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High-concentration glutaraldehyde fixation of bovine pericardium in organic solvent and post-fixation glycine treatment: *in vitro* material assessment and *in vivo* anticalcification effect☆

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Abstract

Objective: Glutaraldehdye (GA)-fixed xenografts are widely used in cardiovascular surgery. The objective of this study was to evaluate the anticalcification effect of glycine treatment and high-concentration GA fixation in organic solvent on GA-fixed bovine pericardium, and to evaluate the possible synergistic effect of combined treatment. **Methods:** Bovine pericardial tissues were divided into four groups according to the methods of treatment. Group 1 consisted of tissues fixed with 0.5% GA (control), group 2 fixed with 0.5% GA and post-treated with glycine, group 3 fixed with 2% GA in organic solvent (65% ethanol + 5% octanol), and group 4 fixed with 2% GA in organic solvent and post-treated with glycine. The material characteristics of the treated tissues were assessed by amino acid analysis, thermal stability test, uniaxial mechanical test and light microscopy. The tissues were subcutaneously implanted into 4-week-old rats for 8 weeks, and the calcium contents of the explanted tissues were measured. **Results:** Differently treated tissues resulted in no significant alterations in material characteristics and morphology as assessed by amino acid analysis, thermal stability test, uniaxial mechanical test, and light microscopy. Median calcium contents of groups 1, 2, 3, and 4 were 80.5 $\mu g\ mg^{-1}$, 1.0 $\mu g\ mg^{-1}$, 0.5 $\mu g\ mg^{-1}$ and 1.7 $\mu g\ mg^{-1}$, respectively. The calcium contents of groups 2, 3 and 4 were all significantly lower than that of group 1 ($p < 0.05$). **Conclusions:** Post-fixation treatment with glycine, high-concentration GA fixation in organic solvent and combined treatment of these all strongly prevented calcification of GA-fixed bovine pericardium in rat subcutaneous implantation model.

Keywords: Glutaraldehyde; Pericardium; Calcification

1. Introduction

Bioprostheses are widely used for a variety of cardiovascular applications such as heart-valve substitutes and patch materials. Bioprosthetic tissues are conventionally cross-linked with glutaraldehyde (GA) to impart tissue stability, reduce antigenicity and to maintain tissue sterility. However, GA-fixed bioprostheses are prone to calcification after long-term implantation in humans, and this is one of the limiting factors in the longevity of bioprostheses. The mechanism of calcification of GA-fixed bioprosthetic tissue is complex, but there are evidences that tissue phospholipids, free aldehyde groups of GA, and residual antigenicity of the bioprosthetic tissue, all play an important role [1–5]. Phosphorus, largely in the form of cell membrane-associated phospholipids, can bind calcium and serve as nucleators of calcium phosphate crystal formation. Residual unbound aldehyde groups of GA can trap host plasma calcium contributing to tissue calcification. Circulating heterograft-specific antibodies can contribute to tissue calcification by immune reaction. Thus, various methods to remove tissue phospholipids or free aldehyde groups and to reduce the antigenicity of bioprosthetic tissue have been investigated to prevent the calcification of bioprostheses. Ethanol or other alcohol solutions were used to extract tissue phospholipids [6,7], and various amino acids or other amino compounds were used to remove the free aldehyde groups of GA [8–16]. Modifications of fixation method using high-concentration GA proved to be effective in the prevention of calcification, presumably by suppressing residual antigenicity of bioprosthetic tissue [8,17–19]. The objective of this study was to

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evaluate the anticalcification effect of glycine treatment and high-concentration GA fixation in organic solvent (mixture of ethanol and octanol) on GA-fixed bovine pericardium, and to evaluate the possible synergistic effect of combined treatment using rat subcutaneous implantation model.

2. Materials and methods

2.1. Experimental design

Bovine pericardial tissues were divided into four groups according to the method of tissue preparation. Group 1 consisted of tissues fixed with 0.5% GA, group 2 consisted of tissues fixed with 0.5% GA and post-treated with glycine, group 3 consisted of tissues initially fixed with 0.5% GA and additionally fixed with 2% GA in organic solvent (65% ethanol + 5% octanol), and group 4 consisted of tissues initially fixed with 0.5% GA, additionally fixed with 2% GA in organic solvent and post-treated with glycine. Assessments of material characteristics (amino acid analysis, thermal stability test and uniaxial mechanical test) were performed for fresh bovine pericardium and groups 1–4, and rat subcutaneous implantations were performed for groups 1–4.

