

Anticalcification effects of decellularization, solvent, and detoxification treatment for genipin and glutaraldehyde fixation of bovine pericardium

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Abstract

OBJECTIVE: Calcification plays a major role in the failure of bioprosthetic and other tissue heart valve substitutes. The objective of this study was to evaluate the anticalcification effect of decellularization and detoxification in glutaraldehyde (GA)/genipin- and solvent-fixed bovine pericardium using a rabbit intramuscular model which is effective for assessing calcification in bioprosthetic tissue.

METHODS: Bovine pericardial tissues were fixed with 0.5% GA/0.4% genipin in organic solvent (75% ethanol + 5% octanol, 75% ethanol + 5% octanediol, or 70% ethanol + 10% isopropanol) and post-treated with glycine, glutamic acid, or sodium bisulfite. Decellularization was performed with 0.25% sodium dodecylsulfate prior to fixation. The material characteristics of the treated tissues were assessed by thermal stability test, uniaxial mechanical test, and light microscopy. Stability of the treated tissue was measured by the resistance to enzymatic degradation using protease. The tissues were intramuscularly implanted into 4-week-old rabbits for 8 weeks, and the anti- α -Gal(Gal[α 1,3-Gal[β 1,4GlcNAc-R) antibodies (immunoglobulin G) were measured at various time intervals after implantation. Explanted tissues were examined by light microscopy and calcium contents of the explanted tissues were measured.

RESULTS: Differently treated tissues resulted in no significant alterations in material characteristics and morphology. GA groups are superior to genipin groups in tissue cross-linking without difference according to addition of decellularization, organic solvent treatment, and detoxification by resistance toward pronase degradation. The titer of anti- α -Gal antibodies gradually increased after implantation in all study groups. The titer of anti- α -Gal antibodies increased less in genipin groups than in GA groups, and less with decellularization than without decellularization. The calcium contents of genipin groups ($n = 75$) were significantly lower than those of GA groups ($n = 118$) ($28.55 \pm 11.22 \mu\text{g mg}^{-1}$ vs $37.16 \pm 7.75 \mu\text{g mg}^{-1}$, $p < 0.001$). Calcium contents decreased with decellularization, organic solvent treatment, and detoxification, irrespective of the type of organic solvent and amino acids. Inorganic phosphorus contents of genipin groups ($n = 66$) were significantly lower than those of GA groups ($n = 103$) ($55.36 \pm 26.98 \mu\text{g mg}^{-1}$ vs $75.67 \pm 23.44 \mu\text{g mg}^{-1}$, $p = 0.000$).

CONCLUSIONS: Genipin fixation is a novel alternative to conventional GA fixation *in vitro* material assessment and *in vivo* anticalcification effect. The addition of decellularization, organic solvent treatment, and detoxification prevented calcification of GA/genipin-fixed bovine pericardium in the rabbit intramuscular implantation model, irrespective of the type of organic solvent and amino acids.

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

INTRODUCTION

Since the primary problem of bioprostheses is structural valve deterioration at long term, mainly due to dystrophic calcification, many improvements have been implemented in order to prolong long-term durability [1]. Cross-linkage of xenograft tissue with glutaraldehyde (GA) was revealed to be successful in suppressing host immunological reactivity and to obtain stabilization of collagen. However, GA fixation promotes dystrophic calcification because of the chemical process between free aldehyde

groups, phospholipids, and residual antigenicity of the bioprosthetic tissue [1–3]. Considerable efforts over many years through basic research have been directed toward developing a tissue treatment process to prevent calcification in GA-fixed xenograft tissue. The main anticalcification strategies aim to extract lipid [4] or to neutralize toxic aldehyde residual [5].

GA-fixed xenografts have cellular/humoral rejection and calcify secondarily [3]. Tissue valve calcification is also initiated primarily within residual cells that have been devitalized [1]. Decellularization approaches proved to be effective in the

prevention of calcification, presumably by suppressing residual antigenicity of bioprosthetic tissue [6,7]. As an alternative other than GA, genipin is known to be a novel tissue cross-linking agent because this naturally occurring cross-linker is less cytotoxic and better *in vivo* biocompatible than GA [8,9].

