

Development of Novel Combined Anticalcification Protocols Including Immunologic Modification for Prolonged Durability of Cardiac Xenograft: Preclinical Study Using Large-Animal Long-Term Circulatory Models

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Cardiac xenografts are conventionally cross-linked with glutaraldehyde (GA) to impart tissue stability, reduce antigenicity, and maintain tissue sterility. However, GA-fixed xenografts are prone to calcification after long-term implantation in humans, because of phospholipids, free aldehyde groups, and residual antigenicity. We evaluated preclinical safety and efficacy using large-animal long-term circulatory models for our novel combined anticalcification protocol including immunological modification, which had been proven effective in small animal experiments. Bovine/porcine xenografts were treated with decellularization, immunological modification with α -galactosidase, GA fixation with organic solvent, and detoxification with glycine. Valve conduits made of these xenografts were transplanted into the pulmonary root of goats, and hemodynamic, radiological, immunohistopathological, and biochemical results were obtained for 12 months after implantation. Evaluation of echocardiography and cardiac catheterization demonstrated good hemodynamic status and function of the pulmonary xenograft valves. Durability of the xenografts was well preserved without calcification by specimen radiography and immunohistopathological examination. The calcium concentrations of the explanted xenografts were lower than the control xenografts. This preclinical study using large-animal long-term circulatory models demonstrated that our synergistic and simultaneous employment of multiple anticalcification therapies and novel tissue treatments, including immunological modifications, have promising safety and efficacy and should be examined further in future clinical studies. *ASAIO Journal* 2015; 61:87–95.

Key words: biomaterials, bioprosthesis, heart valve, tissue engineering, xenograft

Cardiac xenografts have been conventionally cross-linked with the “standard” chemical fixative glutaraldehyde (GA) to increase tissue mechanical strength, decrease antigenicity, and maintain tissue sterility.^{1,2} However, the fixation of cardiac xenografts with GA is highly detrimental toward dystrophic calcification and degradation in the mid-to-long-term implantation in humans because of phospholipids, free aldehyde groups, and residual antigenicity.^{1,2} We³ demonstrated that cardiac xenografts prepared using GA fixation alone resulted in severe calcification more than 300 days after a pig to goat pulmonary root xenotransplantation and should be managed using proper anticalcification treatment and novel preservation methods.

We applied novel combined anticalcification steps to GA-fixed cardiac xenografts. First, an organic solvent can reduce calcification by extracting cholesterol and phospholipids or permanent altering the collagen structure and binding to hydrophobic residues within collagen and elastin.^{2,4,5} Second, a detoxification process with amino acids postfixation can neutralize residual unbound aldehyde groups of GA by forming Schiff bases.^{2,4,5} Third, decellularization can suppress residual antigenicity, and remove the major immunogenic cellular components without compromising the integrity of the extracellular matrix proteins.^{2,6} Our previous study² showed *in vivo* anticalcification effects of organic solvent use, detoxification, and decellularization treatment for GA fixation of cardiac xenografts in a rabbit intramuscular implantation model.

The fourth step is immunological modification, in which complete enzymatic removal of the Gal α 1,3-Gal β 1-4GlcNAc-R(α -Gal) epitope is achieved by α -galactosidase treatment.^{6–8} Because the α -Gal-antiGal immune reaction can result in chronic rejection, inflammation, and calcification, we also introduced recombinant *Bacteroides thetaiotaomicron* α -galactosidase to effectively remove the α -gal epitopes still present on the decellularized xenografts.^{6–8} Our previous study⁹ showed the *in vivo* efficacy of α -galactosidase as a promising agent for prolonged durability of cardiac xenografts using α 1,3-galactosyltransferase knockout mice to mimic the human immunological environment.

We evaluated preclinical safety and efficacy using large-animal long-term circulatory models for our novel combined anticalcification protocol including immunological modification, which was demonstrated to be effective in small-animal experiments.

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Materials and Methods

Tissue Preparation

Fresh bovine and porcine pericardium were obtained from a 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.

Construction and Preparation of α 1,3-Galactosidase from *B. thetaiotaomicron*

Genomic DNA from *B. thetaiotaomicron* was provided by Dr. Yoon-Hoh Kook from 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 polymerase chain reaction and was introduced into the pET28a vector for expression of the His₆-tagged proteins using appropriate restriction sites for protein expression in *Escherichia coli* Rosetta2 (DE3) (Novagen, Madison, WI) as previously reported.¹⁰ *Escherichia coli* was grown in Luria-Bertani media supplemented with 34 μ g/ml chloramphenicol, 30 μ g/ml kanamycin, induced at $A_{600nm} \sim 0.6$ with 1 mM isopropyl-1-thio- β -D-galactopyranoside. The harvested cell pellet was lysed in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole) using an Ultrasonicator (Misonix Inc., Farmingdale, NY). The crude lysates were centrifuged (13,000 rpm, 20 min, 4°C) and the expressed protein was purified using a nickel-NTA agarose column (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Decellularization and Treatment of α -Galactosidase

