Original Article

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A Comparative Study on Mechanical and Biochemical Properties of Bovine Pericardium After Single or Double Crosslinking Treatment

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Background and Objectives: Glutaraldehyde (GA) has been used as a representative method of tissue preservation in cardiovascular surgery. However, GA has showed limited durability including calcification, mechanical failure and toxicity. To overcome those unsolved problems, we analyzed the crosslinking differences of primary amines, GA and genipin in their mechanical and biochemical properties with a single or double crosslinking agent for clinical application.

Materials and Methods: Samples were divided into 3 groups; control, single crosslinking fixation and double crosslinking fixation after decellurarization using bovine pericardium. For analysis of the biochemical and mechanical properties of each crosslinking method, tensile strength, percentage strain, thermal stability, resistance to pronase, nynhydrin and cytotoxicity test were studied.

Results: Combined hexamethylene diamine and suberic acid in the carbodiimide hydrochloride/N-hydroxysucinimide solution (EDC/NHS) after decellurarization, tensile strength and strain percentage were not statistically significant compared to the single crosslinking treated groups ($p>0.05$). Tissue crosslinking stability was weak in single treatment of diphenylphosphoryl azide, suberic acid, low concentration of EDC, hexamethylene diamine and procyanidin groups, but thermal stability and resistance to the pronase and ninhydrin were markedly increased in concentrated EDC/NHS or after combined double treatment with low concentration of GA or genipin ($p<0.001$).

Conclusion: Single or double crosslinking with low concentration of carbodiimide, diphenylphosphonyl azide, procyanidin, suberic acid and hexane diamine were not as effective in mechanical, biochemical, cytotoxic and crosslinking properties compared to GA or genipin fixation, but their mechanical and chemical properties were much improved when combined with low concentrations of GA or genipin in the double crosslinking process. **(Korean Circ J 2012;42:154-163)**

KEY WORDS: Bioprosthesis; Glutaraldehyde.

Introduction

At present, there is no ideal method for the application of collagen-derived bioprostheses. Although glutaraldehyde (GA) fixation has been used as a representative material during cardiovascular surgery, those characteristics of fixed pericardium still have many unsolved problems. Representative problems of GA fixation are calcification, stiffness after fixation, toxicity and slow release of GA due to the dissociation of the unstable Schiff's base.[1)2)] Therefore, it is necessary to identify another crosslinking agent for suitable use.

The ideal crosslinking agents should have low toxicity, low calcification levels, enhanced mechanical tensile strength, stability and durability. Many crosslinking agents and techniques have been developed involving acylazide,[3-5)] carbodiimide,[6)7)] hexamethylene-diisocyanate,[8)] diamines[9)] and polyepoxidic[10)11)] resins for improving physical and chemical properties.

Representative pericardial fixation material, GA, genipin and epoxy compounds have been known to connect amino groups of ly-

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sine or hydroxylysine residues of different polypeptide chains by crosslinking as an intra-molecular or intermolecular bridge.[4)12-14)] However, other agents such as carbodiimide hydrochloride (EDC) or diphenylphosphonyl azide (DPPA) do not introduce foreign cross-linking molecules. Accordingly, when the glutamic and aspartic acid residues themselves are activated, they are crosslinked by the free amino groups of lysine or hydroxyline residues.[15)]

We can assume that tissue fixation with two different crosslinking types may improve mechanical and chemical tissue properties. Therefore, we investigated the effectiveness of many chemical crosslinking agents in single or in combination such as low concentration of GA, genipin, EDC, DPPA, procyanidins, dihexane amine, and suberic acid to determine the ideal crosslinking agents and their combination methods.

To improve poor GA fixation, studies on the effects of mechanical properties such as tensile strength, elasticity, crosslinking stability, permeability and compliance after treatment with solvent, decellularization and detoxification were performed in our laboratory.[16-19)]

We investigated whether combined crosslinking treatment due to differences in crosslinking mechanisms may improve the mechanical and chemical properties on the xenograft using bovine pericardium.

