TISSUE ENGINEERING: Part A
Volume 19, Numbers 21 and 22, 2013

DOI: 10.1089/ten.tea.2013.0062

In Vivo Efficacy of Alpha-Galactosidase as Possible Promise for Prolonged Durability of Bioprosthetic Heart Valve Using Alpha1,3-Galactosyltransferase Knockout Mouse

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The immune response due to Galα1,3-Galβ1–4GlcNAc-R(α-Gal) epitopes is an important factor in bioprosthetic heart valve failure. The aim of this study was to evaluate the immune reaction and anticalcification effect of α-galactosidase and decellularization for glutaraldehyde (GA)/genipin fixed bovine pericardium using α1,3-galactosyltransferase knockout(α-Gal KO) mouse(C57BL/6). Bovine pericardial tissues were decellularized and treated with α-galactosidase before fixation with 0.25% GA/0.4% genipin in organic solvent (75% ethanol and 5% octanol) and treatment with glycine. The removal of α-gal epitope from the bovine pericardium was analyzed by 3,3′-Diaminobenzidine staining intensity. The bovine pericardial tissues were subcutaneously implanted into wild type mice ($n=19$) and α-Gal KO mice ($n=66$), which had been presensitized with rabbit red blood cells to maximize immunologic response or not, and anti α-Gal antibodies were measured at various time intervals. Calcium contents of the explanted tissues ($n=104$) were measured 3 months after implantation. The treatment of α-galactosidase effectively removed the α-gal epitopes expressed on bovine pericardial tissues. In both GA and genipin groups, titers for both anti α-Gal IgM and IgG of α-Gal KO mice increased according to the duration of implantation, and were lower in the groups with decellularization than without decellularization, and were lower in the groups with α-galactosidase + decellularization than with decellularization. The calcium contents of GA/genipin fixed tissues were lower in the groups with decellularization than without decellularization, and were lower in the groups with α-galactosidase + decellularization than with decellularization. Treatment of α-galactosidase with decellularization is useful for removal of the immunogenicity, and reduced calcification in both GA and genipin fixed bovine pericardia, supporting the hypothesis that the immune reaction may cause the calcification. Treatment of α-galactosidase has possible promise to enhance durability of bioprosthetic heart valve. To our knowledge, this is the first report that demonstrates the *in vivo* efficacy of α-galactosidase using presensitized α-Gal KO mouse to mimic the human immunologic environment.

Introduction

Since nonviable bioprosthetic valve implantation has been performed for four decades in cardiac surgery, our reports have recently shown the means to improve the durability of nonviable bioprosthetic valve implantation in cardiac surgery.[1–8] It is generally accepted that the degeneration of bioprostheses is multifactorial, and includes immunologic reactions, foreign body reactions, blood-surface interactions, chemical factors, infection, mechanical factors, material fatigue, and surgical factors.[9] Although the mechanism leading to premature degeneration of the implanted bioprosthesis is not yet fully understood, the immune response has been considered to play an important role as an initial trigger of the degeneration process,[10] and the xenoreactive Galα1,3-Galβ1–4GlcNAc-R (α-Gal) epitope has been noted as the major antigen.

Recently, it has been found that the α-Gal epitopes are still present in commercial bioprosthetic valves treated with glutaraldehyde (GA).[10] Additionally, Park *et al.*[7] reported that patients who received these xenogenic tissue valves exhibited increased levels of natural anti-Gal antibody titers against α-Gal epitopes.[10] Therefore, it is believed that the animal immune response may play an important role in structural damage of the commercially available, GA fixed tissue valves.

Removing α-Gals from the surface of the xenogenic tissue valves can improve the durability of the xenogenic tissue valves. Decellularization approaches proved to be effective in the prevention of calcification, presumably by suppressing residual

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antigenicity of bioprosthetic tissue.[11,12] Lately, our group[2,4,5,8] proved that decellularization is not enough for removal of α-Gal epitopes, and α-galactosidase can effectively remove the α-gal epitopes from xenograft heart valves and pericardium using both qualitative and quantitative analysis without affecting the mechanical properties of the tissues. Therefore, in addition to decellularization, we used enzymatic method using α-galactosidase to remove α-Gal epitopes efficiently from cardiac valvular and pericardial tissues in this study.[2,4,5,8,13–15]

To investigate the xenoresponse in immunological environment similar to human, our group[1] have inactivated the α1,3-galactosyltransferase gene in mice by gene targeting, and have generated mice that do not synthesize the Gal epitope.[16–19] Lee *et al.*[1] proved that bovine pericardium implanted into the α-Gal KO mice caused significant increase in anti-α-Gal antibodies, and showed some histologic evidences of chronic rejection and that α-Gal KO mouse subcutaneous implantation model might be a feasible animal model for testing efficacy of anticalcification treatments incorporating immunologic approach, such as alpha-galactosidase.

The aim of this study was to evaluate the immune reaction and anticalcification effect of alpha-galactosidase and decellularization of GA/genipin fixed bovine pericardium implanted into alpha-Gal KO (alpha-1,3-galactosyltransferase knockout) mouse(C57BL/6), which had been presensitized with rabbit red blood cells to maximize immunologic response.

Materials and Methods

Experimental design and tissue preparation

Fresh bovine pericardium was obtained from the local slaughterhouse, placed in phosphate-buffered saline (PBS, 0.1M, pH 7.4) and immediately transported to our laboratory. On arrival, they were rinsed with normal saline and were freed from adherent fat.

Group 1: GA fixation in organic solvent + detoxification
Group 2: genipin fixation in organic solvent
Group 3: decellularization + GA fixation in organic solvent + detoxification
Group 4: decellularization + genipin fixation in organic solvent
Group 5: decellularization + α-galactosidase + GA fixation in organic solvent + detoxification
Group 6: decellularization + α-galactosidase + genipin fixation in organic solvent

(A) GA groups
Bovine pericardial tissues were initially fixed with 0.25% GA for 3 days at room temperature, and additionally fixed with 0.25% GA in organic solvent of 75% ethanol + 5% octanol for 2 days at room temperature, and finally fixed with 0.25% GA for 7 days at room temperature.

(B) Genipin groups
Bovine pericardial tissues were initially fixed with 0.4% genipin for 3 days at room temperature, and additionally fixed with 0.4% genipin in organic solvent of 75% ethanol + 5% octanol for 2 days at room temperature.

Detoxification

After completion of fixation, tissues were treated with 0.2 M glycine solution (PBS, pH7.4) at 4°C for 24 h in GA groups.

Decellularization

Bovine pericardial tissues were washed with 0.9% normal saline, and then 0.1% peracetic acid with 4% ethanol in distilled water for 1 h and washed for 2 h with distilled water. These tissues were initially treated with hypotonic buffered solution with 0.25% sodium dodesyl sulfate (SDS) for 24 h at 4°C, and washed with distilled water for 1 h. They were then treated with hypotonic solution with 0.5% TritonX-100 for 24 h at 4°C, and washed with distilled water for 12 h at 4°C, followed by isotonic solution for 24 h at 4°C. The tissues were finally treated with hypertonic buffered solution (II) for 6 h at 4°C, and washed with PBS for 1 h at 4°C.

