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Development of a next-generation tissue valve using a glutaraldehyde-fixed porcine aortic valve treated with decellularization, α -galactosidase, space filler, organic solvent and detoxification

Hong-Gook Lim^{a,b}, Gi Beom Kim^{a,c}, Saeromi Jeong^a and Yong Jin Kim^{a,b,*}

^a Seoul National University Hospital Clinical Research Institute, Xenotransplantation Research Center, Seoul, Republic of Korea

^b Department of Thoracic and Cardiovascular Surgery, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea

^c Department of Pediatrics, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea

* Corresponding author. Department of Thoracic and Cardiovascular Surgery, Seoul National University Hospital, Seoul National University Hospital Clinical Research Institute, Xenotransplantation Research Center, 101 Daehak-ro, Jongno-gu, Seoul 110-744, Republic of Korea. Tel: +2-2072-3638, fax: +2-745-5209; e-mail: kyj@plaza.snu.ac.kr (Y.J. Kim).

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Abstract

OBJECTIVES: Conventional crosslinking with glutaraldehyde (GA) renders cardiac xenografts inert, non-biodegradable and non-antigenic, but is a main cause for dystrophic calcification due to phospholipids, free aldehyde groups and residual antigenicity. A significant immune reaction to the galactose- α -1,3 galactose β -1,4-*N*-acetylglucosamine (α -Gal) of a GA-fixed cardiac xenograft occurs, leading to calcification. We developed a next-generation α -Gal-free tissue valve with GA-fixed cardiac xenografts, treated using a novel combined anticalcification protocol including immunological modification, which was demonstrated effective in a small animal study.

METHODS: Porcine aortic valves were decellularized with 1% sodium dodecyl sulphate, 1% Triton X-100 and 1% sodium lauroyl sarcosinate and immunologically modified with α -galactosidase. The valves were treated by a polyethylene glycol space filler, fixed with GA in 75% ethanol + 5% octanol and detoxified with glycine. We manufactured the tissue valve with the porcine aortic valve mounted on a Nitinol (nickel–titanium memory alloy) plate. The tissue valve was placed under *in vitro* mock circulation, and durability from mechanical stress was evaluated for 100 days. Ten sheep underwent mitral valve replacement with the tissue valve, and haemodynamic, radiological, immunohistopathological and biochemical results were obtained for 18 months after implantation.

RESULTS: The *in vitro* circulation experiment demonstrated that the valve functioned well with good morphology. Eight sheep survived for 1, 2, 5, 10, 14, 53, 546 and 552 days after mitral valve replacement, but two sheep did not survive. An evaluation by echocardiography and cardiac catheterization demonstrated good haemodynamic status and function of the mitral valve at 18 months after implantation. The xenografts were well preserved without a α -Gal immune reaction or calcification based on the immunological, radiographic, microscopic and biochemical examinations.

CONCLUSIONS: We developed a next-generation α -Gal-free tissue valve with simultaneous use of multiple anticalcification therapies and novel tissue treatments such as decellularization, immunological modification with α -galactosidase, space filler, an organic solvent and detoxification. Future investigations should evaluate α -Gal-free substitutes such as our tissue valve, and a future clinical study is warranted based on these promising preclinical results.

Keywords: Xenograft • Heart valve • Bioprosthesis • Bioengineering • Biomaterials • Calcification

INTRODUCTION

Crosslinking with glutaraldehyde (GA) renders a cardiac xenograft inert, non-biodegradable and non-antigenic, and has been commonly applied to bioprosthetic heart valves. However, GA crosslinking does not guarantee complete biocompatibility of cardiac xenografts, and paradoxically causes dystrophic calcification due to phospholipids, free aldehyde groups and residual antigenicity [1–3]. A significant immune reaction to the cardiac xenograft

occurs despite GA crosslinking, leading to calcification, rapid structural deterioration and failure [4]. Commercially available bioprosthetic heart valves are made from porcine and bovine cardiac xenografts, and retain the galactose- α -1,3 galactose β -1,4-*N*-acetylglucosamine (α -Gal) antigen despite the current anticalcification process. However, humans have high levels of anti- α -Gal antibody which is responsible for calcifying bioprosthetic heart valves [5]. Inactivating the α -Gal epitope in the next-generation bioprosthetic heart valves is mandatory to meet the requirements

for safe and durable clinical applications [3]. We applied an immunological modification with α -galactosidase in addition to complete decellularization of GA-fixed cardiac xenografts to eliminate immune stimulation to this major xenoreactive antigen and to reduce the potential for immune-mediated calcification and degeneration [6–8].

Our five treatment steps targeted to prevent calcification of GA-fixed cardiac xenografts are as follows: (i) complete decellularization with 1% sodium dodecyl sulphate, 1% Triton X-100 and 1% sodium lauroyl sarcosinate to suppress residual antigenicity [9]; (ii) α -galactosidase treatment to effectively inactivate α -Gal epitopes expressed on decellularized xenografts [6, 8]; (iii) use of a space filler to fill the interstitial void spaces in GA-pretreated tissue with a macromolecular substance [10]; (iv) use of an organic solvent to reduce the calcification potential of aldehyde-fixed tissues by extracting phospholipids and conformational changes in collagen [9, 11, 12]; and (v) detoxification to neutralize residual GA aldehyde groups [9, 11, 12]. We also developed a Nitinol (nickel–titanium memory alloy) plate to manufacture the tissue valve [13], and created a next-generation tissue valve with the GA-fixed porcine aortic valve treated with our novel anticalcification protocol, which has been demonstrated effective in a small animal experiment [6, 9].

Tissue valves in the mitral position are preferred for elderly patients, particularly in whom successful repair is unlikely and anticoagulation is contraindicated, but the durability of current bioprosthetic mitral valves is much less than that of aortic valves [14]. Therefore, a more durable bioprosthetic valve is needed in the mitral position and must be evaluated in a reliable animal model [15]. Several species such as sheep, dogs and pigs have been used to evaluate valves, and the sheep model is the most common [16, 17]. We evaluated the safety and efficacy of our next-generation tissue valve during *in vitro* mock circulation and in the mitral position of sheep [15].

MATERIALS AND METHODS

Tissue preparation

Fresh porcine aortic valves were obtained from a local slaughterhouse, placed in phosphate-buffered saline (PBS, 0.1M, pH 7.4) and transported immediately to our laboratory. They were rinsed with normal saline and freed from adherent fat.

