Interactive CardioVascular and Thoracic Surgery 24 (2017) 402–406
doi:10.1093/icvts/ivw356 Advance Access publication 15 December 2016

ORIGINAL ARTICLE

Cite this article as: Lee C, Lim H-G, Lee C-H, Kim YJ. Effects of glutaraldehyde concentration and fixation time on material characteristics and calcification of bovine pericardium: implications for the optimal method of fixation of autologous pericardium used for cardiovascular surgery. Interact CardioVasc Thorac Surg 2017;24:402–6.

Effects of glutaraldehyde concentration and fixation time on material characteristics and calcification of bovine pericardium: implications for the optimal method of fixation of autologous pericardium used for cardiovascular surgery

Cheul Lee[a], Hong-Gook Lim[b], Chang-Ha Lee[c] and Yong Jin Kim[b,*]

[a] Department of Thoracic and Cardiovascular Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea
[b] Department of Thoracic and Cardiovascular Surgery, Seoul National University College of Medicine, Seoul National University Hospital Clinical Research Institute, Xenotransplantation Research Center, Seoul, Republic of Korea
[c] Department of Thoracic and Cardiovascular Surgery, Sejong General Hospital, Bucheon, Republic of Korea

* Corresponding author: Department of Thoracic and Cardiovascular Surgery, Seoul National University College of Medicine, Seoul National University Hospital Clinical Research Institute, Xenotransplantation Research Center, 101 Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea. Tel: +82-2-20723638; fax: +82-2-7455209; e-mail: kyj@plaza.snu.ac.kr (Y.J. Kim).

Received 1 May 2016; received in revised form 5 September 2016; accepted 20 September 2016

Abstract

OBJECTIVES: Autologous pericardium, which is widely used in the field of cardiovascular surgery, is usually fixed with glutaraldehyde (GA) to improve handling and provide biomechanical stability. However, an optimal method of GA fixation of autologous pericardium is not known. The objective of this study was to evaluate the effects of GA concentration and fixation time on material characteristics and calcification of bovine pericardium.

METHODS: Bovine pericardial tissues were fixed with different concentrations of GA (0.3, 0.4, 0.5 and 0.6%) for different exposure times (10 and 20 min). Material characteristics of the fixed tissues were assessed by mechanical test, thermal stability test and pronase test. The tissues were subcutaneously implanted into 3-week-old rats for 2 months, and the calcium contents of the explanted tissues were measured. Differences between the groups were evaluated by two-way analysis of variance.

RESULTS: Differently treated tissues showed no significant differences in tensile strength. The mean elongation at break of the pericardial tissues fixed with 0.5 and 0.6% was significantly higher compared with 0.3 and 0.4% when fixed for 20 min. The mean elongation at break of the pericardial tissues fixed for 20 min was significantly higher compared with 10 min when fixed with 0.5 and 0.6%. Thermal stability test revealed significantly higher mean shrinkage temperature of the pericardial tissues fixed with 0.6% compared with lower concentrations irrespective of fixation time. The mean shrinkage temperature of the pericardial tissues fixed for 20 min was significantly higher compared with 10 min irrespective of GA concentration. Pronase test revealed significantly lower mean percent remaining weight of the pericardial tissues fixed with 0.3% compared with higher concentrations irrespective of fixation time. The mean percent remaining weight of the pericardial tissues fixed for 20 min was significantly higher compared with 10 min irrespective of GA concentration. The mean calcium content of the pericardial tissues fixed with 0.6% was significantly lower than that of the pericardial tissues fixed with 0.4% irrespective of fixation time.

CONCLUSIONS: Fixation of bovine pericardium with 0.5 and 0.6% GA for 20 min produced superior results with regard to material characteristics (mechanical properties, degree of fixation and resistance to enzymatic degradation) and postimplantation calcification. These results may have implications for optimal fixation of autologous pericardium used for cardiovascular surgery.

Keywords: Calcification • Fixation • Glutaraldehyde • Pericardium

INTRODUCTION

Autologous pericardium is widely used for patches, valve substitutes and conduits in the field of congenital and acquired cardiovascular surgery. Before implantation, autologous pericardium is usually fixed with glutaraldehyde (GA) to improve handling and provide biomechanical stability to the tissue by producing cross-linking between collagen helices. However, GA-fixed autologous or xenogenic tissues are prone to calcification after long-term implantation in humans, and this is one of the factors

limiting durability of cardiovascular implants made of GA-fixed tissues [1–3]. The mechanism of calcification of GA-fixed tissue is complex, but there are evidences that tissue phospholipids, free aldehyde groups of GA, and residual antigenicity of the xenogenic tissue, all play an important role [1–3].