- Hypotonic buffered solution (distilled water 1000 mL; Tris 10 mM; pH 8.0)
- Isotonic buffered solution (distilled water 1000 mL; Tris 50 mM; NaCl 0.15 M; EDTA 0.05%; aprotinin 10 KIU/mL; Neomycin trisulfate 50 mg; pH 8.0)
- Hypertonic buffered solution II (distilled water 1000 mL; Tris 200 mM; NaCl 0.6 M; pH 8.0)
- Distilled water (distilled water 1000 mL; EDTA 0.05%; aprotinin 10 KIU/mL)

Construction and preparation of α1,3 galactosidase from Bacteroides thetaiotaomicron

The genomic DNA of *Bacteroides thetaiotaomicron* was kindly provided by Dr. Yoon-Hoh Kook of the Department of Microbiology and Immunology at the Seoul National University College of Medicine. The gene for the α-galactosidase from *B. thetaiotaomicron* (designated BtGal110B) was amplified from the corresponding genomic DNAs by PCR and was introduced into the pET28a vector for expression of the His_6-tagged proteins using appropriate restriction sites for protein expression in *Escherichia coli* Rosetta2 (DE3) (Novagen). *E. coli* was grown in Luria-Bertani media supplemented with 34 μg/mL chloramphenicol, 30 μg/mL kanamycin, induced at an $A_{600\ nm} \sim 0.6$ with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The harvested cell pellet was lysed in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole) using Ultrasonicator (Misonix, Inc.). The crude lysates were centrifuged for 20 min at 13,000 rpm, 4°C, and the expressed protein was purified using Nickel-NTA agarose column (Qiagen) according to manufacturer's instruction.

Decellularization and treatment of α-galactosidase

Bovine pericardial tissues were washed with 0.9% normal saline, and then 0.1% peracetic acid with 4% ethanol in distilled water for 1 h and washed for 2 h with distilled water. These tissues were initially treated with hypotonic buffered solution with 0.25% SDS for 24 h at 4°C, and washed with distilled water for 1 h. These tissues were treated with hypotonic solution with 0.5% TritonX-100 for 24 h at 4°C. These tissues were treated with isotonic solution with α-galactosidase for 24 h at 4°C, and washed with distilled water for 12 h at 4°C. These tissues were finally treated with hypertonic buffered solution (II) for 6 h at 4°C, and washed with PBS for 1 h at 4°C.

Microscopic examination (preimplantation)

Representative tissue samples from each group were examined with light microscopy. Tissue samples were fixed in

10% formalin, embedded in paraffin wax and 2–4-μm-thick sections were stained with hematoxylin-eosin.

The sectioned tissue samples were also incubated with 1 μg/mL biotin-GSIB$_4$ lectin (Vector Lab). Tissues were washed with PBS and treated with 5 μg/mL avidin-horseradish peroxidase (*Molecular Probes)*. The α-gal eitope on the bovine pericardium was visualized by using DAB (3,3′-Diaminobenzidine; Vector) as a substrate. DAB staining intensity of the valve tissues was captured under a light microscopy.

Rabbit red blood cell immunization

To increase anti-α-gal antibody titers in α-gal$^{-/-}$ mice, rabbit red blood cells (5×10^8) were injected i.p. on days −14 and −7 before xenograft implantation according to the method of McKenzie *et al.*[20] On day 0, mice were implanted with 6-mm^2 pieces of bovine pericardium. Serum α-gal antibody levels were measured in blood obtained from anesthetized animals by retro-orbital puncture at various times before, and after pericardium implantation.

Mouse subcutaneous implantation

This study was approved by Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No. 10-0061). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Animal care and use was performed in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 2011.

Male α-Gal KO ($n=66$) and wild-type ($n=19$) mice (C57BL/6, 6 weeks old, 14–27 g) were used. All surgery was performed by using aseptic techniques. After anesthetizing and shaving, four subcutaneous pouches were created at the dorsal area for each mouse. Pericardial samples were implanted into the pouches with the minimum distance of at least 10 mm between two implanted patches and the wounds were closed with 6/0 nylon sutures. The mice were sacrificed by CO_2 asphyxiation after 12 weeks. The tissue samples were harvested, freed of adherent mouse tissues, and rinsed with normal saline. A major portion of each sample was used for quantitative calcium analysis, whereas representative samples were used for microscopic examinations.

Enzyme-linked immunosorbent assay

To determine the activity of IgM and IgG isotypes of the anti α-Gal(Gal[alpha]1,3-Gal[beta]1,4GlcNAc-R) antibodies, blood samples were taken from the mouse on the 28th (the immunization date), 42nd (the implantation date), 56th, 70th, 98th, and 126th date after birth (the 14th, 28th, 56th, and 84th postimplantation date), and an Enzyme-linked immunosorbent assay was performed. Bovine serum albumin conjugating synthesized α-Gal (Genechem) (α-Gal-BSA) was used as a solid phase antigen. Microtiter plates were coated with 100 μL per well of α-Gal-BSA in PBS (pH 7.4) (at a concentration of 1 μg/mL for IgM and IgG isotypes), and incubated for 1 h at 37°C. Then, the plates were washed with PBS containing 0.05% (v/v) Tween 20. The sera (100 μL per well) of mouse were added to the α-Gal-BSA immobilized wells at a serial two-fold dilution from 1:40 to 1:2560 in BSA-Triton X-100 (PBS, pH 7.4, 3% BSA, 0.01% Triton X-100), then the plates were incubated for 1 h at 37°C. Rabbit anti-mouse IgM antibodies (Jackson Immunoresearch) and goat anti-mouse IgG antibodies (Jackson Immunoresearch) were used as a secondary antibody (1:5000) at a dilution for IgM and IgG in BSA-Triton X-100. The reaction was developed with tetramethylbenzidine solution (PIERCE). Optical density (OD) was measured at 450 nm using the Thermo Electron-Lab Systems (Labsystems).

Immunohistochemistry staining

Representative tissue samples from each group were stained for mouse macrophages. The primary antibodies used were anti-mouse F4/80 antigen (eBioscience) at 1:300 dilution (marker for mouse macrophages). The secondary antibody used was horseradish peroxidase-conjugated donkey anti-rat IgG (Jackson ImmunoResearch) at 1:500 dilution. DAB was used as a chromogen and hematoxylin was used for counterstaining.

Calcium analysis

Harvested tissue samples were washed with normal saline, dried at 70°C for 24 h, and weighed. Samples were

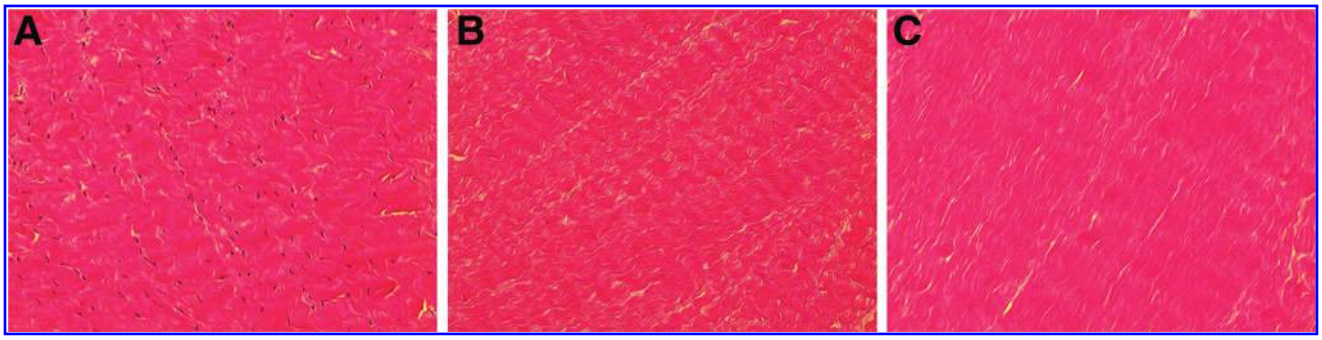

FIG. 1. Light microscopy of unimplanted bovine pericardium (hematoxylin-eosin stain, ×100). Collagen fibers appear well preserved with a normally banded structure in all groups without significant difference among groups. After decellularization, there was no visible cell, and no specific matrix derangement was noticeable **(B, C)**. **(A)** Glutaraldehyde fixation, **(B)** Decellularization before glutaraldehyde fixation, **(C)** Decellularization and α-galactosidase treatment before glutaraldehyde fixation. Color images available online at www.liebertpub.com/tea

then hydrolyzed with 5.0N HCl solution. The calcium content of the hydrolysate was measured colorimetrically by the o-cresolphthaleincomplex-one method, as previously described,[21] using an automatic chemistry analyzer (Hitachi 7070). Calcium contents were expressed as ug/mg dry weight.

