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The Anti-calcification Effect of Dithiobispropionimidate, Carbodiimide and Ultraviolet Irradiation Cross-linking Compared to Glutaraldehyde in Rabbit Implantation Models

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Background: Glutaraldehyde (GA) is a widely used cross-linking agent for improving mechanical properties and resistance to enzymatic degradation of collagenous tissue, but it has several drawbacks such as calcification and cytotoxicity. The aim of this study was to find the alternative effective cross-linking methods to GA. ***Materials and Methods:*** Bovine pericardium was processed with GA with ethanol+octanol and glycine detoxification, and polyethylene glycol (PG) space filler, dimethyl 3,3'-dithiobispropionimidate (DTBP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) treatment, and the physical fixation of ultraviolet irradiation were done. The biologic material properties of variously treated pericardial tissues were assessed by biochemical, mechanical and histological tests. Treated pericardial tissues were also implanted subcutaneously or intramuscularly into the rabbit for 10 weeks to assess the xenoreactive antibody response of immunoglobulin G and M, their anti-calcification effect. ***Results:*** The biochemical and mechanical properties of EDC fixed pericardial tissues were comparable to the GA fixed tissue. The cytotoxicity was lowest in space filler treated GA fixed group. In rabbit subcutaneous or intramuscular implantation models, decellularization, space filler, EDC treatment group showed significantly lower calcium content than GA only and DTBP treatment group ($p < 0.05$, analysis of variance). The titer of anti Galα1-3Galβ1-4GlcNAc-R antibodies did not change in the postimplantation serial enzyme-linked immunosorbent assay. Hematoxylin and eosin and von Kossa staining showed that decellularization, space filler, EDC, and ultraviolet treatment had less inflammatory cell infiltration and calcium deposits. ***Conclusion:*** The decellularization process, PG filler, and EDC treatments are good alternative cross-linking methods compared to GA only fixation and primary amine of DTBP treatment for cardiovascular xenograft preservation in terms of the collagen cross-linking stability and in vivo anti-calcification effects.

Key words: 1. Pericardium
2. Bioprosthesis
3. Polyethylene glycols
4. Glutaraldehyde
5. Ultraviolet

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INTRODUCTION

Glutaraldehyde (GA) fixed collagenous tissue derived from bovine pericardium have been used for manufacturing bioprosthetic heart valves and for cardiovascular repair and reconstruction. GA fixation improves mechanical properties and resistance to enzymatic degradation of collagenous tissue, but has several drawbacks such as calcification and cytotoxicity. Among the drawbacks, calcification is one of the major causes of tissue failure. It is reported that calcification of GA-fixed collagenous tissue is induced by the phosphorus of cell membrane-associated phospholipids, the free aldehyde groups of GA, and the immune response of the host [1-6]. Several processing techniques and cross-linking methods to prevent calcification, other than GA, have been reported: 1) removal of cellular phospholipids using ethanol and sodium dodecyl sulfate (SDS) [4,7], 2) inhibition of calcification by combining free aldehyde groups of GA and amino acid such as glycine and L-glutamic acid [5,6], 3) removal of the cellular components involved in the immune response through decellularization (DC) [4], 4) filling in the void space with synthetic hydrogels formed by DC [8], and 5) use of cross-linking substances such as dimethyl 3,3'-dithiobispropionimidate (DTBP) [9,10], 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [11,12], and ultraviolet (UV) [11,12].

The aim of this study was to find alternative cross-linking methods to GA that are similarly effective in collagenous tissue fixation and in overcoming major drawbacks such as tissue calcification. This study was conducted to assess the usefulness of the primary amine of DTBP, the EDC among the functional protein groups compared with the effects of combining cross-linking substances, and the combined processing techniques including DC, polyethylene glycol (PG) space filling, glycine detoxification, and a mixture of ethanol and 1-octanol treatment.

New Zealand white (NZW) rabbit subcutaneous and intramuscular models were used to assess the effects of the various treatment methods in preventing calcification on bovine pericardium.

MATERIALS AND METHODS

1) Tissue preparation and decellularization

Fresh bovine pericardium obtained from a slaughterhouse were immersed in 4°C normal saline (0.9% w/v of NaCl) and quickly transported to the laboratory. After removing the fat tissue and damaged sites, the pericardial tissue was cut into a 10×10 cm piece and washed with normal saline. In order to achieve disinfection, all of the cut tissues were treated with distilled water containing 0.1% peracetic acid and 4% ethanol for 2 hours at room temperature (RT) and washed with normal saline for 1 hour at RT.

DC was conducted in four consecutive steps: 1) treatment with a hypotonic solution (0.01 M Tris-hydrochloric acids [HCl], pH 8.0) containing 0.25% SDS for 24 hours at 4°C; 2) a hypotonic solution (0.01 M Tris-HCl, pH 8.0; 0.05% ethylenediaminetetraacetic acid [EDTA]; 10 KIU/mL aprotinin; 50 mg/L neomycin) containing 0.5% triton X-100 for 24 hours at 4°C; 3) an isotonic solution (0.05 M Tris-HCl, pH 8.0; 0.15 M NaCl; 0.05% EDTA; 10 KIU/mL aprotinin; 50 mg/L neomycin) for 24 hours at 4°C; and 4) a hypertonic solution (0.2 M Tris-HCl, pH 8.0; 0.6 M NaCl) for 4 hours at 4°C. The decellularized tissues were then washed with normal saline for 1 hour at 4°C. Between each step the tissue was washed with distilled water. All of the DC steps were carried out under continuous shaking at 200 rpm.

2) Tissue processing methods

The tissues were divided into six groups according to the processing method. All of the groups except the GA group were treated by DC before cross-linking.

(1) The glutaraldehyde group (control group): The pericardial tissues were fixed with a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (Hepes, 0.05 M; pH 7.4) containing 0.5% GA for 2 days at RT, and then treated with a mixture of 75% ethanol and 5% 1-octanol containing 0.25% GA for 2 days at RT, and additionally fixed with a Hepes buffer solution containing 0.25% GA for 5 days at RT. After fixation, the pericardial tissues were detoxified in phosphate buffered saline (PBS; 0.01 M, pH 7.4) containing 0.1 M glycine for 1 day at RT.

(2) The decellularization+glutaraldehyde group: After

the DC process, the pericardial tissues were fixed using the same fixation process as the GA group.

