Interactive CardioVascular and Thoracic Surgery 25 (2017) 391–399
doi:10.1093/icvts/ivx131 Advance Access publication 12 May 2017

ORIGINAL ARTICLE

Cite this article as: Park CS, Kim YJ, Lee JR, Lim H-G, Chang J-E, Jeong S *et al*. Anticalcification effect of a combination of decellularization, organic solvents and amino acid detoxification on glutaraldehyde-fixed xenopericardial heart valves in a large-animal long-term circulatory model. Interact CardioVasc Thorac Surg 2017;25:391–9.

Anticalcification effect of a combination of decellularization, organic solvents and amino acid detoxification on glutaraldehyde-fixed xenopericardial heart valves in a large-animal long-term circulatory model

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Received 2 November 2016; received in revised form 13 March 2017; accepted 21 March 2017

Abstract

OBJECTIVES: We aimed to investigate the effect of a combination of anticalcification treatments, which were effective for preventing calcification in a small animal experiment, on glutaraldehyde-fixed xenopericardial valves using a large-animal long-term circulatory model.

METHODS: Valved conduits were made of porcine pericardium as a leaflet and bovine pericardium as a conduit and were implanted into the right ventricular outflow tract of goats under cardiopulmonary bypass. The goats were divided into study (glutaraldehyde + combined anticalcification treatment, $n = 6$) and control (glutaraldehyde alone, $n = 9$) groups. Upon euthanization at 1 year, echocardiography and cardiac catheterization were performed. Explanted tissues were microscopically examined and analysed for measuring the calcium content.

RESULTS: Haemodynamic data were obtained from 3 and 2 goats in the study and control groups, respectively. All valves, except 1, which was limited in motion, were functioning well on echocardiography; pressure gradients across the right ventricular outflow tract were lower in the study group on cardiac catheterization. On gross inspection, all leaflets remained mobile without calcific deposits in the study group, while most leaflets were heavily calcified in the control group. The calcium content in the leaflets remained low (≤4 μg/mg) in the study group. Among the leaflets explanted from goats that survived longer (>3 months), the calcium concentration was higher in the control group than in the study group [15.1 μg/mg ($n = 5$) vs 2.7 μg/mg ($n = 5$), respectively; $P = 0.008$).

CONCLUSIONS: Porcine pericardial leaflets treated with our anticalcification protocol showed better function and less calcification than those treated with glutaraldehyde alone in the pulmonary position.

Keywords: Xenograft • Bioprosthesis • Anticalcification

INTRODUCTION

Patients who undergo right ventricular outflow tract (RVOT) surgical repair for congenital heart disease are at a risk of pulmonary valve replacement due to valve incompetence and subsequent ventricular dilatation and dysfunction. Recently, the prosthesis of choice in the pulmonary position has been a bioprosthetic valve that is made of xenogeneic tissues; however, it has limited durability due to leaflet degeneration caused by calcification [1, 2]. Given the theoretical advantages in durability, mechanical prostheses might be an alternative, but they have inherent drawbacks such as haemodynamic disadvantages; requirement of long-term anticoagulation, which increases the risk of thromboembolic or haemorrhagic complications; and worse quality of life in childbearing women or active people. Ideal prosthetic valves should function as long as possible without lifelong anticoagulation; bioprosthetic valves might be ideal options if their durability can be dramatically improved.

Currently available bioprosthetic valves are made of porcine aortic valves or bovine pericardia pretreated with glutaraldehyde (GA) and various anticalcification treatments. Although GA treatment has been used to reduce immunogenicity, decrease the

possibility of biodegradation and enhance stability and durability with irreversible molecular collagen cross-linking in xenogeneic tissue [3–5], it is a major cause of prosthesis degeneration, which may be attributed to cell debris with residual antigenicity, phospholipids and free aldehyde residues after collagen cross-linking. To mitigate prosthesis degeneration caused by GA, many efforts have been taken, including decellularization to eliminate residual antigenicity [6, 7], organic solvent preincubation to remove phospholipids [8, 9] and post-treatment with amino acids to neutralize free aldehyde residues [10–13].

In vivo anticalcification effects of decellularization, the use of organic solvents and detoxification with amino acids have been demonstrated in a rabbit intramuscular implantation model [14]. The present study aimed to evaluate the effect of a combination of these anticalcification treatments on GA-fixed xenopericardial valves using a large-animal long-term circulatory model.

MATERIALS AND METHODS

Study design

Valved conduits made of porcine pericardium as a leaflet and bovine pericardium as a conduit were implanted on the RVOT of goats under cardiopulmonary bypass. According to tissue preparation methods for the implanted valved conduits, the goats were divided into study (GA + combined anticalcification treatment, $n = 6$) and control (GA only, $n = 9$) groups.

Tissue preparation

Bovine and porcine pericardia were harvested from a local slaughterhouse and transferred to our laboratory in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) with an icebox. Upon arrival, the tissues were washed, and unnecessary tissue residues were removed to proceed with tissue preparation.