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 (SPSS, Inc.). Data were expressed as mean ± standard error. Comparison between groups was performed using the *t*-test and one-way analysis of variance (ANOVA) with *post*

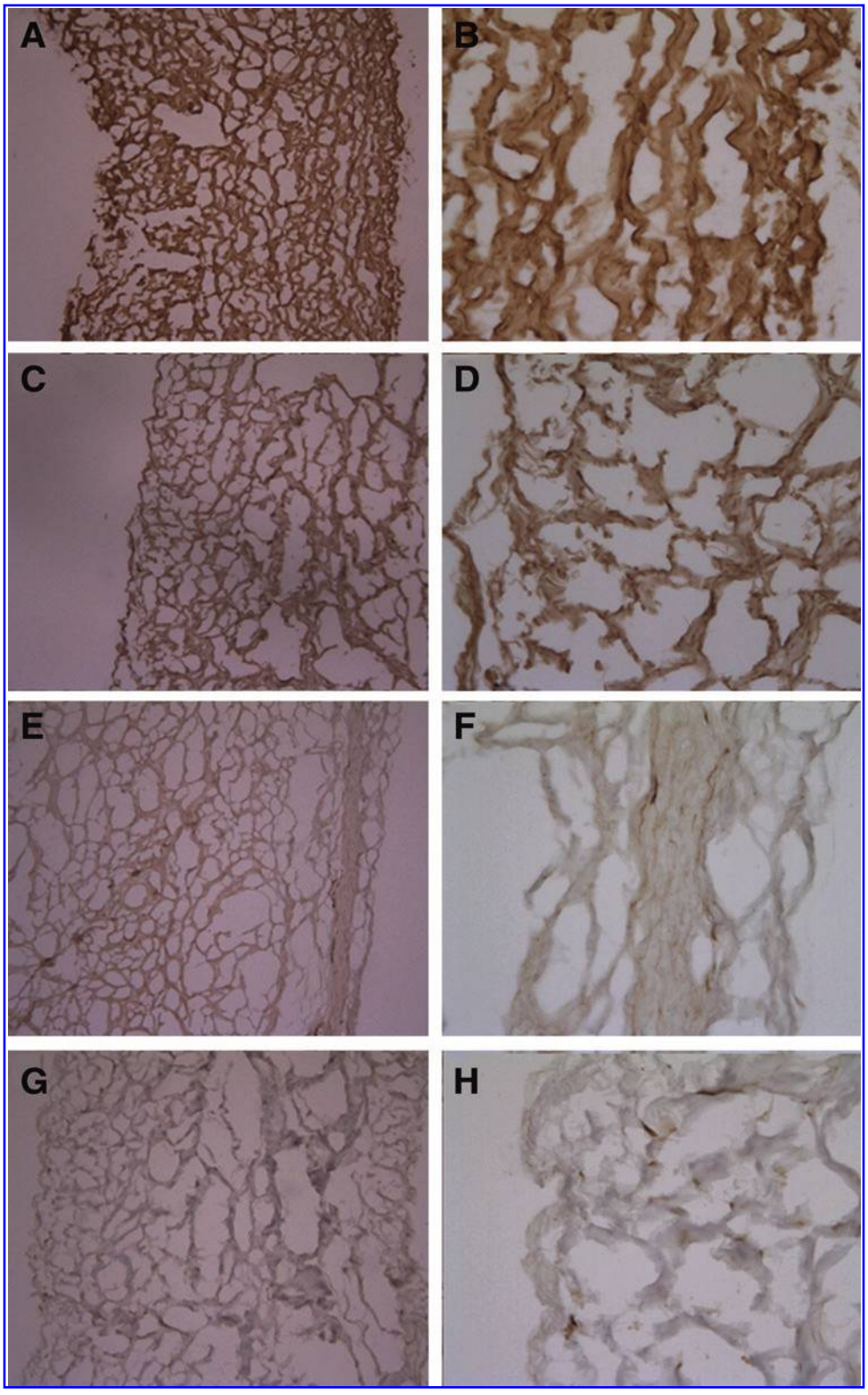

FIG. 2. Staining of α-gal epitope on unimplanted bovine pericardium by lectin histochemistry. The signal of α-gal epitope is brown. Decellularization weakened α-gal intensity on the surface of bovine pericardium **(C, D)**. The treatment of α-galactosidase effectively removed the α-gal epitopes still expressed on decellularized bovine pericardium **(E, F)**. **(A, B)** glutaraldehyde fixation, **(C, D)** decellularization before glutaraldehyde fixation, **(E, F)** decellularization and α-galactosidase treatment before glutaraldehyde fixation, **(G, H)** negative control, **(A, C, E, G:** ×100, **B, D, F, H:** ×400**).** Color images available online at www.liebertpub.com/tea

hoc test or Kruskal-Wallis and Mann-Whitney U tests. The change in the titer of anti α-Gal antibodies after implantation was compared between the groups by the analysis with the repeated measures ANOVA. Probability values of less than 0.05 were considered statistically significant.

Results

Microscopic examination (preimplantation)

In all groups, light microscopy showed optimal preservation of collagen fibers that had multidirectional orientations and regular periodicity without significant difference among groups in hematoxylin-eosin stain. Efficient decellularization was also apparent without significant extracellular matrix destruction in hematoxylin-eosin stain (Fig. 1).

The staining of α-gal epitope on bovine pericardium by lectin histochemistry demonstrated that decellularization weakened α-gal intensity on the surface of bovine pericardium. The treatment of α-galactosidase also effectively removed the α-gal epitopes still expressed on decellularized bovine pericardium (Fig. 2).

Anti-α-Gal antibodies in alpha-Gal KO mice with presensitization

In GA or genipin groups, titers for both anti-alpha-Gal IgM and IgG of alpha-Gal KO mice increased according to the duration of implantation, and are lower in the groups with decellularization than without decellularization (anti-alpha-Gal IgM: $p < 0.05$, anti-alpha-Gal IgG: $p > 0.05$), and are lower in the groups with alpha-galactosidase + decellularization than with decellularization (anti-alpha-Gal IgM: $p < 0.05$, anti-alpha-Gal IgG: $p < 0.05$) (Fig. 3).

Anti-α-Gal antibodies in alpha-Gal KO mice without presensitization

In GA groups, titers for both anti-alpha-Gal IgM and IgG of alpha-Gal KO mice increased according to the duration of implantation, and are lower in the groups with decellularization than without decellularization (anti-alpha-Gal IgM: $p < 0.05$, anti-alpha-Gal IgG: $p < 0.05$), and are not significantly lower in the groups with alpha-galactosidase + decellularization than with decellularization (anti-alpha-Gal IgM: $p > 0.05$, anti-alpha-Gal IgG: $p > 0.05$) (Fig. 4).

