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Detoxification of Glutaraldehyde Treated Porcine Pericardium Using L-arginine & NABH$_4$

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Background: Calcification is the most frequent cause of clinical failure of bioprosthetic tissues fabricated from GA-fixed porcine valves or bovine pericardium. A multi-factorial approach using different mechanisms was recently developed to reduce the calcification of bioprosthetic tissues. The purpose of the present study was to evaluate the synchronized synergism of using L-arginine and NaBH$_4$, compared with ethanol and L-lysine, in glutaraldehyde treated porcine pericardium from the standpoint of calcification and tissue elasticity. **Materials and Methods:** Porcine pericardium was fixed at 0.625% GA (7 days at room temperature after 2 days at 4°C). An interim step of ethanol (80%; 1 day at room temperature) or L-lysine (0.1 M; 2 days at 37°C) or L-arginine (0.1 M; 2 days at 37°C) was followed by completion of the GA fixation. A final step of NaBH$_4$ (0.1 M; 2 days at room temperature) was followed. Their tensile strength, thickness, and thermal stability were measured. Treated pericardia were implanted subcutaneously into three-week-old Sprague-Dawley rats for 8 weeks. Calcium content was assessed by atomic absorption spectroscopy and histology. **Results:** L-arginine and NaBH$_4$ pretreatment (1.81±0.39 kgf/5 mm $p=0.001$, 0.30±0.08 mm $p<0.001$) significantly increased tensile strength and thickness compared with the control (0.53±0.34 kgf/5 mm, 0.10±0.02 mm). In a thermal stability test, L-arginine and NaBH$_4$ pretreatment (84.25±1.12°C, $p=0.023$) caused a significant difference from the control (86.25±0.00°C). L-lysine and NaBH$_4$ pretreatment (183.8±42.6 ug/mg, $p=0.804$), and L-arginine and NaBH$_4$ pretreatment (163.3±27.5 ug/mg, $p=0.621$) did not significantly inhibit calcification compared to the control (175.5±45.3 ug/mg), but ethanol and NaBH$_4$ pretreatment did (38.5±37.3 ug/mg, $p=0.003$). **Conclusion:** The combined pretreatment using L-arginine and NaBH$_4$ after GA fixation seemed to increase the tensile strength and thickness of porcine pericardium, fixed with GA. Additionally, it seemed to keep thermal stability. However it could not decrease the calcification of porcine pericardium fixed with GA. NaBH$_4$ pretreatment seemed to decrease the calcification of porcine pericardium fixed with GA, but only with ethanol.

Key words: 1. Ethanol, L-lysine, NABH$_4$
2. Bioprosthetic calcification
3. Porcine pericardium

INTRODUCTION

Due to a limited supply of frozen homograft tissues, bioprosthetic tissues fabricated from glutaraldehyde (GA)-fixed porcine valve or bovine pericardium are commonly used in cardiovascular surgery. The major cause of clinical failure of

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these bioprosthetic tissues is known to be calcification [1,2]. However, the underlying mechanism of calcification is yet to be fully identified despite of its clinical importance. Until now, it has been suggested that several components may be responsible for the process of calcification: phospholipids that cause calcium deposits within tissues, cavities that are caused by the elimination of proteoglycans during the process of tissue treatment, necrosis, various chemical treatments [3], the immune response [4], mechanical stress [5], and several proteins and inflammatory cells [6] in blood.

Generally, chemical treatments with metal salts such as diphosphonates [7], allumininum chloride, ferric chloride [8], or with a surfactant such as sodium dodecyl sulfate [9] or with amino oleic acid [10] are used to reduce calcification of bioprosthetic tissues. Besides using GA, there is currently ongoing research into using chemical treatments to modify collagen structure [11,12] or using cross linkers [13] such as epoxy compounds.