(3) The decellularization+polyethylene glycol+glutaraldehyde group: During the DC process, pericardial the tissues were immersed in PBS containing space filler of 50% PG for 1 day at 4°C. After the DC process with space filler, the pericardial tissues were fixed using the same fixation process as the GA group.

(4) The decellularization+dithiobispropionimidate group: After the DC process, the pericardial tissues were fixed with borate buffered saline (0.025 M, pH 9.0) containing 0.5% DTBP for 2 days at RT, and then treated with a mixture of 75% ethanol and 5% 1-octanol for 2 days at RT.

(5) The decellularization+1-ethyl-3-(3-dimethylaminopropyl) carbodiimide group: After the DC process, the pericardial tissues were fixed with 2-(N-morpholino) ethanesulfonic acid buffer solution (0.05 M, pH 5.5) containing EDC (0.05 M) and N-hydroxysuccinimide (NHS, 0.01 M) for 2 days at RT, and then treated with a mixture of 75% ethanol and 5% 1-octanol for 2 days at RT.

(6) The decellularization+ultraviolet group: After the DC process, pericardial the tissues were irradiated using two 15 W UV (254 nm) lamps in a custom-made UV chamber for 24 hours at 4°C. The distance between the UV lamps and the pericardial tissues was 10 cm apart, and the UV-fixed pericardial tissues were then treated with a mixture of 75% ethanol and 5% 1-octanol for 2 days at RT.

3) Quantification of DNA

To verify the efficacy of the DC process, the nuclear acids were isolated and purified from untreated and decellularized bovine pericardium. The lyophilized pericardial tissues were mechanically disrupted using a homogenizer in PBS and by adding lysis buffer and proteinase K, and then incubated at 55°C until completely lysed. Saturated NaCl was added to the lysate and was centrifuged. Supernatant and isopropanol were mixed by inversion, and then it was centrifuged. The pellet was washed with 70% ethanol and centrifuged, and the supernatant was then discarded, dried in air, and dissolved in 0.01 M Tris containing RNase. After overnight incubation at 4°C, the DNA contents were measured using a nanodrop spectrophotometer (ND-1000; Thermo Scientific Inc., Wilmington, DE, USA) at an optical density (OD) of 260 nm. The DNA content was expressed as ng of DNA per mg of dry weight.

4) Shrinkage temperature

Shrinkage temperature was measured using a custom-made apparatus. The tissue samples were cut to an 8×40 mm size and were mounted between the two clips. The distance between the two clips was 30 mm. One of the clips was loaded with 95 g weights to continually provide tension on the strip. The tissue strips were immersed in a bath that contained deionized water. The bath was heated at a rate of 2°C/min from 40°C to 100°C. The temperature at which shrinkage occurred was measured using a digital thermometer.

5) Resistance to enzymatic degradation

The resistance to enzymatic degradation of tissue was performed using pronase. The tissue samples were cut into 1×1 cm sizes, dried for 24 hours at 80°C, and weighed (dry weight 1). The dried tissues were incubated in pronase solution (0.1 M Tris-HCl, pH 7.5; 0.5 mg/mL pronase; 0.01 M $CaCl_2$) for 24 hours at 50°C. After incubation, the tissue samples were washed with deionized water, dried, and weighed (dry weight 2). The resistance to enzymatic degradation was expressed as a percentage of remaining tissue (%=dry weight 2/dry weight 1×100).

6) Tensile strength test

The tissue samples (6×6 cm) were cut into a rectangular shape of 5×25 mm at a different angle of 30° to overcome the pericardial tissue anisotropy of the collagen fiber orientation. After measuring the thickness of the tissue center, the sample strips were mounted between the sandpaper attached to the stainless grips to prevent slipping. The tensile strength was measured using a tensile testing machine (Push and Pull Electronic Stand, K-ML-1000N; M-Tech, Bucheon, Korea) equipped with a digital force gauge (DS2-200N; Imada Inc., Tokyo, Japan). The gauge length was 10 mm and the preload was set at 0.0 N. The tissue strips were pulled at an extension rate of 100 mm/min, and the slipped or fractured gripping sites during the test were excluded. The peak load, ultimate tensile strength (UTS), and elonga-

tion at UTS were calculated from the recorded stress-strain curves.

7) Contact cytotoxicity assay

The cultured porcine fibroblasts isolated from a porcine aorta were seeded into each well of 6-well cell culture plates. The cells (8−10 passage) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic antimycotic solution. The cell culture was performed with incubation at 37°C and 5% CO_2 in the air until 70% to 80% confluency was achieved. After replacing the DMEM, the tissue sample discs (diameter 20 mm) were directly attached to the center of each well. The dimethyl sulfoxide (DMSO)-treated DMEM and DMEM alone were used as the positive and negative control, respectively. The tissue-attached plates were incubated for a further 48 hours at 37°C with 5% CO_2 in the air. The morphology and confluency of the cells were examined using phase contrast microscopy. After tissue separation, the cells were stained using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen; BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions and then analyzed using flow cytometry (BD FACSCalibur, BD Biosciences) within 1 hour.

8) Rabbit subcutaneous and intramuscular implantation

This experiment was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital Biomedical Research Institute (no. 11-0047). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Twelve 6-week-old male NZW rabbits (1,405.6±141.4 g) were used. The NZW rabbits were anesthetized using a mixture of 15 mg/kg of Zoletil 50 (tiletamine＋zolazepam) and 5 mg/kg of xylazine. The pericardial tissue discs were washed with normal saline before implantation for 1 hour to remove the residual toxic effect. After shaving and disinfecting the hind legs of the rabbits, the five-subcutaneous pouches were made in one leg at the femoral region and the tissue sample discs (diameter 10 mm) were implanted into the pouches, aseptically. Another batch of five identically treated tissue samples was implanted in the posterior femoral muscle layers of the other leg site. The skin incisions were closed with 4-0 nylon sutures. The NZW rabbits were humanely euthanized 10 weeks after implantation to obtain the tissue samples. The explanted pericardial tissue discs were studied for staining (hematoxylin and eosin [H&E] and von Kossa), calcium (CA), and inorganic phosphorus (IP) analysis.