Various methods for rapid GA fixation of autologous pericardium have been used in clinical practice according to the surgeon's preference (GA concentration of 0.2–1%, fixation time of 1–30 min) [4–12]. However, optimal GA fixation of autologous pericardium is not clearly defined. Performing experiments using human pericardium to find out the optimal method of GA fixation is practically impossible. The objective of this study was to evaluate the effects of GA concentration and fixation time on material characteristics and calcification of bovine pericardium. The results of this study may have implications for defining optimal GA fixation of autologous pericardium used for cardiovascular surgery.

MATERIALS AND METHODS

Tissue preparation and experimental design

Fresh bovine pericardia were obtained from local slaughterhouse and prepared for fixation as have been described in our previous studies [2, 3]. Tissues were then fixed with GA solutions (pH 7.4) of four different concentrations (0.3, 0.4, 0.5 and 0.6%) for two different exposure times (10 and 20 min) at room temperature. For reference, the composition of 0.5% GA solution is as follows: HEPES 11.9 g/l, $MgCl_2{\cdot}6H_2O$ 5.55 g/l, 5 M NaCl 16 ml/l, 50% GA 10 ml/l, $NaHCO_3$ 0.5 g/l, and deionized water 974 ml. The volume of GA used for fixation was ~15–20 times the weight of the pericardium. Fixed tissues were used for assessment of material characteristics (mechanical test, thermal stability test and pronase test) and subcutaneously implanted into rats to induce calcification. Because many samples were required for various *in vitro* and *in vivo* tests, samples were cut from more than one pericardium.

Mechanical test

Mechanical test was performed as described in our previous study [2]. Rectangular tissue strips (5 × 50 mm, 12 strips for each group) were used for measurement of tensile strength and elongation at break.

Thermal stability test

Thermal stability test was performed as described in our previous study (Fig. 1) [2]. Five tissue strips (8 × 30 mm) per each group were used for measurement of shrinkage temperature.

Pronase test

Pronase (Roche, Germany) test was performed as have been described in our previous study [13]. Five samples (10 × 10 mm) per each group were used for measurement of resistance to enzymatic degradation. Resistance to enzymatic degradation was determined by the weight of remaining tissue, expressed as a percentage of predigested tissue weight.

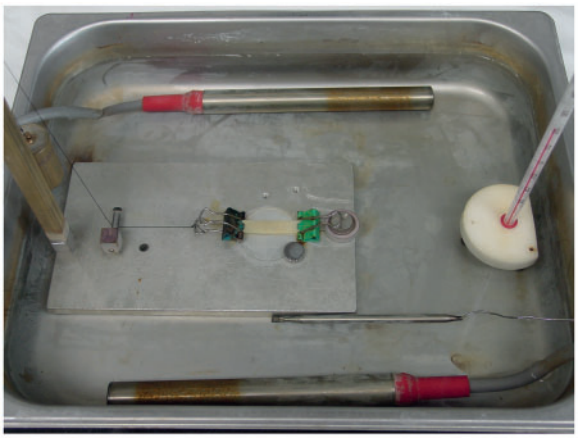

Figure 1: A custom-built extensometer used for thermal stability test.

Rat subcutaneous implantation

This study was approved by the Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No. 12-0147). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Twenty-four male Sprague-Dawley rats (3 weeks old) were used. After anaesthetizing and shaving, five subcutaneous pouches were created at the dorsal area for each rat. Round pericardial samples (diameter of 1 cm) were implanted into the pouches and the wounds were closed with 4/0 nylon sutures. Pericardial samples have been rinsed prior to implantation to reduce the residual GA. No detoxification process was used. The rats were sacrificed by CO_2 asphyxiation after 2 months. The pericardial samples were harvested, freed of adherent rat tissues and rinsed with normal saline. The edges of the pericardial samples were trimmed. The harvested samples were used for quantitative calcium analysis.

Calcium analysis

Quantitative calcium analysis was performed as have been described in our previous studies [2, 3]. Eleven to 14 samples per each group were used for the analysis.