Our approach investigated the most promising preventive strategies which included improvement or modification of GA fixation through reduction reactivity of residual chemical groups (e.g., glycine, glutamic acid, and sodium bisulfite), removal or modification of calcifiable components such as surfactants (e.g., sodium dodecylsulfate (SDS)), organic solvents (e.g., ethanol, octanol, and octanediol), and decellularization, and use of tissue cross-linking agents other than GA (e.g., genipin). The subcutaneous model has been widely used to screen potential strategies for calcification inhibition. In this study, promising approaches have been investigated further in a rabbit intramuscular model. The New Zealand white (NZW) rabbit intramuscular model is an effective model for assessing calcification in bioprosthetic tissue [10] because this model is robust for detecting calcification in a shorter duration (14 days), with less infection complications, more space to implant tissue groups (thereby reducing animal use numbers), and a more metabolically and mechanically dynamic environment than the rat subcutaneous model.

This study evaluates the anticalcification effect of decellularization and detoxification in GA/genipin- and solvent-fixed bovine pericardium using the rabbit intramuscular model. The major purpose of the investigation was to test a new anticalcification treatment effective enough to justify subsequent investigation in the large animal circulatory implant.

MATERIALS AND METHODS

Experimental design and tissue preparation

Bovine pericardial tissues were divided into 33 groups (one group of fresh bovine pericardium as control, 20 groups of GA fixation, and 12 groups of genipin fixation) according to the method of tissue preparation (Table 1).

A. Control: fresh bovine pericardium

B. GA groups ($n = 20$)

Bovine pericardial tissues were initially fixed with 0.5% GA for 3 days at room temperature, and additionally fixed with 0.5% GA in organic solvent of 75% ethanol + 5% octanol, 75% ethanol + 5% octanediol, or 70% ethanol + 10% isopropanol for 2 days at room temperature, and finally fixed with 0.25% GA for 7 days at room temperature.

C. Genipin groups ($n = 12$)

Bovine pericardial tissues were initially fixed with 0.4% genipin for 5 days at room temperature and additionally fixed with 0.4% genipin in organic solvent of 75% ethanol + 5% octanol, 75% ethanol + 5% octanediol, or 70% ethanol + 10% isopropanol for 2 days at room temperature.

Decellularization

Bovine pericardial tissues were initially treated with hypotonic-buffered solution for 14 h at 4 °C and hypotonic-buffered solution with 0.25% SDS for 24 h at room temperature. These tissues were then washed with isotonic-buffered solution for 30 min at 4 °C, then with distilled water for 12 h at 4 °C, and then with

isotonic-buffered solution for 12 h at 4 °C. The tissues were finally treated with hypertonic-buffered solution (II) for 6 h at 4 °C and isotonic-buffered solution for 3 h at 4 °C.

Detoxification

After completion of fixation, tissues were treated with 0.2 M glycine solution (phosphate-buffered saline (PBS), pH 7.4) at 4 °C for 24 h, 0.12 M L-glutamic acid solution (PBS, pH 7.4) at room temperature for 48 h, or 40% sodium bisulfite saturated solution at 4 °C for 24 h.

Thermal stability test

Shrinkage temperature measurements, which assess the degree of fixation (cross-linking) of pericardial tissue, were performed by the hydrothermal method using a custom-built extensometer. Tissue strips (8 mm × 30 mm, 15 strips for fresh pericardium and five strips for each experimental group) were loaded to 95 g, held at constant extension along the long axis, and placed in a water bath. The temperature of the water bath was increased by approximately 2.5–5 °C min⁻¹ and the width of the strip was measured using a microscope. By plotting graphically the width against temperature, the sharp deflection point at which shrinkage occurred was identified as the shrinkage temperature.

Mechanical test

Uniaxial test was used to compare the mechanical properties of differently treated tissues. Tissue strips (5 mm × 10 mm, 50 strips for fresh bovine pericardium and 12 for each experimental group) were cut in different directions to overcome material anisotropy. Tissue thickness was measured at the center of the strip using a Mitutoyo thickness gauge (Quick-Mini 700-117, Mitutoyo, Japan). Tensile properties were evaluated using a tensile testing machine (K-ML-1000N, M-TEC, Republic of Korea) equipped with digital force gauge (DS2-200N, IMADA, Japan) operating at an extension rate of 100 mm min⁻¹ with a preload of 0.0 N. Ultimate strength and strain at fracture were evaluated from the recorded stress–strain curves.