Calcium analysis according to the mouse type

Calcium contents of harvested bovine pericardia implanted into the α-Gal KO mouse group with presensitization, α-Gal KO mouse group without presensitization, and wild type mouse group were $1.4801 \pm 0.38343\ \mu g\ mg^{-1}$ (0–$13.1250\ \mu g\ mg^{-1}$, $n = 55$), $1.0870 \pm 0.19053\ \mu g\ mg^{-1}$ (0–$5.0000\ \mu g\ mg^{-1}$, $n = 25$), and $0.2813 \pm 0.11290\ \mu g\ mg^{-1}$ (0–$2.0370\ \mu g\ mg^{-1}$, $n = 24$), respectively.

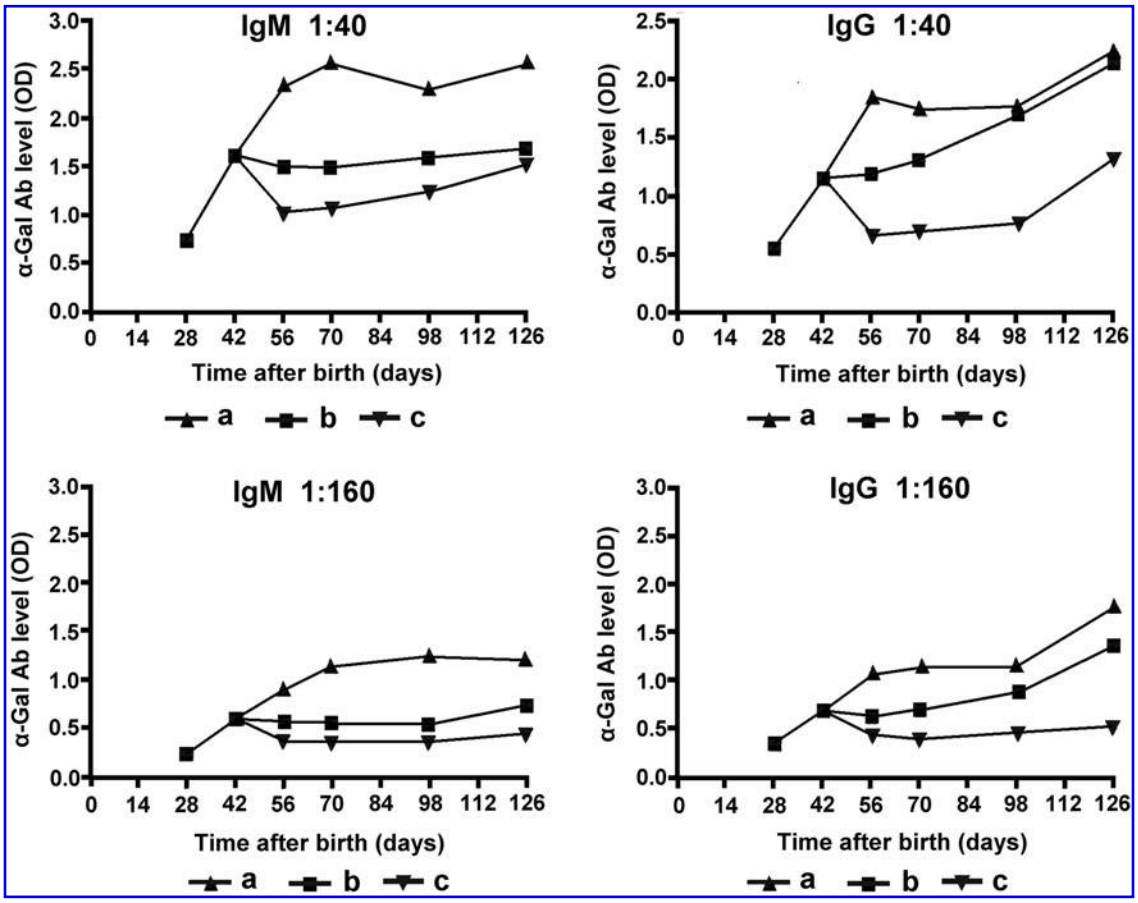

FIG. 3. Titers of anti-α-Gal antibodies in α-Gal KO mouse with immunization. α-Gal KO mouse was immunized 28 days after birth, and bovine pericardial tissues were implanted to α-Gal KO mouse 42 days after birth. (a) GA or genipin fixation, (b) Decellularization before GA or genipin fixation, (c) Decellularization and α-galactosidase treatment before GA or genipin fixation. IgM, IgG–a versus c: $p < 0.05$, b versus c: $p < 0.05$. α-Gal, Galα1,3-Galβ1–4GlcNAc-R; KO, knockout; GA, glutaraldehyde.

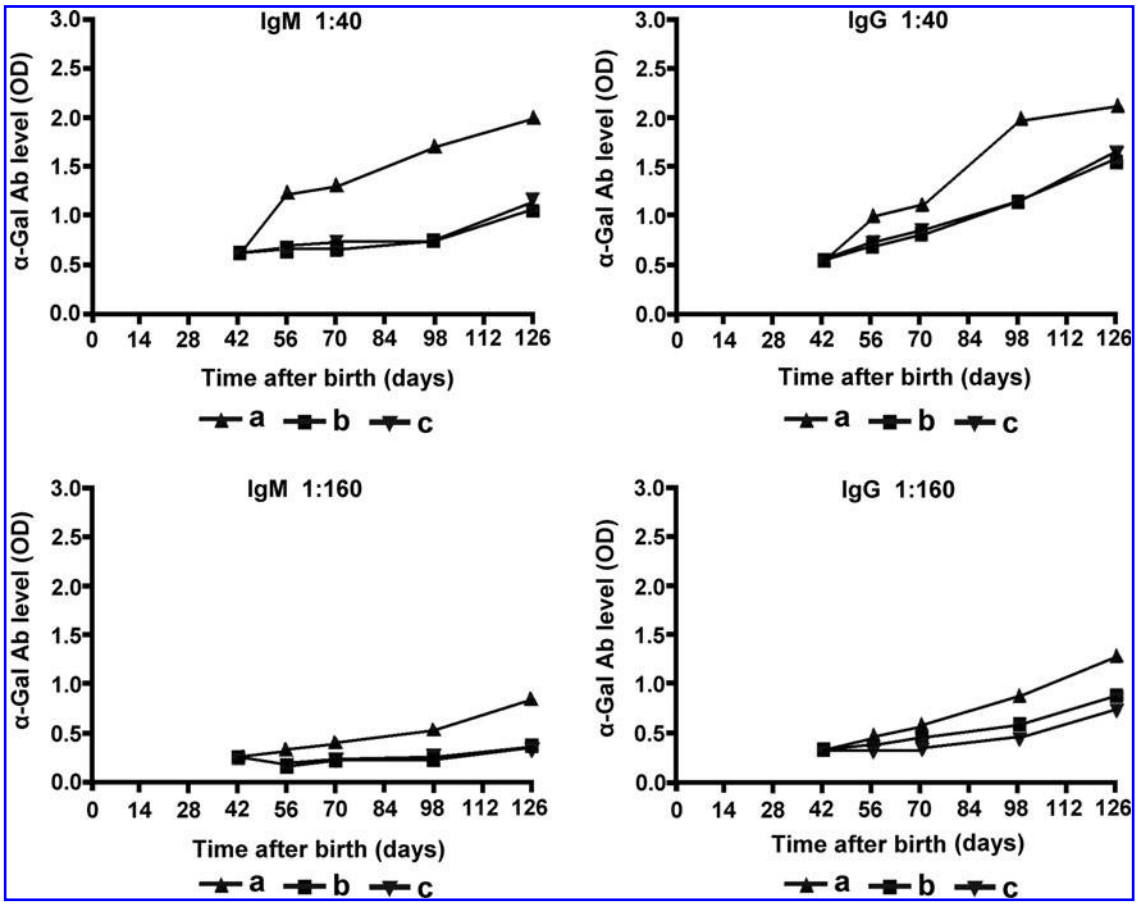

FIG. 4. Titers of anti-α-Gal antibodies in α-Gal KO mouse without immunization. Bovine pericardial tissues were implanted to α-Gal KO mouse 42 days after birth. (a) GA or genipin fixation, (b) Decellularization before GA or genipin fixation, (c) Decellularization and α-galactosidase treatment before GA or genipin fixation. IgM, IgG–a versus b: $p < 0.05$, b versus c: $p > 0.05$.