Materials and Methods

Sample preparation

We have previously reported harvest methods of bovine pericar-

Table 1. Comparison of crosslinking methods used in this study

Group	No.	Methods
I	0	Fresh bovine pericardium
	1	0.25% GA for 3 days → 0.25% GA+75% ethanol+5% octanol for 2 days → 0.25% GA for 1 week → 0.2 M glycine for 2 days
	2	0.3% genipin for 3 days → 0.3% genipin+75% ethanol+5% octanol 2 days
II	3	Decellularization → 0.25% GA for 3 days → 0.25% GA+75% ethanol+5% octanol for 2 days → 0.25% GA for 1 week → 0.2 M glycine for 2 days
	4	Decellularization → 0.3% genipin for 3 days → 0.3% genipin+75% ethanol+5% octanol 2 days
	5	Decellularization → low concentration EDC and NHS (20 mM EDC/NHS)
	6	Decellurarization → medium concentration EDC and NHS (50 mM EDC/NHS)
	7	Decellularization → high concentration EDC and NHS (80 mM EDC/NHS)
	8	Decellularization → DPPA
	9	Decellularization → DIA
	10	Decellularization → SUA
	11	Decellularization → epicatechin (procyanidin)
III	12	Decellularization → butanal and $NaCNBH_3$ → Jaffmine
	13	Decellularization → Jaffmine → GA fixation
	14	Decellularization → low concentration EDC and NHS → genipin fixation
	15	Decellularization → low concentration EDC and NHS → GA fixation
	16	Decellularization → high concentration EDC and NHS → GA fixation
	17	Decellularization → epicatechin (procyanidin) → GA fixation
	18	Decellularization → DPPA → GA fixation
	19	Decellularization → low concentration EDC and NHS → DIA → GA fixation
	20	Decellularization → low concentration EDC and NHS → SUA → GA fixation
	21	Decellularization → low concentration EDC and NHS → DIA → SUA → GA fixation
	22	Decellularization → low concentration EDC and NHS → DIA
	23	Decellularization → low concentration EDC and NHS → SUA
	24	Decellularization → low concentration EDC and NHS → DIA → SUA
	25	Decellularization → butanal and $NaCNBH_3$ → Jaffmine → GA fixation
	26	Decellularization → butanal and $NaCNBH_3$ → Jaffmine → Genipin
	27	Decellularization → butanal and $NaCNBH_3$ → Jaffmine → 10 mM EDC/NHS

Group I: control, Group II: single crosslinking agent fixation with decellurarization, Group III: double crosslinking agent fixation with decellurarization. DIA: hexane-1,6-diamine, DPPA: diphenylphosphonyl azide, EDC: carbodiimide hydrochloride, GA: glutaraldehyde, NHS: N-hydroxysucinimide, SUA: suberic acid

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dium in our laboratory.[16-19] Samples were divided into 3 categories: group I {the control group with a low concentration of GA (0.25%) or genipin crosslinking (0.3%)}, group II (a group with single crosslinking agent fixation after decellurarization), and group III (a group with double crosslinking agent fixation after decellurarization). Each method of treatment on the bovine pericardium is summarized in Table 1.

Decellularization methods of bovine pericardium

All tissues were disinfected in 0.1% (v/v) peracetic acid with 4% ethanol in distilled water for 2 hours. They were then washed vigorously for 1 hour with distilled water. Hypotonic solution (distilled water 10 mL; Tris, 10 mmol/L; ethylendiamine tetraacetic acid (EDTA), 0.1%; and aprotinin, 10 KIU/mL; pH 8) with 0.25% sodium dodecyl sulfate was added for 24 hour at 4°C, washed with distilled water for 1 hour at 4°C, and then the hypotonic solution was added with 0.5% Triton X-100 for 24 hours at 4°C and washed with distilled water for 12 hours at 4°C. Next, isotonic solution (distilled water 10 mL; Tris, 50 mmol/L; NaCl, 0.15 mol/L; EDTA, 0.1%; and aprotinin, 10 KIU/mL; pH 8) was added for 24 hours at 4°C, after which hypertonic solution (II) (distilled water 10 mL; Tris, 200 mmol/L; NaCl, 0.6 mol/L; EDTA, 0.1%; and aprotinin, 10 KIU/mL; neomycin trisulfate 10 mL/L; pH 8) was added for 6 hours at 4°C. After that, the samples were washed with phosphate buffered solution (PBS, 0.1 M, pH 7.4) for 1 hour at 4°C.

Double crosslinking agent preparation

We used 16 mM EDC and 1 mM N-hydroxysucinimide solution (NHS) [2-{N-morpholinol ethanesulfonic acid (MES)} buffer, pH 5.5] in low concentration solution (0.05 M) of EDC and 0.01 M NHS (MES, pH 5.5), 81 mM EDC and 6 mM NHS (MES, pH 5.5) as a high concentration solution. When we prepared other types of crosslinking agents, we used 1.25% DPPA (PBS, pH 7.4), 11.25 mM hexamethylenediamine (MES, pH 5.5), 10 mM suberic acid (MES, pH 5.5), 10 mg/mL epicatechin (PBS, pH 7.4), 8 mL butanal and 2 mL $NaCNBH_3$ (100 mL MES, pH 6.5) and 0.06 M Jaffmine (0.25 M MES, pH 5) solutions.

Tensile strength test and elasticity test

We measured tensile strength by the uniaxial test using 5×10 mm sized bovine pericardium with 5 mm width, measured at a different angle of 30 degrees because of irregular collagen fibrous arrangement of bovine pericardium. A tensile strength machine (K-ML-1000N, M-TECH, Seoul, Korea) was used with a digital force gauge (DS2-50N, Imada, Japan) for measuring the tensile strength and strain percentage and a Quick-mini (PK-1012SU, Mitutoyo, Japan) was employed for measuring tissue thickness. Bovine pericardium was loaded at a speed of 100 mm/min. Tissue thickness was measured at one point using a thickness gauge after a contact of 5 seconds with the sample tissue.