9) Enzyme-linked immunosorbent assay

To determine the activity of the immunoglobulin G (IgG), immunoglobulin M (IgM) isotype of the anti Galα1-3Galβ1-4GlcNAc-R (α-Gal) antibodies, the blood samples were taken from the central ear artery of the rabbit on preimplantation. Enzyme-linked immunosorbent assay (ELISA) and the 14th, 28th, 56th, and 70th postimplantation time points were performed. The 96-well plates were coated with α-Gal conjugated to bovine serum albumin (α-Gal-BSA; Dextra Ltd., Reading, UK) in PBS at a concentration of 0.5 μg per well, and were incubated overnight at 4°C. The wells were blocked with 1% BSA for 2 hours at RT. The sera of the rabbits were serially diluted to concentrations of 1:20 and 1:40, were added to plates at 100 μL per well, and were incubated overnight at 4°C. Peroxidase-conjugated donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) were used as a secondary antibody at 1:20,000 dilutions, and were incubated for 1 hour at RT. Substrates of 50 μL of 3,3,',5,5'-tetramethylbenzidine were added for color reaction. The color development was stopped using 50 μL of 2 N sulfuric acid. The OD was measured at 450 nm using an ELISA microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

10) Histology

The preimplantation pericardial tissue samples were fixed in 10% neutral buffered formalin for 24 hours, dehydrated, and embedded in paraffin. Sections, 4 μm thick, were stained with H&E for identification of cell removal and collagen fiber structure to compare with post-implant. The explanted pericardial tissue discs were cut in half, and the one half was fixed, dehydrated, embedded, and stained with H&E using the von Kossa method for identification of inflammatory-cell infiltration and calcium deposits.

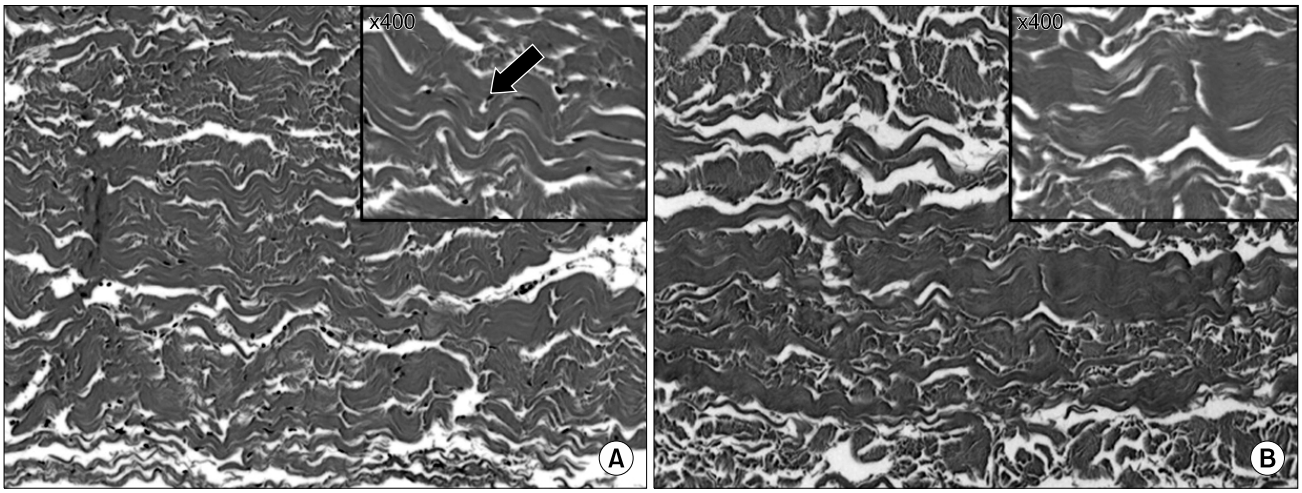

Fig. 1. Verification of decellularization by hematoxylin and eosin staining (×100). (A) Untreated bovine pericardium; the black arrow indicates the nuclei. (B) Decellularized bovine pericardium; the nuclei is invisible.

11) Quantification of calcium and inorganic phosphorus

The explanted pericardial discs (n=10 per group) were washed with normal saline, dried for 24 hours at 80°C, and weighed. The dried tissues were dissolved in 5 N HCl for 24 hours at 80°C, the HCl was evaporated, and the precipitate was dissolved in deionized water. The CA and IP contents were measured using a chemistry analyzer (Hitachi 7070; Hitachi Ltd., Tokyo, Japan). The CA and IP contents were expressed as μg of CA and IP per mg of dry weight.

12) Statistical analysis

Statistical analyses were performed using statistical software IBM SPSS ver. 19.0 (IBM Co., Armonk, NY, USA). The results were presented as mean±standard deviation). The comparison of averages between each group was carried out using one-way analysis of variance (ANOVA) and the *post hoc* test (Scheffe, Dunnett's T3) or the Kruskal-Wallis test. A p-value of <0.05 was considered statistically significant.

RESULTS

1) Decellularization

The bovine pericardium was decellularized using detergents (SDS and Triton X-100) and osmotic shock (hypotonic and hypertonic solution). To validate the DC, untreated and decellularized bovine pericardium was stained with H&E and underwent DNA quantitative analysis. Based on the histological study of the H&E staining, the nuclei were removed completely in the decellularized bovine pericardium (Fig. 1). The DNA contents of the decellularized bovine pericardium showed a 95.6% reduction compared with the untreated bovine pericardium (Fig. 2).

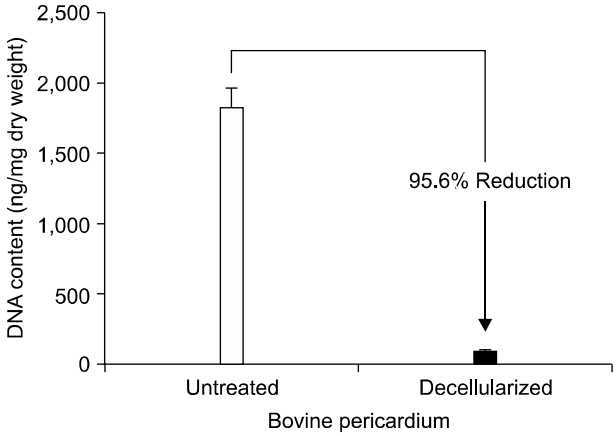

Fig. 2. DNA contents in untreated (1,820.05±143.47 ng/mg) and decellularized (79.94±16.09 ng/mg) bovine pericardium.