2.2. Tissue preparation

Fresh bovine pericardium was obtained from the local slaughterhouse, placed in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and immediately transported to our laboratory. On arrival, they were rinsed with normal saline and were freed from adherent fat. In group 1, tissues were fixed in PBS-buffered 0.5% GA solution (pH 7.4) at room temperature for 14 days. In group 2, tissues were fixed in 0.5% GA solution for 14 days. After fixation, tissues were treated in acetic acid-buffered 0.1 M glycine solution (pH 4.5) at 37 °C for 48 h. After glycine treatment, tissues were treated in PBS-buffered 0.1 M $NaBH_4$ solution (pH 7.4) at room temperature for 24 h. In group 3, tissues were initially fixed in 0.5% GA solution 11 days. After initial fixation, tissues were placed in 2% GA solution in organic solvent (mixture of 65% ethanol and 5% 1-octanol) and were kept on a shaker bath at room temperature for 3 days. In group 4, tissues were fixed by the method used for group 3. After completion of fixation, tissues were treated with glycine by the method used for group 2. Bovine pericardial tissues for each analysis were obtained from the same animal (no differences between experimental groups) to avoid clustering effect.

2.3. Microscopic examination (preimplantation)

Representative tissue samples from each group were examined with light microscopy. Tissue samples were fixed in 10% formalin, embedded in paraffin wax and 2–4 μm-thick sections were stained with hematoxylin–eosin (H–E).

2.4. Amino acid analysis

Prepared tissue samples and fresh bovine pericardial tissues (five 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. Amino acid content of the hydrolysate was measured by liquid chromatography/mass spectrometry (LC/MS, Waters, USA).

2.5. 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 × 30 mm, 15 strips for fresh pericardium and five strips for groups 1–4) 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.

2.6. Mechanical test

Uniaxial test was used to compare the mechanical properties of differently treated tissues. Tissue strips (5 × 50 mm, 30 strips for fresh bovine pericardium and each experimental group) were cut in different directions to overcome material anisotropy. Tissue thickness was measured at three points 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 (DS2-50N, IMADA, Japan) operating at an extension rate of 100 mm min^{-1} with a pre-load of 0.01 N. Tensile strength and elongation at break were evaluated from the recorded load–elongation curves.

2.7. Rat subcutaneous implantation

This study was approved by Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No. 08-0196). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Female Sprague-Dawley rats (4 weeks old, 76–93 g) were used. After anesthetizing and shaving, four subcutaneous pouches were created at the dorsal area for each rat. Pericardial samples (1 × 1 cm, eight samples for each group) were implanted into the pouches and the wounds were closed with 3/0 nylon sutures. The rats were sacrificed by CO_2 asphyxiation after 8 weeks. The tissue samples were harvested, freed of adherent rat 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.

2.8. Microscopic examinations (postimplantation)

Representative tissue samples from each group were examined with light and transmission electron microscopy (TEM). For light microscopy, harvested tissue samples were fixed in 10% formalin, embedded in paraffin wax and 2–4 μm-thick sections were stained with H–E and von Kossa. For TEM,

small pieces were fixed in 2.5% PBS-buffered GA, postfixed in 1% osmium tetroxide (OsO_4) and embedded in Epon. Semithin sections were stained with toluidine blue and observed under the light microscope. Ultrathin sections of selected fields were stained with tannic acid and uranyl acetate and examined with JEM-1400 (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.9. 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-cresolphthalein complexone method, as previously described [20], using an automatic chemistry analyzer (Hitachi 7070, Japan). Calcium contents were expressed as $\mu m\ mg^{-1}$ dry weight.