Decellularization. Porcine and bovine pericardial tissues were washed with 0.9% normal saline, prepared in distilled water with 4% ethanol and 0.1% peracetic acid for 1 h to reduce the bioburden and washed in distilled water for 30 min. These tissues were treated in a hypotonic buffer solution (distilled water, 1000 ml; Tris, 10 mmol/l; pH 8.0) for 14 h at 4°C, a hypotonic-buffered solution with 0.1% sodium dodecyl sulphate for 24 h at room temperature, a hypertonic buffer solution (distilled water, 1000 ml; Tris, 200 mmol/l; sodium chloride, 0.6 mol/l; pH 8.0) for 8 h at 4°C and an isotonic buffer solution (distilled water, 1000 ml; Tris, 50 mmol/l; sodium chloride, 0.15 mol/l; ethylenediaminetetraacetic acid, 0.05%; aprotinin, 10 KIU/ml; neomycin trisulphate, 50 mg; pH 8.0) for 12 h at 4°C in series.

Glutaraldehyde fixation in organic solvents. The decellularized tissues were fixed in 0.5% GA (PBS, pH 7.4) for 3 days at room temperature, in a solution containing 0.25% GA, 75% ethanol and 5% octanol for 2 days at room temperature and then in 0.25% GA for 1 week at room temperature.

Detoxification with glycine. The tissues were detoxified in 0.1 M glycine (0.01 M PBS, pH 7.4) for 48 h at 37°C. Treated tissues were stored after they were washed in normal saline with 2% benzyl alcohol.

Glutaraldehyde fixation in the control group. Porcine and bovine pericardial tissues were washed with 0.9% normal saline, prepared in distilled water with 4% ethanol and 0.1% peracetic acid for 1 h to reduce the bioburden and washed in distilled water for 30 min. Then, these tissues were fixed in 0.5% GA (PBS, pH 7.4) for 14 days at room temperature.

Valve design and valved conduit manufacturing

A specially designed mould was produced to make the pericardia have bulging sinuses. As the valve should be competent in the 15-mm diameter conduit, which would fit the RVOT of an adult goat, the mould was designed to have appropriate protrusions that can form sinuses in a bovine pericardium and valves in a porcine pericardium [15]. The bulging degree was 0.4 times longer than the mould radius, and the bulging sinus height was 1.45 times longer than the mould radius. Bovine and porcine pericardia were tightly wound on the mould and prepared using the assigned protocol. After tissue preparation, 3 sinus-shaped leaflets were excised from the treated porcine pericardium and attached to the corresponding edge of the sinuses in the treated bovine pericardium using non-absorbable sutures (6-0 Prolene; Ethicon, Somerville, NJ, USA). The bovine pericardium was rolled up and shaped into a conduit by suturing each end. The entire manufacturing process is shown in Fig. 1.

Valved conduit implantation using cardiopulmonary bypass

This study was approved by the Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No.10-0057). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

The valved conduits made of xenopericardia treated with the aforementioned methods in both groups were implanted into the RVOT of goats (*Capra aegagrus hircus*) using cardiopulmonary bypass under general anaesthesia [16, 17]. The median body weight of the goats was 34.7 (28–45) kg without an intergroup difference ($P = 0.145$). Following anaesthetic induction with intramuscular Zoletil (5 mg/kg) and Rompun (0.25 mg/kg) injections, endotracheal intubation was accomplished; enflurane (1.3–2.0%) was then inhaled through an endotracheal tube for maintaining anaesthesia. During the entire procedure, direct arterial pressure, electrocardiography and pulse oximetry were continuously monitored. Before opening the chest, prophylactic antibiotics (cefazolin 1.0 g) were intravenously infused.

With the animal in the right lateral decubitus position, the heart and descending aorta were clearly exposed through the fourth intercostal space by a left thoracotomy incision. Once an adequate anticoagulation level was achieved, normothermic cardiopulmonary bypass was initiated after cannulating the descending aorta and right atrium. Pulmonary valve leaflets were removed through the transected main pulmonary artery; the xenopericardial valved conduit was interposed between the proximal and distal segments of the transected main pulmonary artery with non-absorbable monofilament sutures (5-0 or 6-0