Decellularization

The porcine aortic valves were washed in 0.9% normal saline, 0.1% peracetic acid with 4% ethanol in distilled water for 1 h, and then washed for 2 h in distilled water. These tissues were treated initially with hypotonic buffered solution containing 1% sodium dodecyl sulphate for 24 h at 4°C, and washed with distilled water for 1 h. The tissues were treated with a hypotonic solution containing 1% Triton X-100 and 1% sodium lauroyl sarcosinate for 24 h at 4°C, and washed with distilled water for 12 h at 4°C. Then, the tissues were treated with isotonic solution for 24 h at 4°C. Finally, the tissues were treated with hypertonic buffered solution (II) for 6 h at 4°C, and washed in PBS for 1 h at 4°C.

Hypotonic buffered solution (1000 ml distilled water; 10 mM Tris; pH 8.0); isotonic buffered solution (1000 ml distilled water; 50 mM Tris; 0.15 M NaCl; 0.05% EDTA; 10 KIU/ml aprotinin and 50 mg neomycin trisulphate, pH 8.0); hypertonic buffered solution II (1000 ml distilled water; 200 mM Tris; 0.6 M NaCl, pH 8.0); and distilled water (1000 ml distilled water; 0.05% EDTA; 10 KIU/ml aprotinin).

Preparation of α -1,3 galactosidase from *Bacteroides 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 α -galactosidase gene from *B. thetaiotaomicron* (designated BtGal110B) was amplified from the corresponding genomic DNAs by polymerase chain reaction and introduced into the pET28a vector to express the His₆-tagged proteins using appropriate protein expression restriction sites in *Escherichia coli* Rosetta2 (DE3) (Novagen, Madison, WI, USA). *E. coli* was grown in Luria-Bertani media supplemented with 34 μ g/ml chloramphenicol and 30 μ g/ml kanamycin and induced at an A_{600 nm} ~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, USA). The crude lysates were centrifuged at 13 K rpm for 20 min at 4°C, and the expressed protein was purified using a nickel-NTA agarose column (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction.

Immunological modification by inactivating xenoantigens (α -Gal)

The porcine aortic valves were treated with 0.1 units/ml α -galactosidase for 24 h at 4°C.

Space-filler treatment

The porcine aortic valves were treated with 30% polyethylene glycol (1000 MW) in 0.01 M PBS (pH 7.4) for 1 day at room temperature.

Glutaraldehyde fixation in organic solvent

The porcine aortic valves were initially fixed in 0.5% GA for 3 days at room temperature, additionally fixed with 0.25% GA in 75% ethanol + 5% octanol for 2 days at room temperature, and finally fixed in 0.25% GA for 7 days at room temperature.

Detoxification

After fixation, the porcine aortic valves were treated with 0.2 M glycine solution (PBS, pH 7.4) at 4°C for 24 h.

Manufacture of stented tissue valve

A Nitinol (nickel–titanium memory alloy) plate of about 0.20 mm thickness was used as a stent, and manufactured by laser cutting and electropolishing according to our data based on the previously measured ratio of each structure in the porcine aortic valve [13]. The ratio of the right-, non- and left coronary cusp was 1 : 0.92 : 1. The hole was made large. The Nitinol plate was covered with a polyester cloth. The entire porcine aortic valve was GA crosslinked, treated using our anticalcification protocols and mounted on this Nitinol stent (Fig. 1).

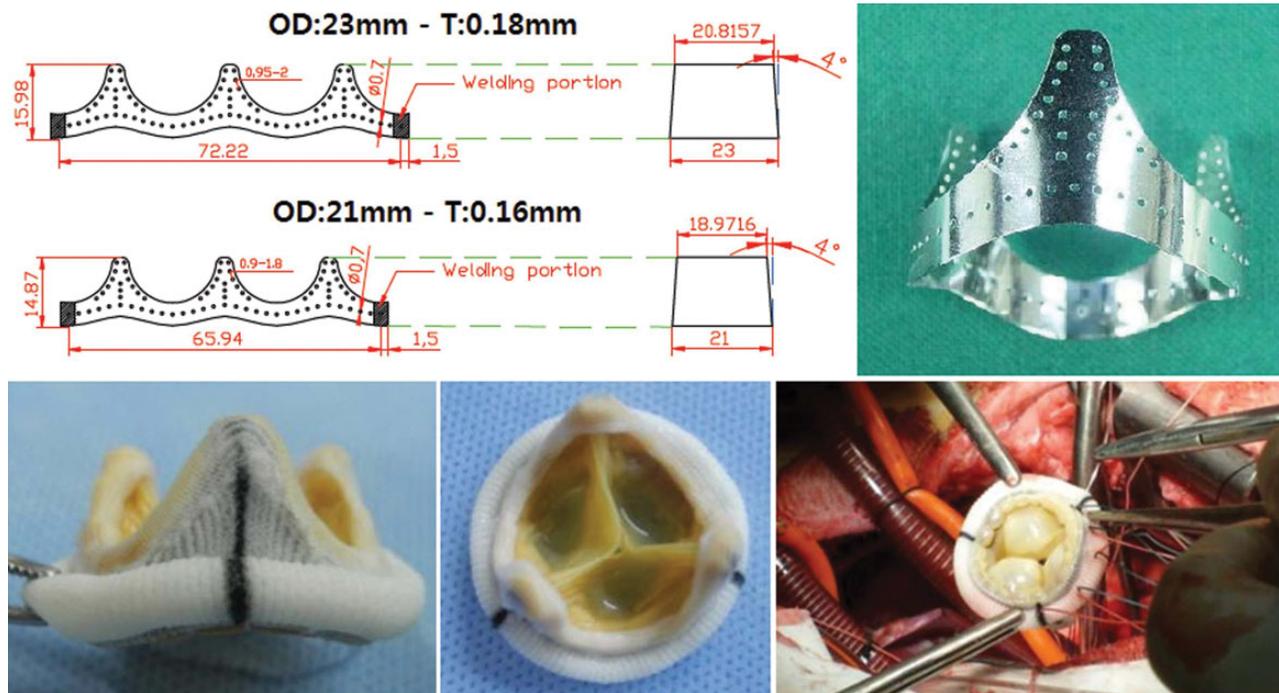


Figure 1: Drawing and photograph of Nitinol (nickel-titanium) plate (upper), tissue valve and operative view of mitral valve replacement (lower). OD, outer diameter; T, thickness [13].