2.10. Statistical analysis

Data were expressed as mean ± standard deviation (amino acid analysis and mechanical test) or median with interquartile range (thermal stability test and calcium analysis because of small sample sizes) as appropriate. Comparison between groups was performed using the Kruskal–Wallis and Mann–Whitney tests (thermal stability test and calcium analysis because of small sample sizes) or one-way analysis of variance (ANOVA) with *post hoc* test (mechanical test). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Microscopic examination (preimplantation)

In all groups, light microscopy showed optimal preservation of collagen fibers that had multidirectional orientations and regular periodicity (Fig. 1).

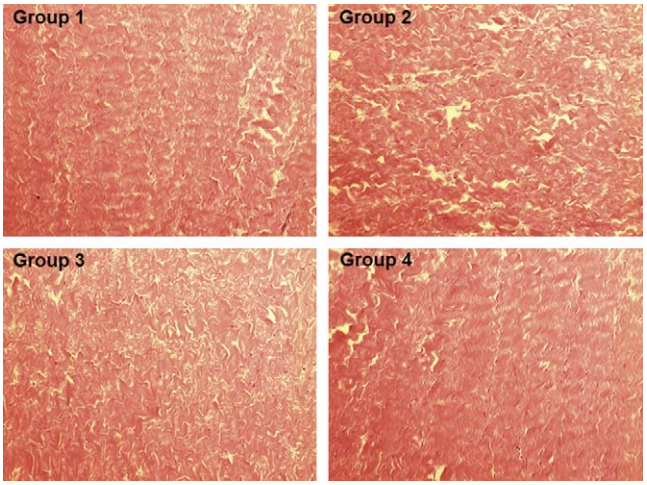

Fig. 1. Light microscopy of unimplanted bovine pericardium (H–E stain, original magnification ×100). Collagen fibers appear well preserved with a normally banded structure in all groups.

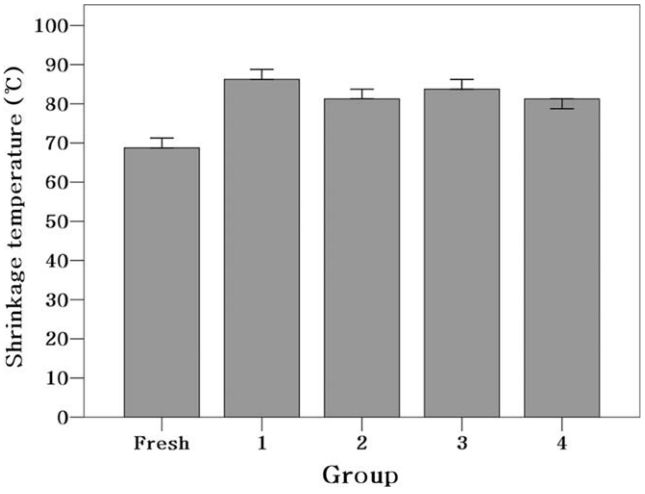

Fig. 2. Shrinkage temperatures of fresh and differently treated bovine pericardial tissues. All data represent median with 95% confidence interval.

3.2. Amino acid analysis

Amino acid analysis of differently treated bovine pericardium showed no significant alteration in amino acid composition, as assessed by percent distribution of major amino acids (Table 1). A significant decrease in lysine and histidine contents of the GA-fixed pericardium (groups 1–4) compared with that of fresh pericardium was noted, and this was due to the stable bonds, which occurred between these amino acids and aldehyde groups of GA.

3.3. Thermal stability test

Shrinkage temperatures for fresh pericardium and groups 1–4 were 68.8 °C (68.8–71.3 °C), 86.3 °C (86.3–87.5 °C), 81.3 °C (81.3–82.5 °C), 83.8 °C (83.8–86.3 °C) and 81.3 °C (80.0–81.3 °C), respectively (Fig. 2). Shrinkage temperatures of groups 1–4 were all significantly higher that that of fresh pericardium ($p < 0.05$). There was no significant difference in shrinkage temperature between differently treated pericardial tissues (groups 1–4).

Table 1. Amino acid analysis of fresh and differently treated bovine pericardium.