Harvested bovine pericardia in the α-Gal KO mouse group with presensitization (a) showed the highest calcium contents, in the α-Gal KO mouse group without presensitization (b) the second highest calcium contents, and in the wild type mouse group (c) the lowest calcium contents (a vs. b: $p > 0.05$, a vs. c: $p < 0.05$, b vs. c: $p < 0.05$) (Fig. 5).

Calcium contents of harvested bovine pericardium implanted into all mouse according to the experimental group

Calcium contents of harvested bovine pericardia in GA or genipin fixation group ($n = 33$) or in decellularization before GA or genipin fixation group ($n = 36$) were significantly higher than in decellularization and α-galactosidase treatment before GA or genipin fixation group ($n = 35$), respectively ($p < 0.05$).

Calcium contents of harvested bovine pericardium implanted into α-Gal KO mouse with presensitization according to the experimental group

Calcium contents of harvested bovine pericardia in GA or genipin fixation group, in decellularization before GA or genipin fixation group, and in decellularization and α-galactosidase treatment before GA or genipin fixation group were $2.3137 \pm 1.01566\ \mu g\ mg^{-1}$ (0–$10.3448\ \mu g\ mg^{-1}$), $1.7574 \pm 0.69598\ \mu g\ mg^{-1}$ (0–$13.1250\ \mu g\ mg^{-1}$), and $0.5739 \pm 0.11105\ \mu g\ mg^{-1}$ (0–$1.6000\ \mu g\ mg^{-1}$), respectively. GA or genipin fixation groups showed the highest calcium contents. Calcium contents of GA or genipin fixation groups decreased with decellularization, and decreased more with decellularization and α-galactosidase treatment (Fig. 6).

Calcium contents of harvested bovine pericardium implanted into α-Gal KO and wild type mouse according to the experimental group

Calcium contents of harvested bovine pericardia in GA fixation group implanted into α-Gal KO and wild type mouse were $1.8540 \pm 0.96744\ \mu g\ mg^{-1}$ (0–$10.3448\ \mu g\ mg^{-1}$, $n = 14$), and $0.4366 \pm 0.21568\ \mu g\ mg^{-1}$ (0–$2.0370\ \mu g\ mg^{-1}$, $n = 10$), respectively. Calcium contents of harvested bovine pericardia in decellularization and α-galactosidase treatment before GA fixation group implanted into α-Gal KO and wild type mouse were $0.3042 \pm 0.05821\ \mu g\ mg^{-1}$ (0–$0.6818\ \mu g\ mg^{-1}$, $n = 14$), and $0.1704 \pm 0.11568\ \mu g\ mg^{-1}$ (0–$1.5556\ \mu g\ mg^{-1}$, $n = 14$), respectively. Calcium contents of harvested bovine pericardia fixed with GA in α-Gal KO mouse are higher than in wild type mouse, and decreased more with decellularization and α-galactosidase treatment compared with in wild type mouse (Fig. 7).

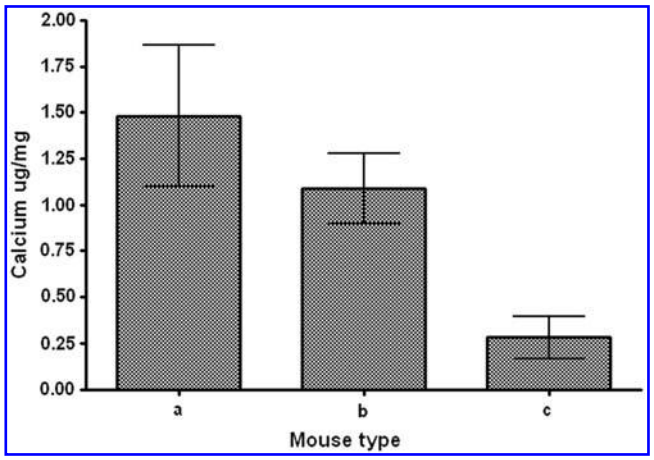

FIG. 5. Calcium contents of harvested bovine pericardium according to the mouse type. (a) α-Gal KO mouse group with presensitization, (b) α-Gal KO mouse group without presensitization, (c) Wild type mouse group. Harvested bovine pericardium in α-Gal KO mouse group with presensitization showed the highest calcium contents, in α-Gal KO mouse group without presensitization the second highest calcium contents, and in wild type mouse group the lowest calcium contents (a vs. b: $p > 0.05$, a vs. c: $p < 0.05$, b vs. c: $p < 0.05$).

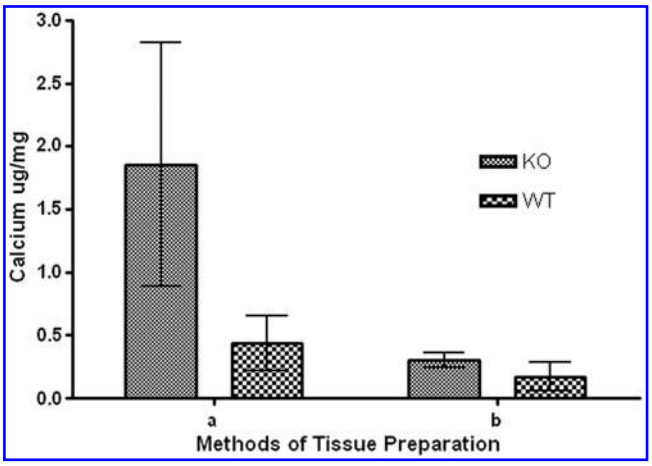

FIG. 7. Calcium contents of harvested bovine pericardium according to the experimental group in α-Gal KO and wild type mouse. (a) GA fixation, (b) decellularization and α-galactosidase treatment before GA fixation. Calcium contents of harvested bovine pericardium fixed with GA in α-Gal KO mouse are higher than in wild type mouse, and decreased more with decellularization and α-galactosidase treatment compared with in wild type mouse. WT, wild type.

Immunohistochemistry

In α-Gal KO mouse with presensitization, immunohistochemistry (IHC) staining showed that macrophages were seen right next to the grafts in GA fixation group. A thick layer of nonstained inflammatory cells was also seen in the external layer of macrophages. In α-Gal KO mouse with presensitization, IHC staining showed that macrophages were less seen right next to the grafts in GA fixation + decellularization + α-galatosidase treatment group compared with GA fixation group. A less thick layer of nonstained inflammatory cells was seen in the external layer of macrophages in GA fixation + decellularization + α-galatosidase treatment group compared with GA fixation group (Fig. 8).