2) Cross-linking efficiency

The shrinkage temperature and resistance to enzymatic degradation were used to assess the degree of cross-linking, and their results are shown in Table 1. The shrinkage temperature

Table 1. Shrinkage temperature and resistance to enzymatic degradation with pronase of the untreated and treated bovine pericardium

Group	Shrinkage temperature[a] (n=15)	Enzymatic degradation[b] (n=20)
Untreated	70.32±0.78[c]	7.12±3.75[c]
GA	87.01±0.59[d]	89.53±2.53[d]
DC+GA	84.45±0.30[c],[d]	84.45±2.32[c],[d]
DC+PG+GA	86.18±0.07[c],[d]	86.85±2.18[c],[d]
DC+DTBP	76.28±0.28[c],[d]	73.45±6.47[c],[d]
DC+EDC	83.33±0.71[c],[d]	83.15±2.66[c],[d]
DC+UV	69.99±1.47[c]	13.65±8.81[c]

Values are presented as mean±standard deviation. GA, treat with glutaraldehyde, ethanol+1-octanol and glycine; DC+GA, decellularize and treat with glutaraldehyde, ethanol+1-octanol, and glycine; DC+PG+GA, decellularize and treat with polyethylene glycol, glutaraldehyde, ethanol+1-octanol, and glycine; DC+DTBP, decellularize and treat with dimethyl 3,3'-dithiobispropionimidate and ethanol+1-octanol; DC+EDC, decellularize and treat with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide+N-hydroxysuccinimide and ethanol+1-octanol; DC+UV, decellularize, irradiate using ultraviolet and treat with ethanol+1-octanol.
GA, glutaraldehyde; DC, decellularization; PG, polyethylene glycol; DTBP, dithiobispropionimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; UV, ultraviolet.
[a]Temperature (°C).
[b]Remaining tissue (%).
[c]$p<0.05$ vs. the GA group.
[d]$p<0.001$ vs. the untreated bovine pericardium.

and resistance to pronase digestion of all the treated groups, except the DC+UV group, were significantly higher than that of the untreated bovine pericardium ($p<0.001$, ANOVA, Kruskal-Wallis test); the GA control group was the highest ($p<0.05$, ANOVA, Kruskal-Wallis test) among the primary amine cross-linking methods.

3) Mechanical properties

The mechanical properties of untreated and treated bovine pericardium are expressed as cross-sectional area (mm^2), peak load (N), UTS (MPa), and elongation at UTS (%). The measured mechanical properties of each group are shown in Table 2. The cross-sectional area was multiplied by the width (5 mm). Bovine pericardium with a thickness of between 0.25 to 0.40 mm was used before cross-linking for the tensile strength test. The space filler treated group was significantly thicker than the untreated bovine pericardium and GA control group ($p<0.05$, ANOVA, Kruskal-Wallis test). The thickness of the DTBP, EDC, and UV treated groups, except the GA-fixed group, were significantly thinner than that of the untreated bovine pericardium ($p<0.05$, ANOVA, Kruskal-Wallis test). The cross-sectional area was widest in the space filler treated group and smallest in the UV treated group ($p<0.05$, ANOVA). There was no significant difference in the peak load, but the UV treated group showed a significantly smaller peak load than the untreated bovine pericardium and GA control group ($p<0.001$, ANOVA, Kruskal-Wallis test). In elongation at UTS, the GA treated groups were significantly more extensible than that of the untreated bovine pericardium ($p<0.05$, ANOVA, Kruskal-Wallis test).

4) Cytotoxicity

The contact cytotoxicity assay for the porcine fibroblast of the various treated bovine pericardium is shown in Fig. 3. No morphological change or lysis was observed in the cells around the tissue samples. The condition of the cells was not different than the negative control (DMEM alone). However, the positive controls (DMSO treated) induced cell death. To measure the percentage of living cells, the cells were assayed using flow cytometry. In the positive control and negative control, 3.92% and 95.5% of the cells survived, respectively. All of the groups were toxic to the cells compared to the negative control. Cytotoxicity was lowest in the DC+PG+GA group (89.38% of the cells survived), and was highest in the GA control group (76.06% of the cells survived).

5) Enzyme-linked immunosorbent assay

The titers for anti-α-Gal IgM, IgG of the rabbit sera did not change significantly according to the duration of implantation (Table 3) in all of the groups with or without DC.

6) Histological studies and quantitation of calcification

The pericardial tissue discs were explanted from the rabbits after 10 weeks and were analyzed using H&E and von Kossa staining along with CA and IP quantitative analysis. The H&E and von Kossa staining results of the explanted tissues

Table 2. Mechanical properties of the untreated and treated bovine pericardium

Group	Cross-sectional area (mm^2)	Peak load (N)	UTS (MPa)	Elongation at UTS (%)
Untreated (n=40)	1.46±0.27	25.26±7.20	17.16±2.86	42.42±7.70[a)]
GA (n=24)	1.74±0.47	25.78±8.11	15.73±6.25	57.50±12.71[b)]
DC+GA (n=24)	1.43±0.28	24.15±9.17	16.82±4.67	52.10±12.01[b)]
DC+PG+GA (n=24)	2.09±0.28[a),b)]	26.43±8.90	12.85±4.39[b)]	52.79±12.92[b)]
DC+DTBP (n=24)	1.22±0.29[a),b)]	24.97±10.19	20.16±9.28	46.35±7.26[a)]
DC+EDC (n=24)	1.28±0.20[a),b)]	23.97±6.18	18.95±5.11	47.20±9.61[a)]
DC+UV (n=24)	0.69±0.12[a),b)]	14.44±6.62[c),d)]	21.34±9.07	38.89±18.29[a)]

Values are presented as mean±standard deviation. GA, treat with glutaraldehyde, ethanol+1-octanol and glycine; DC+GA, decellularize and treat with glutaraldehyde, ethanol+1-octanol, and glycine; DC+PG+GA, decellularize and treat with polyethylene glycol, glutaraldehyde, ethanol+1-octanol, and glycine; DC+DTBP, decellularize and treat with dimethyl 3,3'-dithiobispropionimidate and ethanol+1-octanol; DC+EDC, decellularize and treat with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide+N-hydroxysuccinimide and ethanol+1-octanol; DC+UV, decellularize, irradiate using ultraviolet and treat with ethanol+1-octanol.
UTS, ultimate tensile strength; GA, glutaraldehyde; DC, decellularization; PG, polyethylene glycol; DTBP, dithiobispropionimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; UV, ultraviolet.
[a)] $p < 0.05$ vs. the GA group.
[b)] $p < 0.05$ vs. the untreated bovine pericardium.
[c)] $p < 0.001$ vs. the untreated bovine pericardium.
[d)] $p < 0.001$ vs. the GA group.