Recently, researchers have taken a multi-factorial approach on synchronized synergism. With fixation at a higher concentration of 2～3% GA than the standard 0.625%, various studies have attempted to prevent calcium binding by increasing cross-link stability and effectively removing the remaining GA or by forming a bond in advance between the free aldehyde group (-CHO) and an amino acid such as L-lysine or L-arginine (Schiff base formation) after GA fixation [14,15]. Various anti-calcification methods such as removing phospholipids within tissue with the aid of ethanol [16] or increasing tissue elasticity [17,18] are expected to further reduce calcification of bioprosthetic tissues. We have previously reported the anti-calcification effect of a diamine bridge using L-lysine resembling that of ethanol through increasing thickness and tensile strength by increasing cross-links [19]. L-arginine shares L-lysine's anti-calcification mechanism. In addition, it exhibits antiplatelet activity, angiogenesis, wound healing promotion, and a nerve regeneration effect [20]. Sodium borohydride ($NaBH_4$) directly reduces the aldehyde group of GA or stabilizes Schiff base formation with amino acids [21].

The aim of this study was to evaluate the synchronized synergism when L-arginine and $NaBH_4$ were treated at once. To compare the synchronized synergism from the standpoint of calcification and tissue elasticity, we measured the thickness, tensile strength, and thermal stability of porcine pericardium that went through anti-calcification treatment prior to transplantation.

MATERIALS AND METHODS

1) Preparation of porcine pericardium

Porcine pericardium from a 3 to 4 month pig was extracted directly at a slaughterhouse. The extracted pericardium was stored in PBS solution (0.1 M, pH 7.4) and was transported to the laboratory in an ice box.

2) Control and experimental group set-up

Porcine pericardial tissues were divided into four groups, each group having five tissue segments. We explored the synchronized synergism as we compared group 1, the control group that was fixed with glutaraldehyde alone, with group 2 containing 80% ethanol and $NaBH_4$, group 3 containing L-lysine and $NaBH_4$, and group 4 containing L-Arginine and $NaBH_4$.

Group 1: 0.625% GA
Group 2: 0.625% GA＋80% Ethanol＋0.1 M $NaBH_4$
Group 3: 0.625% GA＋0.1 M L-lysine＋0.1 M $NaBH_4$
Group 4: 0.625% GA＋0.1 M L-arginine＋0.1 M $NaBH_4$

3) Porcine pericardium fixation

The fixation process of porcine pericardium was carried out with 0.625% GA fixation1 (PBS buffer, pH 7.4) for 2 days at 4^{o}C, followed by the additional 7 days fixation at room temperature. After the fixation, the pericardium was cut into 1×1 cm pericardial segments. The pericardial segments that did not need any additional treatments were washed with PBS solution at room temperature and were implanted in rats. The pericardial segments that required further anti-calcification treatments were used directly for the next step.

4) Ethanol & $NaBH_4$ pre-treatment

0.625% GA-fixed pericardium segments were stored in a shaker bath containing 80% ethanol solution (PBS buffer, pH 7.4) for 24 h at room temperature. Then, the segments were rinsed with PBS solution and were treated in 0.1 M $NaBH_4$ (PBS buffer, pH 7.4) for 48 h at room temperature. After the treatment, the samples were washed with PBS solution at room temperature, and were stored in PBS solution at 4°C until the transplantation.

5) L-lysine & $NaBH_4$ pre-treatment

After rinsing 0.625% GA-fixed pericardium segments with PBS solution, the segments were soaked in 0.1 M L-lysine solution (acetic acid buffer, 0.5 M, pH 7.6) for 48 h at 37°C. The segments were rinsed with PBS solution, then were treated in 0.1 M $NaBH_4$ (PBS buffer, pH 7.4) for 48 h at room temperature. After that, the segments were washed again with PBS solution, and were stored in PBS solution at 4°C until the transplantation.

6) L-Arginine & $NaBH_4$ pre-treatment

After rinsing 0.625% GA-fixed pericardium segments with PBS solution, the segments were soaked in 0.1 M L-Arginine solution (acetic acid buffer, 0.5 M, pH 11) for 48 h at 37°C. The segments were rinsed with PBS solution, then were treated in 0.1 M $NaBH_4$ (PBS buffer, pH 7.4) for 48 h at room temperature. After that, the segments were washed again with PBS solution, and were stored in PBS solution at 4°C until the transplantation.