Bovine and porcine 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 (distilled water 1,000 ml; Tris 10 mmol/L; pH 8.0) with 0.25% sodium dodecyl sulfate (SDS) for 24 h at 4°C, and washed with distilled water (distilled water 1,000 ml; ethylenediaminetetraacetic acid (EDTA) 0.05%; aprotinin 10 kallikrein inhibitor units (KIU)/ml) for 1 h. The tissues 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 treated with isotonic solution (distilled water 1,000 ml; Tris 50 mmol/L; NaCl 0.15 mol/L; EDTA 0.05%; aprotinin 10 KIU/ml; neomycin trisulfate 50 mg; pH 8.0) with α -galactosidase for 24 h at 4°C. Then, they were finally treated with hypertonic buffered solution II (distilled water 1,000 ml; Tris 200 mmol/L; NaCl 0.6 mol/L; pH 8.0) for 6 h at 4°C, and washed with PBS for 1 h at 4°C.

GA Fixation in Organic Solvent

Bovine and porcine 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.

Detoxification

After completion of fixation, tissues were treated with 0.2 M glycine solution (PBS, pH 7.4) at 4°C for 24 h.

Design and Manufacture of Pericardial Valve Conduit with Bulging Sinuses

Bulging sinuses were formed on a pericardial valve conduit of 15 mm diameter using our specially designed mold.¹¹ The mold was made of silicone and epolene wax to create a valve conduit with bulging sinuses. Bovine and porcine pericardium was furled and fixed around the mold. Bovine pericardium with three bulging sinuses was used as a conduit wall, and three valve leaflets were made by anastomosing sinus-shaped valves made of porcine pericardium to the edge of the bulging sinuses. So, the wall of the pericardial valve conduit was made of bovine pericardium treated with our anticalcification protocols, and the leaflet of pericardial valve conduit was made of porcine pericardium treated with our anticalcification protocols.

Open Heart Surgery for Pulmonary Root Xenotransplantation in the Right Ventricular Outflow Tract Using Cardiopulmonary Bypass

This study was approved by Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No. 11-0097), and supported with strict Animal Care Protocols. This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The goat model is the most common in the pulmonary position. The body weights of the five goats were 33, 28, 25.5, 27, and 33.5 kg. The pericardial valve conduit of 15 mm diameter also fit into the goats in the pulmonic position. Under general anesthesia, a goat was placed in the right decubitus position, and left thoracotomy was performed through the 4th intercostal space. After 300 IU/kg of heparin was injected intravenously, aortic cannulation was performed through the descending aorta and venous cannulation through the right atrial auricle. Cardiopulmonary bypass was started. The distal main pulmonary artery was transected and distal anastomosis was performed between distal main pulmonary artery and the xenograft valve conduit. The main pulmonary artery was excised, and the native pulmonary valve was also completely excised. Proximal anastomosis was performed between xenograft valve conduit and proximal main pulmonary artery. Cardiopulmonary bypass was weaned, and modified ultrafiltration was performed. After the pulmonary root xenotransplantation, goats survived to 130, 357, 357, 357, and 366 days.

Immunoassay

Blood samples were taken from the goats before, just after, 2 weeks after, 12 weeks after, and 1 year after xenotransplantation. The enzyme-linked immunosorbent assay (ELISA) technique was used to measure the specific antibody concentration (IgG) in serum of goats against porcine and bovine collagen Type I, and against α -Gal.

Collagen Type I antibody assay: Microtiter plates were coated with 100 μ l per well of collagen Type I (Abcam, Cambridge, MA) at 4°C overnight. Plates were incubated with diluted serum at 37°C for 2 h, washed, and treated with a

secondary antibody using horseradish peroxidase-conjugated rabbit antimouse IgG (Santa Cruz Biotechnology, Inc., CA) at 1:1,000 dilution at 37° C for another 2 h.

Alpha-Gal antibody assay. Microtiter plates coated with 100 μ l per well of bovine serum albumin conjugate synthesized α -Gal (Dextra Laboratories, Reading, UK) (α -Gal-BSA) in PBS (pH 7.4, at a concentration of 1 μ g/ml for IgG isotype), and incubated at 37° C for 1 h. Then, the plates were washed with PBS containing 0.05% (v/v) Tween 20. The goat sera (100 μ l per well) were added to the α -Gal-BSA-immobilized wells in serial twofold dilutions from 1:40 to 1:10,000 in BSA-Tween 20 (PBS, pH 7.4, 3% BSA, 0.01% Tween 20), then the plates were incubated for 1 h at 37° C. Donkey antigoat IgG antibodies (Jackson ImmunoResearch, Baltimore, MD) were used as a secondary antibody (1:10,000) at a dilution for IgG in BSA-Tween 20.

Finally, a color reaction was developed using 3,3',5,5'-tetramethylbenzidine solution (TMB; BD Biosciences, San Diego, CA). The absorbance was measured in an ELISA reader at 450 nm.