are shown in Fig. 4C-I and Fig. 5, respectively. In the rabbit subcutaneous and intramuscular implantation models, the DC+PG+GA, DC+EDC, and DC+UV groups had less inflammatory-cell infiltration and calcific deposits than the GA, DC+GA, and DC+DTBP groups. The subcutaneous models showed less cell infiltration and calcific deposits than the intramuscular models. In the subcutaneous model, the DC+PG+GA and DC+EDC groups showed good preservation of the collagenous fibers (Fig. 4D, F), and the calcific deposits were invisible (Fig. 5B, C) while degeneration of the collagenous fibers and multiple scattered calcific deposits were observed in the intramuscular model (Figs. 4H, 5E). In the GA, DC+GA, and DC+DTBP groups, heavy calcific deposits were observed throughout all of the area (Fig. 5A, D), and collagenous fiber degeneration due to calcification was shown (Fig. 4C, G) in both the subcutaneous and intramuscular models. The collagen fibers of the DC+UV group were degraded in both the subcutaneous and intramuscular models (Fig. 4I), but the calcific deposits were invisible (Fig. 5F).

The CA and IP quantitative analysis of explanted the tissues are shown in Table 4. The CA and IP contents of the subcutaneous model were lower than that of the intramuscular model. In the subcutaneous model, the CA and IP contents of the DC+PG+GA, DC+EDC, and DC+UV groups were significantly lower than those of the GA, DC+GA, and DC+DTBP groups ($p < 0.05$, ANOVA). There was no significant difference in the CA and IP content between the DC+PG+GA, DC+EDC, and DC+UV groups ($p=0.932$ in CA, $p > 0.999$ in IP, ANOVA) or between the GA, DC+GA, and DC+DTBP groups ($p > 0.999$ in CA, $p=0.999$ in IP, ANOVA). In the intramuscular model, the CA contents of the DC+PG+GA, DC+EDC, and DC+UV treated groups were significantly lower than those of the GA, DC+GA, and DC+DTBP groups ($p < 0.05$, ANOVA). There was no significant difference in the CA contents between the DC+PG+GA, DC+EDC, and DC+UV groups ($p > 0.999$, ANOVA). The IP contents of the DC+EDC and DC+UV groups were significantly lower than those of the DC+GA and DC+DTBP groups ($p < 0.05$, ANOVA).

DISCUSSION

Many factors causing degeneration and calcification in GA fixed bioprosthetic collagenous tissue have been reported when implanted *in vivo*. This study investigated four general strategies for preventing calcification of bovine pericardium fixed with GA alone, and assessed the alternative cross-linking substances such as primary amine of the functional pro-

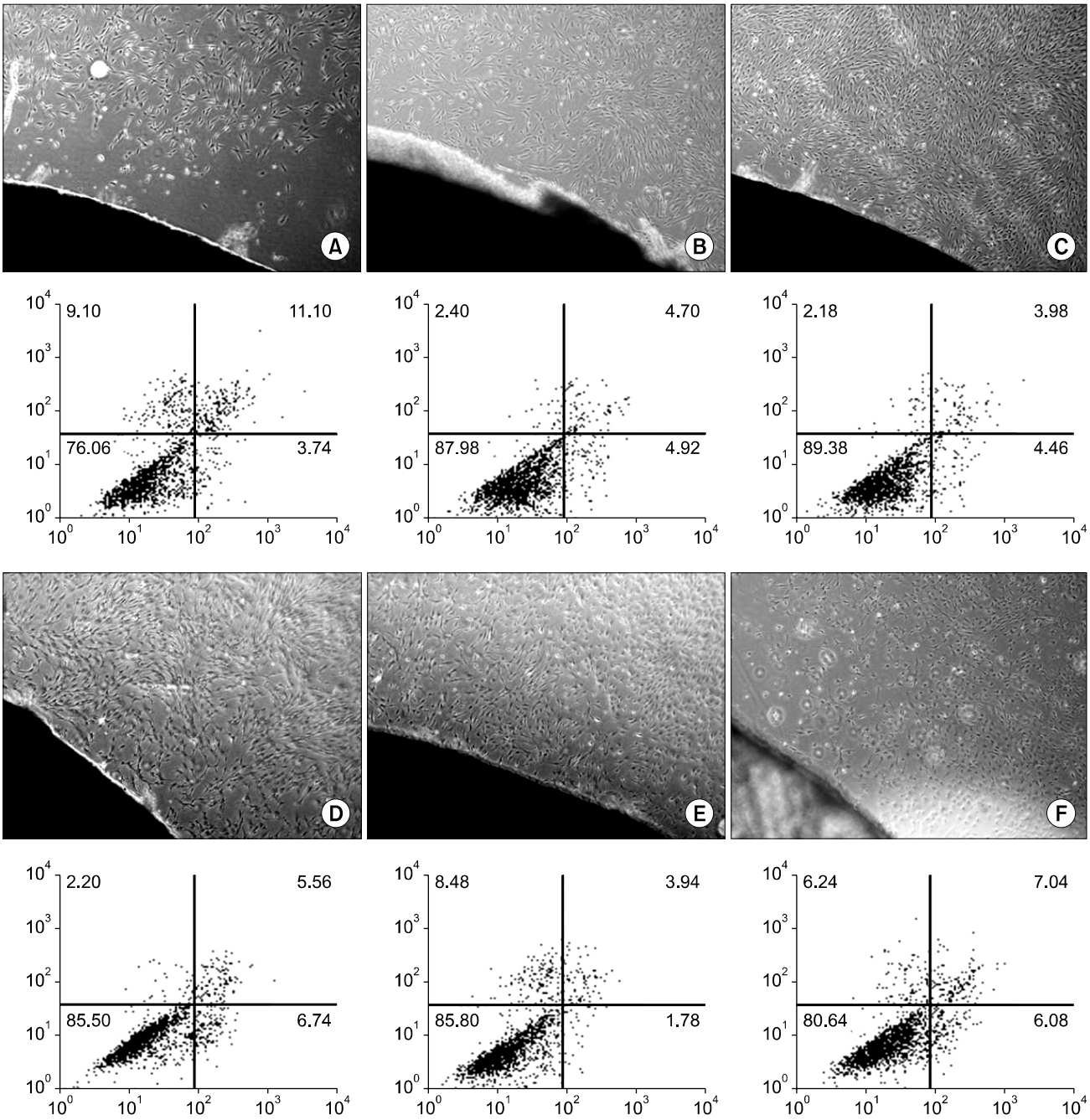

Fig. 3. Contact cytotoxicity assay for porcine fibroblast of treated bovine pericardium. The cells around the tissues samples are recorded as photomicrographs (original magnification, ×40) using phase contrast microscopy. (A) The GA group; (B) the DC+GA group; (C) the DC+PG+GA group; (D) the DC+DTBP group; (E) the DC+EDC group; (F) the DC+UV group. The graphs represent the cells that were analyzed using flow cytometry after fluorescein isothiocyanate (FITC) annexin V and PI staining. The x-axis and y-axis are FITC annexin V and PI, respectively. GA, glutaraldehyde; DC, decellularization; PG, polyethylene glycol; DTBP, dithiobispropionimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; UV, ultraviolet; PI, propidium iodide.