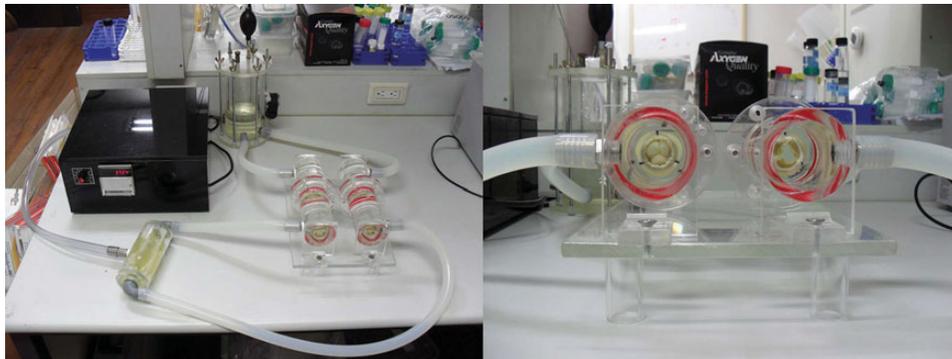


Figure 2: Photograph of *in vitro* mock circulation.

***In vitro* mock circulation**

We developed a specially designed mock circulation model to evaluate mechanical stress and durability of the tissue valve *in vitro*. The tissue valves of outside diameter (OD) 27 mm were fixed to the circuit and placed under *in vitro* circulation. The circulation solutions contained normal saline (0.9% NaCl) and 0.1% GA. A pulsatile pressure of 120/80 mmHg was repetitively provided to the valve at a constant interval of 60 rpm in one direction for 100 days (8 325 306 cycles) to reproduce *in vivo* circulation (Fig. 2).

Open heart surgery to implant the tissue valve in the mitral position of sheep

This study was approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute, Seoul National University Hospital (IACUC No. 11-0355). This facility is accredited

by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The body weights of the 10 sheep were 45.5, 44, 33, 42, 55, 50, 46.5, 46.5, 40.5 and 45 kg. The sheep were injected intramuscularly with 5 mg/kg Zoletil and 0.25 mg/kg Rompun, as preanaesthetics for surgical procedures and for short-term restraint. The electrocardiogram and peripheral capillary oxygen saturation (SpO₂) were monitored continuously using the tongue to place the SpO₂ sensor. The sheep were endotracheally intubated and anaesthetized with 1.3–2.0% enflurane in O₂ and instrumented for haemodynamic monitoring. Respiration was controlled throughout the study. Prophylactic cefazolin was administered intravenously at 20 mg/kg. The sheep were placed in the left lateral decubitus position under general anaesthesia. The right common carotid artery and internal jugular vein were catheterized to measure blood pressure and central venous pressure, to withdraw blood to determine the partial pressure of carbon dioxide and oxygen and arterial pH, and to administer drugs and fluid. A right thoracotomy was performed through

the fourth intercostal space under sterile conditions. The pericardium was opened and tented, and the heart was exposed. Heparin (300 IU/kg) was injected intravenously. Aortic and venous cannulation was performed through the ascending aorta and right atrium. Partial cardiopulmonary bypass was performed using a membrane oxygenator and cooling was started. The inferior vena cava was cannulated. A left atrial vent catheter was inserted through the right upper pulmonary vein, and an aortic root cannula was inserted through the aortic root. The aorta was cross-clamped, and an antegrade, cold cardioplegic was infused via the aortic root. A left atriotomy was performed, and the mitral valve was excised under moderate hypothermia. The mitral valve was replaced with a porcine aortic tissue valve of OD 21 mm in three, 23 mm in six and 25 mm in one sheep, respectively, using Teflon-pledgeted 2-0 Ethibond (ETHICON, Somerville, NJ, USA) and horizontal mattress sutures (Fig. 1). The left atriotomy site was closed using 4-0 Prolene (ETHICON) in a continuous manner. After de-airing, the aortic cross-clamp was released, and warming was started. The sheep were weaned from cardiopulmonary bypass, and modified ultrafiltration was performed. Protamine (3 mg/kg) was injected intravenously. After a chest drain was placed in the thoracic cavity, the thorotomy incision was closed in layers. After the operation, prophylactic cefazolin (20 mg/kg, once/day for 3 days) was administered intravenously. Routine anticoagulation was not administered. Postoperative care consisted of continuous routine monitoring with electrocardiogram, SpO₂, blood pressure and central venous pressure and administration of drugs and fluid. After extubation, the chest tube was removed. The sheep were transferred to the cage after vital signs were stabilized. The clinical courses such as dietary intake, systemic conditions, thromboembolic events and heart failure were closely observed.

Immunoassay

Blood samples were taken from the sheep before, just after, 2 weeks after and 18 months after mitral valve replacement. An enzyme-linked immunosorbent assay (ELISA) was used to measure the specific serum antibody concentrations (IgM and IgG) against α -Gal in the sheep. Microtiter plates were coated with 100 μ l per well of bovine serum albumin conjugating synthesized α -Gal (Dextra Laboratories, Reading, UK) (α -Gal-BSA) in PBS (pH 7.4) (at 1 μ g/ml for the IgM and IgG isotypes), and incubated at 37°C for 1 h. Then, the plates were washed with PBS containing 0.05% (v/v) Tween 20. Sheep sera (100 μ l per well) were added to the α -Gal-BSA immobilized wells in BSA-Tween 20 (PBS, pH 7.4, 3% BSA, 0.01% Tween 20), and the plates were incubated for 1 h at 37°C. Horseradish peroxidase-conjugated rabbit anti-sheep IgM and IgG (AbD SeroTec, Oxford, UK) were used as the secondary antibody (1 : 10 000) at the IgM and IgG dilution in BSA-Tween 20. A colour reaction was developed with 3,3',5,5'-tetramethylbenzidine solution (BD Biosciences, San Diego, CA, USA). Absorbance was measured at 450 nm in an ELISA reader.

Echocardiography and cardiac catheterization

Transthoracic echocardiography and cardiac catheterization were performed for up to 18 months post-transplantation to evaluate haemodynamic changes. The morphology and competency of the leaflets were investigated.

Radiological confirmation by quantifying calcification

After sacrificing the sheep, the tissue valves were tested for radiological confirmation with simple X-rays.

Microscopic examination

Representative tissue samples were examined under light microscopy. The tissue samples were fixed in 10% formalin, embedded in paraffin and 2- to 4- μ m-thick sections were stained with haematoxylin-eosin, Masson's trichrome and the von Kossa method.