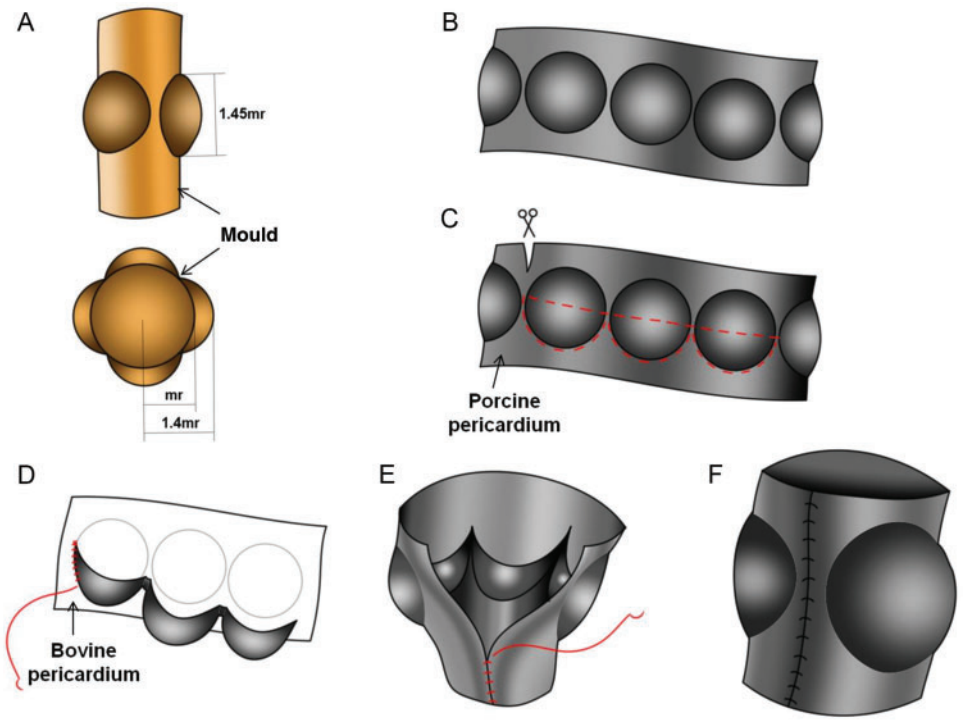

Figure 1: Process of valve manufacturing. (**A**) Specially designed mould for the leaflet and conduit. The length of bulging is 0.4 times longer than the mould radius (mr). The height of the bulging sinus is 1.45 times longer than the mr. (**B**) After they are prepared on the mould, the porcine or bovine pericardium should obtain structures, like a bulging sinus, that will be used as leaflets in the porcine pericardium (**C**) and sinuses in the bovine pericardium (**D**). (**D**–**F**) After completing leaflet attachment (**D**), the bovine pericardium is rolled up and sutured at both ends to form a valved conduit (**E** and **F**).

Prolene; Ethicon). After cardiopulmonary bypass, protamine (3 mg/kg) was infused for heparin reversal. Cardiopulmonary bypass was conducted with a membrane oxygenator; steroids were not used during bypass. Modified ultrafiltration was not performed following bypass.

The chest was closed using 1-0 Vicryl (Ethicon Ltd, Edinburgh, UK) with a chest tube temporarily placed in the thoracic cavity. If an arterial gas was acceptable on spontaneous breathing, the animal could be extubated and then transferred to a cage. Two additional antibiotics dosages were postoperatively administered.

Postimplant examination

As a rule, the goats were to be euthanized 1 year after implantation. Before scheduled euthanasia, transthoracic echocardiography and cardiac catheterization were performed. In case of scheduled euthanization, the procedure was commenced following an intravenous injection of 1 mg/kg of heparin and 2 M potassium chloride. Goats that did not survive for 1 month or had never fully recovered from the operation until death were designated as having an early death.

Transthoracic echocardiography and right heart catheterization. Under general anaesthesia, a cardiologist evaluated the pulmonary valve function and degree of RVOT obstruction by transthoracic echocardiography before euthanasia. Cardiac catheterization was performed through the left internal jugular vein for visualizing the RVOT and measuring the right ventricle and pulmonary artery pressures; the arterial pressure was simultaneously recorded.

Gross inspection. The explanted valved conduits were longitudinally incised and unrolled to examine for any deformity, disruption or calcification and mobility of leaflets.

Microscopic examination. Tissue segments, including the leaflet, wall and anastomotic junction, were obtained and prepared with 10% formalin fixation and paraffin wax embedding. They were sliced at 2–4 μm thickness; stained with haematoxylin and eosin, Masson's trichrome and the von Kossa method; and examined under a light microscope.

Quantification of calcification. Representative tissue samples taken from each explant were washed in saline, dried at 80°C for 24 h and weighed *in toto*. Dried samples were hydrolyzed with 5 eq/l (N) hydrogen chloride at 80°C for 24 h. Using a concentrator (Savant AES 1010 SpeedVac system; Thermo Electron Corporation, Madison, WI, USA), hydrogen chloride was vaporized and pellets were obtained. The samples were dissolved in distilled water and analysed in an automatic chemical analyser (Hitachi 7070; Hitachi, Tokyo, Japan) to quantify the calcium content, which was colorimetrically measured by the *o*-cresolphthalein complexone method, as previously described [18]. The calcium content was expressed as micrograms per milligram dry weight.

Statistics

Data are expressed as a median with range. The Mann–Whitney *U*-test was used to evaluate the intergroup difference of continuous variables because variables followed a non-normal distribution. A *P*-value of <0.05 was considered statistically significant.

Statistical analyses were performed using SPSS 21.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

General outcomes

In the study group, 3 goats could be euthanized as scheduled at 1 year after implantation. There was 1 early death; a goat that survived for 41 days had suffered from poor oral intake and weakness until death, and autopsy showed a large amount of foreign materials likely to disturb the passage and digestion of food. The other 2 goats survived the operation but suddenly died at 115 and 175 days after implantation (Table 1). At autopsy, valve leaflets were mobile without any evidence of thrombus, dehiscence or defects on the leaflets, which suggested the lack of correlation of deaths with valved conduits. In the control group, 2 goats could be electively sacrificed at 224 days and 362 days after implantation (Table 1); the goat sacrificed at 224 days after implantation was euthanized earlier than scheduled because of ongoing weight loss (-3 kg). There were 4 early deaths (2, 3, 3 and 17 days) following sustained dyspnoea, loss of appetite and weakness after implantation. The other 3 goats suddenly died at 147, 264 and 340 days after implantation without any signs or symptoms suggestive of a decline in condition (Table 1). Autopsy could not identify any specific finding related to their sudden deaths.