Statistical analysis

Data are presented as means with standard deviations. Two-way analysis of variance was used to determine whether there was an interaction effect between two independent variables (GA concentration and fixation time) on a continuous dependent variable (tensile strength, elongation at break, shrinkage temperature, percent remaining weight and calcium content). When appropriate, post hoc test was used to evaluate the differences of dependent variables between the groups. All statistical analyses were performed using the SPSS software version 18.0 (SPSS, Inc., Chicago, IL). Statistical significance was defined as $P < 0.05$.

Table 1: Mechanical test ($N = 12$ for each group)

GA concentration (%)	Fixation time (min)	Tensile strength (MPa)	Elongation at break (%)
0.3	10	19.7 ± 5.7	53.4 ± 6.9
	20	19.1 ± 12.1	54.3 ± 17.7
0.4	10	18.0 ± 8.1	55.9 ± 11.1
	20	18.6 ± 7.5	55.6 ± 7.1
0.5	10	13.7 ± 4.1	57.5 ± 7.0
	20	16.6 ± 5.5	77.9 ± 13.6[a,b]
0.6	10	13.3 ± 6.5	64.4 ± 14.3
	20	16.3 ± 4.5	75.2 ± 16.0[a,b]

Data are presented as means with standard deviations.
GA: glutaraldehyde.
[a]Significantly higher compared with 0.3 and 0.4% when fixed for 20 min.
[b]Significantly higher compared with 10 min when fixed with 0.5 and 0.6% concentrations.

Table 2: Thermal stability test ($N = 5$ for each group)

GA concentration (%)	Fixation time (min)	Shrinkage temperature (°C)
0.3	10	70.8 ± 3.2
	20[b]	73.9 ± 2.2
0.4	10	72.3 ± 0.0
	20[b]	73.1 ± 1.8
0.5	10	73.1 ± 1.8
	20[b]	76.3 ± 0.0
0.6[a]	10	76.5 ± 2.7
	20[b]	80.8 ± 1.1

Data are presented as means with standard deviations.
GA: glutaraldehyde.
[a]Significantly higher compared with lower concentrations irrespective of fixation time.
[b]Significantly higher compared with 10 min irrespective of GA concentration.

RESULTS

Mechanical test

There was no significant interaction between GA concentration and fixation time on tensile strength ($F = 0.36$, $P = 0.78$). There was no significant main effect of GA concentration and fixation time on tensile strength ($F = 2.45$, $P = 0.069$; $F = 0.97$, $P = 0.33$, respectively) (Table 1).

There was significant interaction between GA concentration and fixation time on elongation at break ($F = 3.68$, $P = 0.015$). The mean elongation at break of the pericardial tissues fixed with 0.5 and 0.6% concentrations was significantly higher compared with 0.3 and 0.4% when fixed for 20 min ($F = 12.30$, $P < 0.001$). When fixed for 10 min, there was no significant difference of the mean elongation at break according to GA concentration ($F = 1.73$, $P = 0.17$). The mean elongation at break of the pericardial tissues fixed for 20 min was significantly higher compared with 10 min when fixed with 0.5 and 0.6% concentrations ($F = 16.35$, $P < 0.001$; $F = 4.58$, $P = 0.035$, respectively). When fixed with 0.3 and 0.4% concentrations, there was no significant difference of the mean elongation at break according to fixation time ($F = 0.035$, $P = 0.85$; $F = 0.005$, $P = 0.95$, respectively) (Table 1).

Thermal stability test

Thermal stability test revealed a trend towards increasing shrinkage temperature with increasing GA concentration and fixation time (Table 2). There was no significant interaction between GA concentration and fixation time on shrinkage temperature ($F = 1.44$, $P = 0.25$). An analysis of the main effect for GA concentration was performed, which indicated that the main effect was significant ($F = 22.17$, $P < 0.001$). The mean shrinkage temperature of the pericardial tissues fixed with 0.6% concentration was significantly higher compared with lower concentrations irrespective of fixation time ($P < 0.001$). An analysis of the main effect for fixation time was performed, which indicated that the main effect was significant ($F = 21.44$, $P < 0.001$). The mean shrinkage temperature of the pericardial tissues fixed for 20 min was significantly higher compared with 10 min irrespective of GA concentration.