Calcium analysis

Harvested tissue samples were washed in normal saline, dried at 70°C for 24 h and weighed. The samples were then hydrolysed with 5.0 N HCl solution. The calcium content of the hydrolysate was measured colorimetrically using the *o*-cresolphthalein complexone method and an automatic chemistry analyser (Hitachi 7070, Tokyo, Japan). Calcium content was expressed as μ g/mg dry weight.

Statistical analysis

Statistical analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean \pm standard deviation. The change in the antibody titer against α -Gal was compared according to the duration after xenotransplantation using repeated-measures analysis of variance. A *P*-value <0.05 was considered significant.

RESULTS

Microscopic findings of glutaraldehyde-fixed porcine aortic valve treated with our anticalcification protocols

The microscopic findings (haematoxylin-eosin staining, $\times 40$, $\times 100$, $\times 400$) of GA-fixed porcine aortic valve treated with our anticalcification protocols, including decellularization and α -galactosidase treatment demonstrated that the collagen fibres appeared well preserved with a normally banded structure, and no specific matrix derangement was noticeable in the distal leaflet, mid-leaflet, junction of the leaflet and wall, or the wall. Because of complete decellularization, cellular nuclei were not observed (Fig. 3).

Gross findings after mock circulation

Gross findings observed from GA-fixed porcine aortic valves treated with our anticalcification protocols after mock circulation for 100 days showed that the tissue valves maintained good mechanical stability without dehiscence around the suture line, and the leaflets remained mobile without tearing (Fig. 4).

Immunological assay for the α -Gal antibody

The α -Gal IgM antibody titers measured in serum collected before, just after, 2 weeks after and 18 months after mitral valve

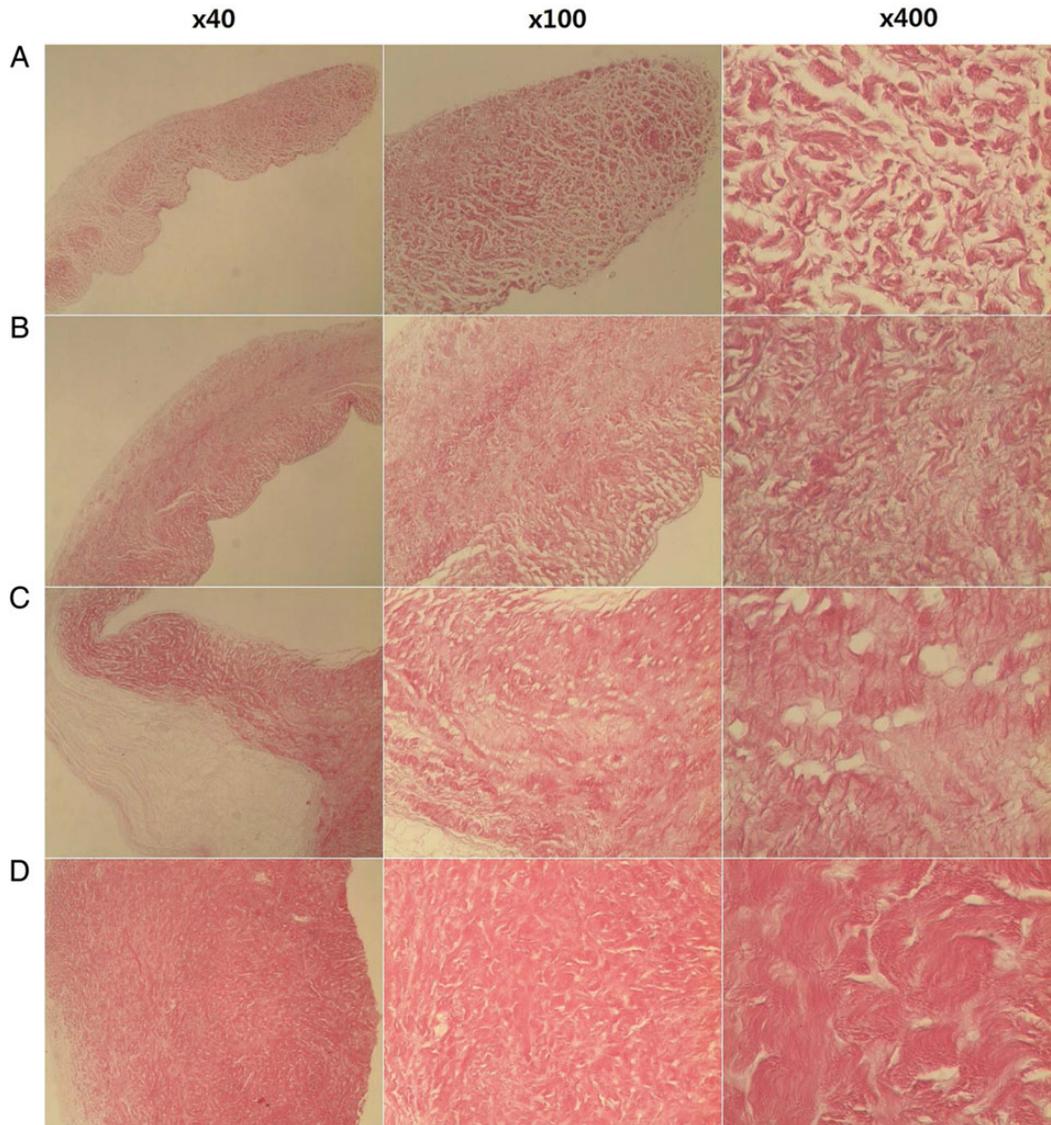


Figure 3: Microscopic findings (haematoxylin and eosin staining, $\times 40$, $\times 100$, $\times 400$) of the GA-fixed porcine aortic valve treated with our anticalcification protocols. GA, glutaraldehyde; **A**, distal leaflet; **B**, mid-leaflet; **C**, junction of leaflet and wall; **D**, wall.

replacement were 0.2001 ± 0.0107 , 0.1971 ± 0.0120 , 0.1989 ± 0.0088 and 0.2133 ± 0.0266 , respectively. No difference in the α -Gal IgM antibody titer was observed according to the duration after mitral valve replacement. The α -Gal IgG antibody titres measured during the same schedule were 0.2412 ± 0.0154 , 0.2293 ± 0.0157 , 0.2343 ± 0.0163 and 0.2348 ± 0.0129 , respectively. No difference in the α -Gal IgG antibody titre was observed based on the time after mitral valve replacement (Fig. 5).