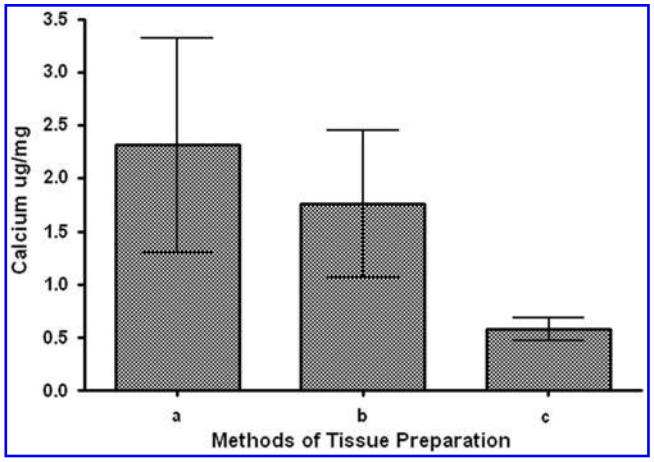

FIG. 6. Calcium contents of harvested bovine pericardium according to the experimental group in α-Gal KO mouse with presensitization. (a) GA or genipin fixation, (b) Decellularization before GA or genipin fixation, (c) Decellularization and α-galactosidase treatment before GA or genipin fixation. GA or genipin fixation groups showed the highest calcium contents. Calcium contents of GA or genipin fixation groups decreased with decellularization, and decreased more with decellularization and α-galactosidase treatment.

Discussion

GA fixation has been introduced to reduce valve antigenicity and to improve its mechanical strength. However, the failure of GA treated xenografts in clinical series has been reported. GA-fixed bioprosthetic deterioration is a complex process because GA fixation is an important issue of dystrophic calcification by the chemical process between free aldehyde groups, and phospholipids, and chronic rejection from residual antigenicity of the bioprosthetic tissue could also be an important other factor.[3,6,10,22,23] Since GA treatment does not remove xenograft antigenicity, GA-fixed xenografts have cellular/humoral rejection and calcify secondarily.[22] As an alternative other than GA, genipin is known to be a novel tissue cross-linking agents and can be obtained from its parent compound geniposide, a compound isolated from the fruit of the gardenia plant (Gardenia jasminoides Ellis). This naturally occurring crosslinker is less cytotoxic, and better *in vivo* biocompatible than GA.[3,24,25] Lim *et al.*[3] proved that genipin fixation is a novel alternative to conventional GA fixation *in vitro* material assessment and *in vivo* anticalcification effect. Decellularization approaches are effective in the prevention of calcification because tissue valve calcification is also initiated primarily within residual cells that have been devitalized.[3,12,23] Our previous study[3,6] demonstrated that the addition of decellularization, organic solvent treatment, and detoxification all strongly prevented calcification of GA/genipin-fixed bovine pericardium in the rabbit intramuscular implantation model, and the best protocol of tissue preservation from our previous study[3,6] were used in this study.

Xenoreactive natural antibodies directed at the Galα1, 3-Galβ1–4GlcNAc-R (α-Gal) epitope are known to be a major barrier in xenotransplantation. In xenotransplantation, anti α-Gal antibodies mediate the hyperacute rejection with

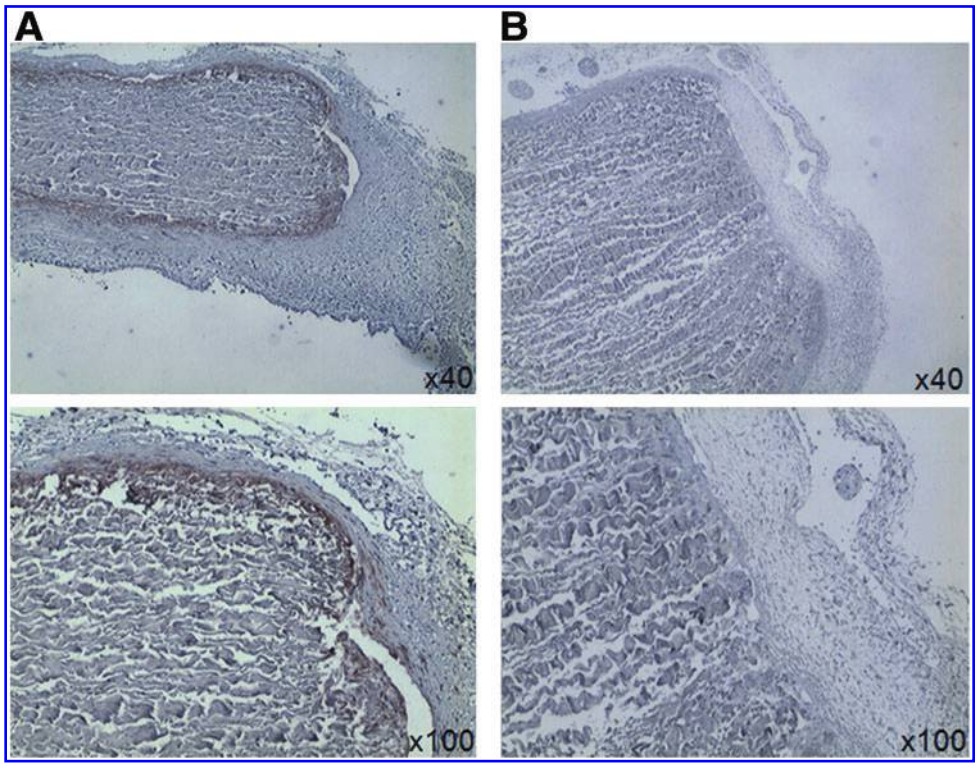

FIG. 8. Representative immunohistochemistry of harvested bovine pericardium (macrophage stain) in α-Gal KO mouse group with presensitization. **(A)** GA fixation group, **(B)** GA fixation + decellularization + α-galatosidase treatment group. Macrophages (dark brown color) were seen right next to the grafts in GA fixation group. A thick layer of nonstained inflammatory cells was also seen in the external surface of macrophages. Macrophages were less seen right next to the grafts in GA fixation + decellularization + α-galatosidase treatment group compared with GA fixation group. A less thick layer of nonstained inflammatory cells was seen in the external surface of macrophages in GA fixation + decellularization + α-galatosidase treatment group compared with GA fixation group. Color images available online at www.liebertpub.com/tea

complement activation, and delayed or chronic rejection with an antibody-dependent cellular cytotoxicity mechanism.[26,27] Today the main concern of cardiac surgeons performing the implantation of a bioprosthesis is not a catastrophic hyperacute rejection but rather a late degeneration of the implanted valve, which often requires reoperation. The chronic rejection elicited by α-Gal antigen can be an important mechanism responsible for bioprosthetic valve failure. In fact, in a model of transplanting porcine and bovine cartilage into cynomolgus monkey, a significant increase of anti-α-Gal IgG antibodies and histological evidence of chronic rejection were demonstrated.[26,28] Lee *et al.*[1] also demonstrated that bovine pericardium implanted into the α-Gal KO mice caused significant increase in anti-α-Gal antibodies, showed some histologic evidences of chronic rejection and revealed a potential toward more calcification.