Pronase test

Pronase test revealed a trend towards increasing resistance to enzymatic degradation with increasing GA concentration and fixation time (Table 3). There was no significant interaction between GA concentration and fixation time on resistance to enzymatic degradation ($F = 0.69$, $P = 0.56$). An analysis of the main effect for GA concentration was performed, which indicated that the main effect was significant ($F = 24.55$, $P < 0.001$). The mean percent remaining weight of the pericardial tissues fixed with 0.3% concentration was significantly lower compared with higher concentrations irrespective of fixation time ($P < 0.001$). An analysis of the main effect for fixation time was performed, which indicated that the main effect was significant ($F = 8.54$, $P = 0.006$). The mean percent remaining weight of the pericardial tissues fixed for 20 min was significantly higher compared with 10 min irrespective of GA concentration.

Calcium analysis

Quantitative calcium analysis revealed a trend towards decreasing calcification with increasing GA concentration (Table 4). There was no significant interaction between GA concentration and fixation time on calcification ($F = 0.027$, $P = 0.99$). An analysis of the main effect for GA concentration was performed, which indicated that the main effect was significant ($F = 3.41$, $P = 0.021$). The mean calcium content of the pericardial tissues fixed with 0.6% was significantly lower compared with 0.4% irrespective of fixation time ($P = 0.030$), and tended to be lower compared with 0.3% ($P = 0.083$). An analysis of the main effect for fixation time was performed, which indicated that the main effect was not significant ($F = 0.088$, $P = 0.77$).

Table 3: Pronase test ($N = 5$ for each group)

GA concentration (%)	Fixation time (min)	Percent remaining weight
0.3[a]	10	59.2 ± 8.0
	20[b]	68.9 ± 6.2
0.4	10	78.4 ± 7.5
	20[b]	84.4 ± 7.0
0.5	10	80.2 ± 7.3
	20[b]	88.6 ± 3.1
0.6	10	88.2 ± 7.9
	20[b]	89.6 ± 7.2

Data are presented as means with standard deviations.
GA: glutaraldehyde.
[a]Significantly lower compared with higher concentrations irrespective of fixation time.
[b]Significantly higher compared with 10 min irrespective of GA concentration.

Table 4: Calcium analysis

GA concentration (%)	Fixation time (min)	*N*	Calcium (μg/mg)
0.3	10	14	51.2 ± 17.2
	20	12	50.8 ± 35.3
0.4	10	14	52.8 ± 10.9
	20	12	53.6 ± 37.1
0.5	10	12	42.4 ± 7.5
	20	13	44.2 ± 7.5
0.6[a]	10	11	34.4 ± 14.6
	20	14	37.3 ± 20.6

Data are presented as means with standard deviations.
GA: glutaraldehyde.
[a]Significantly lower compared with 0.4% irrespective of fixation time and tended to be lower compared with 0.3%.

DISCUSSION

The ideal characteristics of GA-fixed autologous pericardial implants used for cardiovascular surgery include maintenance of mechanical tissue stability, prevention of autolysis, resistance to enzymatic degradation and minimal calcification after long-term implantation. Although rare, fresh or inadequately fixed autologous pericardium can undergo aneurysmal dilatation, especially when it is used as a patch in the high-pressure circulation [14–16]. Calcification is a major factor limiting durability of the GA-fixed pericardial implants, especially when they are used as valve substitutes. In clinical practice, GA concentration and fixation time are two main variables that we can control for optimal fixation of autologous pericardium. Various GA concentration and fixation time have been used according to the surgeon's preference [4–12]. However, to our knowledge, there have been no studies examining the effects of various conditions for GA fixation on both material characteristics and calcification of pericardial tissue.

The findings of this study can be summarized as follows: (i) Tensile strength was not affected by fixation conditions. (ii) Fixation with 0.5 or 0.6% GA for 20 min produced superior material extensibility. (iii) Fixation with 0.6% GA for 20 min produced superior degree of fixation. (iv) Fixation with 0.3% GA was inferior and fixation for 20 min was superior regarding resistance to enzymatic degradation. (v) Fixation with 0.6% GA produced less calcification compared with lower concentrations (0.3 and 0.4%) while the degree of calcification was not affected by fixation time.

Tensile strength was not affected by the different fixation conditions used in our study. It seems that GA fixation does not have a significant effect on tensile strength of pericardial tissue. In our previous study, the tensile strength of bovine pericardium was not affected by different fixation conditions and anticalcification treatments [2]. Furthermore, the tensile strength of fresh bovine or autologous pericardium was not significantly different from that of GA-fixed bovine or autologous pericardium [2, 17]. Meanwhile, fixation with higher concentrations of GA for longer duration produced superior extensibility (elongation at break) of pericardial tissue. Presumably, this was due to the greater degree of 'shrinkage' of pericardial tissue by escalating intensity of fixation. Although clinical benefits of improved extensibility of pericardial implants remain to be determined, it is likely that an extensible material is more suitable for reconstruction of vascular structures compared with rigid materials.

