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In vivo efficacy for novel combined anticalcification treatment of glutaraldehyde-fixed cardiac xenograft using humanized mice

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Abstract

The animal immune response against Galα1,3-Galβ1-4GlcNAc-R(α-Gal) epitopes gives an important cause for the failure of glutaraldehyde(GA)-fixed cardiac xenografts. This study aimed to assess the in vivo effect of our novel combined anticalcification treatment, which includes immunologic modification, using α1,3-galactosyltransferase knock-out mice to mimic human immunologic environment. Bovine pericardia were cross-linked with GA and treated with decellularization, immunologic modification with α-galactosidase, space-filler with polyethylene glycol, organic solvent, and detoxification. The bovine pericardia were subcutaneously implanted into humanized and wild type mice, and titers of anti α-Gal IgM and IgG were evaluated at various time intervals. In vivo calcification and immunohistochemistry staining was assessed for the explanted xenografts several months after implantation. In humanized mice, titers for anti α-Gal IgM and IgG increased as the period of implantation increased, and reduced with our anticalcification treatments. The humanized mice had more in vivo calcification in GA-fixed xenografts treated with our anticalcification protocol compared with wild type mice. In humanized mice, in vivo calcification reduced with our combined anticalcification treatment, and the immunohistochemistry of the harvested xenografts proved the compatible findings with the results of in vivo immunogenicity and calcification. Humanized mice are effective model for the assessment of in vivo calcification, and our combined anticalcification treatments reduced in vivo calcification as well as in vivo immunogenicity in humanized mice group, suggesting that the animal immune reaction is the cause for calcification. Our novel combined anticalcification strategies of decellularization, immunologic modification, space-filler, organic solvent, and detoxification have possible promise to prolong the lifespan of cardiac xenograft.

Keywords

Xenograft, heart valve, bioprosthesis, bioengineering, biomaterials, calcification

Introduction

Glutaraldehyde (GA) generates a cross-link of xenografts to obtain tissue stability, and to attenuate antigenicity, but long-term durability of GA-fixed xenograft is disappointing because tissue phospholipids, free aldehyde groups of GA, and residual antigenicity result in calcification.[1] Our group investigated the promising anticalcification strategies which included (1) decellularization,[1] (2) alpha-galactosidase,[2,3] (3) space filler,[4] (4) organic solvent,[1,5,6] and (5) detoxification.[1,5,6]

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(1) We decellularized the xenografts with sodium dodesyl sulfate (SDS) to suppress residual antigenicity, and remove the immunogenic cellular components.[1] (2) Since the Galα1,3-Galβ1-4GlcNAc-R(α-Gal)-anti-Gal immune reaction result in chronic rejection, inflammation, and calcification, we also introduced recombinant *Bacteroides thetaiotaomicron* α-galactosidase to effectively remove the α-gal epitopes still expressed on decellularized xenografts.[2,3] (3) We added space filler with polyethylene glycol since it reacts with free aldehyde groups of GA to inactivate, and mask platelet receptor sites.[4] (4) We used the organic solvent with 75% ethanol and 5% octanol to reduce calcification by removal of tissue phospholipids or conformational changes in collagen.[1,5,6] (5) We employed the detoxification process with glycine to neutralize free aldehyde groups of GA, forming Schiff base.[1,5,6]

The animal immune reaction due to α-Gal epitopes may play an important role in the failure of xenografts.[7–9] The wild type (WT) mice do not have the similar immunologic environment to human in terms of α-Gal antigen. To mimic the human immunologic environment as an α-Gal-deficient animal model, our group[2,10] have inactivated the α1,3-galactosyltransferase gene in mice by gene targeting, and have generated mice that do not synthesize the Gal epitope.

This study aimed to evaluate the in vivo effect of various anticalcification treatments of GA-fixed xenograft such as decellularization, alpha-galactosidase, space filler, organic solvent, and detoxification using α1,3-galactosyltransferase knock-out(α-Gal KO) mice (C57BL/6) to mimic human immunologic environment.

Materials and methods

Tissue preparation

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

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% SDS for 24 h at 4°C, and washed with distilled water for 1 h. Later, they were 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. These tissues were then treated with isotonic solution for 24 h at 4°C and 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 mmol/L; pH 8.0).
- Isotonic buffered solution (distilled water 1000 mL; Tris 50 mmol/L; NaCl 0.15 mol/L; EDTA 0.05%; aprotinin 10 KIU/mL; Neomycin trisulfate 50 mg; pH 8.0).
- Hypertonic buffered solution II (distilled water 1000 mL; Tris 200 mmol/L; NaCl 0.6 mol/L; pH 8.0).
- Distilled water (distilled water 1000 mL; EDTA 0.05%; aprotinin 10 KIU/mL).

Construction and preparation of α1,3 galactosidase from B. thetaiotaomicron

The genomic DNA of *B. 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 BtGal110 B) 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, Madison, WI, USA). *E. coli* was grown in Luria-Bertani media supplemented with 34 μg/mL chloramphenicol, 30 μg/mL kanamycin, induced at an $A_{600\,nm}$~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., Farmingdale, NY, USA). The crude lysates were centrifuged for 20 min at 13,000 rpm, at 4°C and the expressed protein was purified using Nickel-NTA agarose column (Qiagen, Valencia, CA, USA) according to the 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. Our decellularization protocol excludes trypsin, which damages extracellular matrix and increases permeability and compliance of the bovine pericardium, and instead of DNAse/RNAse, the duration of decellularization with SDS was increased, and α-galactosidase was added.

Space-filler treatment

Bovine pericardial tissues were treated with 50% polyethylene glycol (1000 MW) in 0.01 M PBS (pH 7.4) for one day at RT.