Pronase test (resistance to enzymatic degradation)

Stability of the treated tissue was measured by the resistance to enzymatic degradation using protease (20 U mg⁻¹, Aldrich) [11]. In this procedure, samples were initially soaked in a HEPES-buffered saline solution (10 mM, pH 7.4) containing NaCl (9 g l⁻¹) and glycine (7.5 g l⁻¹) for 4 h at 37 °C. Samples were then transferred to a solution of the same buffer containing the enzyme (3 ml with 12 mg enzyme per sample). To assure optimal enzymatic activity of the solution, the enzyme was added along with CaCl₂ (96 mg to 180 ml solution). Samples were incubated for 4 h, washed, and freeze-dried. After renewed determination of the dry weight, the actual weight loss due to enzymatic degradation was calculated.

Table 1: Study groups

Group	Cross-linker	Organic solvent	Decellularization	Detoxification
1	Glutaraldehyde	-	-	-
2	Glutaraldehyde	75% ethanol + 5% octanol	-	-
3	Glutaraldehyde	75% ethanol + 5% octanediol	-	-
4	Glutaraldehyde	70% ethanol + 10% isopropanol	-	-
5	Glutaraldehyde	-	SDS	-
6	Glutaraldehyde	75% ethanol + 5% octanol	SDS	-
7	Glutaraldehyde	75% ethanol + 5% octanediol	SDS	-
8	Glutaraldehyde	70% ethanol + 10% isopropanol	SDS	-
9	Glutaraldehyde	-	-	Glycine
10	Glutaraldehyde	75% ethanol + 5% octanol	-	Glycine
11	Glutaraldehyde	75% ethanol + 5% octanediol	-	Glycine
12	Glutaraldehyde	70% ethanol + 10% isopropanol	-	Glycine
13	Glutaraldehyde	-	-	Glutamic acid
14	Glutaraldehyde	75% ethanol + 5% octanol	-	Glutamic acid
15	Glutaraldehyde	75% ethanol + 5% octanediol	-	Glutamic acid
16	Glutaraldehyde	70% ethanol + 10% isopropanol	-	Glutamic acid
17	Glutaraldehyde	-	-	Sodium bisulfite
18	Glutaraldehyde	75% ethanol + 5% octanol	-	Sodium bisulfite
19	Glutaraldehyde	75% ethanol + 5% octanediol	-	Sodium bisulfite
20	Glutaraldehyde	70% ethanol + 10% isopropanol	-	Sodium bisulfite
21	Genipin	-	-	-
22	Genipin	-	-	Sodium bisulfite
23	Genipin	-	-	Glycine
24	Genipin	75% ethanol + 5% octanol	-	-
25	Genipin	75% ethanol + 5% octanediol	-	-
26	Genipin	70% ethanol + 10% isopropanol	-	-
27	Genipin	-	SDS	-
28	Genipin	-	SDS	Sodium bisulfite
29	Genipin	-	SDS	Glycine
30	Genipin	75% ethanol + 5% octanol	SDS	-
31	Genipin	75% ethanol + 5% octanediol	SDS	-
32	Genipin	70% ethanol + 10% isopropanol	SDS	-

SDS: sodium dodecylsulfate.

Rabbit intramuscular implantation [10]

This study was approved by the Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No. 09-0230). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Male New Zealand white rabbits (6–7 weeks old, 1,169–1,595 g) were used. All surgery was performed using aseptic techniques. After anesthetizing and shaving, an incision was made through the skin and into the muscle and the pericardial samples were inserted between the muscle layers. The rabbits were euthanized by CO₂ asphyxiation after 8 weeks. The tissue samples were harvested, freed of adherent rabbit tissues, and rinsed with normal saline. A major portion of each sample was used for quantitative calcium analysis, whereas representative samples were used for microscopic examinations.

Enzyme-linked immunosorbent assay

To determine the activity of immunoglobulin G (IgG) isotypes of the anti- α -Gal(Gal[α 1,3-Gal[β 1,4Glc-NAc-R) antibodies, blood samples were taken from the rabbit on the preimplantation date, 12th, and 60th postimplantation date, and an ELISA was performed. Bovine serum albumin (BSA) conjugating synthesized α -Gal (Genechem, Seoul, Korea) (α -Gal-BSA) was used as a

solid phase antigen. Microtiter plates were coated with 100 μ l per well of μ -Gal-BSA in PBS (pH 7.4) (at a concentration of 1 μ g ml⁻¹ for IgG isotype), and incubated for 1 h at 37 °C. Then, the plates were washed with PBS containing 0.05% (v/v) Tween-20. The sera (100 μ l per well) of rabbit were added to the α -Gal-BSA-immobilized wells at a serial twofold dilution from 1:40 to 1:2560 in BSA-TritonX-100 (PBS, pH 7.4, 3% BSA, 0.01% Triton X-100), then the plates were incubated for 1 h at 37 °C. Donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch, Baltimore, MD, USA) were used as a secondary antibody (1:10,000) at a dilution for IgG in BSA-Triton X-100. The reaction was developed with tetramethylbenzidine (TMB) solution (PIERCE, Rockford, USA). Optical density (OD) was measured at 450 nm using the Thermo Electron-Lab Systems (Labsystems, Vienna, VA, USA).

