The Immune Responses and Calcification of Bioprostheses in the α1,3-Galactosyltransferase Knockout Mouse

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Background and aim of the study: **The study aim was to evaluate the immune reaction, difference of degenerative calcification, and anti-calcification effect of decellularization with or without α-galactosidase in bovine pericardium and porcine heart valves, using an α1,3-galactosyltransferase (α-Gal) knockout (KO) mouse model.**

Methods: **In order to elucidate the anti-calcification effect of decellularization with or without α-galactosidase, bovine pericardium and porcine heart valve tissues were assigned to four groups according to the tissue preparation method: (i) glutaraldehyde (GA) fixation only; (ii) decellularization + GA fixation (Decell); (iii) α-galactosidase + GA fixation (α-galactosidase); and (iv) decellularization +α-galactosidase + GA fixation (Decell + α-galactosidase). Each prepared tissue was implanted subcutaneously into α-Gal KO mice. Anti-α-Gal immunoglobulin (Ig) G and IgM antibody titers were monitored prior to implantation and at four, eight and 12 weeks after implantation using an enzyme-linked immunosorbent assay. Calcium contents of explanted tissues were measured at 12 weeks after implantation.**

Results: **There were no significant differences in the anti-α-Gal IgG antibody titers according to the type of bioprosthetic material or tissue preparation method ($p > 0.05$). The calcium content was significantly lower in porcine heart valves than in bovine pericardium when implanted in α-Gal-KO mice ($p < 0.001$). Calcium contents in bovine pericardium and porcine heart valves were significantly lower in the Decell, α-galactosidase and Decell + α-galactosidase groups than in the GA group (all $p < 0.05$).**

Conclusion: **The porcine heart valve induced lower levels of calcium deposition than did the bovine pericardium, but the anti-α-Gal IgG antibody titers did not differ significantly between the bioprosthetic tissues. Decellularization had significant anti-calcification effects in both the bovine pericardium and porcine heart valves, though there was no significant difference in the anti-α-Gal IgG antibody titers among tissue preparation methods.**

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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 (1). Lee et al. (2) 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 towards increased calcification. Furthermore, α-Gal epitopes have reportedly remained present in commercial glutaraldehyde (GA)-treated bioprosthetic heart valves (3). Therefore, an immunologic approach to reducing this degenerative calcification might improve the duration and function of implanted bioprostheses.

Bioprosthesis decellularization reduces immune responses and suppresses early calcium nucleation by eliminating tissue components (4). Decellularization has the potential to reduce immune response, but unfortunately until now only a few decellularized treatments have been shown capable of ensuring

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a complete decellularization together with a good preservation of the extracellular matrix. Treatments capable of completely removing the α-Gal epitope are even fewer in number (5,6). Therefore, the use of α-galactosidase (a glycoside hydrolase that catalyzes the hydrolysis of melibiose into galactose and glucose) or the removal of α-1,3 galactosyl transferase (which catalyzes synthesis of the α-Gal antigen epitope) in an α-Gal KO mouse model can be used to remove the α-Gal antigenic epitope (7), and would be expected to decrease the α-Gal antigen-antibody-mediated immune response.

Bovine pericardium and porcine heart valve leaflets are representative tissues that are frequently used during cardiovascular surgery. Histological and chemical differences in the compositions, characteristics and post-implantation durabilities of these two materials have been reported (8). Previous reports have stated that bovine pericardium (3) and porcine heart valves (5,9), 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 the present authors' knowledge, the immune responses of these α-Gal KO mice and the effects of anti-calcification treatments on bovine pericardium and porcine heart valves have not been compared. Hence, the study aim was to evaluate the effects of decellularization with or without α-galactosidase on immune responses and anti-calcification in GA-fixed bovine pericardium and porcine heart valves, using an α-Gal KO mouse subcutaneous implantation model.

Materials and methods

Animals

α-Gal KO mice (n = 72; C57BL/6 background; aged 6-7 weeks, mean bodyweight 29.8 ± 3.0 g) were used as the experimental animals. All animals 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). The study was approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute (No. 12-0219). The authors' facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Tissue preparation

Following sacrifice of the mice, fresh bovine pericardium and porcine heart valve tissues were removed, washed with normal saline and placed into phosphate-buffered saline (PBS; 0.1 M, pH 7.4). All tissues treated in accordance with the tissue preparation methods were cut into round disc-shaped pieces and stored in a 2% propylene oxide solution before use. The tissues were implanted subcutaneously into α-Gal KO mice and the animals maintained thereafter for a 12-week period.