Echocardiography and cardiac catheterization

The sheep survived for 1, 2, 5, 10, 14, 53, 546 and 552 days after mitral valve replacement, but two sheep did not survive, which was not related with acute-type rejection or device malfunction. The sheep that survived for 18 months were sacrificed. The evaluation of echocardiography and cardiac catheterization

demonstrated good haemodynamic status and function of the mitral valve 18 months after implantation. After implantation for 546 days the echocardiography evaluation demonstrated good ventricular function, good leaflet motion, no mitral regurgitation, minimal mitral stenosis (mean pressure gradient, 3 mmHg) and anteroposterior internal diameter of 19.6 mm. After implantation for 552 days the echocardiographic evaluation demonstrated good ventricular function, good leaflet motion, trivial mitral regurgitation, mitral stenosis (mean pressure gradient, 6 mmHg) and anteroposterior internal diameter of 19.3 mm.

Gross findings

All the explanted mitral valve leaflets did not show calcium deposits or plaque and remained mobile on gross inspection (Fig. 6).

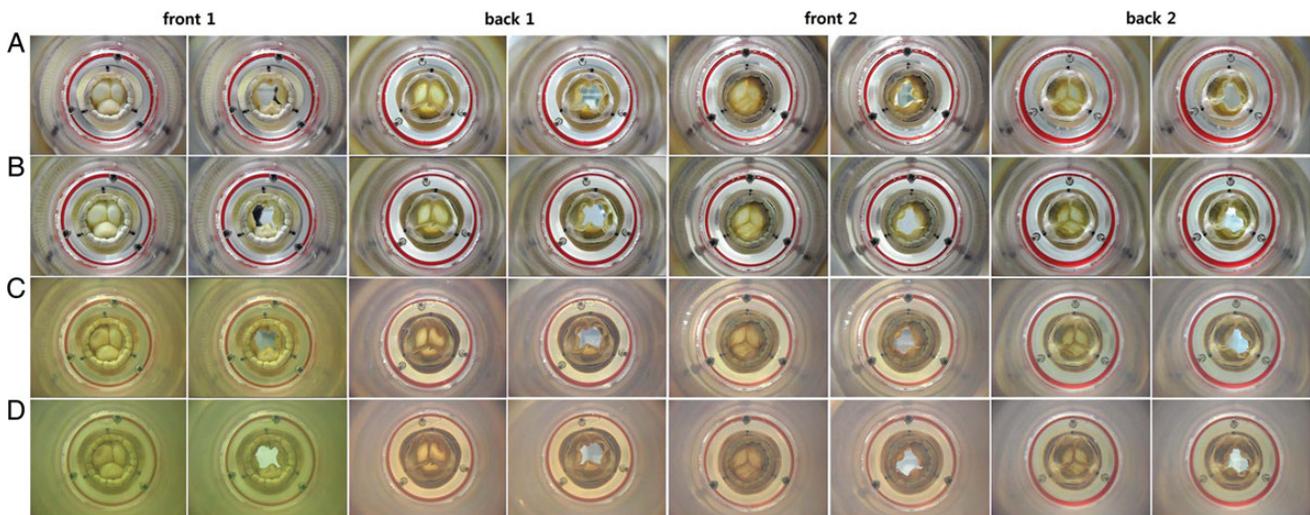


Figure 4: Gross findings of GA-fixed porcine aortic valve treated with our anticalcification protocols 0 (A), 2 (B), 5 (C) and 8 weeks (D) after *in vitro* mock circulation. GA, glutaraldehyde.

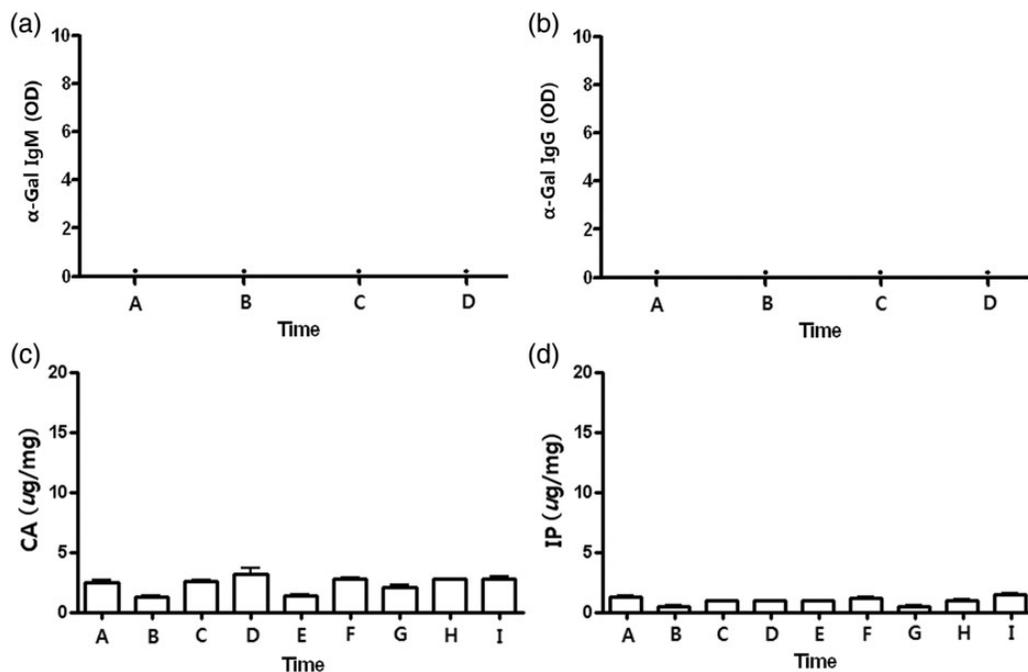


Figure 5: Immunological assay for α -Gal IgM (a) and IgG (b) antibody before (A), just after (B), 2 weeks after (C) and 18 months after (D) mitral valve replacement. OD, optical density. Calcium (CA, c) and inorganic phosphorus (IP, d) analysis of all the explanted mitral valve leaflets 0 (A), 1 (B), 2 (C), 5 (D), 10 (E), 14 (F), 53 (G), 546 (H) and 552 (I) days after mitral valve replacement.

Specimen radiography

Specimen radiography was taken from tissue valves explanted 0, 1, 2, 5, 10, 14, 53, 546 and 552 days after implantation, and demonstrated no calcification (Fig. 7).