Shrinkage temperature provides the degree of fixation (cross-linking between collagen molecules) of pericardial tissue. Adequate fixation of pericardial tissue is of paramount importance for maintaining material stability. As expected, the shrinkage temperature of pericardial tissue increased with increasing GA concentration and fixation time, producing the best result when fixed with 0.6% GA for 20 min. A previous study demonstrated, by measuring shrinkage temperature, that even as little as 15 min of GA fixation could make up to 80% of the total cross-linking that was achieved in 2 weeks [18]. In our previous study, the shrinkage temperature of the bovine pericardium fixed with 0.5% GA for 2 weeks was 86.3°C [2]. In this study, the shrinkage temperature of the bovine pericardium fixed with 0.6% GA for 20 min was 80.8°C. Comparing these results, we think that fixation with 0.6% GA for 20 min is adequate in terms of the degree of fixation (∼94% of the total cross-linking that was achieved in 2 weeks using 0.5% GA). Pronase test revealed that fixation with 0.3% GA was inferior compared with higher concentrations in terms of resistance to enzymatic degradation (biodegradation). Furthermore, there was an obvious trend towards increasing resistance to biodegradation with increasing GA concentration. Longer fixation time increased the resistance to biodegradation. Based on the results of thermal stability test and pronase test, we suggest that inappropriately low concentrations of GA and too short fixation time should be avoided during rapid fixation of autologous pericardium to guarantee material stability.

In an effort to minimize calcification, commercially available tissue valves and pericardial patches are manufactured using various anticalcification processes [1, 19]. However, such processes are complex and require long treatment time, and therefore impractical for rapid fixation of autologous pericardium within the time limit of a surgical procedure. In our study, fixation with 0.6% GA produced less calcification compared with 0.3 and 0.4% while the degree of calcification was not affected by fixation time. Residual free aldehyde groups or polymerized forms of GA are cytotoxic and are known to contribute to the calcification of bioprostheses [1–3, 20]. Therefore, inappropriately high concentrations of GA may be detrimental in terms of calcification potential. Sinha *et al.* [21], in their study of effects of GA concentration and fixation time on postimplantation calcification, found significantly greater calcium levels at

1.2% compared with lower concentrations (0.3125 and 0.625%) for both porcine and bovine pericardia. Although some investigators reported that high-concentration GA fixation proved to be effective in prevention of calcification presumably by suppressing residual antigenicity of bioprosthetic tissue [22], we believe that post-fixation detoxification process is essential in order to neutralize the intrinsic calcification potential of high-concentration GA fixation [3, 23]. Inappropriately low concentrations of GA also may be detrimental in terms of calcification potential. It is generally thought that incomplete fixation (cross-linking) cannot suppress antigenicity of the xenogenic tissue adequately, although this is not an issue when we use autologous pericardium. Residual antigenicity of the xenogenic tissue is one of the factors contributing bioprosthetic calcification [3, 24]. Fixation time is another important factor to minimize calcification of pericardial implants. Liao *et al.* [18] recommended that GA treatment for autologous pericardium should be no more than 60 min to avoid excessive calcification. Based on the results of calcium analysis, we think that GA concentrations of less than 0.5% within the usual time frame (10–20 min) of treatment are detrimental in terms of calcification.

Limitations

We used bovine pericardium, which is much thicker than human pericardium, as a surrogate for human pericardium and sample sizes of our experiments were small. Data regarding postimplantation calcification obtained from rat subcutaneous implantation study may not hold true in human. Also, it is important to acknowledge that the lack of control data (untreated pericardium) is another limitation of our study. Further studies using large-animal long-term circulatory model to verify our results are mandatory.

CONCLUSIONS

Fixation of bovine pericardium with 0.5 and 0.6% GA for 20 min produced superior results with regard to material characteristics (mechanical properties, degree of fixation and resistance to enzymatic degradation) and postimplantation calcification. These results may have implications for optimal fixation of autologous pericardium used for cardiovascular surgery.

Funding

This study was supported by a grant of the Korea Health 21 Research and Development Project, Ministry of Health and Welfare, Republic of Korea [A040004-006].

Conflict of interest: none declared.

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