Thermal stability test

To assess the degree of fixation of bovine pericardium, we measured the tissue shrinkage temperature by the hydrothermal method. Tissue strips sized 8×30 mm were loaded to 95 g weight, held at constant extension along the long axis and placed in a water bath. The temperature of water bath increased at approximately 2.5-5°C/min and the width of the strip was measured using a microscope. We used a water proof digital thermometer (-50°C to 200°C/-58°F to 392°F, Alla France, France) to measure the shrinkage temperature. The sharp deflection point at which shrinkage occurred was identified as the shrinkage temperature by plotting graphics.

Pronase test

We measured the dry weight of about 10×10 mm sized bovine pericardium samples as the baseline value. Next, 0.5 mg/mL pronase was added in HEPES 0.01 M (pH 7.4), glycine 0.1 M and $CaCl_2$

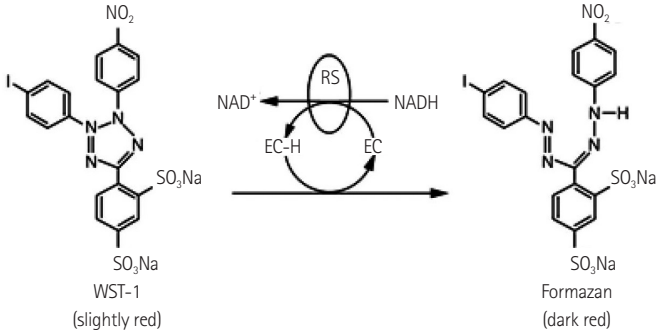

Fig. 1. Cleavage of the tetrazolium salt (WST-1) to formazan.

Fig. 2. Color changes after double crosslinking treatment. GA: glutaraldehyde, De: decellurarization, EDC: ethyl-dimethylaminopropyl carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphoryl azide, Je: Jaffmine, DIA: hexane-1-6 diamine, SUA: suberic acid, Pro: procyanidin.

0.01 M shaking (150 rpm) at 50°C for 24 hours. We measured the ratio of the weight before and after adding pronase.

Ninhydrin test

We extracted the solution from the 5×5 mm sized pericardium tissue by adding 0.1 mg/mL pronase at 50°C for 4 hours, and then added 50 μL ninhydrin. We measured the dissolved free amino acids. When ninhydrin reacts with these free amines, the ninhydrin color changes to deep blue or purple, and this was measured by an enzyme linked immuno sorbent assay reader (Labsystem, Vienna, Austria).

Cytotoxicity test

We used the tetrazolium salt (WST-1) assay (Fig. 1) to measure cytotoxicity and prepared a 96 well plate. A total of 2.5×10^3 cells were included in each well. The pericardium was incubated in Dulbecco's modified Eagle's medium for 72 hours at 37°C as extracting medium after 24 hours serum starvation. We added WST-1 at a concentration of 10 μL/well and incubated it for 4 hours after a 48 hours pre-incubation. Next, the absorbance was measured using a microplate reader (max 440 nm).

Statistics

Data were expressed as mean±standard deviation. We used the one-way analysis of variance and Mann-Whitney test as a comparison method. We also used the Bonferrini and Tamhene method as a post hoc method. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) ver. 15.0 (SPSS software for Windows, SPSS Inc., Chicago, IL, USA). Statistical signific-

Table 2. Tensile strength and strain (%)

Group (n)	Treatment characteristics	Tensile strength & strain			
		Sample size	Thickness (mm)	Ultimate strength (MPa)	Strain at fracture (%)
I (3)	Fresh bovine pericardium	n=50	0.29±0.06	17.53±4.58	42.47±7.55
	GA	n=10	0.27±0.04	16.37±5.18	59.00±14.58
	Genipin	n=10	0.27±0.03	14.58±3.28	65.00±23.00
II (9)	De-GA	n=10	0.23±0.06	14.36±2.86	38.67±9.71
	De-Genipin	n=10	0.38±0.05	14.51±3.55	57.33±8.43
	De-20 mM EDC/NHS	n=10	0.23±0.03	13.74±3.22	40.33±7.11
	De-50 mM EDC/NHS	n=10	0.23±0.05	13.30±3.95	41.34±6.89
	De-80 mM EDC/NHS	n=10	0.30±0.06	17.87±3.76	44.33±4.46
	De-DPPA	n=10	0.36±0.04	13.23±3.91	50.00±10.54
	De-DIA	n=10	0.26±0.05	25.69±6.41	50.33±8.67
	De-SUA	n=10	0.22±0.05	22.65±7.26	38.33±6.53
	De-procyanidin	n=10	0.28±0.04	16.57±4.64	48.33±6.33
III (16)	De-B-Jaffmine	n=10	0.36±0.06	14.63±2.80	59.34±9.00
	De-Jaffmine-GA	n=10	0.46±0.04	12.08±3.67	66.33±9.74
	De-20 mM EDC/NHS-Genipin	n=10	0.42±0.03	11.13±0.90	58.67±9.45
	De-20 mM EDC/NHS-GA	n=10	0.50±0.06	11.47±3.26	55.33±14.16
	De-80 mM EDC/NHS-GA	n=10	0.38±0.05	12.24±1.75	48.33±10.09
	De-procyanidin-GA	n=10	0.39±0.05	11.51±4.59	56.67±19.44
	De-DPPA-GA	n=10	0.32±0.08	11.00±2.49	35.67±12.08
	De-EDC/NHS/DIA-GA	n=10	0.48±0.06	10.27±1.23	58.67±8.64
	De-EDC/NHS/SUA-GA	n=10	0.33±0.04	14.14±3.54	42.67±9.40
	De-EDC/NHS/DIA-SUA-GA	n=10	0.40±0.06	10.46±1.92	58.67±9.71
	De-EDC/NHS/DIA	n=10	0.34±0.03	13.25±7.29	61.00±14.83
	De-EDC/NHS/SUA	n=10	0.26±0.05	12.98±5.17	42.67±4.66
	De-EDC/NHS/DIA-SUA	n=10	0.32±0.04	17.64±5.64	46.00±6.05
	De-B-Jaffmine-GA	n=10	0.39±0.07	15.82±4.58	56.00±7.67
	De-B-Jaffmine-Genipin	n=10	0.38±0.04	11.04±2.90	44.33±5.89
	De-B-Jaffmine-10 mM EDC/NHS	n=10	0.47±0.05	15.48±5.23	55.67±9.17