Table 1: Characteristics of the goats

Group	Serial number	Weight (kg)	Survival duration (days)	Fate
Study	1	38	41	Death (early)
Study	2	39.5	115	Death
Study	3	30	175	Death
Study	4	29	355	Elective sacrifice
Study	5	30.8	356	Elective sacrifice
Study	6	28	363	Elective sacrifice
Control	1	33	362	Elective sacrifice
Control	2	36	264	Death
Control	3	35	340	Death
Control	4	41	3	Death (early)
Control	5	45	147	Death
Control	6	32	2	Death (early)
Control	7	37.5	17	Death (early)
Control	8	35.5	3	Death (early)
Control	9	32	224	Elective sacrifice

Postimplant examination

Transthoracic echocardiography and right heart catheterization. Transthoracic echocardiography and right heart catheterization could be performed in 5 goats (3 and 2 in the study and control groups, respectively) that were electively euthanized. On transthoracic echocardiography, all pulmonary valves of goats in the study group remained mobile and had no more than mild regurgitation; however, 1 valve in a goat in the control group was restricted in motion despite its competence (Table 2). At cardiac catheterization, the pressure ratio of the right ventricle to the systemic artery was lower in the goats in the study group than in those in the control group (0.36, 0.48 and 0.59 vs 0.79 and 0.75, respectively); the pressure gradient across the pulmonary valve also was lower in the goats in the study group than in those in the control group (0, 14 and 9 vs 27 and 18 mmHg, respectively; Table 2).

Gross inspection. In the study group, calcific deposits in or fixation of the leaflets were not found in any leaflets or walls (Fig. 2). In the control group, thickening or calcification was not observed in leaflets from goats that died within 1 month after implantation, but severe leaflet thickening, calcification with large calcific deposits and adhesion to the adjacent wall were seen in leaflets obtained from the other goats (Fig. 2).

Microscopic examination. On haematoxylin and eosin staining, cellular nuclei were not stained owing to decellularization in the study group (Fig. 3). Collagen fibres seemed to be well preserved with a normally banded structure; no specific matrix derangement was evident in both groups (Figs 3 and 4). On Masson's trichrome staining, collagen fibres had preserved structural integrity (Figs 3 and 4) in both groups. On von Kossa staining, calcific deposits were observed in the leaflets from the goats surviving for 355 and 356 days in the study group (Fig. 3) and those surviving for 147 and 362 days in the control group (Fig. 4).

Calcium quantification. In the porcine pericardial leaflets, the calcium content remained low (≤4 μg/mg), regardless of the survival duration in the study group (Fig. 5); however, it was significantly higher in leaflets explanted from goats that survived longer (>3 months) than the others in the control group [15.1 μg/mg (4.8–86.4 μg/mg) vs 3.1 μg/mg (2.0–4.0 μg/mg), respectively; $P = 0.016$; Fig. 6]. Among all the leaflets explanted from goats that survived longer (>3 months), the calcium content was lower in

Table 2: Results of transthoracic echocardiography and right heart catheterization in goats electively euthanized

Group	Serial number	Echocardiography		Right heart catheterization				
		Leaflet motion	PR	SAP (mmHg)	PAP (mmHg)	RVP (mmHg)	ΔP(RV - PA) (mmHg)	pRV/LV
Study	4	Mobile	Trivial	58	21	21	0	0.36
Study	5	Mobile	Mild	67	18	32	14	0.48
Study	6	Mobile	No	61	27	36	9	0.59
Control	1	Fixed	No	56	17	44	27	0.79
Control	9	Mobile	No	55	23	41	18	0.75

PR: pulmonary regurgitation; SAP: systemic arterial pressure; PAP: pulmonary arterial pressure; RVP: right ventricular pressure; ΔP(RV - PA): pressure gradient between the right ventricle and the pulmonary artery; pRV/LV: pressure ratio of the right ventricle to the left ventricle.