Experimental design

The bovine pericardial and porcine heart valve tissues were assigned to the following four groups according to the tissue preparation method: Group 1: simple GA fixation treatment (GA group); group 2: decellularization + GA fixation treatment (Decell group); group 3: α-galactosidase + GA fixation treatment (α-galactosidase group); and group 4: decellularization + α-galactosidase + GA fixation treatment (Decell + α-galactosidase group).

Tissue preparation methods

GA fixation treatment

Tissues were fixed in 0.5% buffered GA for three days at room temperature, and then in 0.25% buffered GA for seven days at room temperature.

Decellularization

All tissues were disinfected in 0.1% peracetic acid, 4% (v/v) ethanol in distilled water for 2 h at room temperature. The decellularization processes were different for the pericardium and heart valve tissues due to their natural differences in composition and mechanical properties. The porcine heart valves were subsequently washed for 30 min in distilled water, and a hypotonic 1.0% sodium dodecyl sulfate (SDS) solution was added to the tissues for 24 h at 4°C, after which a hypotonic 1.0% Triton X-100 solution was added for 24 h at 4°C. The latter step was repeated five to six times. The tissues were then washed in PBS for 24 h at 4°C. The bovine pericardium tissues were subsequently washed for 30 min in distilled water, and a hypotonic 0.5% SDS solution was then added for 24 h at 4°C, followed by a hypotonic 0.5% Triton X-100 solution for 24 h at 4°C. The tissues were then washed in PBS for 24 h at 4°C.

α-Galactosidase treatment

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

Mouse subcutaneous implantation protocol

Intraperitoneal injections of zolazepam + tiletamine (0.2 ml Zoletil®; Virbac, France) and xylazine (0.2 ml 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. A round tissue disc (6 mm diameter) was

then implanted into each of the four pouches and the wounds were closed with 4-0 monofilament sutures. Antibiotics (including cefazoline; 20 mg/kg; Yuhan, Korea) were administered for three days after tissue implantation. The mice were sacrificed by CO_2 gas inhalation at 12 weeks post-implantation without any issues, and tissue samples were then harvested.

Analytical assessments

Microscopic examinations

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

Immunohistochemistry

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

Immunostaining of frozen tissue sections

To ensure correct fixation, fresh tissue blocks were immersed in acetone that had been pre-cooled to -20°C for 10 min. The solution was then discarded, and residual acetone allowed to evaporate from the tissue sections for more than 20 min at room temperature. The tissue sections were rinsed twice with 300 ml 10 mM PBS at neutral pH for 5 min per wash, and then incubated in a 0.3% H_2O_2 solution in PBS at room temperature for 10 min to block endogenous peroxidase activity. The tissue sections were again rinsed with 300 ml PBS in 3- to 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 room temperature and rinsed in 300 ml PBS three times for 5 min each. Subsequently, two drops (50 ml/drop) of avidin and d-biotin (Invitrogen Corporation, Camarillo, CA, USA) were applied to the tissues, followed by 10-min incubation at room temperature. After rinsing, 100 ml of an appropriately diluted primary antibody (1:300) was applied to each section, followed by incubation overnight at 4°C. On the following day, 100 ml 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 a 1-h incubation at room temperature. Next, 100 ml of a diaminobenzidine (Lab Vision Corporation, Fremont, CA, USA) substrate solution was applied to 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 six graduated alcohol solutions (70%, 80%, 90%, 95%, 100%, and 100%) for 5 min each. After clearing the tissue slides in three changes of xylene for 5 min and attaching coverslips with a mounting solution, antibody staining was observed using 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 h, and weighed. These tissue samples were hydrolyzed in 5.0 N HCl and dried again at 75°C for 24 h. Tissue calcium contents were then measured using colorimetry according to the O-cresolphthalein complexone method, using an automatic chemistry analyzer (Hitachi 7070; Hitachi High Technologies Corporation, Japan). Tissue calcium and phosphorus contents were expressed in units of μg/mg tissue dry weight.