7) Tensile strength-thickness test

When lying flat, longitudinal segments (0.5×5 cm) could be obtained by cutting the pre-treated porcine pericardium from six different directions, differentiated by a 30° angle. Then, the tensile strength of each 5-mm-wide segment was measured, and the average of the six segments was calculated so that the value represented the tensile strength of the porcine pericardium. Tensile strength was measured by a Japan Tech & Manufacture, Digital Force Gauge, Model 5FGN, automated materials testing system with a loading speed of 100 mm/min. The measurements were recorded in kgf/5 mm. Also, in order to observe the relationship between thickness and tensile strength of pericardium, the thickness within one sample was measured multiple times using a vernier caliper Mitutoyo Thickness Gauge (Digimatic 543-122-15, Mitutoyo, Japan).

8) Thermal stability test

For thermal stability assessment, pre-treated pericardium samples were cut into 8×30 mm cuboidal segments from six different directions, differentiated by a 30° angle. Using a 95 g pendulum, constant tensile force was applied to the segments. Then, the segments were soaked in distilled water at 55°C, and the water was heated at a rate of 1～2.5°C/min until remarkable shortening occurred for the heterologous graft pericardium segments. The temperature at which the maximal change in the rate of pericardium segment shortening occurred was measured for each group to compare the thermal stability differences [22].

9) In vivo studies: rat subcutaneous implantation

After anesthetizing a rat with Zoletil (0.2 cc IP) and Rompun (0.1 cc IP), four subcutaneous pouches were created at subdorsal subcutaneous tissue. The pretreated pericardium segments were then implanted in these pouches, and the incision was closed with sutures. The perdicardium segments remained implanted for 8 weeks. After 8 weeks of implantation, the rat was euthanized by CO_2 gas, and the segments were extracted by dissecting the subcutaneous pouches that were made at the time of implantation. The extracted perdicardium segments were then analyzed.

10) H&E, and von Kossa staining

For optical microscopy examinations, the extracted pericardium was cut into 2- to 3-mm-thick tissue sections and was put in Duboscq-Brasil solution for 1 h. After post-fixation with 10% formalin, the tissue was embedded in paraffin, cut into 2- to 4-um segments, and was stained with hematoxylin-eosin and vonkossa. Then, the integrity of microscopic structure and the calcium deposition of the segments were evaluated.

11) Quantitative analysis of calcium content

Calcium content was assayed to evaluate the degree of calcification of the segments in each group. For quantitative analysis of calcium, the pericardium segments were rinsed with saline, dried in a hot dryer for more than 24 h, and weighed. The segments were put in a glass tube, and 3 mL of 1 M HCl solution was added. Then, the sample was heated in a 75°C dry oven (Fisher Scientific) for 24 h or more until completely dissolved. The solution was transferred to an e-tube and was dried for more than 12 h using an automatic environmental speedvac system. After pellet formation, the pellet was put in 1 mL PBS (phosphate buffered solution), and the calcium content was assessed using an Automatic chemistry I.S.E machine (Hitachi).

12) Statistical analysis

Statistical tests were performed using Microsoft Excel and SPSS 14.0K, Data are expressed as mean values±standard deviation. The statistical significance of the differences between mean values of each group was found using the Student's t-test, and intergroup comparison for unpaired data of each group was carried out by ANOVA and a post-hoc test (Tukey's test). The statistical significance was accepted at $p<0.05$.