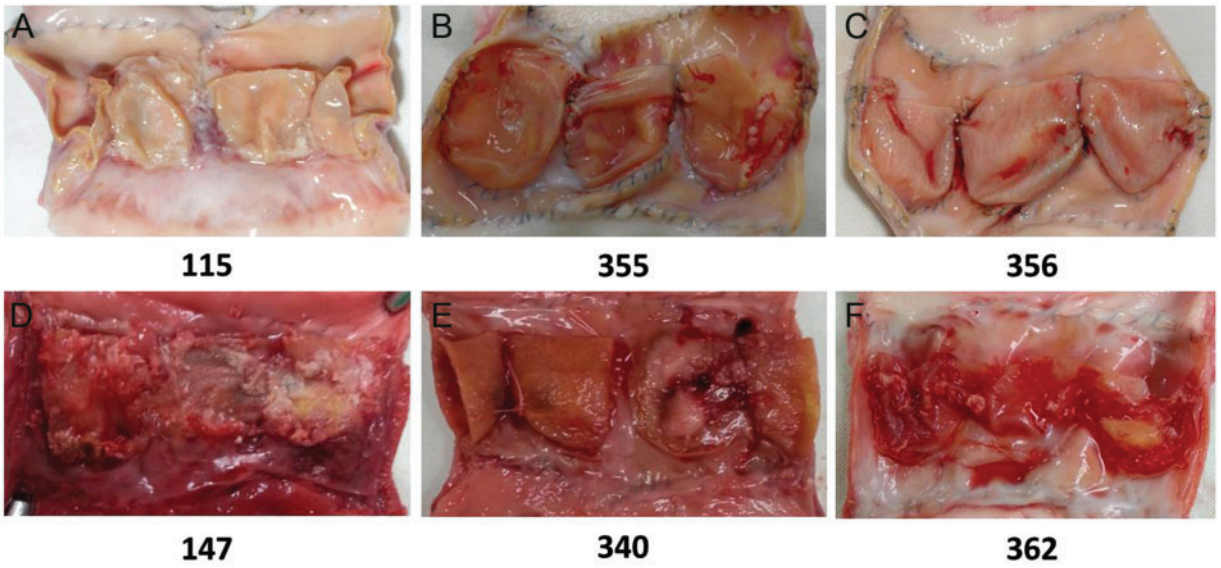

Figure 2: Gross findings of representative valved conduits in each group. (**A**–**C**) Calcific deposits or plaques are not evident in leaflets explanted from goats in the study group. (**D**–**F**) Leaflets explanted from goats in the control group showed calcific deposits and plaques. The numbers represent the surviving days after implantation.

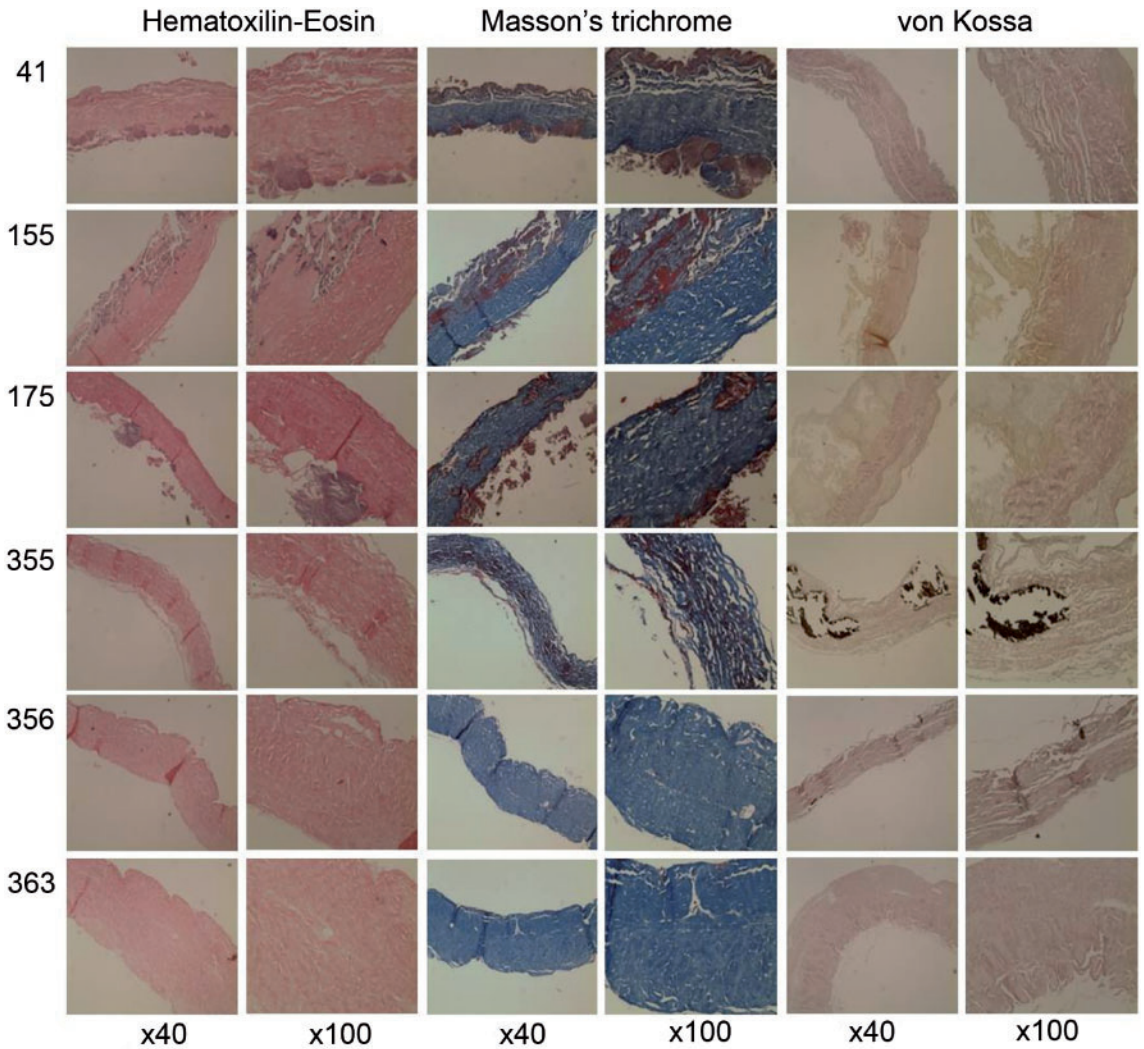

Figure 3: Light microscopy of explanted porcine pericardial leaflets in the study group (Haematoxylin and eosin, Masson's trichrome and von Kossa staining; x40, x100). The numbers on the left column represent the surviving days. The numbers at the bottom represent magnification.