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

ELISA was used to monitor the activities of serum IgM and IgG antibodies against anti-α-Gal in the α-Gal KO mice (n = 70). Results were obtained at pre-implantation and at four, eight and 12 weeks post-implantation. Approximately 0.2-0.4 ml of blood was 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 ml per well of 1 mg/ml α-Gal-BSA in PBS, pH 7.4, for both the IgM and IgG isotypes, and the plates were incubated for 1 h at 37°C. 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™; Invitrogen Corporation, New Zealand) and 0.01% Tween 20),

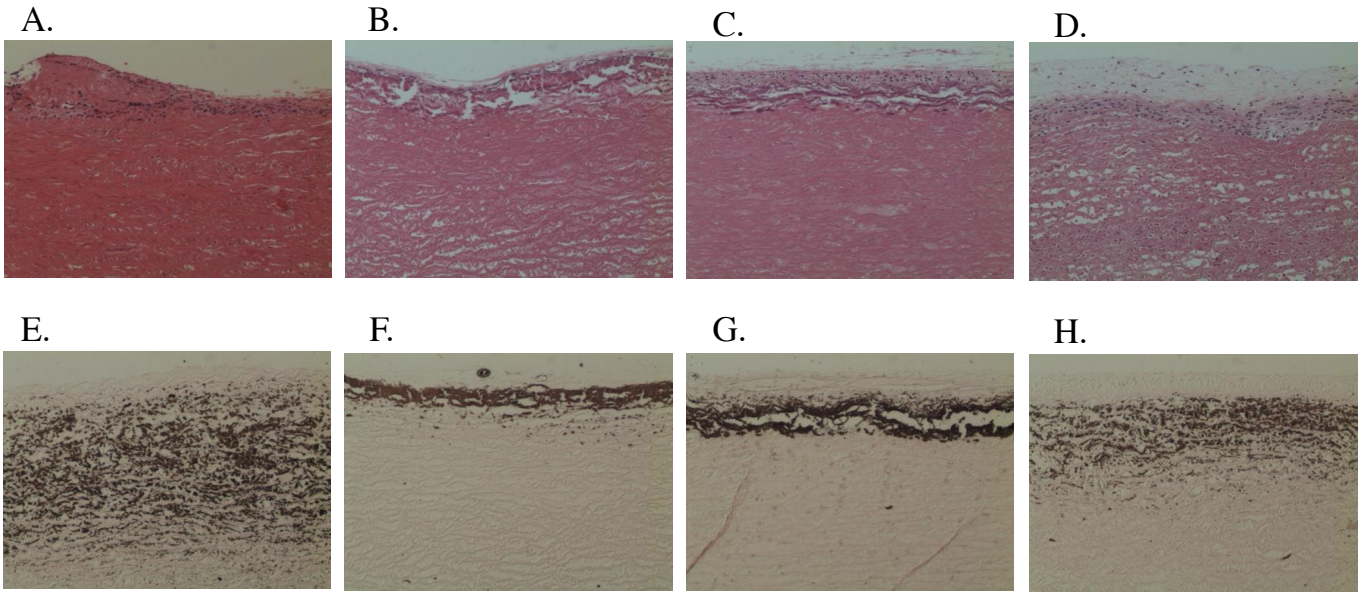

Figure 1: Representative microscopic examination in α-Gal KO mice Large numbers of inflammatory cells were observed in the glutaraldehyde (GA) group. However, a lower degree of inflammatory cell infiltration was observed in the decellularization (Decell), α-galactosidase and Decell + α-galactosidase groups. Additionally, calcium deposition was reduced in the Decell, α-galactosidase and Decell + α-galactosidase groups compared to that in the GA group. A-D) H&E staining. E-H) Von Kossa staining. A,E) GA group. B,F) Decell group. C,G) α-Galactosidase group; D,H) Decell + α-galactosidase group.