Microscopic examination of an explanted xenograft after transplantation

The collagen fibres appeared well preserved with a normally banded structure, and no specific matrix derangement was

noticeable on the haematoxylin–eosin stained specimens. No cellular nuclei were observed because of complete decellularization. The mitral valve leaflets on Masson's trichrome-stained specimens had a compact array of collagen fibres with preserved structural integrity. The mitral valve leaflets on von Kossa-stained specimens showed no evidence of calcification (Fig. 8).

Calcium analysis

The calcium concentrations of the explanted mitral valve leaflets made of GA-fixed porcine aortic valves treated with our anticalcification protocol were 2.51 ± 0.23 , 1.27 ± 0.14 , 2.60 ± 0.15 ,

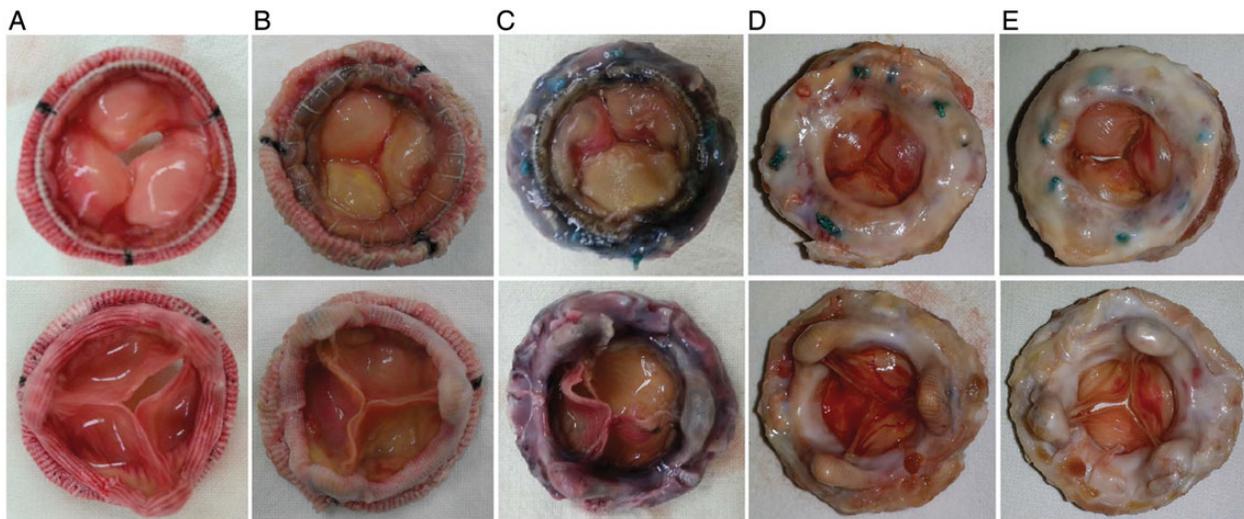


Figure 6: Gross findings taken from an explanted mitral valve 1 (A), 14 (B), 53 (C), 546 (D) and 552 (E) days after mitral valve replacement.

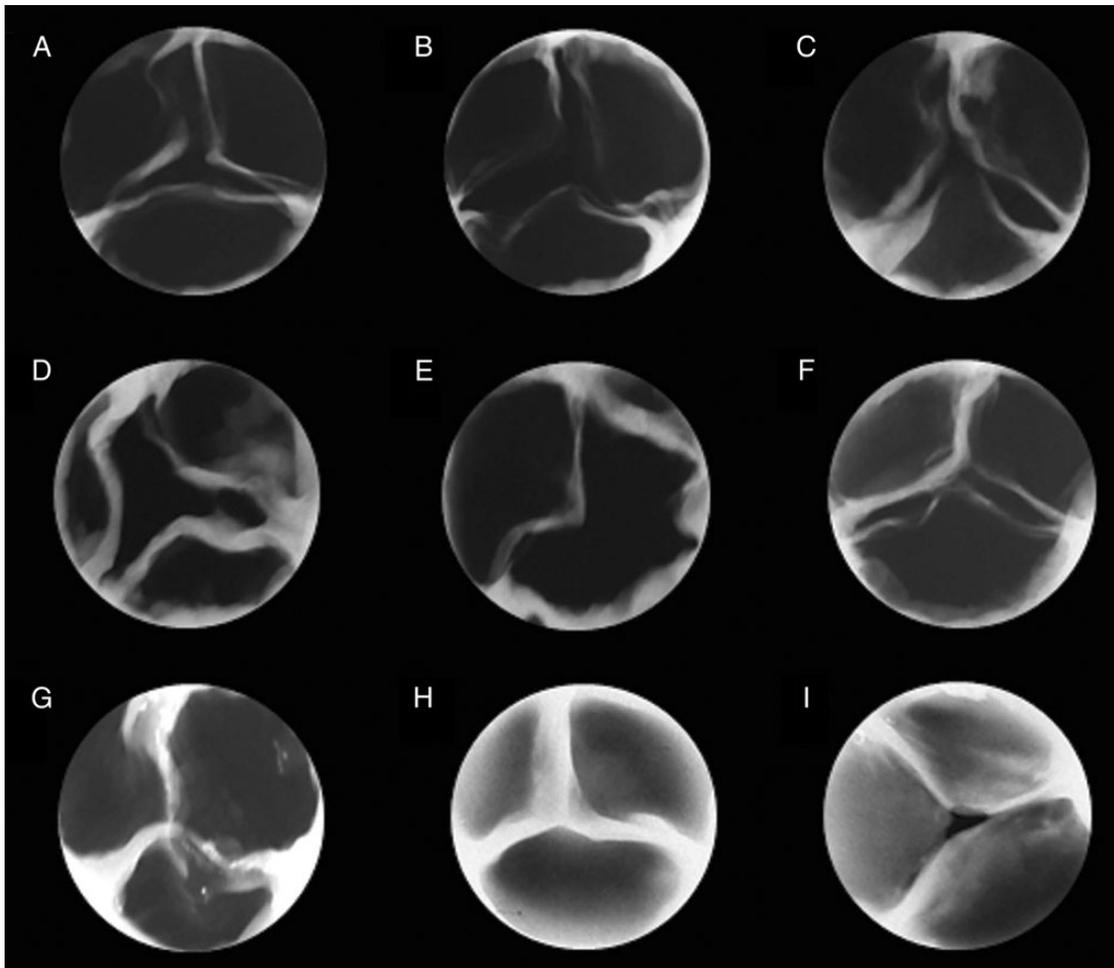


Figure 7: Specimen radiographic findings taken from an explanted mitral valve 0 (A), 1 (B), 2 (C), 5 (D), 10 (E), 14 (F), 53 (G), 546 (H) and 552 (I) days after mitral valve replacement.