Calcium analysis

Harvested tissue samples (six to eight samples for each group) were washed with normal saline, dried at 70 °C for 24 h, and weighed. Samples were then hydrolyzed with 5.0 N HCl solution. The calcium content of the hydrolysate was measured colorimetrically by the o-cresolphthaleincomplex- one method, as previously described [12], using an automatic chemistry analyzer (Hitachi 7070, Japan). Calcium contents were expressed as μ g mg⁻¹ dry weight.

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 17.0 (SPSS, Inc, Chicago, IL, USA). Data were expressed as mean \pm standard deviation. Comparison between groups was performed using the *t*-test and one-way analysis of variance (ANOVA) with *post-hoc* test or Kruskal–Wallis and Mann–Whitney *U* tests. The change in the titer of anti- α -Gal antibodies (IgG) after implantation was analyzed by the Wilcoxon signed ranks test. Probability values of <0.05 were considered statistically significant.

RESULTS

Microscopic examination (preimplantation)

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

Thermal stability test

Shrinkage temperatures of study groups ($n = 160$) were all significantly higher than that of fresh pericardium ($n = 15$) (82.05 ± 4.64 °C vs 69.92 ± 1.29 °C, $p < 0.05$). Shrinkage temperatures for GA groups ($n = 100$) were significantly higher than genipin groups ($n = 60$) (85.08 ± 2.62 °C vs 77.0 ± 2.27 °C, $p = 0.000$). There was no significant difference in shrinkage temperature between differently treated pericardial tissues according to addition of decellularization, organic solvent treatment, and detoxification within GA groups (groups 1–20) and genipin groups (groups 21–32), respectively.

Mechanical test

There was no significant difference in tensile strengths between fresh bovine pericardium ($n = 50$) and differently treated pericardial tissues ($n = 384$) (17.53 ± 4.58 MPa vs 17.44 ± 7.07 MPa, $p = 0.993$). Tensile strengths for GA groups ($n = 240$) were significantly higher than genipin groups ($n = 144$) (18.32 ± 7.65 MPa vs 15.96 ± 5.69 MPa, $p = 0.002$). There was no significant difference in tensile strengths between differently treated pericardial tissues according to addition of decellularization, organic solvent treatment, and detoxification within GA groups (groups 1–20) and genipin groups (groups 21–32), respectively.

Mean tensile strain at break for fresh bovine pericardium ($n = 50$) was significantly smaller than those for study groups ($n = 384$) ($42.47 \pm 7.55\%$ vs $49.92 \pm 15.52\%$, $p = 0.001$). Mean tensile strain at break for GA groups was significantly smaller than that for genipin groups ($45.92 \pm 13.67\%$ vs $56.60 \pm 15.37\%$, $p = 0.000$). There was no significant difference in tensile strain at break between differently treated pericardial tissues according to addition of decellularization, organic solvent treatment, and detoxification within GA groups (groups 1–20) and genipin groups (groups 21–32), respectively.

Pronase test

In study groups ($n = 160$), resistance toward pronase degradation was significantly increased more than fresh bovine pericardium ($77.50 \pm 8.50\%$ vs $47.63 \pm 8.31\%$, $p = 0.000$). In GA groups ($n = 100$), resistance toward pronase degradation was significantly increased more than genipin groups ($n = 60$) ($81.58 \pm 0.82\%$ vs $70.69 \pm 2.97\%$, $p = 0.000$), which suggested that GA groups are superior to genipin groups in tissue cross-linking. However, there was no significant difference in resistance toward pronase degradation according to addition of decellularization, organic solvent treatment, and detoxification within GA groups (groups 1–20) and genipin groups (groups 21–32), respectively.

ELISA

The titer of anti- α -Gal antibodies (IgG) gradually increased with statistical significance on the preimplantation date, 12th, and 60th postimplantation date in all study groups (GA and genipin-fixed groups). The titer of anti- α -Gal antibodies (IgG) gradually increased with statistical significance after implantation in GA groups, but not in genipin groups (Fig. 2A).