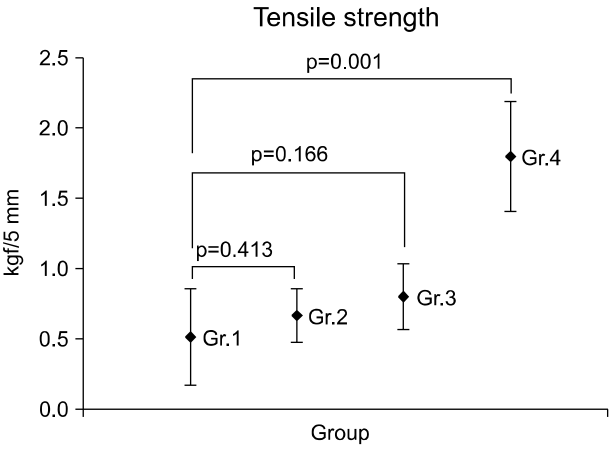
RESULTS

1) Tensile strength

The tensile strength was 0.53±0.34 kgf/5 mm for the control group (0.625% GA-fixed only), 0.68±0.19 kgf/5 mm for the group treated with ethanol and $NaBH_4$, 0.81±0.26 kgf/5 mm for the group treated with L-lysine and $NaBH_4$, and 1.81±0.39 kgf/5 mm for the group treated with L-arginine and $NaBH_4$. All three experimental groups were fixed with 0.625% GA prior to each different chemical treatment. An ANOVA test was used to detect statistically significant differences among treatments ($p<0.01$). Compared to the control group that was fixed with alone, the group treated with L-arginine and $NaBH_4$ after 0.625% GA fixation was the only group that showed statistically significant differences ($p=0.001$) (Fig. 1).

2) Thickness

The thickness of the samples were 0.10±0.02 mm for the control group that was fixed with 0.625% GA alone, 0.11±0.02 mm for the group treated with ethanol and $NaBH_4$, 0.19±0.02 mm for the group treated with L-lysine and $NaBH_4$, and 0.30±0.08 mm for the group treated with L-arginine and $NaBH_4$. All three experimental groups were fixed with 0.625% GA prior to each different chemical treatment. An ANOVA test was used to detect statistically significant differences among treatments ($p<0.01$). Compared to the control group that was fixed with 0.625% GA alone, the group treated with L-lysine and $NaBH_4$ and the group treated with L-arginine and $NaBH_4$ were the only two groups that were statistically significant ($p<0.001$) (Fig. 2).

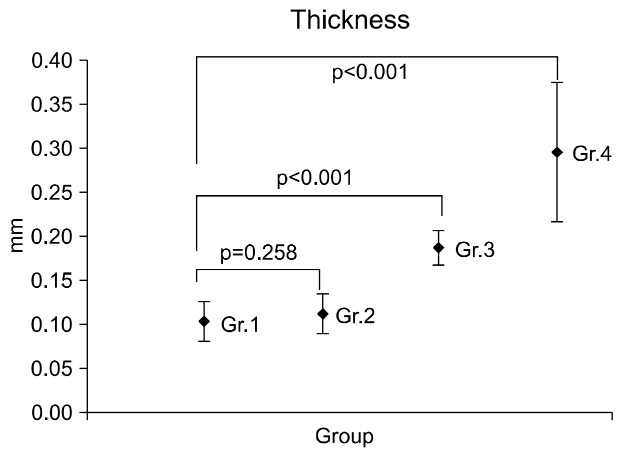


n=5/Group	Gr.1	Gr.2	Gr.3	Gr.4	p-value*
Tensile strength (kgf/5 mm)	0.53±0.34	0.68±0.19	0.81±0.26	1.81±0.39	<0.01
T†	a	a	a	b	

Fig. 1. The tensile strength of porcine pericardium after Gr.1) GA (0.625%) fixation, Gr.2) GA (0.625%) fixation+ethanol (80%)+$NaBH_4$ treatment, Gr.3) GA (0.625%) fixation+L-lysine+$NaBH_4$ treatment, Gr.4) GA (0.625%) fixation+L-arginine+$NaBH_4$ treatment. *=Statistical significance was tested by one-way analyses of variance among groups. †=The same letters indicate a non-significant difference between groups based on Tukey's multiple comparison test.