MPa: tensile strength at break kgf/width 5 mm, Strain (%): tensile strain at break (%). De: decellurarization, GA: glutaraldehyde, EDC: ethyl-dimethylaminopropyl carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphoryl azide, SUA: suberic acid, Pro: procyanidin

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ance was defined as $p<0.05$.

Results

External morphology after crosslinking

Color changes after crosslinking are illustrated in Fig. 2. The color changed to dark-yellow after GA treatment and deep blue after genipin treatment. The color remained whitish with primary amines crosslinking if there was no GA or genipin treatment.

Tensile strength

Tensile strength and strain values are illustrated in Table 2. When we compared low concentration of EDC/NHS (0.01 M EDC+0.001 M NHS), medium concentration of EDC/NHS (0.05 M EDC+0.01 M NHS) and high concentration of EDC/NHS (0.081 M EDC+0.006 M NHS), there was no statistically significance ($p=0.786$) in tensile strength (group 2). When combined with hexamethylenediamine and suberic acid in the EDC/NHS solution after decellurarization, there was no statistically significant mechanical improvement compared to the hexamethylenediamine or suberic acid only treated group

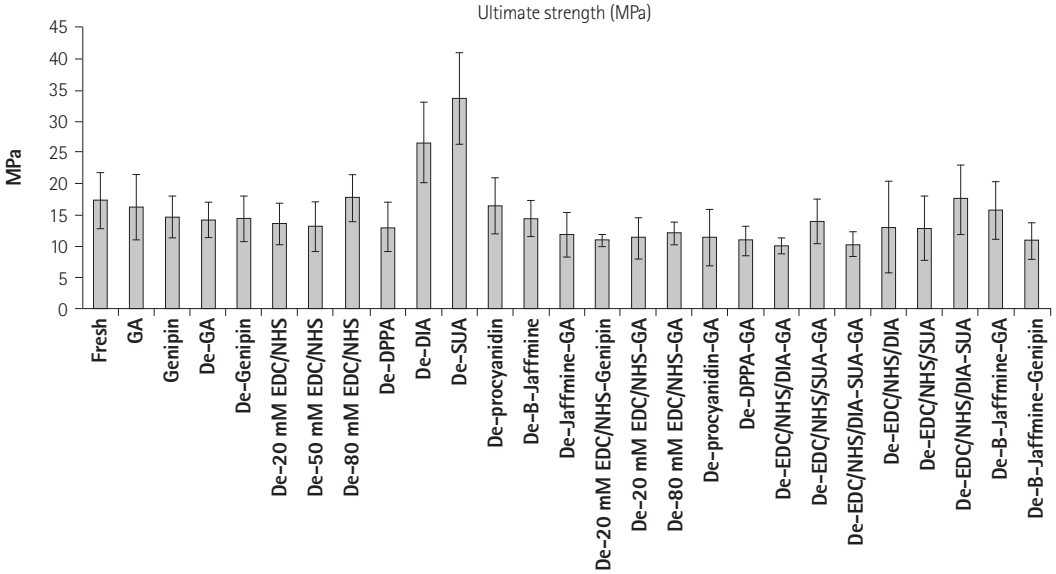

Fig. 3. Tensile strength after double crosslinking. MPa: tensile stress at break (kgf/width 5 mm). GA: glutaraldehyde, De: decellurarization, EDC: ethyl-dimethylaminopropyl carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphoryl azide, DIA: Hexane-1-6 diamine, SUA: suberic acid, Pro: procyanidin.