The titer of anti- α -Gal antibodies (IgG) gradually increased with statistical significance on the preimplantation date, 12th, and 60th postimplantation date in the groups without decellularization, but not in the groups with decellularization (Fig. 2B). The titer of anti- α -Gal antibodies (IgG) gradually increased with statistical significance after implantation in GA groups without decellularization, but not in GA groups with decellularization. The titer of anti- α -Gal antibodies (IgG) gradually tended to increase after implantation in genipin groups without decellularization, but not in genipin groups with decellularization.

Microscopic examination (postimplantation)

In all groups, light microscopy also showed preservation of collagen fibers. In H–E stain, GA groups had more inflammatory cells than genipin groups. With decellularization, organic solvent treatment, and detoxification, bovine pericardium tended to have less inflammatory cells. The arrays of collagen fibers are denser in bovine pericardium without decellularization than with decellularization.

In von Kossa stain, GA groups had more calcific deposits (dark brown color) than genipin groups. With decellularization, organic solvent treatment, and detoxification, bovine pericardium tended to have less calcific deposits.

Calcium analysis (Fig. 3)

Calcium contents of genipin groups ($n = 75$) were significantly lower than those of GA groups ($n = 118$) (28.55 ± 11.22 $\mu\text{g mg}^{-1}$ vs 37.16 ± 7.75 $\mu\text{g mg}^{-1}$, $p < 0.001$). Calcium contents of the GA groups with decellularization were significantly lower than those without decellularization (35.45 ± 11.47 $\mu\text{g mg}^{-1}$ vs 40.40 ± 5.54 $\mu\text{g mg}^{-1}$, $p = 0.002$). Calcium contents of the genipin groups with decellularization were significantly lower than those without decellularization (24.93 ± 9.50 $\mu\text{g mg}^{-1}$ vs 31.89 ± 11.75 $\mu\text{g mg}^{-1}$, $p = 0.006$).