GA fixation in organic solvent

Bovine pericardial tissues were initially fixed with 0.25% GA for three days at room temperature, and additionally fixed with 0.25% GA in organic solvent of 75% ethanol+5% octanol for two days at room temperature, and finally fixed with 0.25% GA for seven 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.

Experimental, positive control, and negative control groups

The bovine pericardia were treated as a step-by-step process with (1) decellularization, (2) alpha-galactosidase, (3) space filler, (4) GA fixation in organic solvent, and (5) detoxification. The bovine pericardia treated with conventional GA fixation were used as a positive control, and monkey pericardia treated with GA fixation in organic solvent and detoxification were used as a negative control.

Mouse subcutaneous implantation

This study was approved by Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No. 11-0107). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Male α-Gal KO ($n = 62$) and wild-type ($n = 55$) mice (C57BL/6, six weeks old, 11–27 g) were used. All surgeries were 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 and the wounds were closed with 6/0 nylon sutures. The mice were sacrificed by CO_2 asphyxiation after 16 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 antibodies, blood samples were taken from the mouse on the implantation date, 2 weeks, 4 weeks, 8 weeks, 12 weeks, and 16 weeks after implantation, and an enzyme-linked immunosorbent assay (ELISA) was performed. Bovine serum albumin conjugating synthesized α-Gal (Genechem, Seoul, Korea) (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 mice 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), and then the plates were incubated for 1 h at 37°C. Rabbit anti-mouse IgM antibodies (Jackson Immunoresearch, Baltimore, MD, USA) and goat anti-mouse IgG antibodies (Jackson Immunoresearch, Baltimore, MD, USA) 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, Rockford, IL, USA). Optical density (OD) was measured at 450 nm using the Thermo Electron-Lab Systems (Labsystems, Vienna, VA, USA).

Microscopic examination (postimplantation)

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.

Immunohistochemistry (IHC) staining

Representative tissue samples from each group were stained for mouse macrophages. The primary antibodies used were anti-mouse F4/80 antigen (eBioscience, San Diego, CA, USA) at 1:300 dilution (marker for mouse macrophages). The secondary antibody used was horseradish peroxidase-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA)

at 1:500 dilution. Diaminobenzidine was used as a chromogen and hematoxylin was used for counterstaining.

Calcium analysis

Harvested tissue samples ($n = 348$) were washed with normal saline, dried at 70°C for 24 h, and weighed. Samples were then hydrolyzed with 5.0 N HCl solution. The calcium content of the hydrolysate was measured colorimetrically by the o-cresolphthaleincomplex-one method, as previously described,[11] using an automatic chemistry analyzer (Hitachi 7070, Japan). Calcium contents were expressed as μg/mg dry weight.

Statistical analysis

Statistical analyses were performed using SPSS version 21.0 (SPSS, Inc, Chicago, IL). 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 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

The change of in vivo calcification according to the duration of implantation and the effect of our anticalcification protocols in wild type mice

Calcium contents of harvested bovine pericardium fixed with GA in organic solvent implanted into wild type mice were 4.55 ± 0.55 μg/mg ($n = 14$), 6.83 ± 0.78 μg/mg ($n = 18$), and 9.64 ± 2.55 μg/mg ($n = 14$) in three, four, and six months after implantation, respectively, and increased according to the duration of implantation ($p < 0.05$) (Figure 1). However, calcium contents of harvested bovine pericardium fixed with GA in organic solvent implanted into wild type mice were less than alpha-Gal KO mice in four months after implantation (6.83 ± 0.78 μg/mg, $n = 18$ vs 9.95 ± 1.20 μg/mg, $n = 27$).

In wild type mice, calcium contents of harvested bovine pericardium treated with our anticalcification protocols (5.29 ± 0.25 μg/mg, $n = 90$) were less than conventional GA fixation in four months after implantation ($p < 0.05$). In wild type mice, inorganic phosphorus contents of harvested bovine pericardium treated with our anticalcification protocols (1.78 ± 0.16 μg/mg, $n = 90$) were less than conventional GA fixation in four months after implantation ($p < 0.05$).

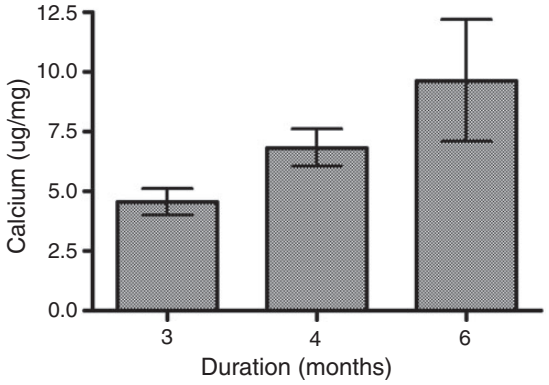

Figure 1. The change of in vivo calcification in wild type mice according to the duration of implantation. Calcium contents of harvested bovine pericardium ($n = 46$) fixed with glutaraldehyde in organic solvent implanted into wild type mice increased according to the duration of implantation ($p < 0.05$). (3 vs 4 months: $p < 0.05$).

The effect of our anticalcification treatment protocols on in vivo immunogenicity in alpha-Gal KO mice

Titers for both anti-alpha-Gal IgM and IgG of alpha-Gal KO mice increased according to the duration of implantation ($n = 59$). Titers for anti-alpha-Gal IgM of alpha-Gal KO mice increased more than anti-alpha-Gal IgG according to the duration of implantation. Titers for both anti-alpha-Gal IgM and IgG of alpha-Gal KO mice increased the highest in conventional GA fixation as a positive control ($n = 6$), the second in GA fixation with organic solvent ± detoxification ($n = 16$), the third in GA fixation with organic solvent + detoxification + decellularization ± alpha-galactosidase ± space filler ($n = 30$), and the lowest in monkey pericardium as a negative control ($n = 7$). There was a statistically significant difference of antibody titers between conventional GA fixation and our combined anticalcification treatment group (GA fixation with organic solvent + detoxification + decellularization ± alpha-galactosidase ± space filler) ($p < 0.05$). There was a statistically significant difference of antibody titers between conventional GA fixation and monkey pericardium as a negative control ($p < 0.05$). Titers for both anti-alpha-Gal IgM and IgG of alpha-Gal KO mice statistically significantly changed according to the duration of implantation ($p < 0.05$), and there was a statistically significant difference of antibody titers among the treatment groups ($p < 0.05$) (Figure 2).