3) Thermal stability test

Thermal shrinkage temperature of the control group, 0.625% GA-fixed porcine pericardium, was 86.25±0.00°C, that of the pericardium post-treated with 80% ethanol and $NaBH_4$ after 0.625% GA fixation was 84.25±1.12°C, that of the pericardium post-treated with L-lysine and $NaBH_4$ after 0.625% GA fixation was 85.25±1.37°C, and that of the pericardium post-treated with L-arginine and $NaBH_4$ after 0.625% GA fixation was 84.25±1.12°C. An ANOVA test was used to detect statistically significant differences among treatments



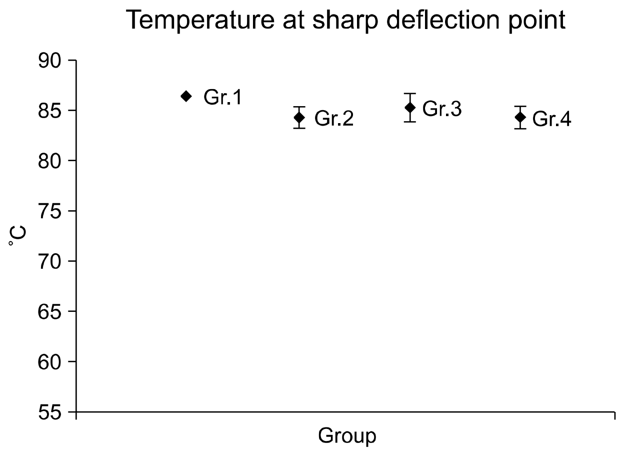
n=5/Group	Gr.1	Gr.2	Gr.3	Gr.4	p-value*
Thickness (mm)	0.10±0.02	0.11±0.02	0.19±0.02	0.30±0.08	<0.01
T†	a	a	b	c	

Fig. 2. The thickness of porcine pericardium after Gr.1) GA (0.625%) fixation, Gr.2) GA (0.625%) fixation+ethanol (80%)+$NaBH_4$ treatment, Gr.3) GA (0.625%) fixation+L-lysine+$NaBH_4$ treatment, Gr.4) GA (0.625%) fixation+L-arginine+$NaBH_4$ treatment. *=Statistical significance was tested by one-way analyses of variance among groups. †=The same letters indicate a non-significant difference between groups based on Tukey's multiple comparison test.

(p=0.023) (Fig. 3).

4) H&E, and von Kossa staining

Pericardium was composed of a serosa layer, fibrous layer, and epicardium connective tissue. The fibrous layer was the thickest layer in the pericardium, and was composed of collagen, elastic fibers, neurons, blood vessels, and lymphatic vessels. Calcium ions were mostly deposited in the connective tissues of the epicardium, and some were found inside the fibrous layer. Calcium deposition was observed in all layers for the group treated with 0.625% GA alone. For the group treated with L-lysine and $NaBH_4$, and for the group treated with L-arginine and $NaBH_4$ after 0.625% GA fixation, calcium deposits were observed inside the fibrous layer. In addition, for the group treated with 80% ethanol and $NaBH_4$ after 0.625% GA fixation, calcium deposits were observed in all three layers with a majority of the calcium deposits being inside the epicardium connective tissue (Fig. 4, 5).



n=5/Group	Gr.1	Gr.2	Gr.3	Gr.4	p-value*
Temperature (°C)	86.25±0.00	84.25±1.12	85.25±1.37	84.25±1.12	0.023
T†	b	a	a, b	a	

Fig. 3. The thermal stability test of porcine pericardium after Gr.1) GA (0.625%) fixation, Gr.2) GA (0.625%) fixation+ethanol (80%)+$NaBH_4$ treatment, Gr.3) GA (0.625%) fixation+L-lysine+$NaBH_4$ treatment, Gr.4) GA (0.625%) fixation+L-arginine+$NaBH_4$ treatment. *=Statistical significance was tested by one-way analyses of variance among groups. †=The same letters indicate a non-significant difference between groups based on Tukey's multiple comparison test.

5) Calcium analysis

Harvested pericardium-four segments in each group-were evaluated for their calcium content. The calcium content of the samples were 175.5±45.3 ug/mg for the control that was fixed with 0.625% GA alone, 38.5±37.3 ug/mg for the group treated with ethanol and $NaBH_4$ after 0.625% GA fixation, 38.5±37.3 ug/mg mm for the group treated with L-lysine and $NaBH_4$ after 0.625% GA fixation, and 163.3±27.5 ug/mg for the group treated with L-arginine and $NaBH_4$ after 0.625% GA fixation. An ANOVA test was used to detect statistically significant differences among treatments ($p < 0.01$). Compared to the control group that was fixed with 0.625% GA alone, the group treated with ethanol and $NaBH_4$ was the only group that was statistically significant (p=0.003) (Fig. 6).