Table 3. The titer of the optical density level for anti-α-Gal immunoglobulin G of the rabbits in all of the groups

Group	Time interval change of the anti-α-Gal immunoglobulin G level					
	Dilution	Before	14 Days	28 Days	56 Days	70 Days
GA (n=2)	1:20	0.079	0.134	0.000	0.064	0.293
	1:40	0.059	0.053	0.000	0.012	0.037
DC+GA (n=2)	1:20	0.003	0.057	0.013	0.028	0.000
	1:40	0.010	0.000	0.034	0.042	0.039
DC+PG+GA (n=2)	1:20	0.031	0.008	0.006	0.015	0.015
	1:40	0.037	0.037	0.050	0.000	0.050
DC+DTBP (n=2)	1:20	0.025	0.075	0.081	0.000	0.046
	1:40	0.067	0.099	0.000	0.086	0.125
DC+EDC (n=2)	1:20	0.000	0.015	0.017	0.022	0.016
	1:40	0.071	0.000	0.017	0.030	0.019
DC+UV (n=2)	1:20	0.007	0.000	0.042	0.026	0.088
	1:40	0.000	0.041	0.054	0.077	0.082

GA, treat with glutaraldehyde, ethanol+1-octanol and glycine; DC+GA, decellularize and treat with glutaraldehyde, ethanol+1-octanol, and glycine; DC+PG+GA, decellularize and treat with polyethylene glycol, glutaraldehyde, ethanol+1-octanol, and glycine; DC+DTBP, decellularize and treat with dimethyl 3,3'-dithiobispropionimidate and ethanol+1-octanol; DC+EDC, decellularize and treat with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide+N-hydroxysuccinimide and ethanol+1-octanol; DC+UV, decellularize, irradiate using ultraviolet and treat with ethanol+1-octanol.
GA, glutaraldehyde; DC, decellularization; PG, polyethylene glycol; DTBP, dithiobispropionimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; UV, ultraviolet.

tein group DTBP, and EDC and UV irradiation to GA fixation.

The calcification process in a bioprosthetic heart valve occurs due to the reaction of the rich phosphorus of the cell membrane-associated phospholipids and the calcium of the extracellular fluid [4]. The phospholipids can be extracted with an ethanol and long-chain alcohol solvent such as 1-octanol, 1,2-octanediol which is structurally similar to phospholipids. Thus, a mixture of ethanol and octanol was found to remove the phospholipids more effectively [7,13]. It has been known that the residual aldehyde groups after GA fixation may react with CA from host plasma and cause cytotoxicity to the surrounding tissue. The amino acid treatment after GA fixation is also effective in mitigating calcification and cytotoxicity [14,15].

For this reason, in our previous studies using a rat subcutaneous model, the bovine pericardial tissues were treated with a mixture of ethanol and 1-octanol, and detoxified with amino acids for preventing calcification in the bovine pericardium fixed with GA alone with good anti-calcification effects [5]. However, there were no significant differences between the groups using ethanol, long-chain alcohol, or detoxification in the CA and IP levels of the rat subcutaneous model [6].

The rabbit implantation model is an effective method for detecting calcification differences between various fixative treatment groups within a relatively short period [16]. Accordingly, in the present study, the anti-calcification effects of the variously treated pericardial tissues were assessed using rabbit subcutaneous and intramuscular models. We compared the degree of calcification when treated with DC, PG as space filler, primary amine cross-linking methods such as DTBP and EDC, and physical fixation of UV irradiation using rabbit implantation models. There was a tendency toward a higher calcification in the intramuscular portion than in the subcutaneous portion of implantation, probably due to the different blood supply effects. The cellular components of implanted tissue have been known to cause accelerated calcification and immune response to the host tissues [17]. In our experiments, almost all of the cellular components were removed by DC using SDS detergent, Triton X-100, and osmotic shock methods, without damage to the collagen fibers. However, the decellularized GA group showed a high calcification similar to that of the GA control group in this study.