Echocardiography and Cardiac Catheterization

Transthoracic echocardiography was performed at 12 months posttransplantation to evaluate hemodynamic changes. The morphologies and competencies of the leaflet were investigated. Cardiac catheterization was also performed to confirm hemodynamics just before sacrificing the goat.

Radiologic Confirmations with Quantification of Calcification

After sacrificing the goat, grafts were tested for radiologic confirmation with simple X-rays.

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 μ m thick sections were stained with hematoxylin and eosin, Masson's trichrome, and the von Kossa method.

Calcium Analysis

The harvested tissue samples 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 calorimetrically by the *o*-cresolphthalein complex-one method, as described previously,¹² using an automatic chemistry analyzer (Hitachi 7070, Hitachi, Tokyo, Japan). Calcium contents were expressed as μ g/mg dry weight.

Statistical Analysis

Statistical analyses were performed using the SPSS software (ver. 21.0; SPSS, Inc., Chicago, IL). Data are expressed as mean \pm standard error. Comparison between groups was performed using the Mann-Whitney *U* test. The change in the titer of antibodies against collagen Type I and α -Gal was compared according to the duration after xenotransplantation by an analysis with repeated measures analysis of variance. *p* values <0.05 were considered to indicate statistical significance.

Results

Immunological Assay of Collagen Type I and α -Gal Antibodies

The antibody titers for collagen Type I measured in the serum collected before, just after, 2 weeks after, 12 weeks after, and 1 year after xenotransplantation were 0.47 ± 0.21 , 0.28 ± 0.04 , 1.12 ± 0.20 , 1.44 ± 0.10 , and 0.89 ± 0.31 , respectively (Figure 1A). Similarly, the α -Gal antibody titers were 0.23 ± 0.02 , 0.23 ± 0.01 , 0.21 ± 0.02 , 0.23 ± 0.01 , and 0.22 ± 0.02 , respectively (Figure 1B). There was no difference in collagen Type I or α -Gal antibody titer according to time after xenotransplantation.

Echocardiography and Cardiac Catheterization

Evaluation of echocardiography and cardiac catheterization data demonstrated good hemodynamic status and function of the pulmonary xenograft valve (Table 1). Echocardiography revealed good valve motion, and no valve stenosis or regurgitation was observed. The pressure ratio of the right ventricle to the aorta was 0.46 ± 0.06 , and the pressure gradient between the right ventricle and the pulmonary artery was 11.3 ± 4.7 mm Hg.

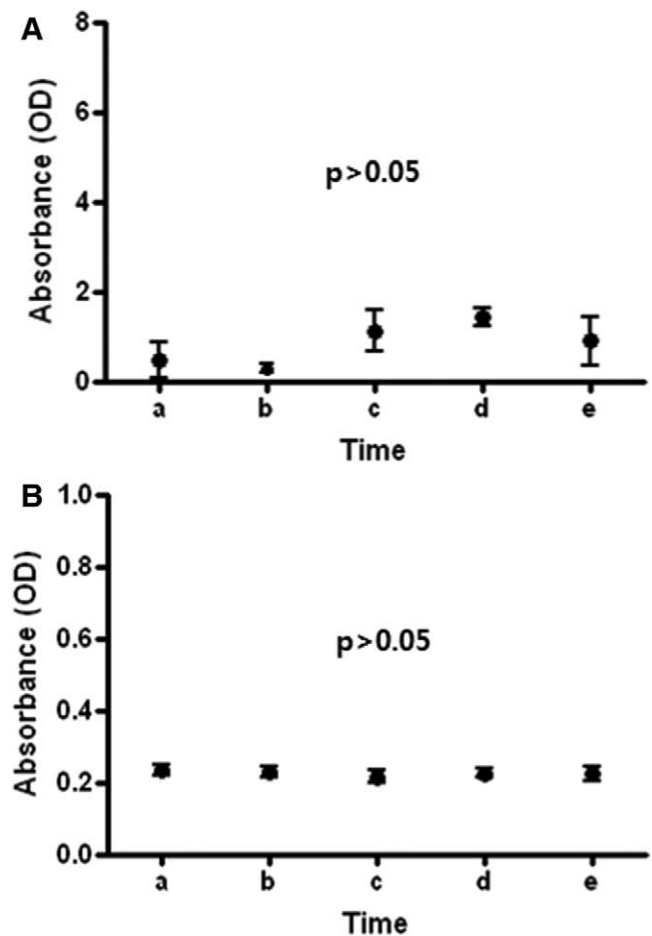


Figure 1. Immunological assay of collagen Type I (A) and α -Gal antibody (B). a: Before xenotransplantation, b: Just after xenotransplantation, c: 2 weeks after xenotransplantation, d: 12 weeks after xenotransplantation, and e: 1 year after xenotransplantation. There was no difference in collagen Type I or α -Gal antibody titer according to time after xenotransplantation. OD, optical density.