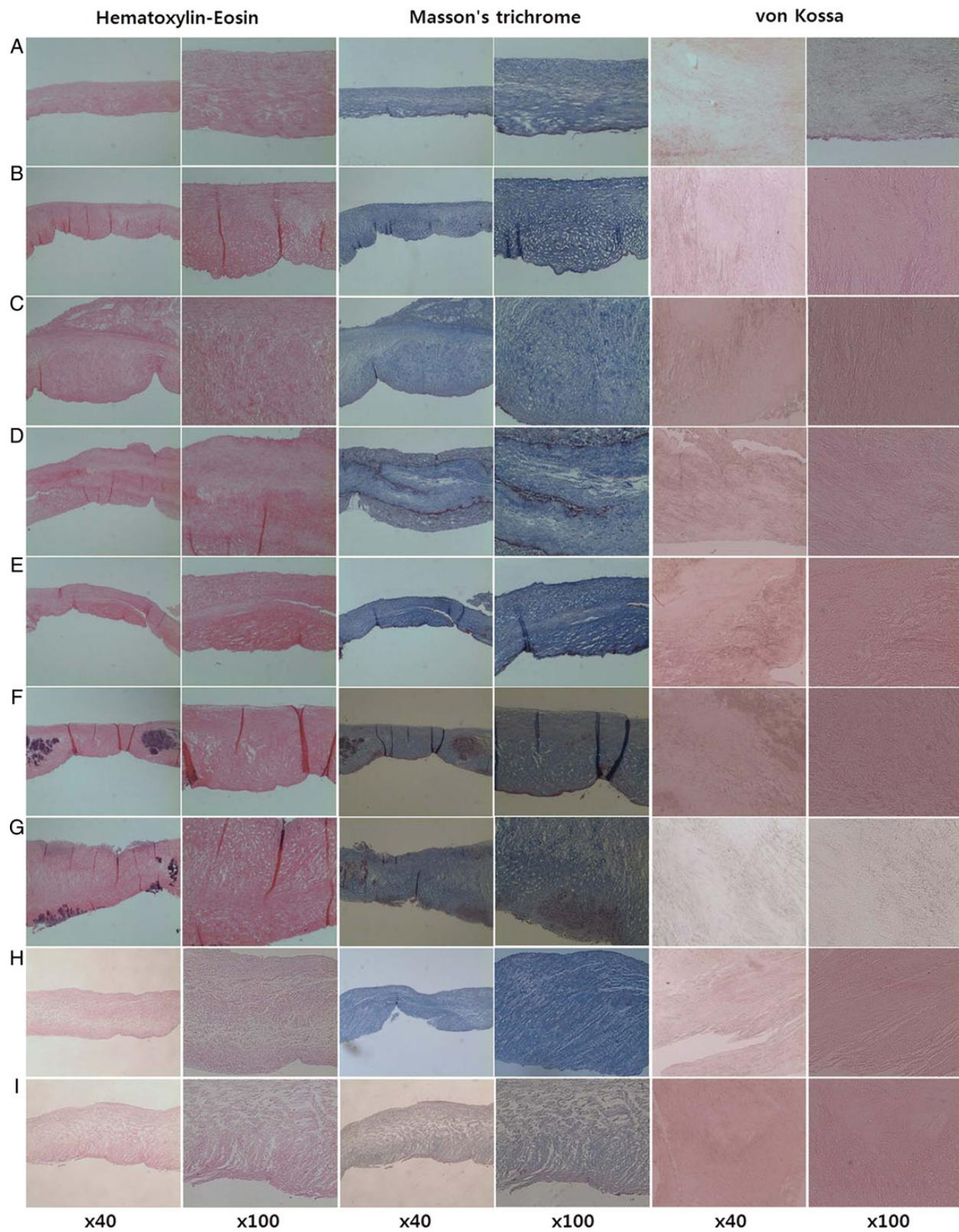


Figure 8: Microscopic findings (haematoxylin-eosin, Masson's trichrome and von Kossa staining, $\times 40$, $\times 100$) taken from an explanted mitral valve leaflet 0 (A), 1 (B), 2 (C), 5 (D), 10 (E), 14 (F), 53 (G), 546 (H) and 552 (I) days after mitral valve replacement.

3.15 ± 1.04 , 1.39 ± 0.22 , 2.80 ± 0.12 , 2.08 ± 0.47 , 2.73 ± 0.06 and 2.80 ± 0.30 $\mu\text{g}/\text{mg}$ at 0, 1, 2, 5, 10, 14, 53, 546 and 552 days after mitral valve replacement, respectively (Fig. 5).

Inorganic phosphorus analysis

The inorganic phosphorus (IP) concentrations of the explanted mitral valve leaflets made of GA-fixed porcine aortic valves treated with our anticalcification protocol were 1.28 ± 0.13 , 0.48 ± 0.26 , 0.98 ± 0.03 , 0.99 ± 0.04 , 0.97 ± 0.02 , 1.17 ± 0.14 , 0.47 ± 0.16 , 1.01 ± 0.10 and 1.48 ± 0.28 $\mu\text{g}/\text{mg}$ at 0, 1, 2, 5, 10, 14, 53, 546 and 552 days after mitral valve replacement, respectively (Fig. 5).

DISCUSSION

The durability of bioprosthetic heart valves is based on their ability to prevent mechanical damage and calcification, and efforts to improve durability have been increased. Two types of tissue valves made of porcine valves and bovine pericardium have been widely used, but the porcine aortic valve remains a three-leaflet device unlike the pericardium type [18]. Although small differences in transprosthetic gradient and indexed aortic valve orifice area exist between porcine and bovine aortic valves, both prostheses allow similar regression of the left ventricular mass during the first year after aortic valve replacement [19]. We measured the geometry and ratio of porcine aortic and pulmonary valves to develop the porcine tissue valves. The ratio of the right, non- and left coronary cusp was 1:0.88:1, and valve height also correlated to leaflet size [13]. Because the valves must be designed to coapt under loading when the stent is deflected and the leaflet material stretches [18], we designed the stented porcine aortic valve (including stent height and width, stent thickness and working thickness) with outer diameters of 19–33 mm according to our data based on the previous measured ratio of each structure [13]. Supporting stents can also influence the mechanical durability of bioprosthetic heart valves. A rigid supporting stent has a risk of early failure at the commissures. In contrast, a flexible stent-post cushions shock loading, which would remedy commissural tearing; therefore, flexible stents are favoured compared with rigid stents. However, a side effect of completely flexible stents is that they can deform during insertion or during cardiac contraction if placed in the mitral position. Stent-post flexure can also cause problems leading to increased commissural stress and early valve failure [18]. We used a Nitinol (nickel–titanium memory alloy) stent to improve bioprosthetic valve performance, which has excellent cardiac pulsatile fatigue and durability properties [20], and developed the tissue valve with the entire porcine aortic valve mounted on this Nitinol stent.