Filling of the interstitial void spaces in GA-fixed collage-

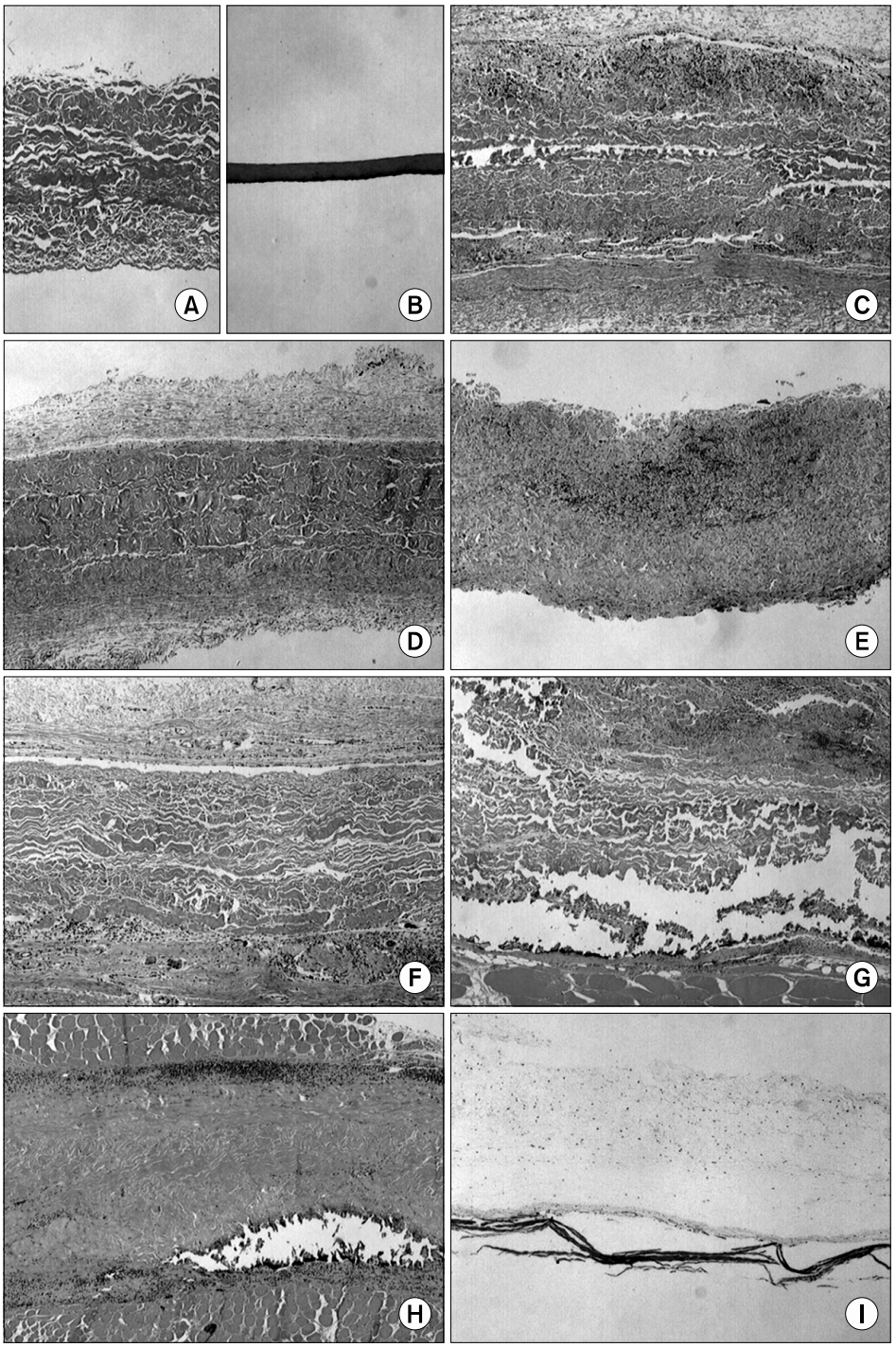

Fig. 4. Representative hematoxylin and eosin staining photomicrographs of the pre-implant samples (A, B) and the subcutaneously (C–F) or intramuscularly (G–I) implanted samples (×40). (A) A representative photomicrograph of the pre-implant samples except of the DC+UV group; (B) the pre-implant DC+UV group; (C) the explanted GA group; (D) the explanted DC+PG+GA group; (E) the explanted DC+DTBP group; (F) the explanted DC+EDC group; (G) the explanted DC+GA group; (H) the explanted DC+EDC group; (I) the explanted DC+UV group. GA, glutaraldehyde; DC, decellularization; PG, polyethylene glycol; DTBP, dithiobispropionimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; UV, ultraviolet.

nous tissue using macromolecular substances have been reported to be effective in preventing calcification. Macromolecules inactivate the free aldehyde groups of GA and prevent the release of residual GA [8,18]. PG is a hydrophilic polymer with a good biocompatibility and non-immunogenicity, and it can reduce calcification, protein adsorption, and platelet adhesion [19,20]. In this study, an additional PG treatment to the DC+GA group (DC+PG+GA group) was found to have reduced calcification compared with the non-space filler treatment groups (GA, DC+GA, and DC+DTBP groups).

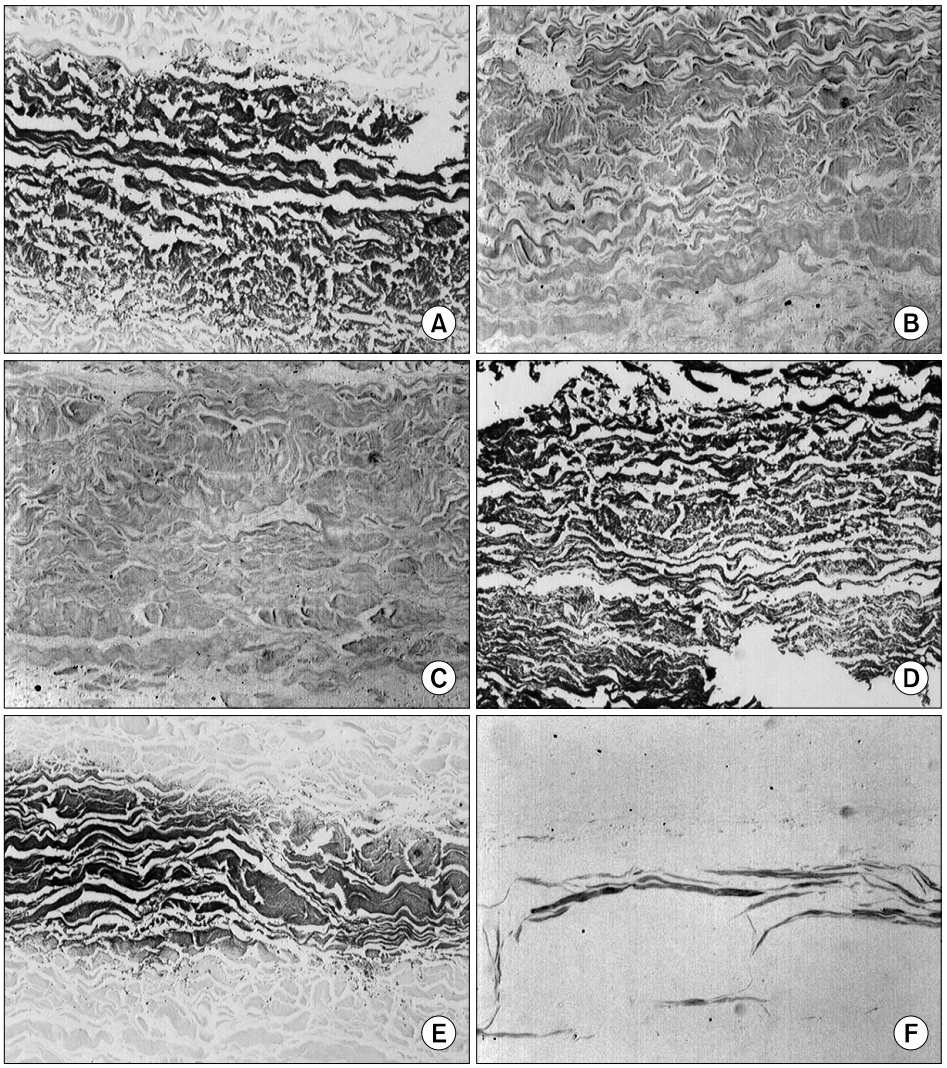

Fig. 5. Representative von Kossa staining photomicrographs of the subcutaneously (A–C) or intramuscularly (D–F) implanted samples (×100). (A) The GA group; (B) the DC+PG+GA group; (C) the DC+EDC group; (D) the DC+GA group; (E) the DC+PG+GA group; (F) the DC+UV group. GA, glutaraldehyde; DC, decellularization; PG, polyethylene glycol; DTBP, dithiobispropionimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; UV, ultraviolet.