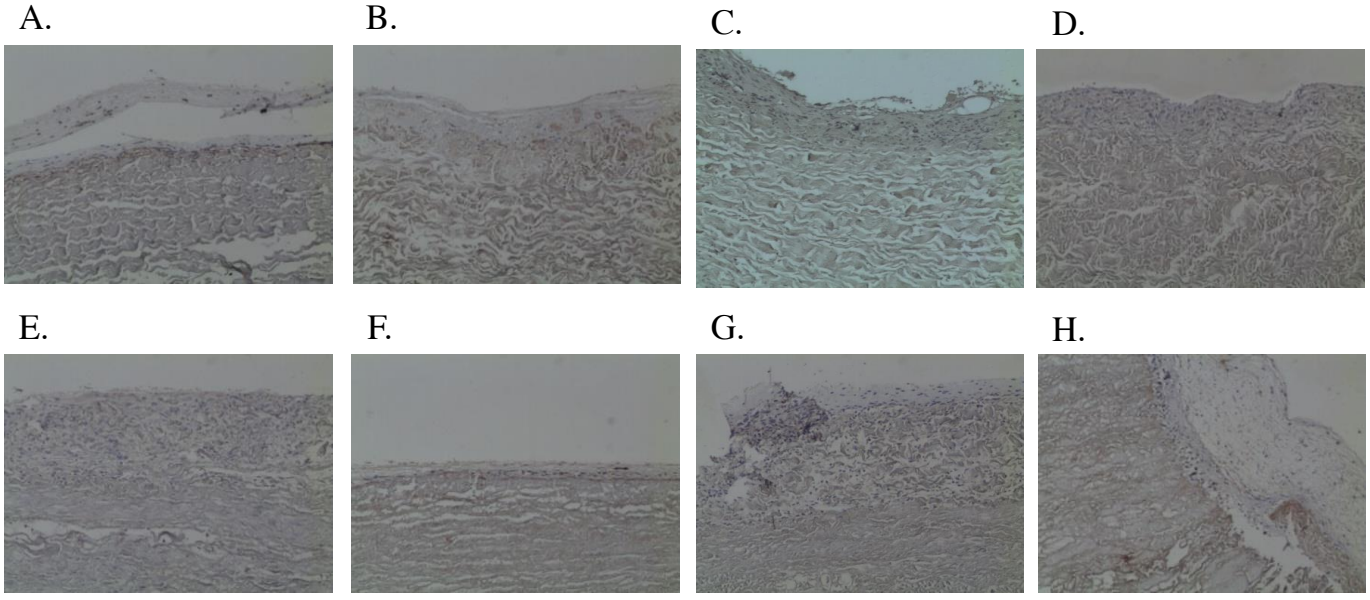

Figure 2: Representative immunohistochemistry of harvested bovine pericardium and porcine heart valves (F4/80 macrophage staining) from α-Gal KO mice (original magnification, ×100). A higher level of macrophage infiltration was observed in the bovine pericardium than in the porcine heart valves. A-D) Bovine pericardium. E-H) Porcine heart valves. A,E) GA group. B,F) Decell group. C,G) α-Galactosidase group; D,H) Decell + α-galactosidase group.

and the plates were incubated for 1 h at 37°C. The secondary antibodies were HRP-conjugated rabbit anti-mouse IgM (Jackson ImmunoResearch) and goat anti-mouse IgG (Jackson ImmunoResearch), used at 1:10,000 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 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.