In our previous study, the porcine aortic valve conduit was preserved using fresh cryopreservation and decellularized cryopreservation, and implanted in a goat aorta. After the xenograft was exposed to the high pressure of systemic circulation, severe calcification, fibrosis and tissue degeneration developed in both groups. Marked neutrophil infiltration was observed in the fresh cryopreservation group, whereas lymphocytes were observed in the decellularized cryopreservation group. Decellularization reduced antigenicity but did not decrease calcification, fibrosis or tissue degeneration. Thus, processing methods should be properly modified for optimal xenograft preservation of the aortic valve conduit [21]. GA has been conventionally used to crosslink porcine tissue to reduce antigenicity and to stabilize it against

proteolytic degradation that would otherwise occur once implanted in the recipient [18]. Unfortunately, GA crosslinking is also a main cause for dystrophic calcification. To solve this problem, we developed novel combined anticalcification treatments for GA-fixed cardiac xenografts and proved their efficacy in small animal experiments [6, 9–12]. However, these anticalcification treatments are not always safe and efficacious if used on valves placed under *in vitro* mock circulation or under large-animal *in vivo* systemic circulation [1]. The aim of this study was to evaluate the preclinical safety and efficacy of our novel combined anticalcification strategies for the GA-fixed porcine aortic valve, which was mounted on Nitinol stents.

Structural tissue valve deterioration occurs due to chemical processes between GA and free calcium ions in the blood and is associated with xenograft rejection [4]. Although GA fixation reduces some immunogenicity and degeneration of a bioprosthetic heart valve, immunogenicity persists, which may trigger calcification [4]. GA treatment is insufficient to eliminate the immune response to the α -Gal epitope, as an immune response-initiated process occurs that is not seen with decellularized valves [22]. However, neither GA fixation nor decellularization procedures ensure a definitive solution because of the persistence of reactive xenoantigen residues. No tissue treatment is able to completely mask or inactivate such epitopes [23]. Current heart valves contain the major xenogeneic antigen α -Gal, and a quantitative evaluation of the α -Gal epitope in commercially available bioprosthetic heart valves, in clinical practice for about 40 years, has been conducted [3]. The clinical use of current α -Gal-positive xenogeneic bioprosthetic materials can induce a recipient anti- α -Gal antibody response, which may contribute to valve calcification and degeneration [7]. We have investigated the xenoreactive immune response directed at α -Gal, and bioprosthetic heart valve implantation in adults elicits increased formation of anti- α -Gal antibodies, with different patterns for each isotype [5]. In other studies, implantation of bioprostheses elicits a specific humoral immune response against α -Gal bearing cells, and the role of the α -Gal antigen and human α -Gal antibodies has been demonstrated during the calcification process of valvular bioprostheses [24].

Valve companies have made significant progress decreasing structural valve deterioration due to calcification by using various valve chemical treatments, but the design of valvular bioprostheses is not resistant to the host's α -Gal immune response [4]. We demonstrated previously that α -galactosidase almost completely removes the α -Gal on xenografts without affecting the mechanical properties [9] and also effectively removes the α -Gal expressed on decellularized xenografts [6]. α -Galactosidase and decellularization are useful for removing immunogenicity and reduced calcification in GA-fixed xenografts in a humanized mouse experiment [6]. In this study, we inactivated the α -Gal epitope in GA-fixed cardiac xenografts by immunological modification using α -galactosidase [6, 8] and performed the first *in vitro* and *in vivo* large-animal study under systemic circulation for this α -Gal-free substitute.

Our mock circulation model was an *in vitro* evaluation for the mechanical stress of pressure and durability. We demonstrated the safety of our combined anticalcification process in this mock circulation, as valve function was well maintained with good gross morphology under systemic pressure for 100 days. This *in vitro* mock circulation trial confirmed the durability of our anticalcification protocol including decellularization and α -galactosidase treatment for the cardiac xenograft, and the feasibility of an *in vivo* systemic circulation study. Our *in vivo* study investigated the immunological response to α -Gal, a major xenoantigen present on

cardiac xenografts. A xenoreactive antibody response may exist between different species even in this α -Gal concordant animal model such as pig to sheep [9]. However, we found little detectable immune response towards α -Gal in sheep that received a cardiac xenograft treated with our combined anticalcification protocols including immunological modification for up to 18 months after mitral valve replacement. Our combined anticalcification strategies efficiently mitigated *in vivo* calcification and degeneration of GA-fixed cardiac xenografts without altering microscopic structure, and calcium content was 2–3 $\mu\text{g}/\text{mg}$ for up to 1.5 years after mitral valve replacement. In contrast, in our previous xenograft failure model study, GA-fixed xenograft without anticalcification treatment showed severe *in vivo* calcification and degeneration, and calcium content increased to $7.93 \pm 5.34 \mu\text{g}/\text{mg}$ 6 months after implantation and was more than 20 $\mu\text{g}/\text{mg}$ 1 year after implantation [25]. Our present results suggest that calcification is not triggered by the absence of an immune response initiated by α -Gal [22]. In this study, *in vitro* and *in vivo* experiments were performed with implantable tissue valves manufactured according to our data based on previous measured ratios of each porcine aortic valve [13]. We also demonstrated that the Nitinol stent device performed well in pre-clinical animal studies as well as *in vitro* mock circulation before being implanted into humans.

The survival of this study may have been better if we had performed adequate blood transfusion for haemodilution and bleeding, added an effective chest tube drain and administered intensive care with cardiac medication after open heart surgery. Inadequate nutritional support, infection and gastrointestinal problems also prevented survival.

In conclusion, we developed a next-generation α -Gal-free tissue valve with simultaneous use of multiple anticalcification therapies and novel tissue treatments such as decellularization, immunological modification with α -galactosidase, a space filler, an organic solvent and detoxification. Future investigations should be directed towards α -Gal-free substitutes such as our tissue valve [3], and a future clinical study is warranted based on our preclinical results using *in vitro* mock circulation and large-animal *in vivo* systemic circulatory models.

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