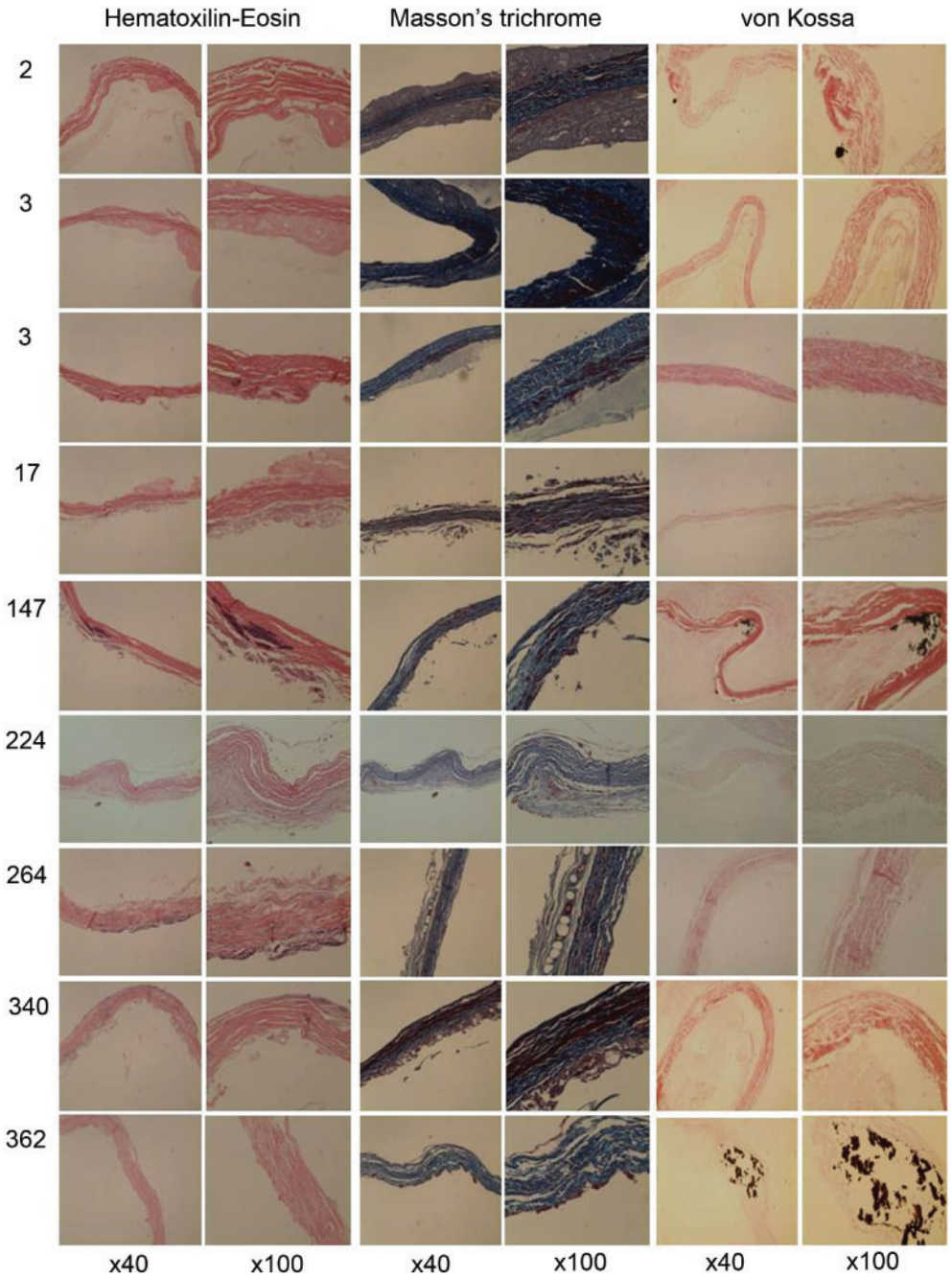

Figure 4: Light microscopy of explanted porcine pericardial leaflets in the control group (Haematoxylin and eosin, Masson's trichrome and von Kossa staining; x40, x100). The numbers on the left column represent the surviving days after implantation. The numbers at the bottom represent magnification.

the study group than in the control group [2.7 μg/mg (0.4–4.1 μg/mg) vs 15.1 μg/mg (4.8–86.4 μg/mg), respectively; $P = 0.008$; Fig. 6].

In the bovine pericardial walls, no significant increase or difference was found in the calcium content (Fig. 5).

DISCUSSION

Currently, GA is the most popular fixative in tissue processing for bioprosthetic valves; however, GA is a major cause of valve failure because of calcification. Treatment with GA will induce cell death and subsequently form cellular debris, which can be foci for calcification [4]. Therefore, the calcification amount can be reduced by achieving cell removal from the tissue before GA treatment. Additional anticalcification effects might also be obtained by reducing antigens (Galα1,3-Galβ1–4GlcNAc-R epitope, α-Gal) on the cell membrane, given the incomplete removal of cells with α-Gal in commercial bioprosthetic heart valves [19], and increasing anti-α-Gal antibody titres following commercial bioprosthetic heart valve implantation in humans [20–22]. Among the methods used for decellularization [6], we developed an optimal decellularization protocol [14], which preserved the microscopic structure and degree of cross-linking and tissue