DISCUSSION

GA has been used extensively for fixation of bioprosthetic

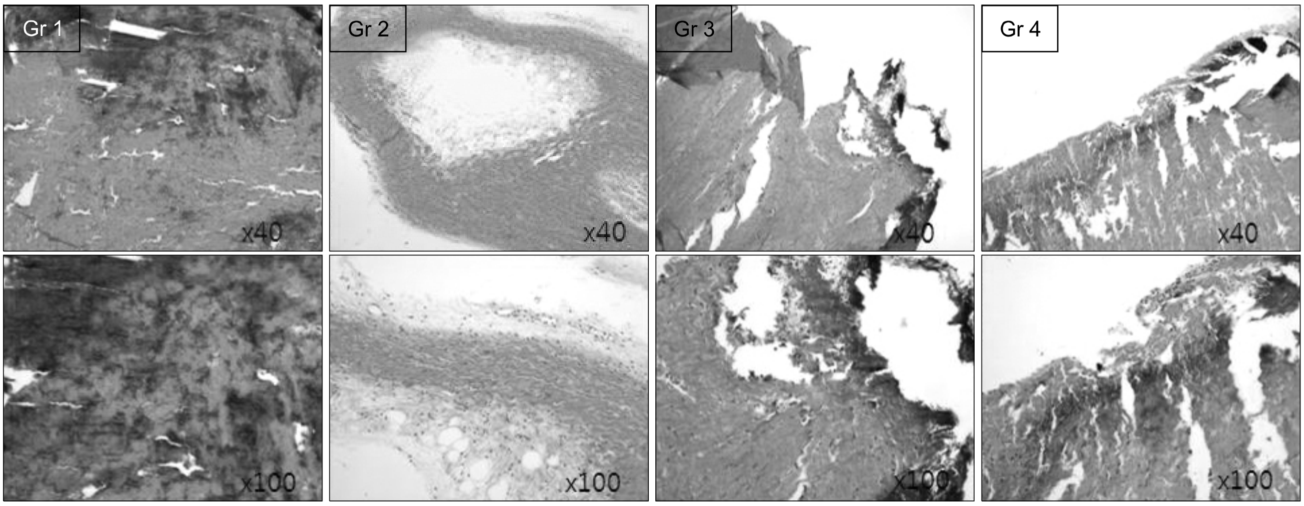


Fig. 4. Harvested porcine pericardium with hematoxylin-eosin staining after Gr.1) GA (0.625%) fixation, Gr.2) GA (0.625%) fixation+ethanol (80%)+$NaBH_4$ treatment, Gr.3) GA (0.625%) fixation+L-lysine+$NaBH_4$ treatment, Gr.4) GA (0.625%) fixation+L-arginine+$NaBH_4$ treatment.