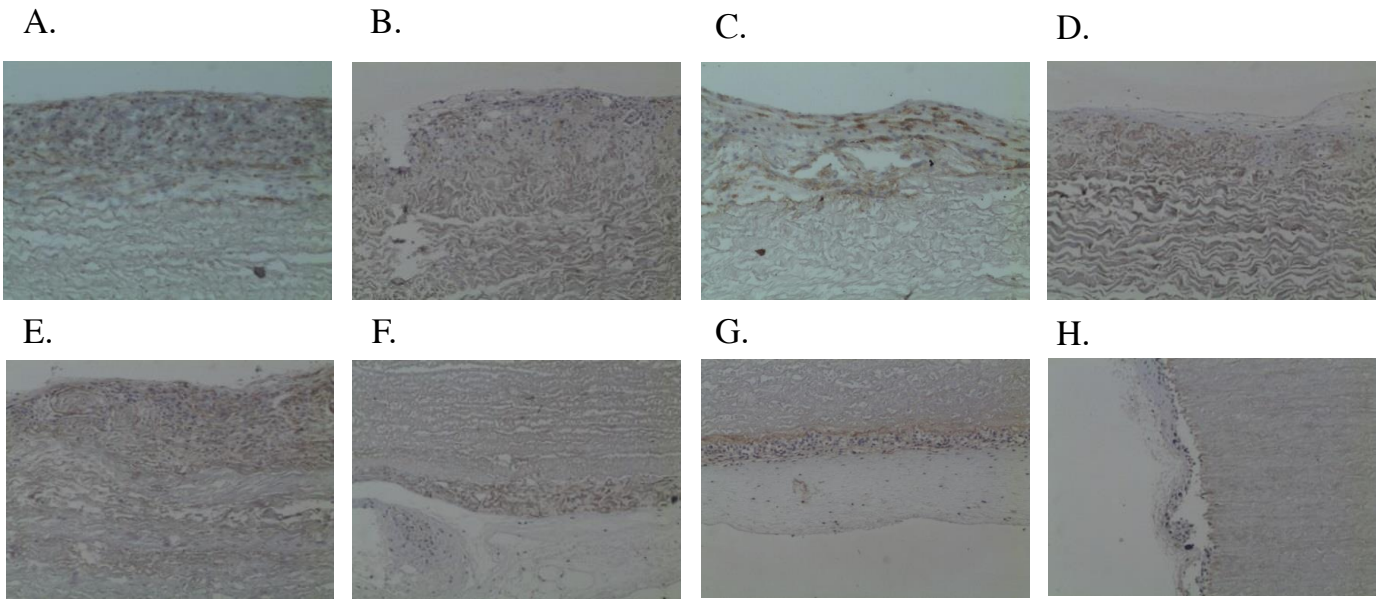

Figure 3: Representative immunohistochemistry of harvested bovine pericardium and porcine heart valves (CD4 T-cell staining) in α-Gal KO mice (original magnification, ×100). Large amounts of T-cell infiltration were observed in the grafted xenogenic tissues. A-D) Bovine pericardium. E-H) Porcine heart valves. A,E) GA group. B,F) Decell group. C,G) α-Galactosidase group. D,H) Decell + α-galactosidase group.

Statistical analysis

Continuous variables were expressed as mean ± SD. Student's *t*-test was used for normally distributed continuous variables. A one-way ANOVA was used for comparisons among more than three 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 to be statistically significant. All statistical analyses were performed using the SPSS statistical software package (SPSS version 19.0; SPSS Inc., Chicago, IL, USA).

Results

Microscopic examination

Inflammatory cell infiltration was identified with H&E staining. Large amounts of inflammatory cell infiltrates were observed in the GA group, but lower levels of inflammatory cell infiltration were observed in the Decell, α-galactosidase and Decell + α-galactosidase groups than in the GA group. Von Kossa staining revealed that calcium deposition was also reduced in the Decell, α-galactosidase and Decell-α-galactosidase groups compared to the GA group (Fig. 1).

Immunohistochemistry

F4/80 staining revealed significant macrophage infiltration. In general, higher levels of macrophage infiltration were observed in bovine pericardium than in porcine heart valves. No difference was observed in the degree of macrophage infiltration with respect to the tissue preparation method (Fig. 2). CD4 staining revealed a high degree of T-cell infiltration in the grafted xenogenic tissues in the α-Gal KO mice. However, no difference was observed in the degree of T-cell infiltration with respect to the type of prosthetic tissue used (bovine pericardium versus porcine heart valves) or the tissue preparation method (Fig. 3).

Anti-α-Gal antibodies

Absolute serum levels of IgG and IgM were increased over time. The IgG titer showed a tendency to increase over time, with a peak at eight weeks followed by a decrease. These changes in anti-α-Gal antibody level (except for the GA and α-galactosidase group of bovine pericardium) were statistically significant (p <0.05). No differences were observed in anti-α-Gal IgG and IgM antibodies with regards to the use of bovine pericardium versus porcine heart valves (all p >0.05). Although the α-galactosidase group showed a tendency to reduce the anti-α-Gal IgG antibodies compared to the GA group (p = 0.118), no significant differences were observed among all four groups (all p >0.05). No significant differences were observed in the anti-α-Gal IgM antibodies with respect to the tissue preparation method and the type of bioprosthetic material (all p = 1.000; Tables I and II; Fig. 4).