The effect of α-Gal KO mice on in vivo calcification in the experimental group

The α-Gal KO mice had more calcium contents in GA-fixed bovine pericardium treated with our

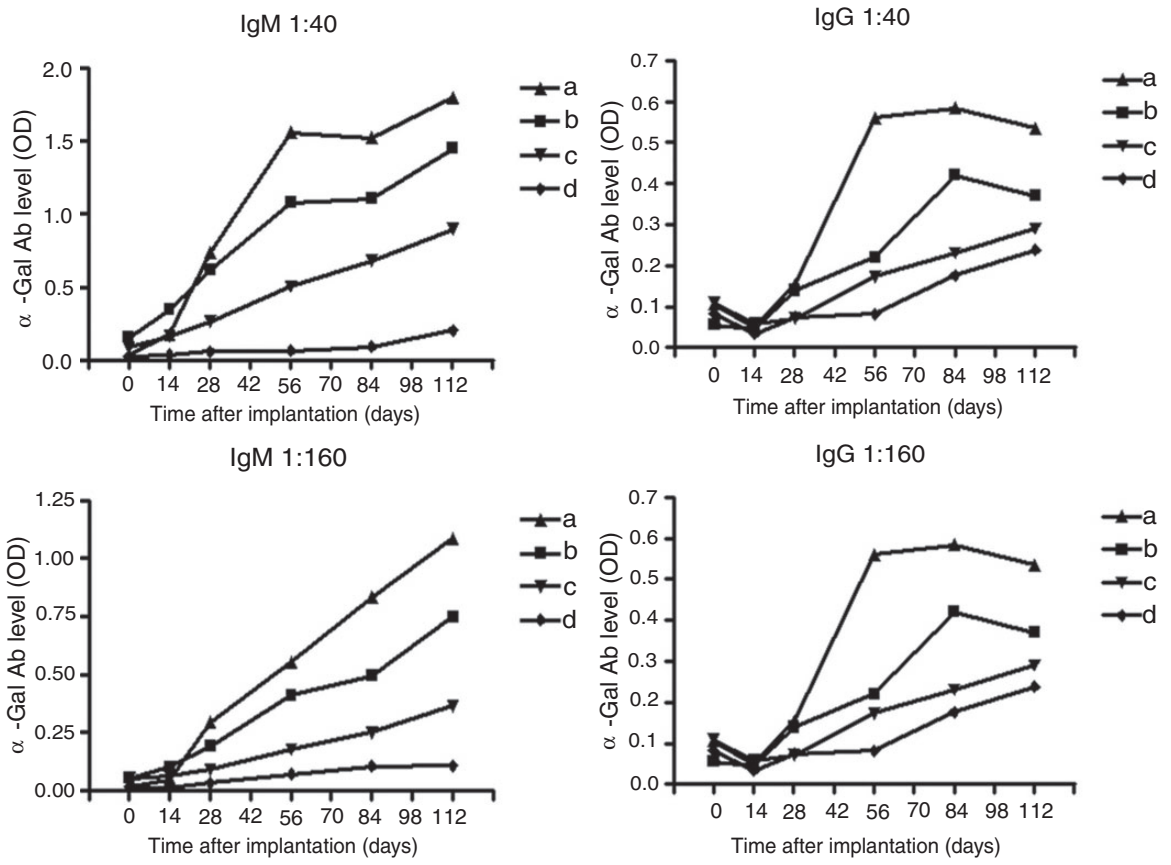

Figure 2. The effect of our anticalcification treatment protocols on in vivo immunogenicity in α-Gal KO mice ($n = 59$). Titers for both anti-α-Gal IgM and IgG of α-Gal KO mice statistically significantly changed according to the duration of implantation ($p < 0.05$), and there was a statistically significant difference of antibody titers among the treatment groups ($p < 0.05$). a vs c : $p < 0.05$, a vs d: $p < 0.05$. (a) Bovine pericardium treated with conventional GA fixation as a positive control ($n = 6$), (b) bovine pericardium treated with GA fixation in organic solvent ± detoxification ($n = 16$), (c) bovine pericardium treated with GA fixation in organic solvent + detoxification + decellularization ± alpha-galactosidase ± space filler ($n = 30$), (d) monkey pericardium treated with GA fixation in organic solvent+detoxification as a negative control ($n = 7$). α-Gal: Galα1,3-Galβ1-4GlcNAc-R; KO: knock-out; GA: glutaraldehyde.

anticalcification protocol than wild type mice in four months after implantation. (7.73 ± 0.45 μg/mg, $n = 163$ vs 5.29 ± 0.25 μg/mg, $n = 90$, $p < 0.05$) (Figure 3(a)).

The α-Gal KO mice had more inorganic phosphorus contents in GA-fixed bovine pericardium treated with our anticalcification protocol than wild type mice in four months after implantation. (3.34 ± 0.23 μg/mg, $n = 163$ vs 1.78 ± 0.16 μg/mg, $n = 90$, $p < 0.05$) (Figure 3(b)).