Table 1. Hemodynamic Results 1 Year After Xenotransplantation

| No | Echocardiography | | | Cardiac Catheterization (mm Hg) | | | | |
|----|------------------|----|----|------------------------------------|----|----|----------|----------------|
| | Leaflet Motion | PR | PS | aorta | PA | RV | RV/aorta | Δ RV-PA |
| B | Good | No | No | 75 | 16 | 41 | 0.55 | 25 |
| C | Good | No | No | 86 | 21 | 30 | 0.35 | 9 |
| D | Good | No | No | 72 | 20 | 25 | 0.35 | 5 |
| E | Good | No | No | 61 | 29 | 35 | 0.57 | 6 |

PA, pulmonary artery; PR, pulmonary regurgitation; PS, pulmonary stenosis; RV, right ventricle.

Gross Findings

On gross visual inspection, the explanted valve conduit showed neither calcific deposits nor plaque and remained mobile (**Figure 2**).

Specimen Radiography

Specimen radiographic findings taken from the explanted graft after xenotransplantation and from the same graft unfolded longitudinally demonstrated no calcification (**Figure 3**). Indeed, much less calcification of the xenografts was observed by specimen radiography than in the control xenografts fixed by GA alone in our previous study.³

Microscopic Examination Taken from the Explanted Graft After Xenotransplantation

With hematoxylin and eosin staining, collagen fibers appeared well preserved with a normally banded structure and no specific matrix derangement was noticeable. Because of complete decellularization, cellular nuclei were not observed, and inflammatory cells were not found (**Figure 4**). In Masson's trichrome staining, the xenografts had compact arrays of collagen fibers with preserved structural integrity (**Figure 5**). In von Kossa staining, the xenografts showed no evidence of calcification (**Figure 6**).

Calcium Analysis

The calcium concentrations of all the explanted pulmonary conduit walls and leaflets made of bovine or porcine pericardium taken at 313.4 ± 45.9 days after xenotransplantation were 1.69 ± 0.31 $\mu\text{g}/\text{mg}$ ($n = 10$) (**Figure 7**). At 1 year after xenotransplantation, the calcium concentrations of the explanted pulmonary conduit walls made of bovine pericardium were 1.2, 1.2, 1.7, and 2.1 $\mu\text{g}/\text{mg}$ ($n = 4$), and the pulmonary leaflets made of porcine pericardium were 1.4, 1.4, 1.4, and 1.8 $\mu\text{g}/\text{mg}$ ($n = 4$), which were lower than from control xenografts fixed with GA alone (>20 $\mu\text{g}/\text{mg}$ at a xenotransplantation duration of more than 1 year in our previous study).³ For longer survivors (>4 months), calcium concentration in this study xenografts with multicalcification strategies was significantly lower than that in control xenografts without multicalcification strategies ($p < 0.05$).

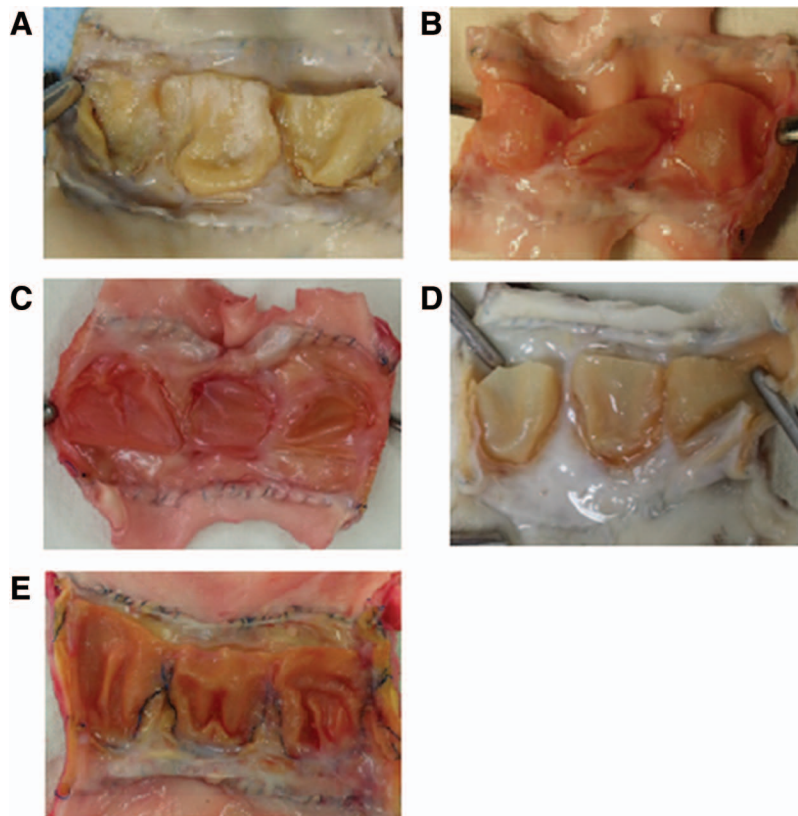


Figure 2. Gross findings from the explanted graft after xenotransplantation. The explanted valve conduit showed neither calcific deposits nor plaque and remained mobile. full color online