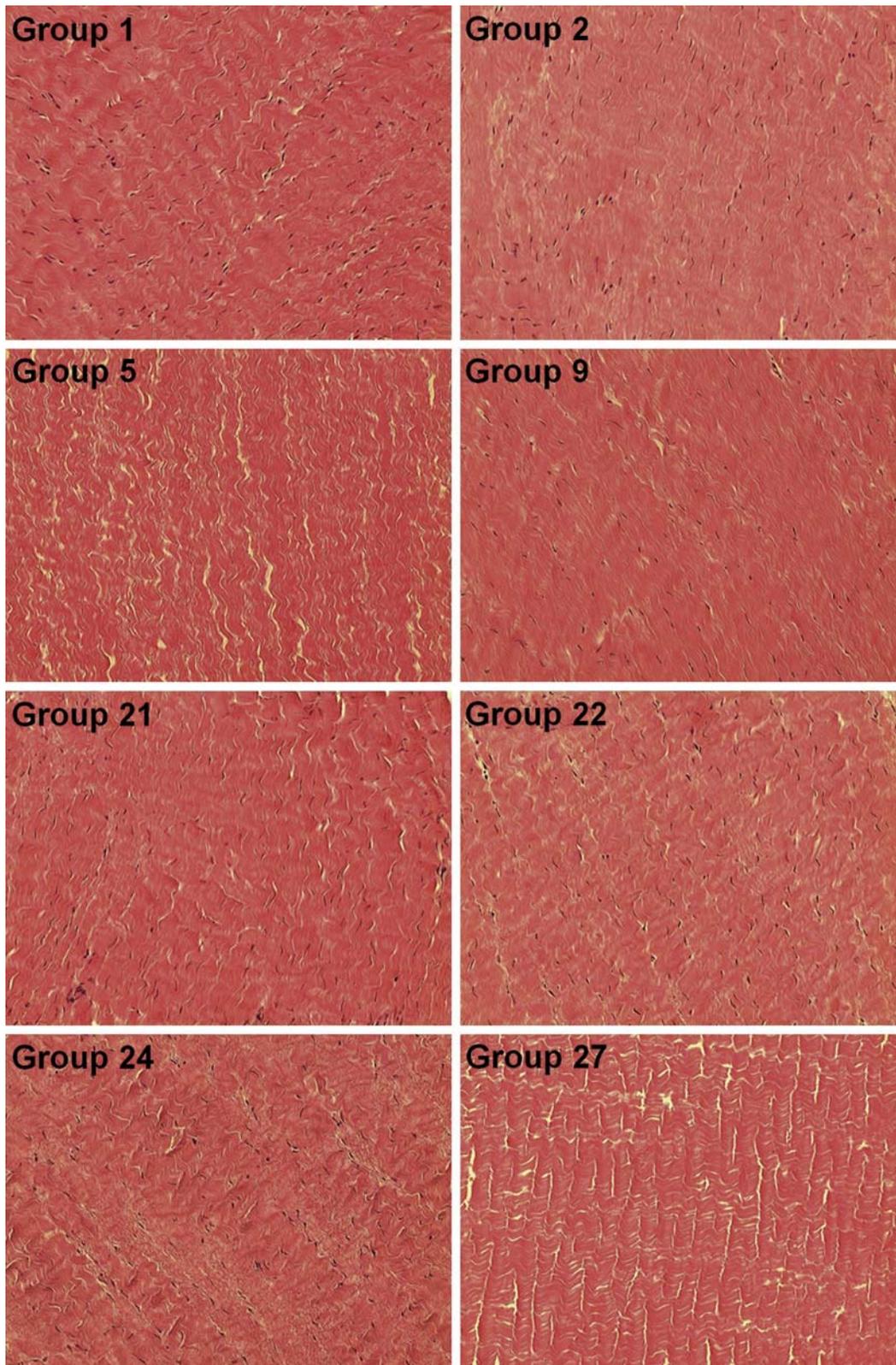


Figure 1: Light microscopy of unimplanted bovine pericardium (hematoxylin–eosin stain, $\times 100$). Collagen fibers appear well preserved with a normally banded structure in all groups without significant difference among groups. In decellularized bovine pericardium groups (groups 5 and 27), there was no visible cell, and no specific matrix derangement was noticeable. See Table 1 for the details of study groups.

Calcium contents of the GA groups with organic solvent treatment were significantly lower than those without organic solvent treatment ($36.16 \pm 5.98 \mu\text{g mg}^{-1}$ vs $40.24 \pm 11.19 \mu\text{g mg}^{-1}$, $p =$

0.017). There was no difference of calcium contents in the GA groups according to the type of organic solvent including octanol, octanediol, and isopropanol ($p > 0.05$). Calcium

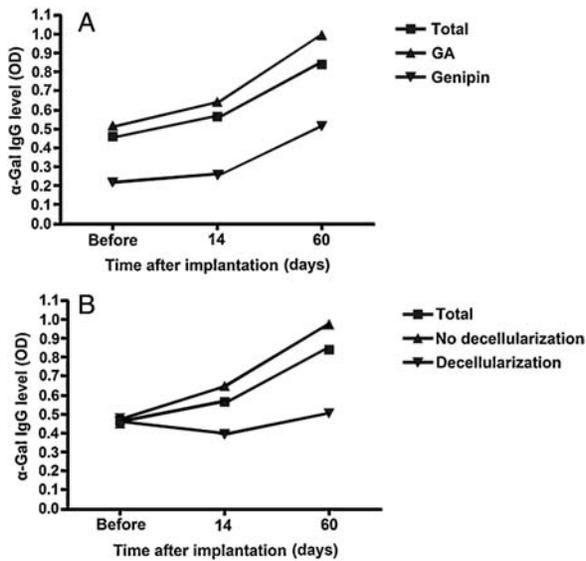


Figure 2 Changes in anti α -Gal activity before and after rabbit intramuscular implantation of bovine pericardium. The titer of anti α -Gal antibodies (IgG) gradually increased with statistical significance on the preimplantation date, 12th, and 60th postimplantation date in total study groups. The titer of anti α -Gal antibodies (IgG) gradually increased with statistical significance after implantation in GA groups, but not in genipin groups (A). The titer of anti α -Gal antibodies (IgG) gradually increased with statistical significance in the groups without decellularization, but not in the groups with decellularization (B). GA: glutaraldehyde-fixed group and Genipin: genipin-fixed group.