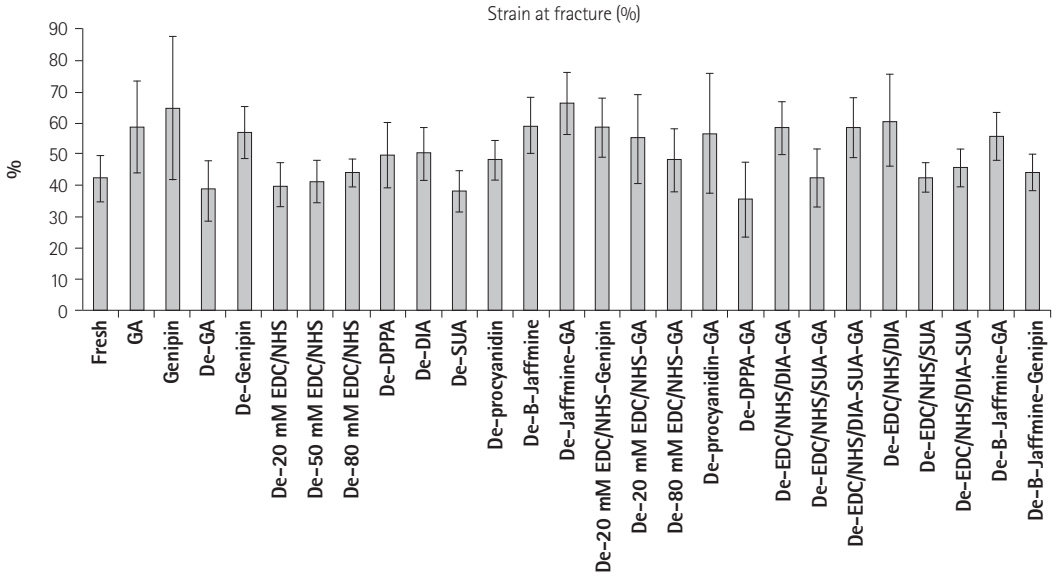

Fig. 4. Strain at break after double crosslinking. Strain (%): tensile strain at break (%). GA: glutaraldehyde, De: decellurarization, EDC: ethyl-dimethylaminopropyl carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphoryl azide, DIA: hexane-1-6 diamine, SUA: suberic acid, Pro: procyanidin.

(p=0.792, p=0.599). In hexamethylenediamine, suberic acid and DPPA groups, tensile strength values were slightly reduced after treatment of GA fixation (p<0.01). There was no statistically significant differences between epicatechin and butanal/$NaCNBH_3$+ jaffmine groups after treatment of GA fixation compared to the epicathechin only and butanal/$NaCNBH_3$+Jaffmine only treated groups (p=0.734, p=0.509). No tendency to decrease tensile strength was observed despite using double cross-linking methods (Fig. 3).

Tensile strain at break

There was also no difference in tensile strain at break (%) between the low and high concentration EDC/NHS groups. In the hexamethyleneamide and suberic acid groups, when they were combined after decellurarization, the tensile strength significantly increase compared to GA fixation and genipin after decellurarization (p<0.001). Hexamethyleneamide and suberic acid showed a weak strain nature, but their properties increased after GA treatment (p<0.001). When treated with GA fixation in hexamethylenediamine, suberic acid, combination group with EDC/NHS, the strain values increased in the hexamethylenediamine and suberic acid group (p=0.011, p=0.001), but decreased in the combination group (p<0.001). In the DPPA group, there was no difference in strain despite adding GA (p=0.267). Strain value decreased after adding GA in the epicathecin group (p=0.004), but increased after adding GA in the butanal/$NaCNBH_3$+Jaffmine group without statistical significance (p=0.051) (Fig. 4).

Thermal stability test

Thermal stabilities themselves were weak when combined with crosslinking agents to the fresh bovine pericardium, especially in only DPPA, suberic acid, hexamethylenediamine (DIA), epicatechin and low concentration EDC/NHS treated groups. Also, they were also unstable in combination of low concentration of EDC/NHS and other crosslinking agents (p<0.001). Thermal stability was also weak in the butanal treated group (Table 3). However, thermal stability became stable at a high concentration of EDC/NHS reaching 80°C. When combined with GA or genipin crosslinking fixation after treatment of Jaffmine or EDC/NHS, the thermal stability markedly increased (p<0.001).

Pronase test

When the pronase test was performed, 50 mM EDC/NHS and 80 mM EDC/NHS showed significantly increased resistance towards pronase digestion. Proteinase resistance was weak in the suberic acid, procyanidin, DPPA and butanal-Jaffmine groups (p<0.001). After enzymatic degradation *in vitro*, the relative weight loss in the GA, genipin and high concentration of EDC and NHS double crosslinking groups was lower than fresh bovine group and other groups (Table 4).