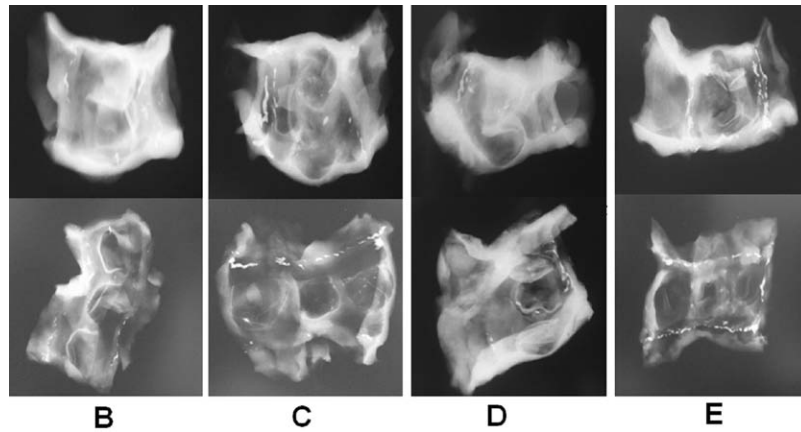


Figure 3. Specimen radiographic findings from the explanted graft after xenotransplantation (upper) and from the same graft unfolded longitudinally (lower). At 1 year after xenotransplantation, the explanted graft showed no calcification.

Inorganic Phosphorus Analysis

The IP concentrations of all the explanted pulmonary conduit walls and leaflets made of bovine or porcine pericardium taken at 313.4 ± 45.9 days after xenotransplantation were 1.20 ± 0.72 $\mu\text{g}/\text{mg}$ ($n = 10$) (**Figure 7**). One year after xenotransplantation, the IP concentrations of the explanted pulmonary conduit walls made of bovine pericardium were 0.1, 0.1, 0.7, and 0.5 $\mu\text{g}/\text{mg}$ ($n = 4$), respectively, and the pulmonary leaflets made of porcine pericardium were 0, 0, 0, and 0 $\mu\text{g}/\text{mg}$ ($n = 4$).

Discussion

A subcutaneous or intramuscular implantation model using small animals has been used widely to screen potential strategies for anticalcification effects. Promising approaches should be investigated further in a large-animal valve xenotransplant model, because potential strategies that appeared efficacious in small-animal experiments have not always proven favorable when used on valves transplanted into the circulation.¹ Thus, the aim of this study was to evaluate the preclinical safety and