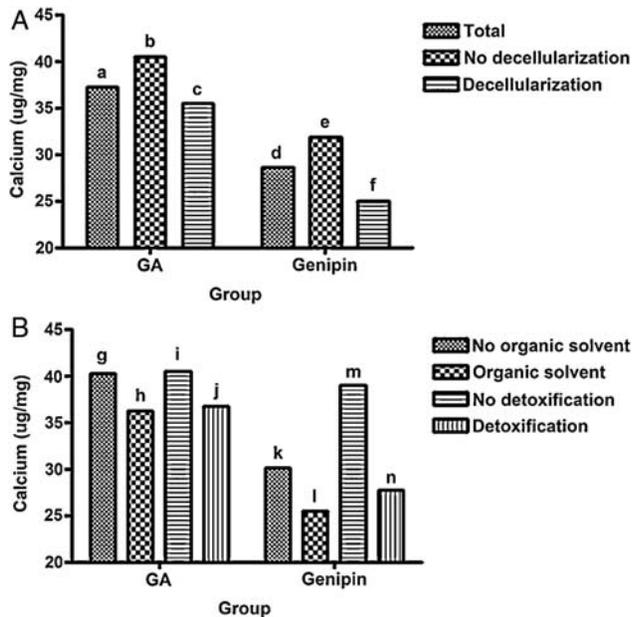


Figure 3: Bar graph showing calcium contents of explanted bovine pericardium. Calcium contents of total genipin groups were significantly lower than that of total GA groups (A). Calcium contents decreased with decellularization (A), organic solvent treatment (B), and detoxification (B) both in GA and genipin groups. GA: glutaraldehyde-fixed group and Genipin: genipin-fixed group (a vs d, b vs c, e vs f, g vs h, i vs j, and m vs n: $p < 0.05$).

contents of the genipin groups with organic solvent treatment tended to be lower than those without organic solvent treatment ($25.43 \pm 10.31 \mu\text{g mg}^{-1}$ vs $31.14 \pm 6.08 \mu\text{g mg}^{-1}$, $p = 0.141$). There was no difference of calcium contents in the genipin groups according to the type of organic solvent including octanol, octanediol, and isopropanol ($p = 0.821$).

Calcium contents of the GA groups with detoxification were significantly lower than those without detoxification ($36.74 \pm 6.54 \mu\text{g mg}^{-1}$ vs $40.40 \pm 5.54 \mu\text{g mg}^{-1}$, $p = 0.009$). There was no difference of calcium contents in the GA groups according to the type of amino acids including glycine, glutamic acid, and sodium bisulfite ($p > 0.05$). Calcium contents of the genipin groups with detoxification were significantly lower than those without detoxification ($27.68 \pm 8.42 \mu\text{g mg}^{-1}$ vs $38.94 \pm 13.09 \mu\text{g mg}^{-1}$, $p = 0.015$). There was no difference of calcium contents in the genipin groups according to the type of amino acids including glycine, glutamic acid, and sodium bisulfite ($p > 0.05$).

Inorganic phosphorus analysis

Inorganic phosphorus contents of genipin groups ($n = 66$) were significantly lower than those of GA groups ($n = 103$) ($55.36 \pm 26.98 \mu\text{g mg}^{-1}$ vs $75.67 \pm 23.44 \mu\text{g mg}^{-1}$, $p = 0.000$).

DISCUSSION

Detoxification

Residual free aldehyde groups or polymerized forms of GA create a locally cytotoxic environment and result in tissue calcification. Amino acid postfixation improves protein cross-linking to increase the mechanical performance of the bioprostheses or to neutralize these free aldehyde groups because the amino groups of these compounds can react with the free aldehyde groups of GA, forming Schiff base [5,13,14]. Sodium bisulfite is a classic reductive reagent which reacts with aldehydes to form alpha-hydroxyl sodium sulfonate, and would react with any free GA in fixed pericardial tissue, thus enabling the mitigation of *in vivo* calcification [15].

In this study, post-treatment with glycine, L-glutamic acid, and sodium bisulfite did not alter the microscopic structure, the degree of cross-linking, and tissue strength. *In vivo* calcification test demonstrated inhibition of calcification in the groups of GA/genipin fixation with detoxification compared with conventional fixation without detoxification, regardless of the types of reducing agents used.

Organic solvent

Short-chain alcohols, such as ethanol at high concentration (>50% in aqueous buffers), reduce calcification potential of aldehyde-fixed tissues [4] by removal of phospholipids or conformational changes in collagen. The alcohols may also be preferentially bound to hydrophobic residues within collagen and elastin [16], which may undergo independent calcification [17]. The removal of phospholipids using a combination of short- and long-chain alcohols (LCAs) may also reduce the calcification potential of fixed tissues, especially given that LCAs are more structurally similar to phospholipids (long alkyl chain and a polar end group) than short-chain alcohols and therefore may remove phospholipids more efficiently [18].