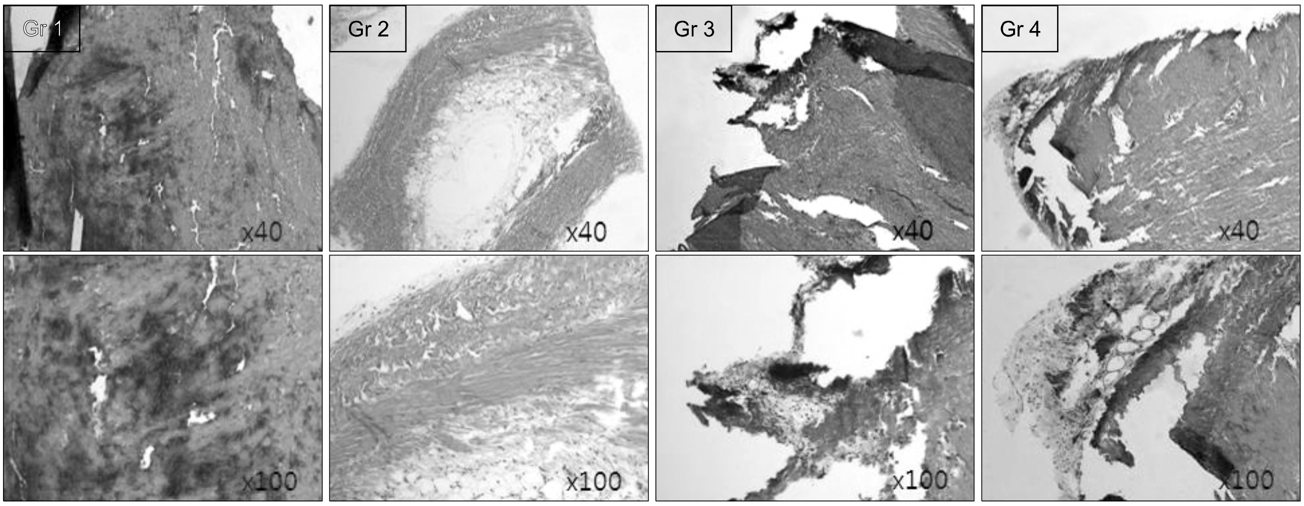
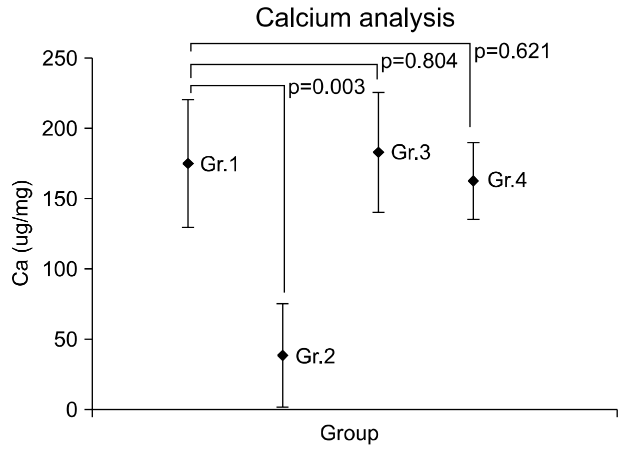


Fig. 5. Harvested porcine pericardium with von Kossa staining after Gr.1) GA (0.625%) fixation, Gr.2) GA (0.625%) fixation+ethanol (80%) +$NaBH_4$ treatment, Gr.3) GA (0.625%) fixation+L-lysine+$NaBH_4$ treatment, Gr.4) GA (0.625%) fixation+L-arginine+$NaBH_4$ treatment.

tissues fabricated from porcine valves or bovine pericardium, because GA reacts with the collagenous fiber of tissue to form stable cross-links during the formation of GA polymer on the surface. Nevertheless, it is believed that calcification occurs mostly due to a bond formation between the free aldehyde groups (-CHO) of GA and calcium ions. In this study, the anti-calcification effect was explored when treated with L-arginine and $NaBH_4$ in both in vivo and in vitro experiments, under the hypothesis that coupling the free aldehyde groups with L-arginine and $NaBH_4$ in advance would further stabilize the bond, which would effectively block the bond formation between free aldehyde groups and calcium ions [21].

The in vitro experiment showed that the combined pretreatment using L-arginine and $NaBH_4$ after GA fixation is statistically significant in increasing tensile strength (1.81±0.39 kgf/5 mm p=0.001) and thickness (0.30±0.08 mm, $p<0.001$), compared to the control group (0.53±0.34 kgf/5 mm,



n=5/Group	Gr.1	Gr.2	Gr.3	Gr.4	p-value*
Ca/Dry weight (ug/mg)	175.5±45.3	38.5±37.3	183.8±42.6	163.3±27.5	<0.01
T†	b	a	b	b	

Fig. 6. Calcium analysis of harvested porcine pericardium after Gr.1) GA (0.625%) fixation, Gr.2) GA (0.625%) fixation+ethanol (80%)+$NaBH_4$ treatment, Gr.3) GA (0.625%) fixation+L-lysine+$NaBH_4$ treatment, Gr.4) GA (0.625%) fixation+L-arginine+$NaBH_4$ treatment. *=Statistical significance was tested by one-way analyses of variance among groups. †=The same letters indicate a non-significant difference between groups based on Tukey's multiple comparison test.