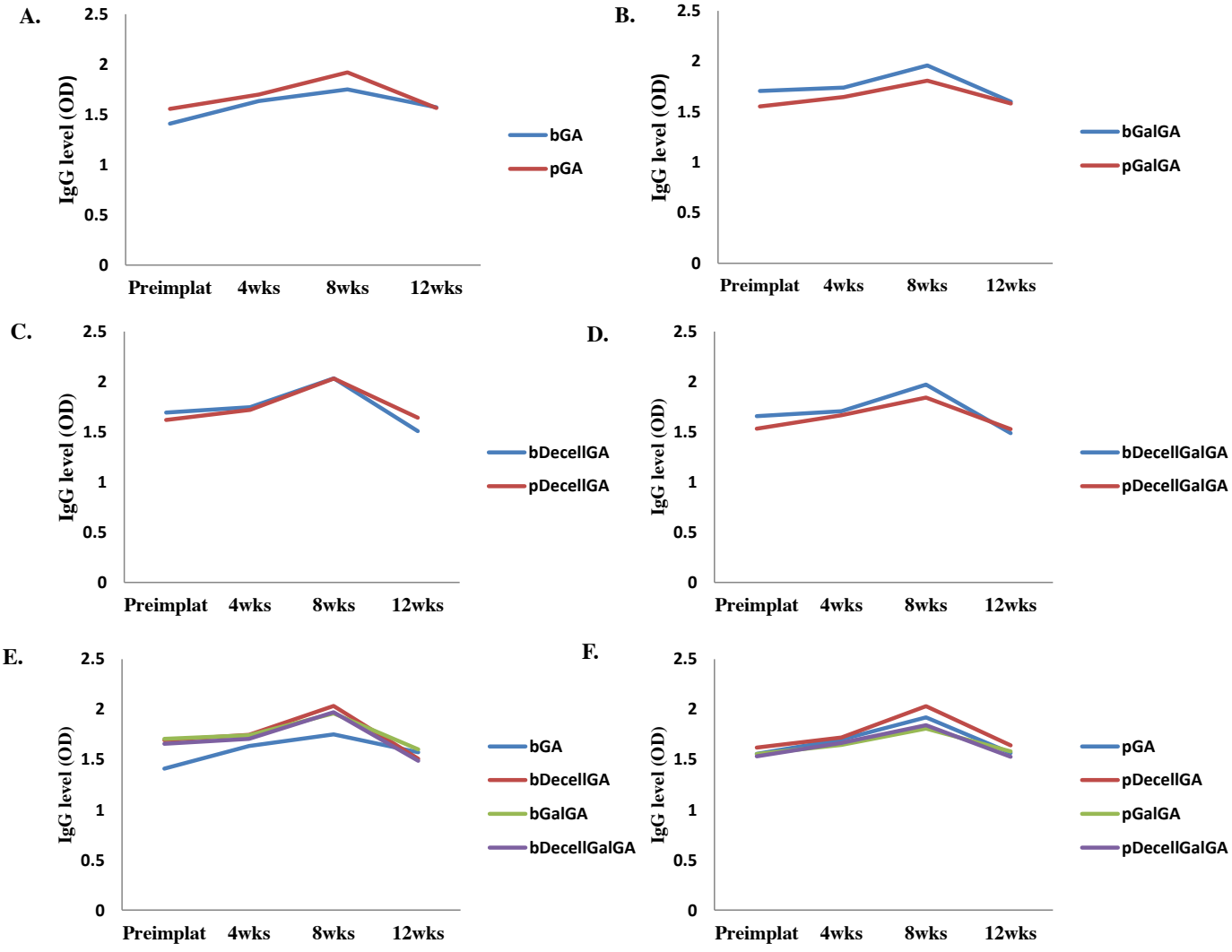

Figure 4: Changes in the anti-α-Gal IgG antibodies (1:40 dilution) over time, according to the mouse type and tissue preparation method. No differences were observed in the anti-α-Gal IgG antibodies with respect to the use of bovine pericardium versus porcine heart valves and tissue preparation methods. A-C) Bovine pericardium. D-F) Porcine heart valve. OD: Optical density.

Calcium content

Comparison between bovine pericardium and porcine heart valves

Among α-Gal KO mice, calcium contents were significantly lower in porcine heart valves than in bovine pericardium in the GA, Decell, α-galactosidase and Decell + α-galactosidase groups (all $p < 0.001$).

Comparison according to tissue preparation method

Bovine pericardium calcium contents were significantly lower in the Decell group (11.1 ± 3.1 μg/mg; n = 23; $p < 0.003$), α-galactosidase group (10.9 ± 2.7 μg/mg; n = 23; $p < 0.001$) and Decell + α-galactosidase group (13.7 ± 3.6 g/mg; n = 22; $p < 0.001$) than in the GA group (16.4 ± 2.8 μg/mg; n = 24). Similarly, calcium contents in the porcine heart valves were significantly lower in the Decell-GA group (6.1 ± 1.5 μg/mg; n = 26; p = 0.010), α-galactosidase group (5.9 ± 2.3 μg/mg; n = 22; p = 0.006) and Decell + α-galactosidase group (5.2 ± 1.6 μg/mg; n = 27; $p < 0.001$) than in the GA group (7.9 ± 3.8 μg/mg; n = 27). There were no significant differences in calcium content among the Decell, α-galactosidase and Decell + α-galactosidase groups in either the bovine pericardium or the porcine heart valves (all $p > 0.05$) (Fig. 5).

Discussion

In general, higher levels of macrophage and T-cell infiltration were observed via immunohistochemistry, and serum IgG antibody titers were elevated in the α-Gal KO mice as determined using ELISA. The study findings were similar to those of previous investigations in which bovine pericardium, when implanted into α-Gal-KO mice, induced significant increases in anti-α-Gal antibody levels and macrophage infiltration of