Amino acids*	Fresh	Group 1	Group 2	Group 3	Group 4
Glycine	34.9 ± 4.0	46.7 ± 5.5	40.2 ± 2.4	38.6 ± 0.9	38.3 ± 2.3
Alanine	11.4 ± 1.4	13.8 ± 1.4	14.6 ± 0.6	14.3 ± 1.0	14.5 ± 0.9
Proline	11.4 ± 0.7	2.4 ± 0.6	4.9 ± 0.5	5.5 ± 0.3	5.7 ± 0.4
Hydroxy-proline	5.1 ± 0.3	9.3 ± 2.1	10.5 ± 0.8	10.9 ± 0.8	11.9 ± 1.0
Lysine	2.7 ± 0.3	0.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
Histidine	2.8 ± 0.3	1.4 ± 0.5	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.1

* Percent residues. All data represent mean ± standard deviation.

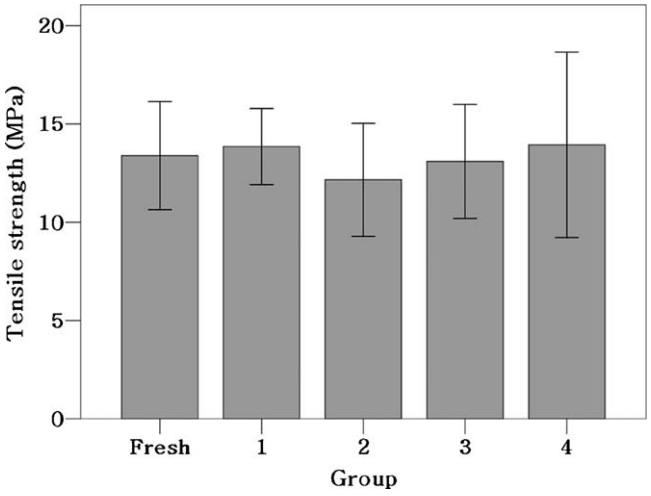

Fig. 3. Tensile strength of fresh and differently treated bovine pericardium. All data represent mean ± standard deviation.

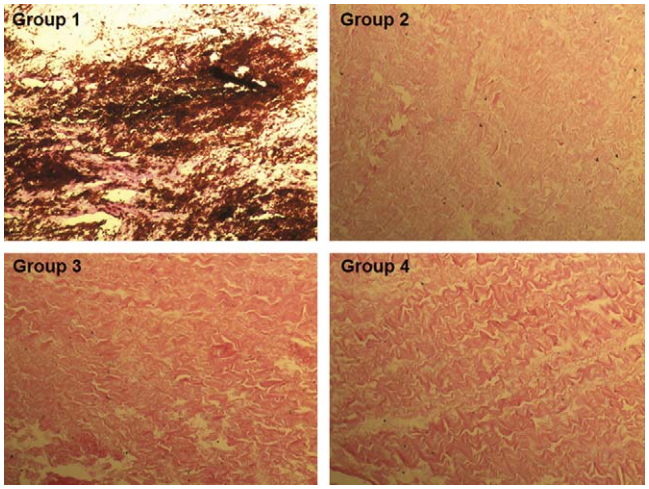

Fig. 5. Light microscopy of harvested bovine pericardium (von Kossa stain, original magnification ×100). In group 1, heavy calcific deposits (dark brown color) were observed throughout the entire field, whereas rare calcific deposits were observed in other groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Mechanical test

Mean tensile strength for fresh bovine pericardium was 13.4 ± 2.8 MPa. Mean tensile strengths for groups 1, 2, 3, and 4 were 13.9 ± 1.9 MPa, 12.2 ± 2.9 MPa, 13.1 ± 2.9 MPa, and 13.9 ± 4.7 MPa, respectively (Fig. 3). There was no significant difference in tensile strengths between fresh bovine pericardium and differently treated pericardial tissues ($p = 0.19$). Mean elongation at break for fresh bovine pericardium was 15.6 ± 3.2%. Mean elongations at break for groups 1, 2, 3, and 4 were 24.1 ± 5.4%, 31.5 ± 11.0%, 30.3 ± 5.6%, and 26.2 ± 5.9%, respectively (Fig. 4). Mean elongation at break for fresh bovine pericardium was significantly smaller than those for groups 1–4 ($p < 0.05$).