Since the α-Gal-anti-Gal immune reaction may play a certain role in valve calcification and degradation after tissue valve replacement, much effort has been expended to avoid the immune response directed against it, including immunoglobulin or enzymatic treatment and genetic manipulation (α1,3-galactosyltransferase knockout).[13,29,30] An α-1,3 galactosyltransferase deficient pigs (α-Gal knockout pigs) created with the recently well established animal cloning methods can be used without a fear of hyperacute rejection.[29,31] Unfortunately, the α1,3-galactosyltransferase knockout pig is not yet available for mass production within a clinical setting. In our previous study,[2,4,5,8] it was proved that enzymatic treatment using α-galactosidase is an effective method of removing α-Gal epitopes, which is easy to use and inexpensive.[13–15] Although α-Gal may reappear in the α-galactosidase treated organs after a certain time,[15] α-Gal would not reappear after enzymatic removal of α-Gal epitopes from cardiac valvular and pericardial tissues. So, enzymatic method was used to remove the α-Gal epitope which have caused a delay in the rejection.[2,4,5,8] Nam *et al.*[4] already demonstrated that recombinant *B. thetaiotaomicron* α-galactosidase effectively removed α-gal from bovine pericardium with a small amount under physiological conditions compared to recombinant human α-galactosidase, which may alleviate the harmful xenoreactive immunologic responses of α-gal. In this study, the staining of α-gal epitope on bovine pericardium by lectin histochemistry demonstrated that the treatment of α-galactosidase also effectively removed the α-gal epitopes still expressed on decellularized bovine pericardium without difference in hematoxylin-eosin stain. Our previous study[2,4,5] demonstrated that α-gal on xenograft heart valves and tissues can be removed almost totally with recombinant α-galactosidase treatment (using both qualitative and quantitative analysis), without affecting the mechanical properties of the tissues. An absence of changes in the mechanical properties was confirmed by compliance, permeability and tensile strength testing, as well as by Masson's trichrome staining and TEM imaging after enzyme treatment.[2,4,5]

In our previous study,[3,6] we have mainly used rat subcutaneous implantation model[6] and rabbit intramuscular implantation model[3], which have been widely used as a small animal model for rapidly screening efficacy of

anticalcification treatments. However, implanting bovine tissues into rats is a kind of concordant xenotranaplantation in terms of α-Gal antigen because both donor and recipient species have α-Gal epitopes, and as such, will not provoke anti-α-Gal immune response. Therefore, it seems rational to use genetically manipulated α-Gal-deficient animals, such as α-Gal KO mice that lack the Gal epitope, and contain high titer antibodies (both IgG and IgM) against the Gal epitope, as an animal model to mimic the human immunologic environment.[1,16–19] Humans do not express the Gal epitope. Instead, human serum contains high titer antibodies (both IgG and IgM) against the Gal epitope, probably due to continual exposure to intestinal bacterial flora that have the Gal epitope.[32] Immunization of α $gal^{-/-}$ mice with sheep erythrocytes to enhance anti-α gal antibody levels led to a more robust early inflammatory response after implantation.[33] In addition to the protocol of our previous study,[1] the α-Gal KO mice has been presensitized with rabbit red blood cells to maximize immunologic response in this study.[20]

Although GA-fixation significantly reduces the antigenicity of bioprosthetic valve xenografts, there was a significant rise in anti α-Gal antibodies (IgM and IgG) of all study groups (GA and genipin fixed groups) in all α-Gal KO mouse groups with or without presensitization, supporting the hypothesis that there is a humoral response to the GA and genipin fixed xenograft. Decellularization suppress the rise of the titer of anti α-Gal antibodies (IgM and IgG) of both GA and genipin groups in all α-Gal KO mouse groups with or without presensitization, suggesting that decellularization approach is useful for removal of the immunogenicity in GA and genipin fixed tissue.[12] Decellularization and treatment of alpha-galactosidase suppress more the rise of the titer of anti α-Gal antibodies (IgM and IgG) of both GA and genipin groups than decellularization in α-Gal KO mouse group with presensitization, suggesting that decellularization and treatment of alpha-galactosidase are more useful for removal of the immunogenicity in GA and genipin fixed tissue than decellularization. *In vivo* calcification results are also consistent with the increase of anti α-Gal antibodies titer, particularly in the α-Gal KO mouse group with presensitization. Comparing *in vivo* calcification results with anti α-Gal antibodies titer, *in vivo* experiment demonstrated that decellularization and treatment of alpha-galactosidase reduced calcification in both GA and genipin groups, supporting the hypothesis that calcification of the bovine pericardium may occur as a "by-product" of the immune response. This study also demonstrated that the α-Gal KO mouse, particulary with presensitization is very useful for the evaluation of the efficacy of α-galactosidase compared with wild type mouse both in immunogenicity and *in vivo* calcification.

The IHC of the harvested grafts demonstrated the compatible findings with the results of *in vivo* immunization and *in vivo* calcification. The IHC staining showed that macrophages (dark brown color) were seen right next to the grafts of GA fixation group implanted into α-Gal KO mouse with presensitization, suggesting that these macrophages played important role on causing the immune reaction to the grafts. However, macrophages were less seen right next to the grafts in GA fixation group with decellularization and α-galatosidase treatment compared with GA fixation group. In the grafts fixed with GA implanted into the α-Gal KO mice with presensitization, a thick layer of nonstained inflammatory cells were seen in the external layer of stained macrophages, but nonstained inflammatory cells were less seen in the grafts treated with GA fixation + decellularization + α-galatosidase. The nonstained inflammatory cells which were seen in macrophage-stained grafts revealed T-cells in our previous study.[1] These IHC results demonstrated that a chronic cellular immune response exist to the α-Gal antigen in α-Gal KO mice.[1,22,28]

Conclusions

The main anticalcification strategies for GA-fixed bioprosthetic deterioration have been to extract lipids or to neutralize toxic aldehyde residuals. In this study, our approach investigated the most promising preventive strategies for chronic rejection from residual antigenicity of the bioprosthetic tissue, which could also be an important other factor for GA-fixed bioprosthetic deterioration. GA/genipin fixed bovine pericardium implanted into presensitized alpha-Gal KO mouse caused increase in anti-alpha-Gal antibodies and calcification, which suggested that the immune reaction cause the calcification. Treatment of decellularization reduced the immunologic response and calcification of GA/genipin fixed bovine pericardium, and treatment of alpha-galactsidase with decellularization reduced more. It was proved the anticalcification effects of alpha-galactsidase and decellularization in GA/genipin fixed bovine pericardium. Thus, the elimination of xenoreactive α-gal epitopes on implantable bioprosthetic heart valves is expected to increase significantly the durability of bioprosthetic heart valves. To our knowledge, this is the first study that demonstrates the efficacy of α-galactosidase using presensitized α-Gal KO mouse to mimic the human immunologic environment. Preclinical approaches to demonstrate safety and efficacy of α-galactosidase and decellularization in addition to synergistic and simultaneous employment of multiple anticalcification therapies or novel tissue treatments using large-animal long-term circulatory models are under investigation.

Acknowledgments

This study was supported by a grant of the Korea Health 21 Research and Development Project, Ministry for Health, Welfare, and Family Affairs, Republic of Korea (Project No.: A040004-006).

Disclosure Statement

No competing financial interests exist.

References

1. Lee, C., Ahn, H., Kim, S.H., Choi, S.Y., and Kim, Y.J. Immune response to bovine pericardium implanted into α1,3-galactosyltransferase knockout mice: feasibility as an animal model for testing efficacy of anticalcification treatments of xenografts. Eur J Cardiothorac Surg **42,** 164, 2012.
2. Choi, S.Y., Jeong, H.J., Lim, H.G., Park, S.S., Kim, S.H., and Kim, Y.J. Elimination of α-gal xenoreactive epitope: α-galactosidase treatment of porcine heart valves. J Heart Valve Dis **21,** 387, 2012.
3. Lim, H.G., Kim, S.H., Choi, S.Y., and Kim, Y.J. Anticalcification effects of decellularization, solvent, and detoxification treatment for genipin and glutaraldehyde fixation of bovine pericardium. Eur J Cardiothorac Surg **41,** 383, 2012.
4. Nam, J., Choi, S.Y., Sung, S.C., Lim, H.G., Park, S.S., Kim, S.H., *et al.* Changes of the structural and biomechanical