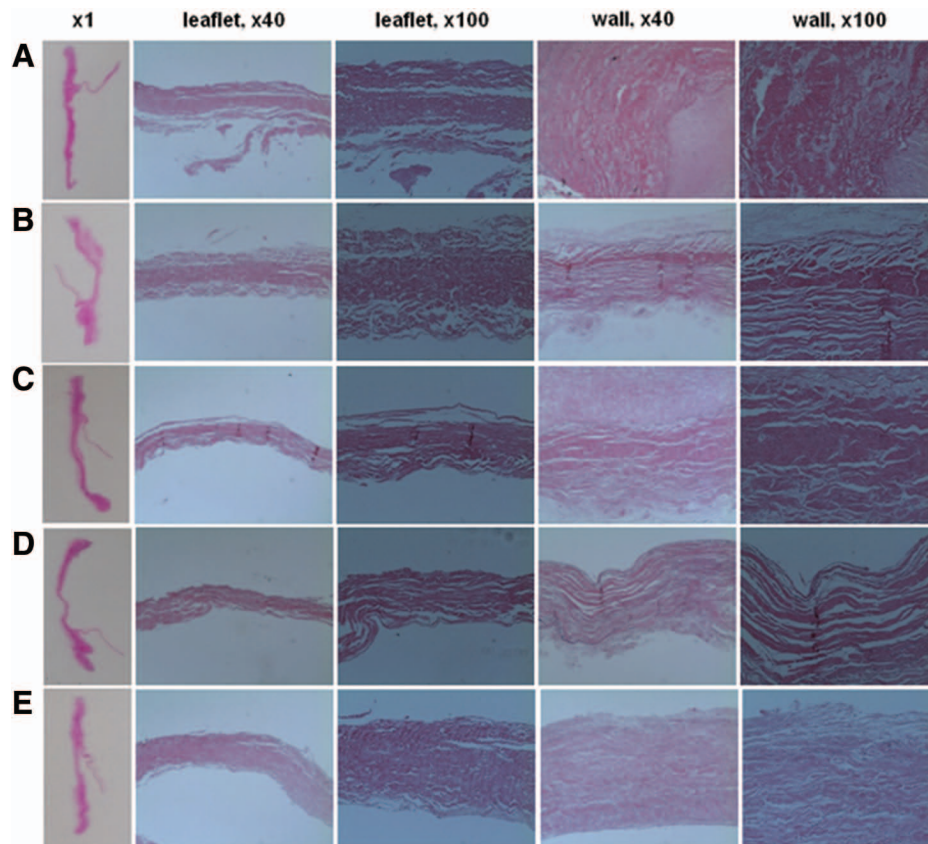


Figure 4. Microscopic findings from the explanted graft after xenotransplantation (hematoxylin and eosin staining, $\times 1$, $\times 40$, $\times 100$). Collagen fibers appeared well preserved with a normally banded structure and no specific matrix derangement was noticeable. Because of complete decellularization, cellular nuclei were not observed, and inflammatory cells were not found. [\[full color online\]](#)

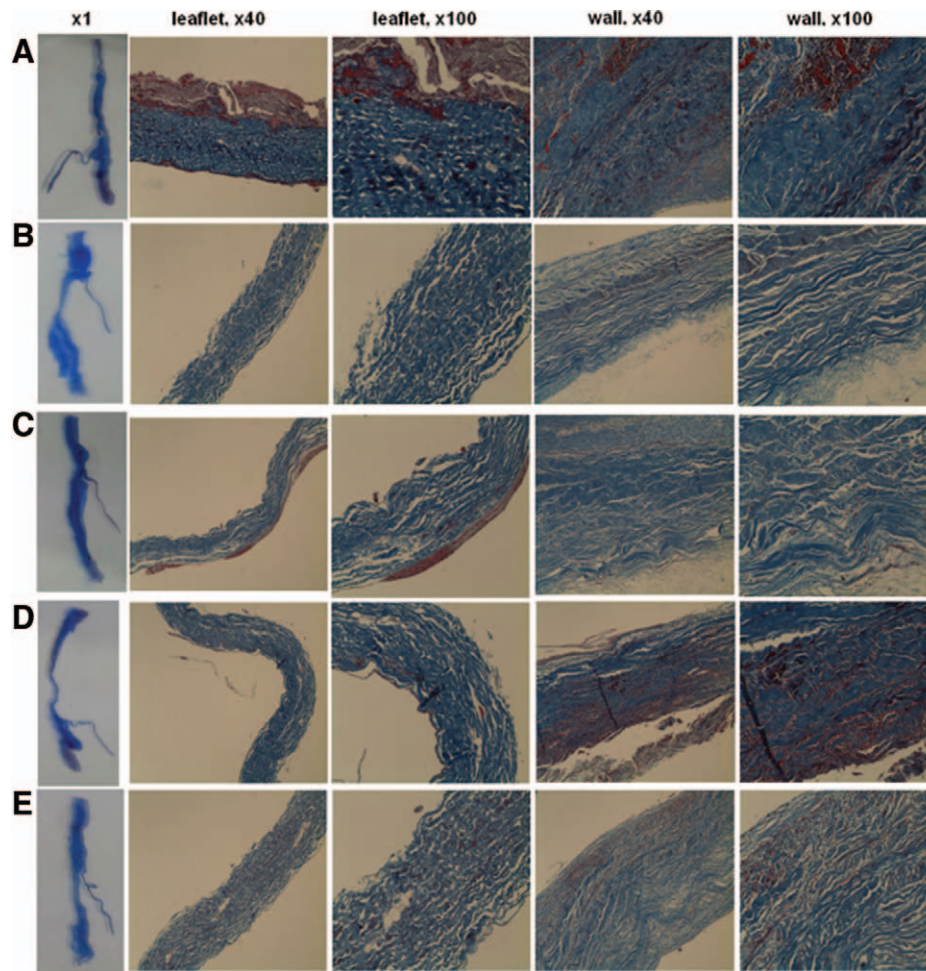


Figure 5. Microscopic findings from the explanted graft after xenotransplantation (Masson's trichrome staining, $\times 1$, $\times 40$, $\times 100$). The leaflet had compact arrays of collagen fibers with preserved structural integrity. full color online

efficacy of our novel combined anticalcification strategies including immunological modification with α -galactosidase for GA-fixed bovine and porcine pericardium in a large-animal study using a goat pulmonary root xenotransplantation model.

Organic Solvent

Cell membrane-associated phospholipids are a key material for the nidus in calcium phosphate crystal formation and are involved in the initial step of *in vivo* calcification after GA fixation. Treatment with organic solvents can remove residual phospholipid contents.^{13,14} We demonstrated that the several combinations of short-chain alcohols at high concentration and long-chain alcohols, which have similarity with phospholipids in structure, effectively prevented *in vivo* calcifications without compromising physical properties.^{2,4,5} In this long-term *in vivo* circulatory model using large animals, we used an 80% concentration of alcohols (a mixture of 75% ethanol and 5% octanol solution) instead of using pure organic solvent for the extraction of tissue phospholipids, conformational changes in collagen, and binding to hydrophobic residues within collagen and elastin, which mitigated efficiently *in vivo* calcification of GA-fixed cardiac xenografts in the pulmonary circulation without alteration of microscopic structure.