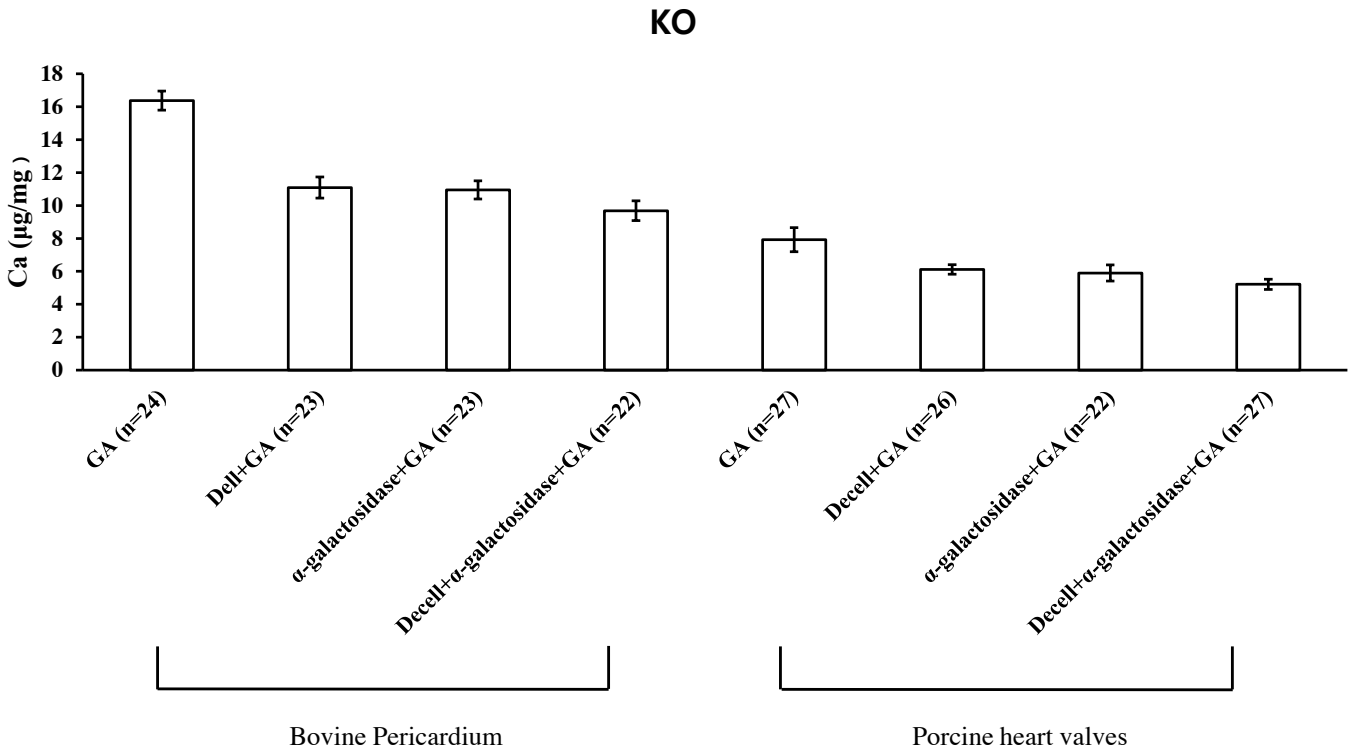

Figure 5: Calcium contents according to the tissue material and preparation method. Calcium contents were significantly lower in porcine heart valves than in bovine pericardium in all groups (all p <0.01). Calcium contents were also significantly lower in the Decell, α-galactosidase and Decell + α-galactosidase groups than in the GA group.

the pericardial tissue (2,9). These findings provided immunological and histological evidence of chronic rejection in a non-viable tissue implantation model, and suggested a potential mechanism for xenograft calcification, although the relationship between chronic rejection and calcification was not clearly demonstrated (10).

The effect of α-galactosidase on reducing calcification has been reported previously, and might improve the durability of bioprostheses after implantation (7,11). However, the calcium contents of both the bovine pericardium and porcine heart valves from the Decell + α-galactosidase-GA groups did not differ significantly from those in the Decell groups. This inferred that the

Table I: Changes in anti-α-Gal IgG antibodies over time according to tissue treatment.