Table 3. Thermal stability after treatment of double crosslinking

Group		Shrinkage temperature (°C)		
		Sample size	Mean	SD
	Fresh	n=15	69.42	±1.48
I	GA	n=5	85.25	±1.37
	Genipin	n=5	79.25	±1.12
	De-GA	n=5	83.75	±1.77
	De-Genipin	n=5	78.75	±0.00
	De-20 mM EDC/NHS	n=5	72.75	±1.37
	De-50 mM EDC/NHS	n=5	77.25	±1.37
II	De-80 mM EDC/NHS	n=5	79.75	±1.37
	De-DPPA	n=5	64.75	±1.37
	De-DIA	n=5	68.25	±1.12
	De-SUA	n=5	66.25	±0.00
	De-procyanidin	n=5	69.75	±1.37
	De-B-Jaffmine	n=5	61.25	±0.00
	De-Jaffmine-GA	n=5	86.25	±0.00
	De-20 mM EDC/NHS-Genipin	n=5	81.75	±1.12
	De-20 mM EDC/NHS-GA	n=5	88.25	±1.12
	De-80 mM EDC/NHS-GA	n=5	86.75	±1.12
	De-procyanidin-GA	n=5	84.75	±1.37
	De-DPPA-GA	n=5	83.75	±0.00
III	De-EDC/NHS/DIA-GA	n=5	83.75	±0.00
	De-EDC/NHS/SUA-GA	n=5	86.25	±0.00
	De-EDC/NHS/DIA-SUA-GA	n=5	86.75	±1.12
	De-EDC/NHS/DIA	n=5	68.75	±0.00
	De-EDC/NHS/SUA	n=5	66.75	±1.12
	De-EDC/NHS/DIA-SUA	n=5	58.25	±1.12
	De-B-Jaffmine-GA	n=5	79.75	±1.37
	De-B-Jaffmine-Genipin	n=5	72.50	±0.00
	De-B-Jaffmine-10 mM EDC/NHS	n=5	80.25	±1.37

GA: glutaraldehyde, De: decellurarization, EDC: ethyl-dimethylaminopropyl carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphoryl azide, DIA: hexane-1-6 diamine, SUA: suberic acid, Pro: procyanidin

Ninhydrin test

Dissolved amounts of free amino acid after 50 mM EDC/NHS and 80 mM EDC/NHS treatment were shown to decrease. The suberic acid, procyanidin, DPPA and butanal-Jaffmine groups showed weak nature to the ninhydrin test similar to the pronase test. However, when we performed double crosslinking, dissolved free amino acids decreased (Table 5).

Cytotoxicity test

The results of the cytotoxicity test are shown in Table 6. EDC/NHS treated groups showed cytotoxicity, but there was a tendency for

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Table 4. Pronase test based on the crosslinking methods

Group		Pronase test		
		Sample size	Mean	SD
I	Fresh	5	10.00	9.56
	GA	5	88.53	4.02
II	Genipin	5	84.99	2.72
	De-GA	5	85.74	2.63
	De-Genipin	5	79.47	2.13
	De-20 mM EDC/NHS	5	44.10	6.50
	De-50 mM EDC/NHS	5	72.84	2.10
	De-80 mM EDC/NHS	5	80.53	5.10
	De-DPPA	5	1.10	0.33
	De-DIA	5	40.46	4.41
	De-SUA	5	36.87	2.89
	De-procyanidin	5	41.26	5.12
III	De-B-Jaffmine	5	14.29	1.84
	De-Jaffmine-GA	5	83.50	3.10
	De-20 mM EDC/NHS-Genipin	5	89.36	4.09
	De-20 mM EDC/NHS-GA	5	88.16	1.94
	De-80 mM EDC/NHS-GA	5	79.50	16.71
	De-procyanidin-GA	5	81.58	3.37
	De-DPPA-GA	5	84.47	1.48
	De-EDC/NHS/DIA-GA	5	90.48	12.34
	De-EDC/NHS/SUA-GA	5	89.21	1.86
	De-EDC/NHS/DIA-SUA-GA	5	82.39	5.66
	De-EDC/NHS/DIA	5	26.19	7.61
	De-EDC/NHS/SUA	5	5.43	7.36
	De-EDC/NHS/DIA-SUA	5	1.14	0.16
	De-B-Jaffmine-GA	5	68.22	3.60
	De-B-Jaffmine-Genipin	5	43.11	8.60
	De-B-Jaffmine-10 mM EDC/NHS	5	67.85	3.31

GA: glutaraldehyde, De: decellurarization, EDC: ethyl-dimethylaminopropyl carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphoryl azide, DIA: hexane-1-6 diamine, SUA: suberic acid

the cytotoxicity to reduce in the double crosslinking treated groups, although these findings were not statistically significant.

Discussion

We have analyzed common crosslinking agents for ideal tissue preservation methods and focused on the effects of mechanical and chemical properties in this experiment after treatment with various single or multiple crosslinking agents. GA was used as a representative fixation method in cardiac surgery, but its toxicity and calcification were found to be disadvantageous. Therefore, we investigated the characteristics of different types of crosslinking agents compared to GA or genipin.