The effect of our anticalcification treatment protocols in α-Gal KO mice

Calcium contents of harvested bovine pericardium with conventional GA fixation (positive control) implanted into α-Gal KO mice were 35.67 ± 6.86 μg/mg ($n = 21$). Calcium contents of harvested bovine pericardium with our anticalcification treatment implanted into α-Gal KO mice were 7.73 ± 0.45 μg/mg ($n = 163$). Calcium contents of harvested monkey pericardium (negative control) implanted into α-Gal KO mice were 10.39 ± 1.84 μg/mg ($n = 23$). Calcium contents of harvested bovine pericardium with our anticalcification treatment implanted into α-Gal KO mice were less than conventional GA fixation (positive control) ($p < 0.05$). Calcium contents of harvested monkey pericardium (negative control) implanted into α-Gal KO mice were less than conventional GA fixation (positive control) ($p < 0.05$) (Figure 3(c)).

Inorganic phosphorus contents of harvested bovine pericardium with conventional GA fixation (positive control) implanted into α-Gal KO mice were 22.41 ± 5.48 μg/mg ($n = 21$). Inorganic phosphorus contents of harvested bovine pericardium with our anticalcification treatment implanted into α-Gal KO mice were 3.34 ± 0.23 μg/mg ($n = 163$). Inorganic phosphorus contents of harvested monkey pericardium

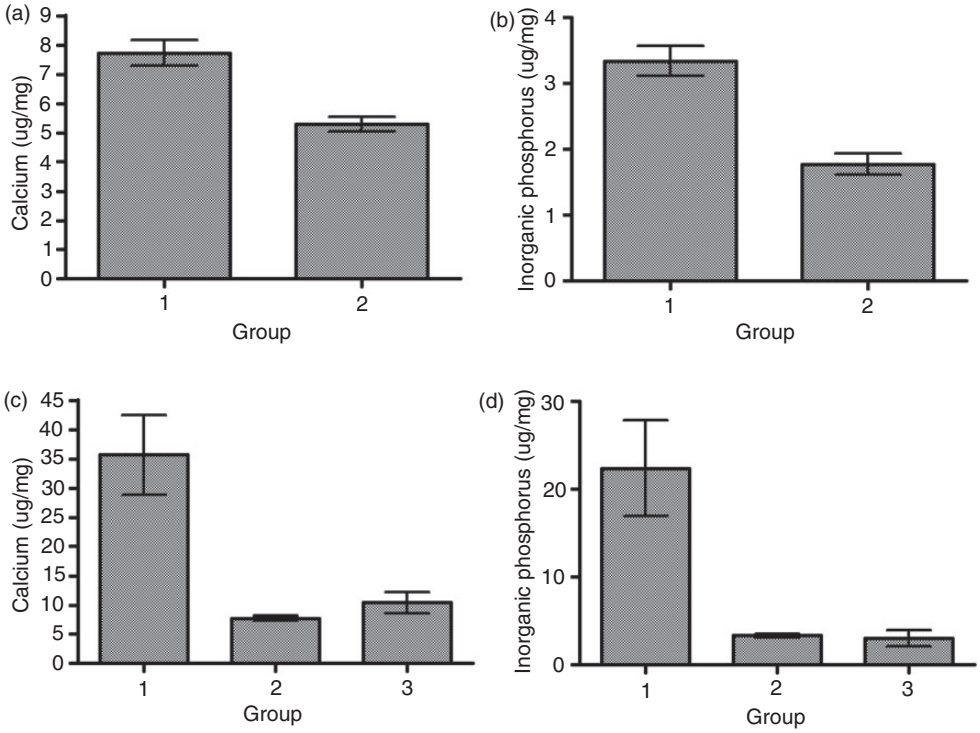

Figure 3. The effect of α-Gal KO mice on in vivo calcification in the experimental group (a, b) and the effect of our anticalcification treatment protocols in α-Gal KO mice (c, d). (a): calcium contents, (b): inorganic phosphorus contents, group 1: α-Gal KO mice ($n = 163$), group 2: wild type mice ($n = 90$), a, b: group 1 vs 2, $p < 0.05$; (c) calcium contents, (d) inorganic phosphorus contents, group 1 : conventional glutaraldehyde fixation as a positive control ($n = 21$), group 2: our anticalcification treatment ($n = 163$), group 3: monkey pericardium as a negative control ($n = 23$), c, d: group 1 vs 2, $p < 0.05$, 1 vs 3, $p < 0.05$. α-Gal: Galα1,3-Galβ1-4GlcNAc-R; KO: knock-out.

(negative control) implanted into α-Gal KO mice were 3.06 ± 0.91 μg/mg ($n = 23$). Inorganic phosphorus contents of harvested bovine pericardium with our anticalcification treatment implanted into α-Gal KO mice were less than conventional GA fixation (positive control) ($p < 0.05$). Inorganic phosphorus contents of harvested monkey pericardium (negative control) implanted into α-Gal KO mice were less than conventional GA fixation (positive control) ($p < 0.05$) (Figure 3(d)).

The effect of organic solvent on GA-fixed bovine pericardium in α-Gal KO mice

Calcium contents of harvested GA-fixed bovine pericardium without organic solvent implanted into α-Gal KO mice were 35.67 ± 6.86 μg/mg ($n = 21$). Calcium contents of harvested GA-fixed bovine pericardium with organic solvent implanted into α-Gal KO mice were 9.95 ± 1.20 μg/mg ($n = 27$) and the calcium contents of harvested GA-fixed bovine pericardium decreased with organic solvent ($p < 0.05$).

Inorganic phosphorus contents of harvested GA-fixed bovine pericardium without organic solvent implanted into α-Gal KO mice were 22.41 ± 5.48 μg/mg ($n = 21$). Inorganic phosphorus contents of harvested GA-fixed bovine pericardium with organic solvent implanted into α-Gal KO mice were 4.32 ± 0.60 μg/mg ($n = 27$) and the inorganic phosphorus contents of harvested GA-fixed bovine pericardium decreased with organic solvent ($p < 0.05$).