3.5. Microscopic examination (postimplantation)

In all groups, light microscopy showed preservation of collagen fibers. In group 1, heavy calcific deposits (dark brown color) were observed throughout the entire field, whereas rare calcific deposits were observed in other groups (von Kossa stain, Fig. 5). TEM also revealed well-preserved collagen fibers in all groups. In group 1, calcific deposits were observed upon cell-membrane fragments (Fig. 6(a)). In groups 3 and 4, devitalized connective-tissue cells with loss of membrane integrity were observed, probably resulting from the lipid extraction effect of organic solvent (Fig. 6(b)).

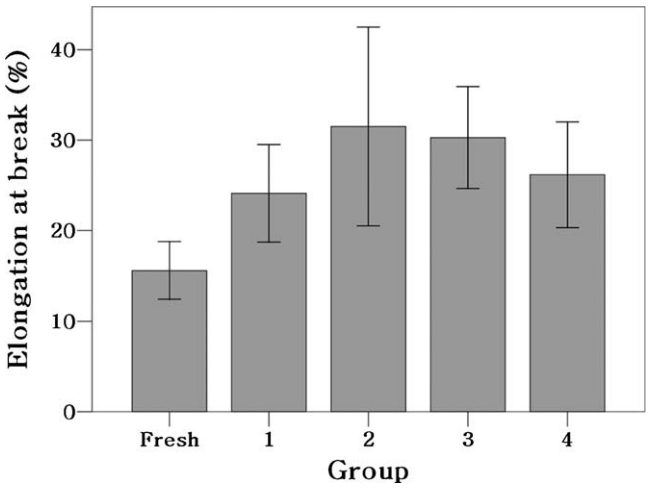

Fig. 4. Elongation at break of fresh and differently treated bovine pericardium. All data represent mean ± standard deviation.

3.6. Calcium analysis

Calcium contents of groups 1, 2, 3, and 4 were 80.5 μm mg^{-1} (70.1–91.3 μm mg^{-1}), 1.0 μm mg^{-1} (0.6–1.3 μm mg^{-1}), 0.5 μm mg^{-1} (0.5–0.9 μm mg^{-1}) and 1.7 μm mg^{-1} (1.1–2.1 μm mg^{-1}), respectively (Fig. 7). Calcium contents of groups 2, 3, and 4 were all significantly lower than that of group 1 ($p < 0.05$). Calcium content of group 4 was not significantly different from that of group 2 ($p = 0.059$). Calcium content of group 4 was significantly higher than that of group 3 ($p = 0.002$). Therefore, we failed to prove the synergistic effect of combined treatment.

4. Discussion

GA-fixed bioprostheses fabricated from either porcine aortic valve or bovine pericardium are widely used in cardiovascular surgery. These bioprostheses ultimately fail, especially rapidly in young children, due to dystrophic calcification. In an effort to prevent or delay the bioprosthetic calcification, various anticalcification strategies have been investigated, and are still being developed. This is due to, in large part, the complex, multifactorial and incompletely understood mechanism of the bioprostheses calcification. There are evidences that tissue phospholipids, free aldehyde groups of GA, and residual antigenicity of the bioprosthetic tissue play an important role [1–5].

In this study, we demonstrated the anticalcification efficacy of glycine treatment and high-concentration GA

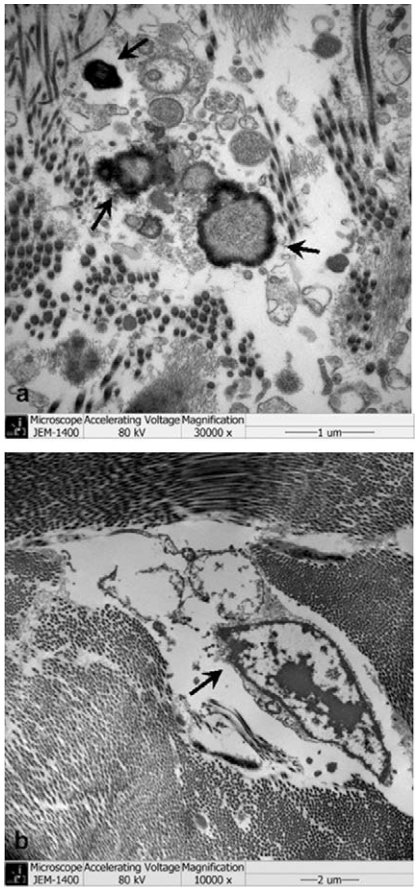

Fig. 6. Transmission electron microscopy of harvested bovine pericardium: (a) tissue from group 1 showing calcific deposits (arrows) upon cell-membrane fragments (original magnification ×30,000), (b) tissue from group 3 showing devitalized connective-tissue cell (arrow) with loss of membrane integrity (original magnification ×10,000).

fixation in organic solvent on GA-fixed bovine pericardium in a subcutaneous rat model.