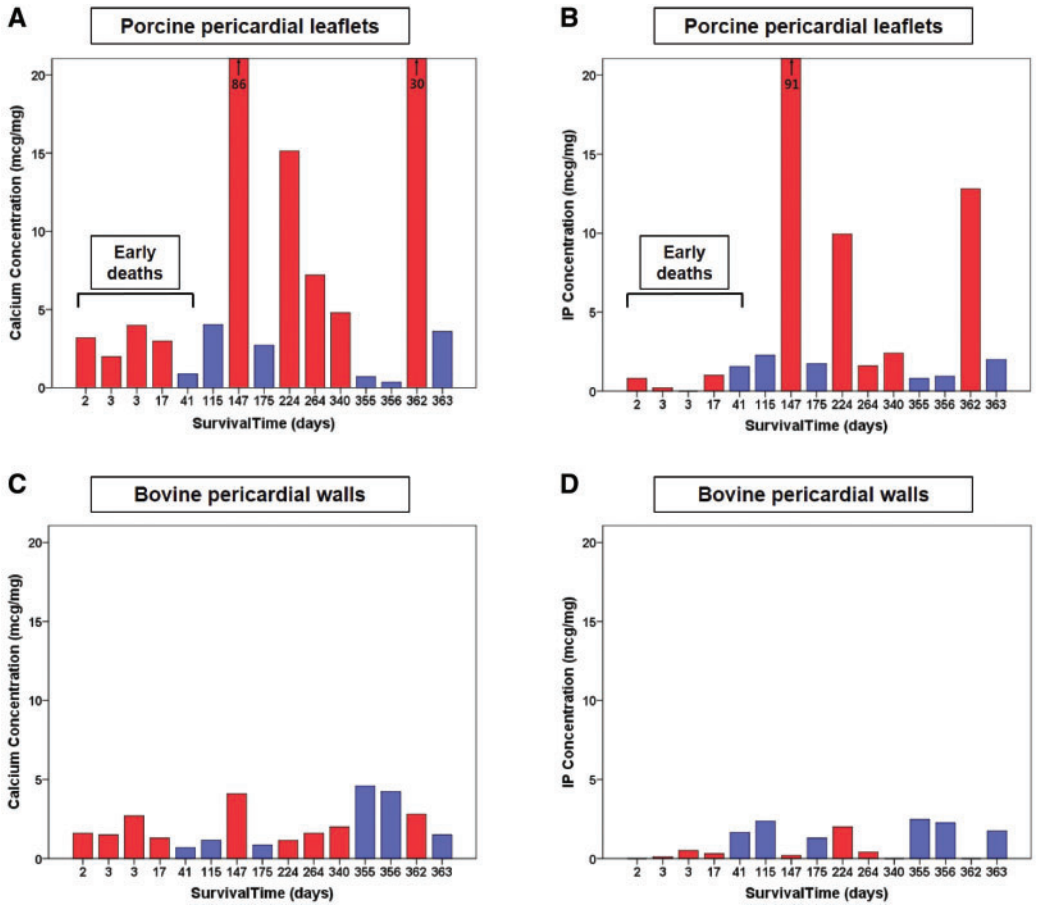

Figure 5: Calcium (left column) and inorganic phosphorus (right column) content of each xenograft. (**A** and **B**) Porcine pericardial leaflets. (**C** and **D**) Bovine pericardial walls. Red and blue bars represent the control and study groups, respectively. The calcium or inorganic phosphorus content exceeding the upper range (20 μg/mg) is presented with an arrow in the bar.

strength, lowered cytotoxicity and inhibited the *in vivo* calcification of GA-fixed xenografts [14, 23]. In our model, our decellularization protocol was effective in complete cell removal and preventing *in vivo* calcification while maintaining collagenous structural integrity.

Free aldehyde groups that failed to form cross-links with tissue collagen after GA treatment can promote tissue calcification. To prevent calcification related to free aldehyde groups, many investigators have attempted to neutralize these groups with various amino acids or amine compounds by forming a Schiff base [10–14, 24–26]; however, the anticalcification effect was diverse. We previously demonstrated that detoxification treatment with glycine prevented *in vivo* calcification without altering the microscopic structure, degree of cross-linking and tissue strength in small animal experiments [11, 14]; this large-animal long-term circulatory model confirmed the previously demonstrated anticalcification effect of detoxification treatment. There is greater resistance to enzymatic digestion in GA-fixed tissues with amino acid treatment than in tissues treated with GA alone [26]. In the present study, denser arrays of collagen fibres in the leaflets of the study group than in those in the control group might be related to the lesser degree of degeneration because of the greater resistance to enzymatic degradation obtained with amino acid treatment.