The effect of detoxification on GA-fixed bovine pericardium in α-Gal KO mice

Calcium contents of harvested GA-fixed bovine pericardium in organic solvent without detoxification implanted into α-Gal KO mice were 9.95 ± 1.20 μg/mg ($n = 27$). Calcium contents of harvested GA-fixed bovine pericardium in organic solvent with detoxification implanted into α-Gal KO mice were 6.26 ± 1.03 μg/mg ($n = 29$). Calcium contents of harvested GA-fixed bovine pericardium decreased with detoxification ($p < 0.05$) (Figure 4(a)).

Inorganic phosphorus contents of harvested GA-fixed bovine pericardium in organic solvent without detoxification implanted into α-Gal KO mice were 4.32 ± 0.60 μg/mg ($n = 27$). Inorganic phosphorus contents of harvested GA-fixed bovine pericardium in

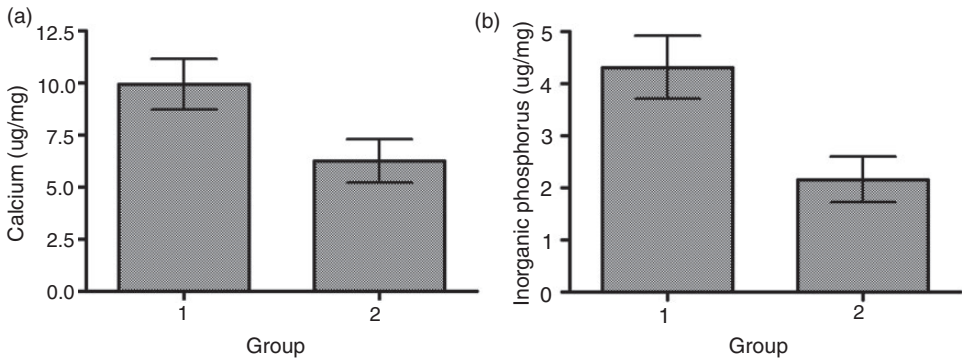

Figure 4. The effect of detoxification on glutaraldehyde-fixed bovine pericardium in α-Gal KO mice. (a) Calcium contents, (b) inorganic phosphorus contents, group 1: no detoxification ($n = 27$), group 2: detoxification ($n = 29$), a, b: group 1 vs 2, $p < 0.05$. α-Gal, Galα1,3-Galβ1-4GlcNAc-R; KO, knock-out.

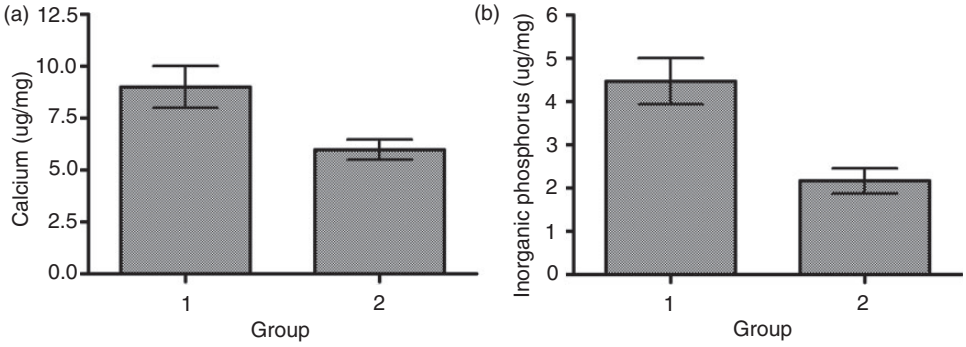

Figure 5. The effect of alpha-galactosidase on our combined anticalcification protocol in α-Gal KO mice. (a) calcium contents, (b) inorganic phosphorus contents, group 1: no alpha-galactosidase ($n = 25$), group 2: alpha-galactosidase ($n = 25$), b: group 1 vs 2, $p < 0.05$. α-Gal: Galα1,3-Galβ1-4GlcNAc-R; KO: knock-out.

organic solvent with detoxification implanted into α-Gal KO mice were 2.17 ± 0.44 μg/mg ($n = 29$). Inorganic phosphorus contents of harvested GA-fixed bovine pericardium decreased with detoxification ($p < 0.05$) (Figure 4(b)).

The effect of alpha-galactosidase on our combined anticalcification protocol in α-Gal KO mice

Calcium contents of harvested bovine pericardium treated with our combined anticalcification protocol without alpha-galactosidase implanted into α-Gal KO mice were 9.00 ± 1.01 μg/mg ($n = 25$). Calcium contents of harvested bovine pericardium treated with our combined anticalcification protocol including alpha-galactosidase implanted into α-Gal KO mice were 5.99 ± 0.48 μg/mg ($n = 25$). Calcium contents of harvested GA-fixed bovine pericardium decreased with alpha-galactosidase (Figure 5(a)).

Inorganic phosphorus contents of harvested bovine pericardium treated with our combined anticalcification protocol without alpha-galactosidase implanted into α-Gal KO mice were 4.48 ± 0.53 μg/mg ($n = 25$). Inorganic phosphorus contents of harvested bovine pericardium treated with our combined anticalcification protocol including alpha-galactosidase implanted into α-Gal KO mice were 2.17 ± 0.29 μg/mg ($n = 25$). Inorganic phosphorus contents of harvested GA-fixed bovine pericardium decreased with alpha-galactosidase ($p < 0.05$) (Figure 5(b)).