properties of the bovine pericardium after the removal of α-Gal epitopes by decellularization and α-galactosidase treatment. Korean J Thorac Cardiovasc Surg **45,** 380, 2012.
5. Min, B.J., Kim, Y.J., Choi, J.W., Choi, S.Y., Kim, S.H., and Lim, H.G. Histologic characteristics and mechanical properties of bovine pericardium treated with decellularization and α-galactosidase: a comparative study. Korean J Thorac Cardiovasc Surg **45,** 368, 2012.
6. Lee, C., Kim, S.H., Choi, S.H., and Kim, Y.J. High-concentration glutaraldehyde fixation of bovine pericardium in organic solvent and post-fixation glycine treatment: *in vitro* material assessment and *in vivo* anticalcification effect. Eur J Cardiothorac Surg **39,** 381, 2011.
7. Park, C.S., Park, S.S., Choi, S.Y., Yoon, S.H., Kim, W.H., and Kim, Y.J. Anti alpha-gal immune response following porcine bioprosthesis implantation in children. J Heart Valve Dis **19,** 124, 2010.
8. Park, S.S., Kim, W.H., Choi, S.Y., and Kim, Y.J. Removal of alpha-Gal epitopes from porcine aortic valve and pericardium using recombinant human alpha Galactosidase A. J Korean Med Sci **24,** 1126, 2009.
9. Gabbay, S., Kadam, P., Factor, S., and Cheung, T.K. Do heart valve bioprostheses degenerate for metabolic or mechanical reasons? J Thorac Cardiovasc Surg **95,** 208, 1988.
10. Konakci, K.Z., Bohle, B., Blumer, R., Hoetzenecker, W., Roth, G., Moser, B., *et al.* Alpha-GAL on bioprostheses: xenograft immune response in cardiac surgery. Eur J Clin Invest **35,** 17, 2005.
11. Courtman, D.W., Pereira, C.A., Kashef, V., McComb, D., Lee, J.M., and Wilson, G.J. Development of a pericardial acellular matrix biomaterial: biochemical and mechanical effects of cell extraction. J Biomed Mater Res **28,** 655, 1994.
12. Bloch, O., Golde, P., Dohmen, P.M., Posner, S., Konertz, W., and Erdbrügger, W. Immune response in patients receiving a bioprosthetic heart valve: lack of response with decellularized valves. Tissue Eng Part A. **17,** 2399, 2011.
13. LaVecchio, J.A., Dunne, A.D., and Edge, A.S.B. Enzymatic removal of alphagalactosyl epitopes from porcine endothelial cells diminishes the cytotoxic effect of natural antibodies. Transplantation **60,** 841, 1995.
14. Stone, K.R., Ayala, G., Goldstein, J., Hurst, R., Walgenbach, A., and Galili, U. Porcine cartilage transplants in the cynomolgus monkey: III Transplantation of α-galactosidase treated porcine cartilage. Transplantation **65,** 1577, 1998.
15. Luo, Y., Wen, J., Luo, C., Cummings, R.D., and Cooper, D.K. Pig xenogeneic antigen modification with green coffee bean alpha-galactosidase. Xenotransplantation **6,** 238, 1999.
16. Thall, A.D., Malý, P., and Lowe, J.B. Oocyte Gal alpha 1,3Gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse. J Biol Chem **270,** 21437, 1995.
17. Tearle, R.G., Tange, M.J., Zannettino, Z.L., Katerelos, M., Shinkel, T.A., Van, Denderen, B.J., *et al.* The alpha-1,3-galactosyltransferase knockout mouse. Implications for xenotransplantation. Transplantation **61,** 13, 1996.
18. Pearse, M.J., Cowan, P.J., Shinkel, T.A., Chen, C.G., and d'Apice, A.J. Anti-xenograft immune responses in alpha 1,3-galactosyltransferase knock-out mice. Subcell Biochem **32,** 281, 1999.
19. Kheir, E., Stapleton, T., Shaw, D., Jin, Z., Fisher, J., and Ingham, E. Development and characterization of an acellular porcine cartilage bone matrix for use in tissue engineering. J Biomed Mater Res A. **99,** 283, 2011.
20. McKenzie, I.F., Li, Y.Q., Patton, K., Thall, A.D., and Sandrin, M.S. A murine model of antibody-mediated hyperacute rejection by galatose-α(1,3)galactose antibodies in Gal o/o mice. Transplantation **66,** 754, 1998.
21. Steitz, S.A., Speer, M.Y., McKee, M.D., Liaw, L., Almeida, M., Yang, H., *et al.* Osteopontin inhibits mineral deposition and promotes regression of ectopic calcification. Am J Pathol **161,** 2035, 2002.
22. Manji, R.A., Zhu, L.F., Nijjar, N.K., Rayner, D.C., Korbutt, G.S., Churchill, T.A., *et al.* Glutaraldehyde-fixed bioprosthetic heart valve conduits calcify and fail from xenograft rejection. Circulation **114,** 318, 2006.
23. Schoen, F.J., and Levy, R.J. Calcification of tissue heart valve substitutes: progress toward understanding and prevention. Ann Thorac Surg **79,** 1072, 2005.
24. Chang, Y., Tsai, C.C., Liang, H.C., and Sung, H.W. *In vivo* evaluation of cellular and acellular bovine pericardia fixed with a naturally occurring crosslinking agent (genipin). Biomaterials **23,** 2447, 2002.
25. Chang, Y., Hsu, C.K., Wei, H.J., Chen, S.C., Liang, H.C., Lai, P.H., *et al.* Cell-free xenogenic vascular grafts fixed with glutaraldehyde or genipin: *in vitro* and *in vivo* studies. J Biotechnol **120,** 207, 2005.
26. Galili, U., LaTemple, D.C., Walgenbach, A.W., and Stone, K.R. Porcine and bovine cartilage transplants in cynomolgus monkey: II. Changes in anti-Gal response during chronic rejection. Transplantation **63,** 646, 1997.
27. Lin, S.S., Kooyman, D.L., Daniels, L.J., Daggett, C.W., Parker, W., Lawson, J.H., *et al.* The role of natural anti-Galα1–3Gal antibodies in hyperacute rejection of pig-to-baboon cardiac xenotransplants. Transpl Immunol **5,** 212, 1997.
28. Stone, K.R., Walgenbach, A.W., Abrams, J.T., Nelson, J., Gillett, N., and Galili, U. Porcine and bovine cartilage transplants in cynomolgus monkey: I. A model for chronic xenograft rejection. Transplantation **63,** 640, 1997.
29. Phelps, C.J., Koike, C., Vaught, T.D., Boone, J., Wells, K.D., Chen, S.H., *et al.* Production of α1,3-galactosyltransferase-deficient pigs. Science **299,** 411, 2003.
30. Schussler, O., Shen, M., Shen, L., Carpentier, S.M., Kaveri, S., and Carpentier, A. Effect of human immunoglobulins on the immunogenicity of porcine bioprostheses. Ann Thorac Surg **71,** S396, 2001.
31. Dai, Y., Vaught, T.D., Boone, J., Chen, S.H., Phelps, C.J., Ball, S., *et al.* Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol **20,** 251, 2002.
32. Galili, U., Mandrell, R.E., Hamedeh, R.M., Shohet, S.B., and Griffiths, J.M. Interaction between natural anti-[alpha]-galactosyl immunoglobulin G and bacterial of the human flora. Immun Infect **56,** 1730, 1988.
33. Raeder, R.H., Badylak, S.F., Sheehan, C., Kallakury, B., and Metzger, D.W. Natural anti-galactose α1,3 galactose antibodies delay, but do not prevent the acceptance of extracellular matrix xenografts. Transpl Immunol **10,** 15, 2002.

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Received: January 28, 2013
Accepted: May 1, 2013
Online Publication Date: June 18, 2013