expected in the nonviable tissue implantation model used in this study. As expected, higher levels of macrophage and T cell infiltration were observed via IHC in the α-Gal KO mice, and the serum IgG antibody titers were elevated in α-Gal-KO, as determined by ELISA. Our findings agreed with those of previous studies in which bovine pericardium, when implanted into α-Gal-KO mice, induced significant increases in the anti-α-Gal antibody levels and macrophage infiltration of the pericardial tissue [11, 15]. These findings provided immunological and histological evidence of chronic rejection in a nonviable tissue implantation model and suggested a potential mechanism for xenograft calcification, although the relationship between chronic rejection and calcification was not clearly demonstrated [47].

With efforts to eliminate or reduce immune reactions using mechanical and biochemical methods based on the complete elimination of xenoreactive α-Gal epitopes through the decellularization process or α-galactosidase enzyme treatment, treated xenografts could be used for tissue engineering and regenerative processing. The effect of α-galactosidase on reducing calcification has been reported in previous studies and might improve the durability of bioprostheses after implantation [23, 48]. However, the calcium contents of both the bovine pericardium and porcine heart valves from the Decell+α-galactosidase-GA groups did not significantly differ from those in the Decell groups. We inferred that the reactions to the implanted treated tissue discs might be insufficient and heterogeneous due to the small sizes of the bioprosthetic tissues.

In the α-Gal KO mice, no differences in IgG levels were observed between tissue preparation methods. We assumed that these results were due to insufficiently strong responses to detect increases in IgG via ELISA or to insufficient sampling numbers to

provide statistical power. We also observed slight increases in the IgM and IgG levels in WT mice, even though the recipient and donor animals had the same α-Gal antigen status in this case. This finding was likely due to non-gal or nonspecific antibody binding. Many studies of the immune mechanisms that contribute to human xenograft rejection have identified two types of antibodies that act as barriers to xenograft transplantation: anti-Gal antibodies and anti-non-gal antibodies. Whereas most previous studies focused on anti-Gal mediated xenograft rejection, anti-non-gal antibodies are not fully understood. Anti-non-gal antibodies in human and monkey xenograft recipients were found to induce complement-dependent and antibody-dependent cell-mediated cytolytic activities. There are multiple anti-non-gal B cell clones, each comprising a small number of lymphocytes. The initial activation of these multiple clones is insufficient to increase the size of each clone to a level at which they can produce detectable amounts of anti-non-gal antibodies. Because fibroblasts undergo rapid anti-Gal-mediated destruction, the period of anti-non-gal B cell antigenic stimulation is very short, and anti-non-gal antibody responses are therefore difficult to detect. However, after a second immune challenge, large numbers of anti-non-gal memory B cells are generated and can produce antibodies [49-51]. *N*-glycolyl neuraminic acid (neu5Gc), generated by the enzyme cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH), is a representative anti-non-gal antibody that is absent in humans due to a mutation in the CMAH gene; this antibody acts as the second major barrier. The absence of Neu5Gc in humans during immunologic education results in the presence of IgG and IgM antibodies that bind to these carbohydrate antigens to fix complement [52, 53].

Porcine heart valves featured lower levels of calcification and macrophage infiltration

than did the bovine pericardium. This finding might be due to differences in the structural elements that comprise the porcine heart valves and bovine pericardium. Unlike the bovine pericardium tissues, which contain collagen with an elastic fiber network, porcine heart valves have three-layered walls (ventricularis, spongiosa, and fibrosa), in which the outermost fibrosa layer comprises very dense collagen bundles that are difficult to penetrate and are resistant to biochemical agents in the implanted tissue environment. The lower levels of calcification and macrophage infiltration in the porcine heart valves might suggest the superiority of porcine heart valves as a bioprosthetic material. A recent report supported the idea that porcine heart valves would have a longer durability than heart valves made from bovine pericardium as pulmonary valve replacements for patients with Tetralogy of Fallot, although there remains controversy regarding the best tissue valve to use and the lack of strong follow-up data [54-57]. However, in this study, there were no significant differences in the α-Gal antibody titers with respect to the implantation of bovine pericardium and porcine heart valves, despite a significant difference in the levels of calcification and macrophage infiltration between the tissue types. This result suggests that the immune response could not account for all of the observed bioprosthetic calcification and indicates a multifactorial mechanism associated with the degenerative calcification.

We found that the tensile strength and elasticity were weaker in the Decell and Decell+α-galactosidase groups than in the GA group. In the porcine heart valve group, the pronase stability was also weaker in the Decell and Decell+α-galactosidase groups than in the GA group. SDS, which was used in the decellularization methods in this study, is an ionic detergent with a very strong decellularization effect. SDS is known to eliminate

acidic phospholid, a cell membrane component, and to completely remove azurophilic substances and cell proteins such as vimentin. However, SDS is also known to have destructive effects on tissue structure, to reduce GAG, and to weaken collagen integrity, all of which might be associated with the lower tensile strength, elasticity, and pronase stability that we observed [58-60]. However, the small sample size failed to allow conclusions to be drawn regarding the mechanical and pronase stability tests in this study.

In this study, we selected IHC methods for staining frozen tissue sections rather than paraffin-embedded tissue sections because the use of frozen tissue sections tends to allow the visualization of structures that are sensitive to aldehyde fixation and tissue processing. Generally, frozen tissue sections allow much better antigen preservation than paraffin sections and are favored by some researchers because the antigens are left in a more 'native' form due to the lack of excessive fixation and processing. However, the tissue morphology is of lower quality in frozen sections than in paraffin-embedded sections, likely due to the higher degree of fixation in paraffin sections. Furthermore, frozen tissue sectioning is notably more technically demanding, especially if the tissue is calcified or has high lipid content [61].