The effect of space filler on our combined anticalcification protocol in α-Gal KO mice

Calcium contents of harvested bovine pericardium treated with our combined anticalcification protocol without space filler implanted into α-Gal KO mice were 8.58 ± 1.40 μg/mg ($n = 30$). Calcium contents of harvested bovine pericardium treated with our combined anticalcification protocol including space filler implanted into α-Gal KO mice were 5.99 ± 0.48 μg/mg ($n = 25$). Calcium contents of harvested GA-fixed bovine pericardium decreased with space filler (Figure 6(a)).

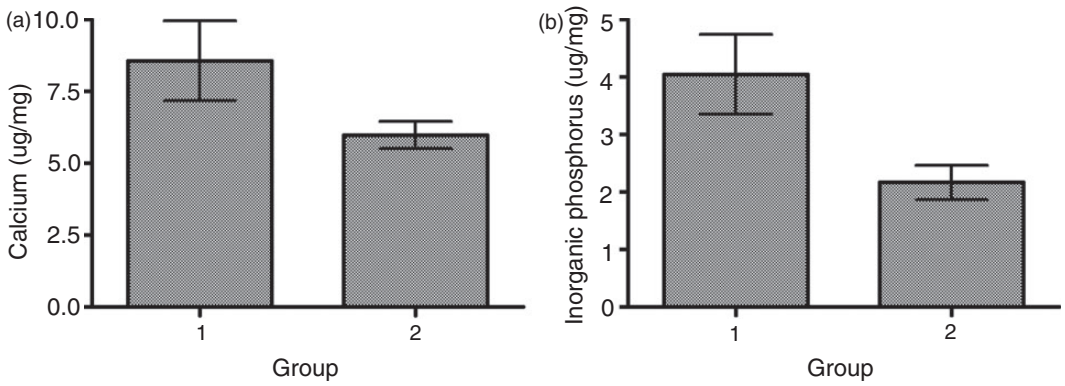

Figure 6. The effect of space filler on our combined anticalcification protocol in α-Gal KO mice. (a) Calcium contents, (b) inorganic phosphorus contents, group 1: no space filler ($n = 30$), group 2: space filler ($n = 25$), b: group 1 vs 2, $p < 0.05$. α-Gal: Galα1,3-Galβ1-4GlcNAc-R; KO: knock-out.

Inorganic phosphorus contents of harvested bovine pericardium treated with our combined anticalcification protocol without space filler implanted into α-Gal KO mice were 4.05 ± 0.69 μg/mg ($n = 30$). Inorganic phosphorus contents of harvested bovine pericardium treated with our combined anticalcification protocol including space filler implanted into α-Gal KO mice were 2.17 ± 0.29 μg/mg ($n = 25$). Inorganic phosphorus contents of harvested GA-fixed bovine pericardium decreased with space filler ($p < 0.05$) (Figure 6(b)).

Hematoxylin-eosin stain of harvested bovine pericardium implanted into α-Gal KO mice

Extensive inflammatory cellular infiltrates disrupting collagen fibers were observed in harvested bovine pericardium treated with GA fixation in organic solvent, whereas less inflammatory cellular infiltrates were observed in harvested bovine pericardium treated with decellularization, alpha-galactosidase, space filler, GA fixation in organic solvent, and detoxification (Figure 7).

IHC stain of harvested bovine pericardium implanted into α-Gal KO mice

In α-Gal KO mice, IHC staining showed that stained macrophages were present right next to the grafts in harvested bovine pericardium treated with GA fixation in organic solvent. A thick layer of nonstained inflammatory cells was also present in the outer surface of stained macrophages. In α-Gal KO mice, IHC staining showed that stained macrophages were less present right next to the grafts in harvested bovine pericardium treated with decellularization, alpha-galactosidase, space filler, GA fixation in organic solvent, and detoxification compared with harvested bovine pericardium treated with GA fixation in organic solvent. A less thick layer of nonstained inflammatory cells was present in the outer surface of stained macrophages in harvested bovine pericardium treated with decellularization, alpha-galactosidase, space filler, GA fixation in organic solvent, and detoxification compared with harvested bovine pericardium treated with GA fixation in organic solvent (Figure 8).

Discussion

We have made considerable efforts to establish the most promising anticalcification treatment strategies of GA-fixed xenografts such as (1) decellularization, (2) alpha-galactosidase, (3) space filler, (4) organic solvent, and (5) detoxification over many years. As previous step of clinical trials, this study proved in vivo efficacy of our combined anticalcification treatment protocols including immunologic modification using α-Gal KO mice as well as WT mice.

(1) Decellularization[1,12–15]: After our decellularization, there was no visible cell, and no specific matrix derangement was noticeable in hematoxylin-eosin stain.[1,2] Our decelluarization did not alter the microscopic structure, the degree of cross-linking as assessed by thermal stability test and pronase test, and the degree of tissue strength as assessed by uniaxial mechanical test.[1,16] In vivo calcification test using rabbit intramuscular model demonstrated inhibition of calcification in GA fixation with decellularization, as assessed by calcium quantification and von Kossa stain.[1] Our decellularization lowered the cytotoxicity in GA fixed xenografts. In rabbit subcutaneous or intramuscular implantation models, decellularization lowered in vivo calcification of GA-fixed xenografts.[17]