Residual free aldehyde groups or polymerized forms of GA, which are used in the fixation of bioprosthetic tissues, are cytotoxic and are known to result in tissue calcification [9,10]. To block these detrimental effects of the free aldehyde group, various amino acids or other amino compounds were used for post-fixation treatment of GA-fixed bioprosthetic tissue. The amino groups of these compounds can react with the free aldehyde groups of GA, forming Schiff base. Detoxification of the GA-fixed porcine aortic root using urazole was effective in reducing tissue calcification in sheep model [8]. Detoxification of GA-fixed bovine pericardium using homocysteic acid was effective in both reducing tissue calcification in a rat model and reducing *in vitro* cytotoxicity [9,10]. L-Glutamic acid treatment of GA-fixed bovine pericardium was effective in both reducing tissue calcification in rat model and reducing *in vivo* cytotoxicity [13,15]. L-Arginine treatment of GA-fixed bovine pericardium was effective in reducing tissue calcification in a rat model [16]. All amino compounds do not equally prevent calcification of GA-fixed bioprosthetic tissue. Gentamicin and albumin, large molecules containing more than one amino group, did not prevent calcification of GA-fixed bovine pericardium in a rat model [21]. L-Glutamic acid and L-arginine, which were reported to be effective in the prevention of calcification, were not effective in the prevention of calcification in another report [11]. Why certain amino compounds are not effective in preventing calcification and why some amino compounds have contradictory anticalcification efficacy are not clear. Possibly, molecular structure of an amino compound, treatment conditions such as concentration, pH, and reaction time will all influence the anticalcification efficacy of a specific treatment. In our study, we used glycine, the simplest form of all amino acids, for detoxification of GA-fixed bovine pericardium. We used acidic condition and warm temperature for optimal detoxification [14]. Post-treatment with glycine did not alter the microscopic structure, the degree of cross-linking as assessed by thermal stability test, and the degree of tissue strength as assessed by uniaxial mechanical test. *In vivo* calcification test using rat subcutaneous model demonstrated almost complete inhibition of calcification, as assessed by calcium quantification and von Kossa stain, compared with conventional GA fixation without glycine treatment.

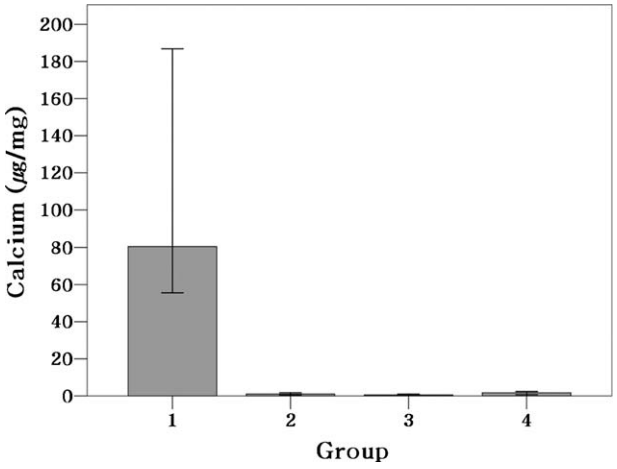

Fig. 7. Bar graph showing calcium contents of explanted bovine pericardium. Calcium contents of groups 2, 3, and 4 were all significantly lower than that of group 1 ($p < 0.05$). All data represent median with 95% confidence interval.