Table 5. The ninhydrin test shows that dissolved amounts of free amino acid after 50 mM EDC/NHS and 80 mM EDC/NHS treatment were decreased. Suberic acid, procyanidin, DPPA and butanal-Jaffmine group showed weak nature to the ninhydrin test similar to the pronase test

Group		Free Aa (ug/mg)	SD
I	Fresh	12.47	2.54
	GA	1.42	
II	Genipin	1.47	
	De-GA	1.21	
	De-Genipin	4.16	0.58
	De-20 mM EDC/NHS	5.77	
	De-50 mM EDC/NHS	1.45	0.28
	De-80 mM EDC/NHS	1.76	
	De-DPPA	14.68	1.47
	De-DIA	4.86	1.66
	De-SUA	5.73	0.09
	De-procyanidin	7.71	0.42
III	De-B-Jaffmine	17.24	3.45
	De-Jaffmine-GA	2.50	0.04
	De-20 mM EDC/NHS-Genipin	0.46	0.12
	De-20 mM EDC/NHS-GA	2.54	0.79
	De-80 mM EDC/NHS-GA	1.27	
	De-procyanidin-GA	2.48	
	De-DPPA-GA	4.37	1.07
	De-EDC/NHS/DIA-GA	0.68	0.21
	De-EDC/NHS/SUA-GA	0.63	0.09
	De-EDC/NHS/DIA-SUA-GA	0.84	0.01
	De-EDC/NHS/DIA	11.72	1.85
	De-EDC/NHS/SUA	9.14	2.10
	De-EDC/NHS/DIA-SUA	19.80	3.25
	De-B-Jaffmine-GA	2.10	0.07
	De-B-Jaffmine-Genipin	6.01	0.59
	De-B-Jaffmine-10 mM EDC/NHS	8.51	0.78

GA: glutaraldehyde, De: decellurarization, EDC: ethyl-dimethylaminopropyl carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphoryl azide, DIA: hexane-1-6 diamine, SUA: suberic acid

When we performed collagen double-crosslinking as a different crosslinking method, we found that there was no significant difference compared to the single crosslinking method in tensile strength and strain. However, tissue thermal stability as stable crosslinking efficiency was weak without GA or genipin crosslinking fixation. High concentration of EDC/NHS showed resistance to the pronase treatment, but relative weakness to the pronase test was showed in the suberic acid, procyanidin and butanal-Jaffmine without GA or genipin crosslinking fixation groups. Ninhydrin (2,2-dihydroxyindane-1,3-dione) is a chemical agent that is used to detect ammonia or primary and secondary amines.[20] We dissolved each cross-

Table 6. The cytotoxic test showes there was a tendency to reduce the cytotoxicity in the double crosslinking treated groups althogh it did not reach the statistical significance

Group		Adipose steml cell (#10 p16)			Fibroblast (A3P3)		
		n	Mean	SD	n	Mean	SD
	Fresh	3	1.77	0.04	2	1.55	0.11
I	GA	3	2.15	0.21	2	1.25	0.32
	Genipin	3	2.17	0.09	2	1.91	0.29
	De-GA	3	1.81	0.23	2	1.95	0.15
	De-Genipin	3	1.58	0.18	2	1.87	0.27
	De-20 mM EDC/NHS	3	0.45	0.61	2	0.39	0.01
	De-50 mM EDC/NHS				2	1.74	0.13
II	De-80 mM EDC/NHS	3	1.12	0.13	2	0.73	0.02
	De-DPPA	3	1.63	0.29	2	1.92	0.11
	De-DIA				2	1.81	0.05
	De-SUA				2	1.88	0.19
	De-procyanidin	3	1.98	0.03	2	1.83	0.13
	De-B-Jaffmine	3	2.20	0.13	2	1.22	0.05
	De-Jaffmine-GA	3	1.79	0.23	2	1.30	0.23
	De-20 mM EDC/NHS-Genipin	3	1.49	0.17	2	1.28	0.12
	De-20 mM EDC/NHS-GA	3	1.84	0.20	2	2.20	0.11
	De-80 mM EDC/NHS-GA	3	1.73	0.09	2	1.98	0.05
	De-procyanidin-GA	3	1.81	0.06	2	2.38	0.01
	De-DPPA-GA	3	1.84	0.06	2	2.25	0.09
III	De-EDC/NHS/DIA-GA	3	1.84	0.07	2	2.15	0.08
	De-EDC/NHS/SUA-GA	3	1.90	0.07	2	1.15	0.09
	De-EDC/NHS/DIA-SUA-GA	3	1.79	0.09	2	2.26	0.05
	De-EDC/NHS/DIA	3	1.47	0.18	2	1.97	0.04
	De-EDC/NHS/SUA	3	1.45	0.05	2	0.36	0.01
	De-EDC/NHS/DIA-SUA	3	1.56	0.09	2	1.84	0.15
	De-B-Jaffmine-GA	3	1.65	0.09	2	1.91	0.04
	De-B-Jaffmine-Genipin				2	1.90	0.09
	De-B-Jaffmine-10 mM EDC/NHS				2	1.87	0.16
	Positive	3	2.40	0.05	2	2.18	0.05
	Negative	3	0.38	1.08	2	0.29	0.00

Positive: 90% Dulbecco's modified Eagle's medium (DMEM)+10%, Negative: negative control: 50% DMEM+50% Dimethyl sulfoxide (DMSO). GA: glutaraldehyde, De: decellurarization, EDC: carbodiimide hydrochloride, NHS: N-hydroxysucinimide, DPPA: diphenylphosphonyl azide, DIA: dihexane amine, SUA: suberic acid

linked pericardial sample for 4 hours and the supernatant reacting with ninhydrin. From this data, we could identify that bovine pericardium of the middle, high concentration EDC/NHS and double crosslinking treated pericardium degraded very slowly. When the cytotoxicity test was performed, cytotoxicity decreased in the double crosslinking treated groups although EDC/NHS groups showed harmful cytotoxicity. Therefore, this can help to prevent harmful effects of chemically fixed pericardial tissues.