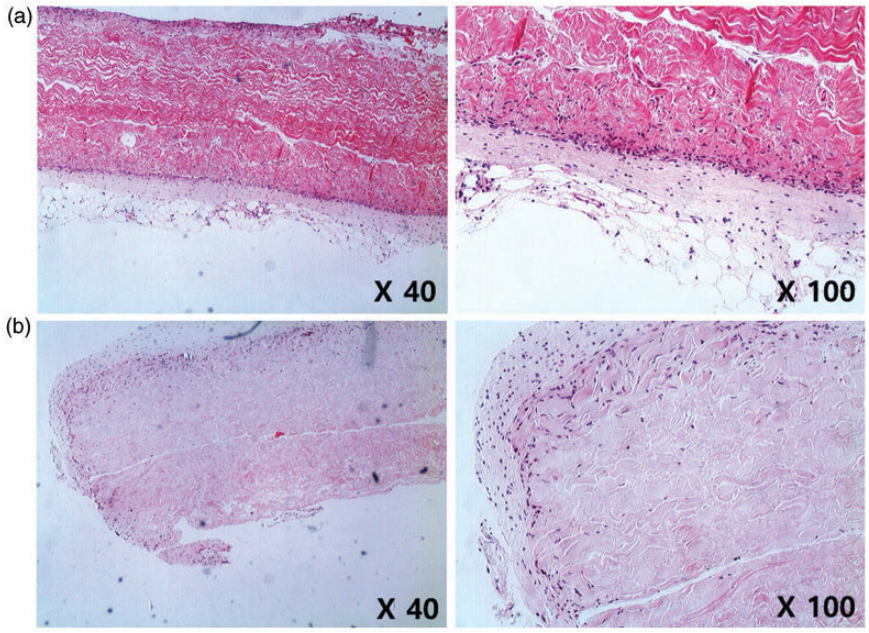

Figure 7. Hematoxylin-eosin stain of harvested bovine pericardium in α-Gal KO mice group. Extensive inflammatory cellular infiltrates disrupting collagen fibers were observed in (a), whereas less inflammatory cellular infiltrates were observed in (b). Bovine pericardium treated with GA fixation in organic solvent (a, upper), and decellularization, alpha-galactosidase, space filler, GA fixation in organic solvent, and detoxification (b, lower). Original magnification× 40 (left), × 100 (right). α-Gal: Galα1,3-Galβ1-4GlcNAc-R; KO: knock-out; GA: glutaraldehyde.

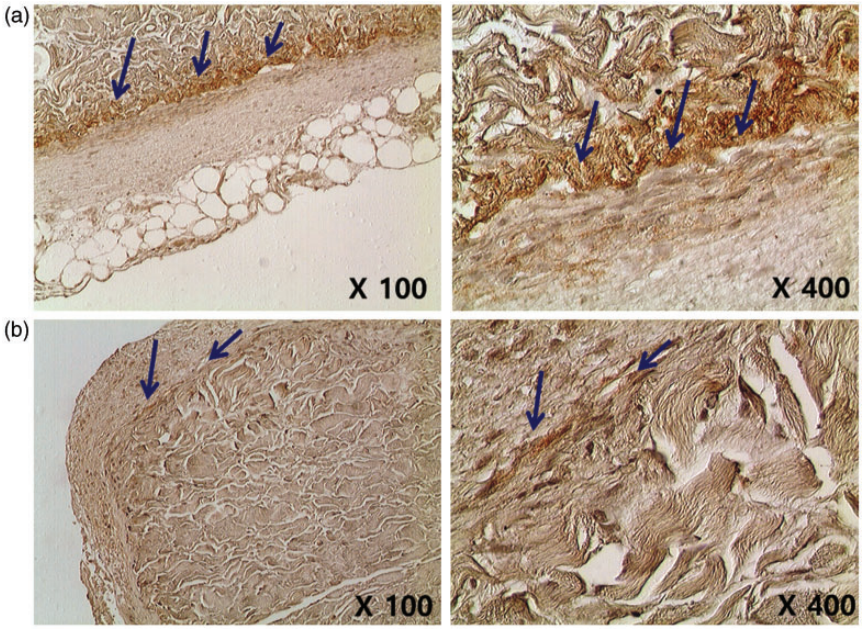

Figure 8. Representative immunohistochemistry of harvested bovine pericardium (macrophage stain) in α-Gal KO mice group. Macrophages (dark brown color, arrow) were present right next to the grafts in (a). A thick layer of nonstained inflammatory cells was also present in the outer surface of macrophages. Macrophages were less present right next to the grafts in (b) compared with (a). A less thick layer of nonstained inflammatory cells was present in the outer surface of macrophages in (b) compared with (a). Bovine pericardium treated with GA fixation in organic solvent (a, upper), and decellularization, alpha-galactosidase, space filler, GA fixation in organic solvent, and detoxification (b, lower). Original magnification× 100 (left), × 400 (right). α-Gal: Galα1,3-Galβ1-4GlcNAc-R; KO: knock-out; GA: glutaraldehyde.

(2) Alpha-galactosidase.[2,3,12,13,18,19]: 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.[12] 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.[2] 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.[3,12,13] Absence of changes in the mechanical properties was confirmed by compliance, permeability and tensile strength testing, as well as by transmission electron microscopy imaging following enzyme treatment.[3,12,13] Lim et al.[2] evaluated the immune reaction and anticalcification effect of α-galactosidase and decellularization for GA/genipin fixed bovine pericardium using α-Gal KO mice, which had been presensitized with rabbit red blood cells to maximize immunologic response, and demonstrated that treatment of α-galactosidase with decellularization are useful for removal of the immunogenicity, and reduced calcification in both GA and genipin fixed bovine pericardia.