Detoxification

Residual unbound aldehyde groups that did not cross-link with collagen after GA fixation may be cytotoxic and combine readily with host plasma calcium. Amino groups in organic molecules, such as amino acids, can remove and neutralize such free aldehyde molecules.¹⁵ We demonstrated that the detoxification treatment with glutamate, urazole, glycine, L-glutamic acid, and sodium bisulfite prevented *in vivo* calcification without worsening the physical properties.^{2,4,5} In this long-term *in vivo* circulatory model using large animals, we used a detoxification process with glycine to neutralize free aldehyde groups of GA, forming Schiff bases,^{2,5} which also mitigated efficiently the *in vivo* calcification of GA-fixed cardiac xenografts in the pulmonary circulation without altering the microscopic structure.

Decellularization

Devitalized cells and cellular remnants can induce immunological responses in the host against residual xenoantigen, which can result in the formation of a nidus for calcification, because GA treatment is not sufficient to completely remove antigenicity.^{2,6,16-18} We have attempted to determine the optimal conditions for decellularization by introducing several

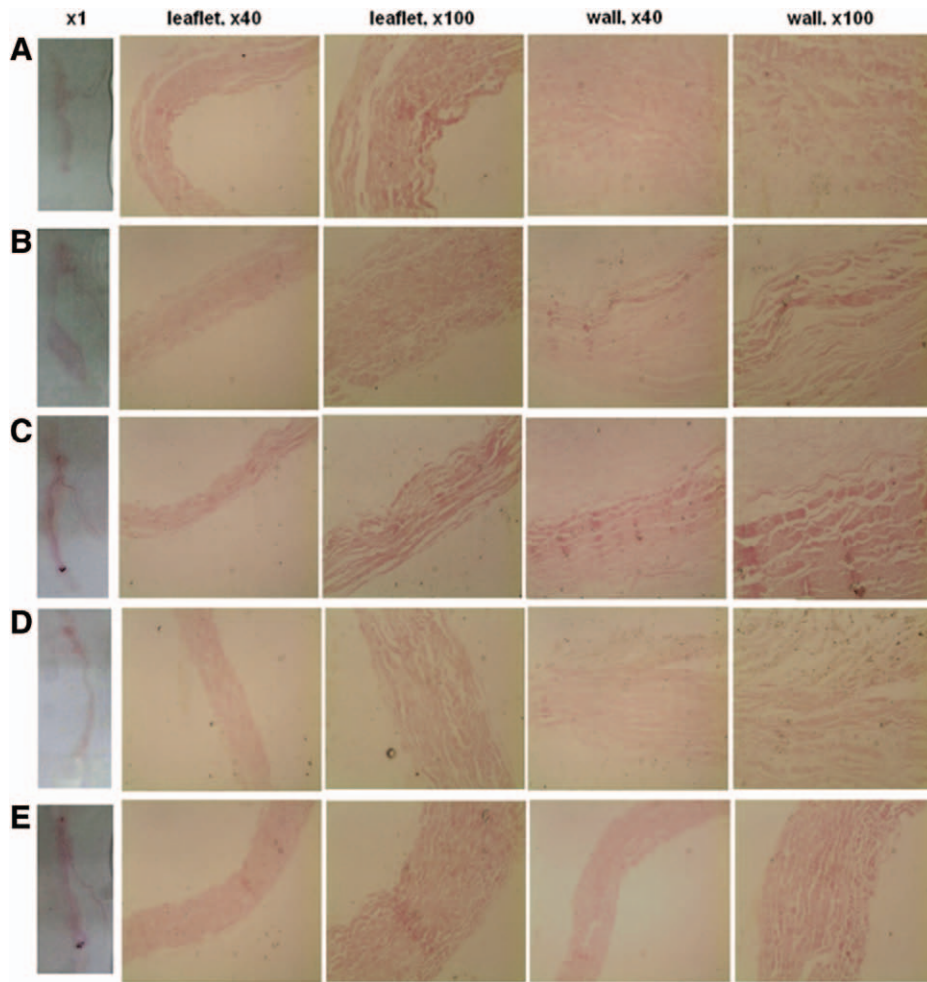


Figure 6. Microscopic findings from the explanted graft after xenotransplantation (von Kossa staining, $\times 1$, $\times 40$, $\times 100$). The leaflet showed no evidence of calcification. full color online

modifications to the appropriate environment, such as incubation temperature, duration, and osmotic pressure using various kinds and concentrations of chemical detergents.¹⁹ Finally, we developed an optimal decellularization protocol involving a multistep method with SDS and Triton X-100 in a hypotonic solution,² which preserved the microscopic structure, the degree of cross-linking, and the degree of tissue strength,² lowered the cytotoxicity,²⁰ and inhibited *in vivo* calcification of GA-fixed xenografts.^{2,20} In this large-animal study, a multistep method

with SDS and Triton X-100 in a hypotonic solution was used for optimal decellularization, preserving the extracellular matrix, which resulted in complete decellularization while keeping collagenous structural integrity and little *in vivo* calcification.