0.10±0.02 mm) that was fixed with 0.625% GA alone. It is believed that L-arginine treatment contributes to the increase in tensile strength and thickness as the treatment enhances cross-linking within the tissue. An ANOVA test was used to detect statistically significant differences among treatments in both tensile strength ($p<0.01$) and thickness ($p<0.01$).

A thermal stability test was performed to evaluate the stability of collagen cross-links within pericardium segments. When heat is applied to collagen until certain temperature, tissue undergoes modification that shrinks the pericardium segments by 1/3 of its initial length. The shrinkage temperature was measured. It is believed that the higher the temperature, the better the durability and biostability of the tissue [20,22]. In the thermal stability test, the temperature of the control group (86.25±0.00°C) that was fixed with GA alone was higher than that of the experimental group (84.25±1.12°C) that was post-treated with L-arginine and $NaBH_4$ after GA fixation. However, since the temperature difference was only 2°C, it was concluded that the L-arginine and $NaBH_4$ treatment did not have a significant effect on thermal stability of pericardium segments.

An in vivo experiment using a rat subcutaneous model was performed to evaluate the synergy effect of L-arginine and $NaBH_4$ post-treatment after GA fixation; however, calcium analysis showed no statistically significant differences among the control (175.5±45.3 ug/mg) that was fixed with GA alone, the group with L-lysine and $NaBH_4$ treatment (183.8±42.6 ug/mg) after GA fixation, and the group with L-arginine and $NaBH_4$ treatment (163.3±27.5 ug/mg) after GA fixation (p=0.804, p=0.621). The only group that was significantly different was the group with ethanol and $NaBH_4$ treatment (38.5±37.3 ug/mg) after GA fixation (p=0.003). From previous studies, treatment with L-lysine alone had a similar anti-calcification effect to ethanol treatment. Instead, calcium deposition increased in the group treated with ethanol, L-lysine and $NaBH_4$ altogether after GA fixation [19]. Another study has also reported an anti-calcification effect for ethanol and $NaBH_4$ treatment. However, progression of calcific degeneration has been reported with $NaBH_4$ treatment alone [21]. Because the mechanism has yet to be elucidated, there are limitations to our current ways of evaluating the effect of $NaBH_4$ in this study. L-arginine is known to exhibit antiplatelet activity, angiogenesis, wound healing promotion, and nerve regeneration effects [20].

Compared to adults, accelerated calcific degeneration in growing children is observed due to a relatively active metabolism. Limitations of the applicability of bioprosthetic tissues to pediatric patients lie in the thickness of bioprosthetic tissues made from porcine valve or bovine pericardium. The bovine pericardial thickness (0.29±0.06 mm) obtained from 28- to 30-month-old cattle was found to be more than twice the porcine pericardial thickness (0.13±0.05 mm) obtained from 3- to 4-month-old pigs [16]; therefore, bioprosthetic tissues made from porcine pericardium appear to be more suitable in pediatric patients. The life span of a mouse is about two to three years. The period of lactation continues for approximately three weeks, and that of growth lasts until 14 weeks. Then, it matures into an adult mouse. We used three-week-old mice because previous studies have observed a faster calcific degeneration rate at younger ages.

CONCLUSION

L-arginine and $NaBH_4$ treatment on GA-fixed porcine pericardium increased tensile strength and thickness and also preserved thermal stability, but no anti-calcification effect was observed. However, combining $NaBH_4$ and ethanol did show an anti-calcification effect.

In the midst of a growing demand for surgical bioprostheses, late calcific degeneration of bioprosthetic tissues made from chemically treated animal tissues after implantation remains to be solved. To mitigate calcific degeneration, we post-treated the porcine pericardium with L-arginine and $NaBH_4$ after 0.625% GA fixation. This potentially has significant implications for future manufacture and commercialization of transplantable bioprosthetic valves for adult or pediatric patients. In order to verify the established anti-calcification strategy, animal studies should be performed as a next step.

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