We selected octanol [19], 1,2-octanediol [18,20], and isopropanol [21] as an LCA and used a buffered ethanolic solution as a solvent and as a carrier for the LCA [18] to improve its solubility in water-based solutions, reduce micelle formation, and possibly improve its

penetration into the thick tissue. Our *in vivo* calcification study clearly demonstrated excellent anticalcification efficacy of this treatment, regardless of the types of organic solvents used.

Decellularization

The key common feature for the pathophysiology of calcification is the involvement of devitalized cells and cellular debris [1]. As for the decellularization, we also intended to suppress residual antigenicity, which was thought to be important for anticalcification efficacy. Decellularization did not alter the microscopic structure, the degree of cross-linking, and tissue strength. *In vivo* calcification test demonstrated inhibition of calcification in both GA and genipin fixation with decellularization.

Genipin

Genipin, a naturally occurring cross-linker, can be obtained from its parent compound geniposide, a compound isolated from the fruit of the gardenia plant (*Gardenia jasminoides* Ellis). Chang and colleagues [8,9] have shown that genipin is less cytotoxic than GA, and also that the *in vivo* biocompatibility of genipin-fixed vascular grafts and bovine pericardium is significantly better than their GA-fixed counterparts.

In this study, genipin-treated tissues resulted in good material characteristics and morphology. Although genipin groups are inferior to GA groups in tissue cross-linking without difference according to addition of decellularization, organic solvent treatment, and detoxification by resistance toward pronase degradation, *in vivo* experiment demonstrated that calcium and inorganic phosphorus contents of genipin groups were significantly lower than those of GA groups.

Immunologic effect

Although there is debate in the literature [22] as to whether or not there is an immune system response to bioprosthetic heart valves, bioprosthetic heart valve (porcine/bovine) implantation is a form of xenotransplantation. Recently, it has been found that the mammalian cell surface xenoantigen called α -Gal epitopes are still present on the commercially available, GA-fixed tissue valves [2]. It has been reported that the immune response due to α -Gal epitopes is an important factor in tissue valve failure. In addition, we [23] reported that patients who received these tissue valves exhibited increased levels of natural anti-Gal antibody titers against α -Gal epitopes [2]. Therefore, it is believed that the animal immune response may play an important role in structural damage of the commercially available, GA-fixed tissue valves. The differences in the fine specificity of natural anti-Gal in various species may cause the multiple B-cell clones to produce anti-Gal antibodies which have specificities that differ slightly from each other, and thus recognize various 'facets' of the α -Gal epitope in its three-dimensional form [24].

We found a significant rise in anti- α -Gal antibodies (IgG) in all study groups (GA>genipin-fixed groups), supporting the hypothesis that there is a humoral response to the GA and genipin-fixed xenograft. Decellularization suppresses the rise of the titer of anti- α -Gal antibodies (IgG) in both GA and genipin groups, suggesting that the decellularization approach is useful for removal of

the immunogenicity. *In vivo* calcification results are also consistent with the increase of anti- α -Gal antibodies titer. Comparing *in vivo* calcification results with anti- α -Gal antibodies titer, the *in vivo* experiment demonstrated that genipin-fixed groups had less calcification than GA groups, and decellularization reduced calcification in both GA and genipin groups, supporting the hypothesis that calcification of the bovine pericardium may occur as a 'by-product' of the immune response.

Although no clinically useful preventive approach is yet available, several strategies based on improvement or modification of GA fixation through reduction reactivity of residual chemical groups (e.g., glycine, glutamic acid, and sodium bisulfite), removal or modification of calcifiable components such as surfactants (e.g., SDS), organic solvents (e.g., ethanol, octanol, and octanediol), decellularization, and use of tissue cross-linking agents other than GA (e.g., genipin) appear to be promising in this study. Interesting approaches to preventing this problem through synergistic and simultaneous employment of multiple anticalcification therapies or novel tissue treatments using large-animal long-term circulatory models are under investigation.

In conclusion, genipin fixation is a novel alternative to conventional GA fixation *in vitro* material assessment and *in vivo* anticalcification effect. Decellularization, GA or genipin fixation in organic solvent, detoxification, and combined treatment all resulted in no significant alterations in bovine pericardial material characteristics and morphology as assessed by light microscopy, thermal stability test, uniaxial mechanical test, and pronase test. The addition of decellularization, organic solvent treatment, and detoxification all strongly prevented calcification of GA/genipin-fixed bovine pericardium in the rabbit intramuscular implantation model, irrespective of the type of organic solvent and amino acids for detoxification.

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