Phosphorus exists largely in the form of cell membrane-associated phospholipids, and they can serve as nucleators of calcium phosphate crystal formation [11]. Notwithstanding decellularization, a significant amount of phosphorus still remains as a form of phospholipid in intercellular structures. Therefore, the process of eliminating phospholipids can prevent calcification. Various organic solvents have been used to extract phospholipid from various tissues [8, 9, 27–30]. Calcification in porcine aortic leaflets was almost completely inhibited when pretreated with ethanol at a concentration ≥50%, but only partial inhibition of calcification was seen when pretreated with chloroform–methanol (2:1) in a 21-day rat subdermal implant model [9]. From these findings, it was suggested that the mechanism for calcification inhibition by ethanol is related to an interaction of lipid extraction, structural protein conformation changes and altered water status. A short- and long-chain alcohol combination can extract most phospholipids from GA-fixed porcine valves and bovine pericardial tissues and reduce *in vivo* calcification in a rat subcutaneous implant model [8]. In that study, a long-chain alcohol was used to effectively eliminate phospholipids due to its structural similarity to phospholipids and a short-chain alcohol was used as a carrier for a less water-soluble long-chain alcohol to improve penetration through thick tissues. Previously, we demonstrated that several combinations of short- and long-chain alcohols effectively prevent *in vivo* calcification without worsening physical properties [11, 14]. In our model, we used a combination of 75% ethanol and 5% octanol as the solvent, which could effectively reduce the *in vivo* calcification of GA-fixed

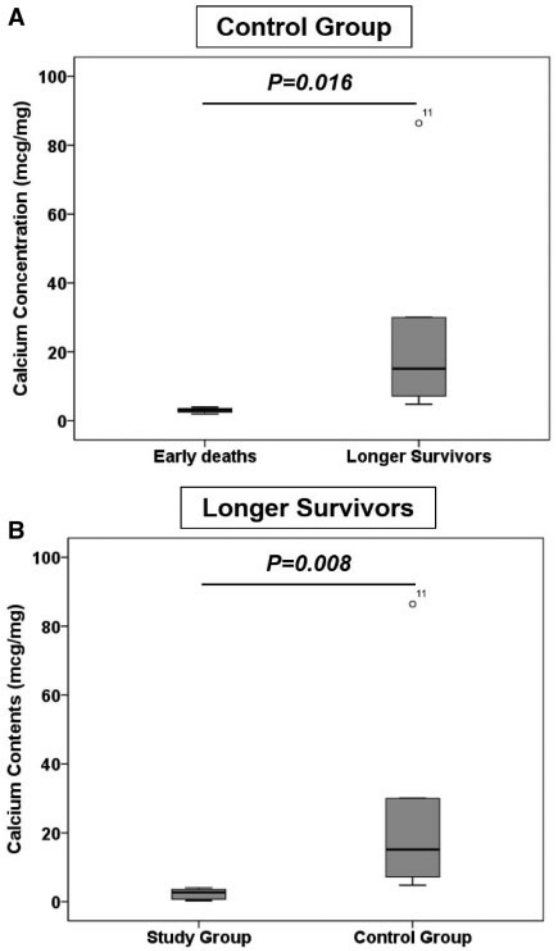

Figure 6: Comparison of calcium content in leaflets. (**A**) In the control group, the calcium content was significantly higher in leaflets explanted from goats that survived longer (>3 months) than the others ($P = 0.016$). (**B**) Among leaflets explanted from goats that survived longer (>3 months), the calcium content was higher in the control group than in the study group ($P = 0.008$).

xenopericardial valve conduits in the pulmonary position without markedly altering the microscopic structure.

In the control group, the calcification in porcine pericardial leaflets was conspicuous in the goats that survived for 147 days or more, although it was not in goats surviving for 17 days or less; this finding suggested that calcification begins somewhere in between. Our anticalcification treatment could prevent calcification and might defer calcification for at least 1 year.

Leaflet fixation in echocardiography, the higher pressure gradients across the RVOT and the higher pressure ratios of the right ventricle to the systemic artery in the control group indicated the higher degree of pulmonary stenosis that was attributed to calcification in leaflets; these findings were confirmed by gross inspection and calcium quantification. In contrast, calcification was not evident in the light microscopic examination with von Kossa stain in some leaflets from goats that survived longer (>3 months) survivors. This discrepancy might be due to the uneven calcification in the leaflets and selectively obtaining tissues from the less calcified areas.

Despite the same anticalcification process, bovine pericardial walls were less calcified than porcine pericardial leaflets. The possible explanations included less manipulation during manufacturing, less *in vivo* haemodynamic stress and less contact surface area with recipient blood, apart from the obvious difference in species.

Due to the nature of the large animal study, our study included a rather small number of animals. Therefore, most results should be only qualitative descriptions rather than statistical conclusions. The fact that a significant number of goats died earlier than expected might be a critical limitation and therefore weaken the power of our study. Although there were no remarkable findings in autopsies, all possible postoperative problems following open-heart surgery, including malnutrition, hypovolaemia, anaemia and infection, might be attributed to early deaths. A lot of stress imposed on the experimental animals during postoperative care in an unfamiliar environment could also be a reason for the deaths. To avoid unexpected deaths, more delicate monitoring and timely postoperative care should have been provided and more efforts for stress mitigation should have been taken; nonetheless, it was possible to evaluate the difference in calcification with time because of the unexpected early deaths.

CONCLUSION

In conclusion, GA-fixed porcine pericardial leaflets treated with our anticalcification protocol, including prefixation decellularization, fixation in organic solvents and postfixation amino acid detoxification, were better functioning and less calcified in the pulmonary position than those treated with GA alone in our large-animal long-term circulatory model.

ACKNOWLEDGEMENTS

The authors would like to thank Enago (http://www.enago.co.kr) for the English language review.

Funding

This work was supported by a grant of the Korea Health 21 Research and Development Project, Ministry for Health, Welfare, and Family Affairs, Republic of Korea (project number: A040004-006).

Conflict of interest: none declared.

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