Glutaraldehyde generates crosslinking between lysine and hydroxylysine residues of tissue proteins, and can be used as an intramolecular and intermolecular bridge.[21] However, GA is cytotoxic and has been implicated in calcification of tissue. Therefore, alternative tissue fixation methods have been developed and several crosslinking methods have been reported.

Primary amines are organic compounds and functional groups that contain basic nitrogen atoms with a lone pair for internal conjugation EDC and DPPA do not introduce foreign crosslinking molecules. Also, glutamic acid and aspartic acid residues when activated, crosslink with free amino groups.

We selected the carbodiimide direct crosslinking of the collagen

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method, involving the activation of the carboxylic acid groups of glutamic or aspartic acid residues in this experiment, because it has reduced toxicity and improved compatibility.[22] We also added NHS to the carbodiimide containing solution because there was a report that NHS solution added to the carbodiimide was effective in increasing the number of crosslinking events.[1] Although we could not detect an increase in tensile strength and strain in our experiment, there was no decrease in mechanical properties. In chemical properties, due to the zero-length nature of carbodiimide crosslinking (collagen molecules directly linked to one another), its use was limited because of thermal instability,[23] and were able to observe this thermal instability in our experiment. However, thermal stabilization recovered in carbodiimide crosslinking after a low concentration GA treatment.

Epicatechin is a polyphenolic antioxidant plant secondary metabolite derived from catechu, which is the juice or boiled extract of Mimosa catechu.[24] Procyanidins are polymer chains of epicatechin. The characteristic features of procyanidin crosslinking are the formation of hydrogen bonds between the phenolic hydroxyl and the matrix protein amide carbonyl.[25][26] Collagen, proline-rich proteins are high affinity to the hydrogen bond and procyanidins have a very high affinity to proline-rich proteins.[27] Therefore, we can inspect the stabilization of mechanical strength in the procyanidin treatment group. It showed no significant difference in mechanical strength and strain compared to other groups and had a moderate degree of pronase resistance.

Suberic acid is a dicarboxylic acid which contains two carboxylic acid groups and is used in the cross-linking of biopolymers found in urine as a product of omega-oxidation of fatty acids.[28] Hexane-1,6-diamine which consists of a hexamethylene hydrocarbon chain also serves as a crosslinking agent in epoxy resins.[29] We were able to observe the effect of hexane-1,6-diamine and suberic acid with EDC and NHS. There was no significant difference when we combined the low concentration of EDC and NHS with hexane-1,6-diamine or suberic acid. However, decreased tensile strength and strain was observed when we combined the hexane-1,6-diamine and suberic acid in low concentration EDC and NHS solution. Therefore, we assumed that triple crosslinking may be harmful to the tissue. Suberic acid and hexamethylene-1,6-diamine showed weakness to the thermal injury and pronase treatment.

Jaffmine is a poly (propylene glycol) bis2-(aminopropyl) ether that contains primary amino groups attached to the terminus of a polyether backbone. This material has some characteristic features. It has a high intrinsic hydrophilicity and flexibility, which means, it can easily penetrate deep in the tissue and reduce stiffness due to its longer chains between crosslinking.[13] Therefore, we used Jaffmine with butanal and $NACNBH_3$. However, we saw that tensile strength decreased and thermal stability and pronase resistance were also weak in Jaffmine combined with butanal and $NACNBH_3$ group.

This study has some limitations. It is an *in vitro* study and the number of each crosslinking experiment is not large enough to cover all the protein functional groups, so it is still difficult to conclude which crosslinking agent is better among the primary amines. Also, we need to conduct an *in vivo* study to compare the results of *in vivo* biologic degradation and calcification in the future.

We found that the double crosslinking method with low concentration of GA or genipin crosslinking showed similar or cumulative effects in mechanical strength and strain compare to the usual single GA or genipin crosslinking methods. Also, double crosslinking agent treatment showed resistance to thermal and proteinase treatment.

We need additional experiments to confirm the results on the double crosslinking methods. Although the low or high concentrations of EDC and NHS with other crosslinking agents increase crosslinking efficacy, single or double crosslinking with carbodiimide, DPPA, procyanidin, suberic acid, and hexane diamine were not as effective in mechanical, biochemical and cytotoxicity properties compared to the usual crosslinking agents of GA and genipin fixation.

Ideal tissue crosslinking methods, which improve the mechanical and biochemical properties that have low cytoxicity and non-calcification characteristics should be sought in the near future.

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