(3) Space filler[4,17]: Lately, we studied the effects of space-filler treatment in order to improve the properties of heart valve xenografts. In the rabbit intramuscular implantation model, space-filler treatment was effective in mitigating calcification of GA fixed xenografts for biological tissue preservation.[4] Space filler lowered the cytotoxicity in GA fixed xenografts, and in vivo calcification of GA-fixed xenografts in rabbit subcutaneous or intramuscular implantation models.[17]

(4) Organic solvent[20]: Anti-calcification treatment with solvents (77.5% ethanol and 2.5% octanol or 2.5% octandiol) did not worsen the physical properties, and prevented in vivo calcifications using rat subdermal implantation model for 12 weeks compared to GA fixation only.[5] Lee et al. demonstrated that 70% concentration of alcohols (65% ethanol and 5% octanol) did not alter the microscopic structure, the amino acid composition as assessed by percent distribution of major amino acids, the degree of cross-linking as assessed by thermal stability test, and the degree of tissue strength as assessed by uniaxial mechanical test. In vivo calcification study of 70% concentration of alcohols (65% ethanol and 5% octanol) using rat subcutaneous implantation model for eight weeks clearly demonstrated excellent anticalcification efficacy of this treatment.[6] We used 80% concentration of alcohols (a mixture of 75% ethanol and 5% octanol solution; 75% ethanol and 5% octandiol solution; and 70% ethanol and 10% isopropanol solution) instead of using pure organic solvent for the extraction of tissue phospholipids. These organic solvent treatments did not alter the microscopic structure, the degree of cross-linking as assessed by thermal stability test and pronase test, and the degree of tissue strength as assessed by uniaxial mechanical test.[1] In vivo calcification study clearly demonstrated excellent anticalcification efficacy of these organic solvent treatment, regardless of what kinds of organic solvents were used.[1]

(5) Detoxification: The deleterious effects of the free aldehyde groups of GA should be blocked with detoxification.[21] Chang et al. demonstrated that anti-calcification treatment with glutamate and urazole did not worsen the physical properties of bovine pericardium, and prevented in vivo calcifications using rat subdermal implantation model for 12 weeks compared to GA fixation only.[5] Post-treatment with glycine did not alter the microscopic structure, the amino acid composition as assessed by percent distribution of major amino acids, the degree of cross-linking as assessed by thermal stability test, and the degree of tissue strength as assessed by uniaxial mechanical test. In vivo calcification test using rat subcutaneous model for eight weeks demonstrated almost complete inhibition of calcification, as assessed by calcium quantification and von Kossa stain, compared with conventional GA fixation without glycine treatment.[6]

Post-treatment with glycine, L-glutamic acid, and sodium bisulfite did not alter the microscopic structure, the degree of cross-linking as assessed by thermal stability test and pronase test, and the degree of tissue strength as assessed by uniaxial mechanical test.[1] In vivo calcification test using rabbit intramuscular model demonstrated inhibition of calcification in the groups of GA fixation with detoxification, as assessed by calcium quantification and von Kossa stain, compared with conventional fixation without detoxification, regardless of what kinds of reducing agents were used.[1]

In this study, we demonstrated that in WT mice, the calcium contents increased according to the duration of implantation, and our anticalcification treatments reduced in vivo calcification compared with conventional GA fixation. However, in WT mice group, we did not demonstrate the difference of in vivo

calcification results according to the various tissue treatments during four months implantation. In WT mice group, the implantation duration of 4 months is not enough to demonstrate the difference of in vivo calcification results according to the various tissue treatments. As the implantation duration increase (3, 4, and 6 months), in vivo calcification results of WT mice gradually increase in this study. The WT mice group of more than four months implantation duration will help us to demonstrate the difference of in vivo calcification results according to the various tissue treatments.

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.[2,10,22–24] Our group[2,10] 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 more calcification than WT mice.

In this study, it was proved that the immune reaction contributes to the cause of calcification and our combined anticalcification treatment protocols including immunologic modification are effective as follows:

(1) The α-Gal KO mice had more in vivo calcification results (calcium and inorganic phosphorus contents) in GA-fixed xenografts treated with our anticalcification protocol than WT mice during four months implantation, and unlike WT mice, the α-Gal KO mice group of the four months implantation duration demonstrate the difference of in vivo calcification results according to the various tissue treatments.
(2) In α-Gal KO mice, titers for both anti α-Gal IgM and IgG increased according to the duration of implantation, and were lower with our anticalcification treatments of organic solvent ± detoxification than conventional GA fixation, and were far lower with our additional anticalcification treatments of decellularization ± alpha-galactosidase ± space filler. In α-Gal KO mice, our combined anticalcification treatment also decreased in vivo calcification, respectively.
(3) In α-Gal KO mice, the hematoxylin-eosin and IHC staining of the harvested grafts demonstrated the compatible findings with the results of in vivo immunogenicity and calcification. The IHC staining showed that stained macrophages were present right next to the GA-fixed grafts implanted into α-Gal KO mice, suggesting that these macrophages contributed to the cause of immune reaction to the grafts. However, stained macrophages were less present right next to the GA-fixed grafts treated with decellularization and alpha-galactosidase compared with the GA-fixed grafts. In the GA-fixed grafts implanted into the α-Gal KO mice, a thick layer of non-stained inflammatory cells was present in the outer surface of stained macrophages, but non-stained inflammatory cells were less present in the GA-fixed grafts treated with decellularization and alpha-galactosidase. Our previous study also demonstrated that the non-stained inflammatory cells in the outer surface of stained macrophages were T-cells.[4,10] These IHC results of α-Gal KO mice proved the presence of a chronic cellular immune response to the α-Gal antigen.[10,25]

Conclusions

Compared with wild type mice, α-Gal KO mice are more effective model for the assessment of in vivo calcification, and our combined anticalcification treatments reduced in vivo calcification as well as in vivo immunogenicity in α-Gal KO mice, suggesting that the animal immune reaction give cause for calcification. Our novel combined anticalcification strategies of decellularization, immunologic modification, space filler, organic solvent, and detoxification have possible promise to prolong the lifespan of cardiac xenograft. Our preclinical approaches to demonstrate safety and efficacy of synergistic and simultaneous employment of multiple anticalcification therapies or novel tissue treatments such as organic solvent, decellularization, α-galactosidase, space filler, and detoxification using large-animal long-term circulatory models are under investigation.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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