Limitations of the study

Some animals were lost during the experiment, and therefore it was difficult to draw appropriate conclusions about the effects of *α*-galactosidase in this study. Furthermore, the maximum magnification power for visualizing the IHC samples in this study was x100. However, this magnification was insufficient to appropriately detect macrophage and T cell morphology. Furthermore, we did not quantitatively analyze macrophage and T

cell infiltration via IHC in this study.

CONCLUSION

The α-Gal KO mice showed significantly greater macrophage/T cell infiltration, anti-α-Gal IgG antibody titers, and calcium deposition than WT mice when transplanted with either bovine pericardium or porcine heart valves. Compared with the bovine pericardium, the porcine heart valves induced lower levels of macrophage/T cell infiltration and calcium deposition, although the anti-α-Gal IgG antibody titers were not significantly different between the bioprosthetic tissues. Decellularization with or without α-galactosidase yielded significant anti-calcification effects in the both bovine pericardium and porcine heart valves in the α-Gal KO mice. However, there were no significant differences in the anti-α-Gal IgG antibody titers between the tissue preparation methods. The discrepancies in the calcium deposition levels and anti-α-Gal IgG antibody titers observed in our study suggest that bioprosthetic calcium deposition might occur through a multifactorial mechanism, although the relationship between calcium deposition and the anti-α-Gal immune reaction was not clarified in our study.

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국문 초록

서론: 퇴행성 석회화는 생체 인공물 손상의 대표적 원인 중 하나이고 알파갈 항원에 의한 면역 반응이 퇴행성 석회화의 중요한 원인 인자로 알려져 있다. 따라서 이 연구의 목적은 알파갈 항원 제거 및 정상생쥐에서 글루타르알데하이드를 고정한 소심낭 심막 및 돼지 판막을 가지고 탈세포화 및 알파갈락토시다제를 포함하거나 포함하지 않은 실험 방법으로 이종간 면역학적 반응과 항석회화의 효과를 비교 분석하고자 한다.

방법: 소심낭 심막 및 돼지 판막에 탈세포화 및 갈락토시다제 처리를 하여 항석회화 효과를 알아보기 위해 조직 처리 방법에 따라 세 그룹으로 나누었다 (글루타르알데하이드 그룹, 탈세포화 그룹, 탈세포화-갈락토시다제 그룹). 소심낭의 기계적 특성의 측정은 조직 이식 전에 측정하였다. 각각의 준비된 조직은 알파갈 생쥐 및 정상 생쥐의 피하에 이식하였고, 대식세포 및 T 세포의 침윤 정도를 면역조직화학법으로 관찰하였다. 면역글로불린 G 와 M 을 이식전, 이식 후 4 주, 8 주, 12 주에 enzyme-linked immunosorbent assay (ELISA) 방법을 이용하여 측정하였다. 칼슘 침착 정도는 이식 후 12 주에 측정하였다.

결과: 탈세포화 그룹 및 탈세포화-갈라토시다제 그룹에서 글루타르알데하이드 그룹과 비교하여 낮은 장력을 보였다($P < 0.001$, $P = 0.002$). 모든 그룹에서 정상 생쥐보다 알파갈 제거 생쥐에서 더 많은 양의 대식 세포 및 T 세포의 침윤이 관찰되었다. 또한 소심낭에서 돼지 판막보다 더 많은 양의 대식

세포의 침윤이 관찰되었다. 양쪽 생체 물질의 조직 처리 방법에 관계없이 알파갈 제거 생쥐에서 시간에 따른 면역글로불린 G의 값이 정상 생쥐보다 높게 관찰되었고($P < 0.001$), 알파갈 생쥐에서 생체 물질 및 조직 처리 방범에 따른 항 알파갈 면역글로불린 G의 차이는 발견되지 않았다($P > 0.05$). 알파갈 제거 생쥐 군에서 폐동맥 판막 그룹은 소심낭 그룹보다 칼슘 침착이 낮게 관찰되었다($P < 0.001$). 또한 모든 그룹에서 탈세포화 그룹과 탈세포화-갈라토시다제 그룹에서 글루타르알데하이드 그룹보다 칼슘 침착 정도가 낮게 관찰되었다(모두 $P < 0.001$). 하지만 탈세포화 그룹과 탈세포화-갈라토시다제 그룹 사이의 차이점은 관찰하지 못하였다(모두 $P > 0.05$).

결론: 알파갈 항원 제거 생쥐에 소심낭과 돼지 판막을 이식하였을 때 조직학적 만성 염증 반응, 항 알파갈 항체 반응, 석회화의 증가가 정상 생쥐보다 뚜렷하게 나타났고, 알파갈 항원 제거 생쥐에서 돼지 판막이 소심낭보다 면역 반응 및 석회화 정도가 적게 관찰되었다. 알파갈락토시다제를 포함하거나 포함하지 않은 탈세포화 처리는 알파갈 생쥐 군에서 뛰어난 항석회화 효과를 보였다. 하지만 조직 처리 방법에 따른 항알파갈 면역글로불린 항체의 역가 차이는 보이지 않았다.

주요어 : 글루타르알데하이드, 석회화, 형질제거생쥐, 돼지판막

학 번 : 2012-30520