Immunological Modification

Despite GA fixation of cardiac xenografts, we showed that the immune reaction caused by α -Gal epitopes is an

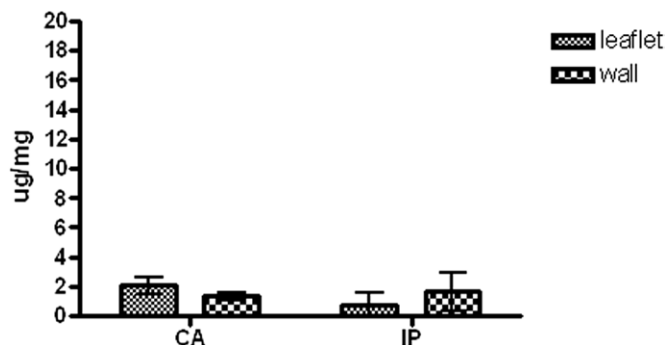


Figure 7. Calcium (CA) and inorganic phosphorus (IP) analyses of porcine pericardial leaflet and bovine pericardial wall with a xenotransplantation period of 313.4 ± 102.6 days.

important factor in cardiac xenograft failure, particularly in young patients who have a more vigorous immune system and metabolism.^{21–23} The nonnegligible amount of the reactive α -Gal xenoantigen discovered in commercially available cardiac xenografts confirms an incomplete biocompatibility status with the potential to stimulate the recipient immune system, with the transplantation of a cardiac xenograft.^{24,25} The design of a cardiac xenograft that is resistant to the recipient immune response would be a major step forward in the transplantation of cardiac xenografts.²³ Complete inactivation of the α -Gal epitope is essential to meet the requirements for safe clinical application, and further researches should be conducted toward the production of α -Gal-free cardiac xenografts.²⁵ Although there is a huge potential market for cardiac xenografts, certain economic factors have to be yet considered and the associated problems need to be resolved for mass production of α -Gal-free long-lasting cardiac xenografts using transgenic animals.¹ So, we introduced the immunological modification with enzymatic methods, with a recombinant *B. thetaiotaomicron* α -galactosidase, and demonstrated that the α -galactosidase effectively removed the α -Gal epitopes still present on decellularized xenopericardium in lectin histochemistry staining for the α -Gal epitope,⁹ and removed almost all α -Gal from cardiac xenografts without affecting the mechanical properties.^{6,7} We also demonstrated that treatment of α -galactosidase with decellularization are useful for removal of the immunogenicity and reduced *in vivo* calcification in GA-fixed xenopericardium using the α 1,3-galactosyltransferase knockout mouse model, which mimics the human immunological environment.⁹ In this large-animal study, immunological modification with α -galactosidase treatment was used for the complete inactivation of the α -Gal epitope, which also mitigated efficiently *in vivo* calcification of GA-fixed cardiac xenografts in the pulmonary circulation without alteration of microscopic structure.

To evaluate the immunological response, a species-specific ELISA against collagen I, the most common protein of the extracellular matrix, and the response to α -Gal, a major xenoantigen present on the cardiac xenografts, were assessed. We were able to show that there was little detectable immune response toward the collagen I or α -Gal in goats that had received a cardiac xenograft treated with our combined anticalcification protocols including immunological modification up to 12 months after xenotransplantation. From the present results, it can be suggested that the process of calcification is not being triggered by the absence of an immune response initiated by collagen I and α -Gal.¹⁷ The xenoreactive antibody response between the different species even in this α -Gal concordant animal model may be suggested to exist with the species- and tissue-specific expression of these conjugated compounds including α -Gal epitopes, and the differences in fine specificity of natural anti-Gal antibodies in various species which recognize various 'facets' of the α -Gal epitope in its 3D form.^{2,26}

In this study, all of the goats survived after xenotransplantation because GA toxicity and immunogenicity were sufficiently attenuated with our anticalcification treatment. However, inadequate nutritional support, infection, and gastrointestinal problem might prevent long-term survival in one goat.

As a next-generation product, these α -Gal-free long-lasting cardiac xenografts treated with α -galactosidase would be expected to enhance the durability of cardiac xenografts. This should be assessed in a human clinical study. To the best of our

knowledge, this is the first report of a large-animal experiment using open heart surgery as a preclinical study to demonstrate the *in vivo* efficacy and safety of novel combined anticalcification protocols including α -galactosidase.

Conclusion

Our preclinical approaches in large-animal long-term circulatory models demonstrated that our synergistic and simultaneous use of multiple anticalcification therapies or novel tissue treatments such as organic solvent, decellularization, α -galactosidase, and detoxification has promising safety and efficacy for future clinical studies.

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