Tissue material	Tissue treatment	Pre-implant	Post-implant			p-value
			4 weeks	8 weeks	12 weeks	
Bovine pericardium	GA	1.4 ± 0.3 (16)	1.6 ± 0.2 (15)	1.8 ± 0.2 (14)	1.6 ± 0.1 (13)	0.124
	Decell	1.7 ± 0.2 (12)	1.7 ± 0.0 (10)	2.0 ± 0.2 (9)	1.5 ± 0.1 (7)	0.016*
	Gal	1.7 ± 0.1 (6)	1.7 ± 0.0 (6)	2.0 ± 0.3 (6)	1.6 ± 0.1 (6)	0.223
	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 ± 0.3 (16)	1.7 ± 0.1 (13)	1.9 ± 0.2 (13)	1.6 ± 0.1 (13)	0.018*
	Decell	1.6 ± 0.3 (16)	1.7 ± 0.1 (15)	2.0 ± 0.3 (12)	1.6 ± 0.3 (11)	0.019*
	Gal	1.6 ± 0.3 (14)	1.6 ± 0.1 (9)	1.8 ± 0.2 (8)	1.6 ± 0.1 (6)	0.037*
	De-Gal	1.5 ± 0.3 (14)	1.7 ± 0.1 (12)	1.8 ± 0.3 (12)	1.5 ± 0.1 (12)	0.021*

Values shown are Optical density (mean ± SD).
Values in parentheses are numbers of mice.
*p <0.05
Decell: Decellurization; De-Gal: Decellurization-α-galactosidase group; GA: Glutaraldehyde; Gal: α-Galactosidase; IgG: Immunoglobulin G.

Table II: Changes in the anti-α-Gal IgM antibodies over time according to tissue treatment.

Tissue material	Tissue treatment	Pre-implant	Post-implant			p-value
			4 weeks	8 weeks	12 weeks	
Bovine pericardium	GA	0.6 ± 0.2 (16)	0.6 ± 0.1 (15)	0.6 ± 0.1 (14)	0.6 ± 0.2 (13)	0.423
	Decell	0.6 ± 0.1 (12)	0.6 ± 0.1 (10)	0.5 ± 0.1 (9)	0.5 ± 0.1 (7)	0.248
	Gal	0.6 ± 0.1 (6)	0.7 ± 0.2 (6)	0.6 ± 0.0 (6)	0.6 ± 0.1 (6)	0.033*
	De-Gal	0.6 ± 0.1 (12)	0.6 ± 0.1 (11)	0.5 ± 0.1 (11)	0.6 ± 0.1 (8)	0.445
Porcine valve	GA	0.6 ± 0.1 (16)	0.7 ± 0.1 (13)	0.6 ± 0.1 (13)	0.6 ± 0.1 (11)	0.110
	Decell	0.6 ± 0.1 (16)	0.6 ± 0.1 (15)	0.6 ± 0.2 (12)	0.6 ± 0.2 (11)	0.149
	Gal	0.6 ± 0.2 (13)	0.7 ± 0.1 (9)	0.6 ± 0.1 (7)	0.7 ± 0.2 (6)	0.820
	De-Gal	0.6 ± 0.2 (14)	0.6 ± 0.1 (13)	0.6 ± 0.1 (13)	0.6 ± 0.2 (12)	0.296

Values shown are Optical density (mean ± SD).
Values in parentheses are numbers of mice.
*p <0.05
Decell: Decellurization; De-Gal: Decellurization-α-galactosidase group; GA: Glutaraldehyde; Gal: α-Galactosidase; IgM: Immunoglobulin M.

reactions to the implanted treated tissue discs might be insufficient and heterogeneous due to the small sizes of the bioprosthetic tissues. In addition, no differences in IgG levels were observed between tissue preparation methods. It was 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.

Porcine heart valves featured lower levels of calcification and macrophage infiltration than did the bovine pericardium, but this finding might be due to differences in the structural elements that comprise the two tissues. 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 glycosaminoglycans (GAGs) maintain hydration and the intrinsic viscoelasticity of the tissue. Finally, the elastin matrix exists to restore collagen structures to their resting states between loading cycles (12,13). 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 (14).

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 controversy persists regarding the best tissue valve to use and there is a lack of strong follow up data (15,16). However, in the present 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. These results suggest that the immune response could not account for all of the observed bioprosthetic calcification, and indicate that a multifactorial mechanism may be associated with the degenerative calcification.

Study limitations

The main limitation was that some animals were lost during the experiment, and consequently it became difficult to draw appropriate conclusions regarding the effects of α-galactosidase. Furthermore, the infiltration of macrophages and T cells, as assessed via immunohistochemistry, were not analyzed quantitatively. Finally, access of the immune system to a subcutaneously grafted tissue in mice is known to differ from that of an implanted graft localized in the heart or bloodstream of a human subject.

In conclusion, compared to bovine pericardium, porcine heart valves induced lower levels of 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 both bovine pericardium and porcine heart valves in the α-Gal KO mice. However, there were

no significant differences in anti-α-Gal IgG antibody levels between the tissue preparation methods. The discrepancies in calcium deposition and anti-α-Gal IgG antibody levels observed in the study suggested 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 the present investigation.

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