The PG processing seems to play a crucial role in reducing calcification. This group's cytotoxicity to the fibroblast showed the lowest level, and the shrinkage temperature and resistance to pronase degradation also showed relatively high levels, while the UTS showed relatively low levels. In general, DC reduces collagen density, and thus affects the tensile strength. However, the peak load of the DC+PG+GA group had no significant difference compared with untreated tissue. Therefore, the UTS was considered to be reduced because the thickness of collagenous tissue was increased by filling the interstitial void spaces with PG in this study.

The amines are organic compounds and protein functional groups that contain a basic nitrogen atom with a lone pair. The primary amine group exists at the N-terminus (NH_2) of each peptide chain and in the side chain of lysine residues. Imidoester crosslinkers form amidine bonds by reacting with primary amines. The DTBP, homobifunctional, imidoester crosslinker containing disulfide bonds, effectively crosslinks at alkaline pH and does not polymerize. The DTBP have mechanical properties and resistance to enzymatic degradation similar to GA. The DTBP treated collagenous tissue has been reported to show less calcification and good biocompatibility compared to GA treated collagenous tissue [9,10]. However, in this study, shrinkage temperature and resistance to enzymatic degradation of the DTBP treated group improved compared to untreated tissue, but improved less than the GA treated groups. In addition, the DTBP treated group showed high inflammatory-cell infiltration and calcification similar to

Table 4. Calcium and inorganic phosphorus quantitative analysis of subcutaneously or intramuscularly implanted samples

Group	Subcutaneous model			Intramuscular model		
	No.	CA (μg/mg)	IP (μg/mg)	No.	CA (μg/mg)	IP (μg/mg)
GA	10	134.48±26.19	62.99±10.76	10	185.81±14.26	89.63±8.46
DC+GA	10	131.77±40.82	66.16±15.52	10	196.59±9.40	95.58±6.26
DC+PG+GA	10	38.74±29.12[a)]	21.14±14.11[a)]	10	130.56±57.24[a)]	68.64±33.44
DC+DTBP	10	133.92±43.95	65.38±22.46	10	195.02±8.74	89.12±4.89
DC+EDC	10	34.88±37.33[a)]	19.88±15.04[a)]	10	123.10±71.09[a)]	61.86±32.17
DC+UV	9	55.76±64.13[a)]	23.85±27.38[a)]	6	115.80±59.51[a)]	59.96±27.51

Values are presented as mean±standard deviation. Some of the tissue discs of the implanted DC+UV group had disappeared because of biodegradation (the subcutaneous model=1 and the intramuscular model=4). GA, treat with glutaraldehyde, ethanol+1-octanol and glycine; DC+GA, decellularize and treat with glutaraldehyde, ethanol+1-octanol, and glycine; DC+PG+GA, decellularize and treat with polyethylene glycol, glutaraldehyde, ethanol+1-octanol, and glycine; DC+DTBP, decellularize and treat with dimethyl 3,3'-dithiobispropionimidate and ethanol+1-octanol; DC+EDC, decellularize and treat with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide+N-hydroxysuccinimide and ethanol+1-octanol; DC+UV, decellularize, irradiate using ultraviolet and treat with ethanol+1-octanol.

CA, calcium; IP, inorganic phosphorus; GA, glutaraldehyde; DC, decellularization; PG, polyethylene glycol; DTBP, dithiobispropionimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; UV, ultraviolet.

[a)]$p<0.05$ vs. the GA, DC+GA, and DC+DTBP groups.

the GA and DC+GA groups, which was contradictory to the previous report.

The EDC, a carbodiimide cross-linker as one of the protein functional groups, reacts with the carboxylic acid groups and activates to form an O-acylisourea intermediate, and then cross-linking is formed by coupling the amine groups to form amide bonds. Adding NHS to EDC to increase cross-linking has been reported to be very effective [11,12]. EDC and NHS treated groups showed less inflammatory cell infiltration and calcification similar to the DC+PG+GA group. The treatment method of the DC+EDC group has been demonstrated to resist calcification in the rabbit implantation models. In addition, the cross-linking efficiency and mechanical properties of the DC+EDC group showed similar levels to that of the GA treated groups.

The UV cross-linked collagenous tissue have been reported to show enhanced mechanical properties and to exhibit resistance to proteinase [21,22]. UV irradiation does not use toxic chemicals such as GA. Therefore, our study was expected to show low cytotoxicity. However, the UV irradiated DC+UV group was shown to be toxic to porcine fibroblast in contact cytotoxicity assay. After implantation into the rabbits, the pericardial tissue disc of the UV treated group was degraded by nonspecific proteinase *in vivo*, because the cross-linking had not formed. The CA and IP contents of the DC+UV group were significantly lower than the GA, DC+GA, and DC+DTBP groups. It may have been due to the loss of interstitial void space caused by in UV irradiation. Removal of interstitial void spaces using PG filling or the drying method is used to prevent calcification. Accordingly, despite the anti-calcification effect of the UV treatment method, UV is not suitable for collagenous tissue due to its low resistance to enzymatic degradation and thermal stability.

CONCLUSION

In conclusion, our study showed that DC, PG as space filler, and primary amine of the EDC treatment used in consecutive steps reduced the calcification of bovine pericardium when implanted in rabbit subcutaneous and intramuscular models. The DC+PG+GA and DC+EDC treatments are good alternative cross-linking methods compared to GA only fixation for xenograft cardiovascular tissue preservation for bioprosthetic tissue stability and in vivo anticalcification effects. Our study had limitations because anticalcification treatments obtained from studies in small animal models should be interpreted cautiously and validated by other studies on intracardiac valvular implantation in large animals.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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