Phospholipids of bioprosthetic tissue can act as a substrate for calcification and various alcohol solutions were used to remove these calcifiable materials. Ethanol treatment of GA-fixed porcine aortic valve resulted in nearly complete extraction of phospholipid and inhibition of calcification in both rat subcutaneous and sheep circulatory model [6]. Inhibition of calcification was noted for ethanol treatment at concentrations of ≥50%. Treatment of GA-fixed bovine pericardium or porcine aortic valve with mixture of 40% ethanol and 5% 1,2-octanediol solution resulted in reduction of tissue phospholipid content and inhibition of calcification in rat subcutaneous model [7]. It was hypothesized that the removal of phospholipids using a combination of these short- and long-chain alcohols might reduce the calcification, given that long-chain alcohols are more structurally similar to phospholipids than short-chain alcohols and therefore might remove phospholipids more efficiently. We used a mixture of 65% ethanol and 5% 1-octanol solution for the extraction of tissue phospholipids based on the hypothesis described above. After initial fixation in 0.5% aqueous GA solution,

additional fixation using 2% GA in organic solvent (mixture of 65% ethanol and 5% 1-octanol) was performed. Cross-linking of bovine pericardium using GA in pure organic solvent environment produced optimal cross-linking and produced materials with tensile mechanical behavior that was very close to that of fresh pericardium, and this phenomenon was hypothesized to be the result of conformational changes in collagen facilitated by polar/non-polar interactions with the solvent that are locked in by the action of glutaraldehyde [22]. In our study, tensile strengths of differently treated bovine pericardial tissue (groups 1—4) were not significantly different from that of fresh pericardial tissue. However, the extensibility (elongation at break) of bovine pericardial tissue after GA fixation (groups 1—4) increased compared with that of fresh pericardial tissue, probably due to 'shrinkage' of tissue in GA. The elastin component of bioprosthetic tissue may undergo independent calcification irrespective of devitalized cell-mediated calcification observed in GA-fixed bioprosthetic tissue [23]. Little evidence for GA cross-linking of elastic tissue has been shown, and cross-linking of elastic tissue might be suspected to be difficult given the hydrophobic structure of elastin, which might inhibit penetration of GA. The possibility of effective cross-linking of the elastin component was suggested using GA fixation in a pure organic solvent environment, and this was hypothesized to be the result of conformational changes in elastin facilitated by polar/non-polar interactions with the solvent that are locked in by the action of glutaraldehyde [24]. Based on these observations and in a hope to improve anticalcification efficacy and mechanical properties, we used additional GA fixation in organic solvent. Instead of using pure organic solvent, we used 70% concentration of alcohols (65% ethanol and 5% octanol). This was because there was a report that GA fixation in a pure alcohol environment resulted in heavy calcification of porcine aortic valve in rat subcutaneous model [25]. Our *in vivo* calcification study clearly demonstrated excellent anticalcification efficacy of this treatment. As for the additional fixation using high-concentration (2%) GA, we intended to suppress residual antigenicity and obtain better cross-linking, which were thought to be important for anticalcification efficacy [8,17—19].

Considering the complex and multifactorial mechanism of calcification of GA-fixed bioprosthetic tissue, combining anticalcification treatments targeting different steps of calcification process may be beneficial through a synergistic effect. In our study, we combined high-concentration GA fixation in organic solvent and detoxification using glycine, and demonstrated its excellent anticalcification effect *in vivo*. Although we failed to prove the synergistic effect of this combined treatment compared with each treatment, this is possibly due to the very low levels of calcification observed after a short duration of implantation using a rat subcutaneous model. We speculate that the synergistic effect of combined treatment will prove to be beneficial in long-term implantation, and further studies using large-animal long-term circulatory implantation model to prove this hypothesis are warranted in the future.

In conclusion, post-fixation treatment with glycine, high-concentration GA fixation in organic solvent, and combined treatment all resulted in no significant alterations in bovine pericardial material characteristics and morphology as assessed by amino acid analysis, thermal stability test, uniaxial mechanical test, and light microscopy. These treatments all strongly prevented calcification of GA-fixed bovine pericardium in rat subcutaneous implantation model. These treatments may be used as an effective anticalcification treatment when manufacturing bioprosthetic heart valves using bovine pericardium. Further studies using large-animal long-term circulatory model are warranted.

5. Limitations of the study

Our experiment was a kind of pilot study for screening effective anticalcification methods and some sample sizes of our experiment were small. Further studies using large-animal long-term circulatory model to verify our results are mandatory.

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