筑　波　大　学

博　士　（医　学）　学　位　論　文

Shrinkage temperature and anti-calcification property of triglycidylamine-crosslinked autologous tissue

（自己組織のトリグリシジルアミンによる架橋処理後の組織強度と抗石灰化効果についての検討）

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筑波大学大学院博士課程人間総合科学研究科

佐藤　真剛

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Biomaterials

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Abstract Since bioprosthetic valve dysfunction may arise due to histological calcification in the crosslinking process by glutaraldehyde (GA), non-GA crosslinking reagents have been investigated. We compared the efficacy of triglycidylamine (TGA), a newly synthesized epoxy compound, and GA as crosslinking reagents for the treatment of autologous tissues. We assessed the strength of crosslinked tissues using shrinkage temperature (Ts) measured by differential scanning calorimetry. We also conducted subdermal allografting of the crosslinked pericardium and thoracic aorta in rats, and verified the anti-calcification efficacy of TGA by histological evaluations with von Kossa stain, and immunological evaluations using tenascin-C (TN-C) or matrix metalloproteinase-9 (MMP-9). TGA treatment resulted in slower increases in Ts of the pericardium, and it required 9–12 h to reach Ts achieved by GA. In subdermal implantation of rat tissues, calcium content was lower in the TGA group than in the GA groups ($p < 0.005$). The expression site of TN-C and MMP-9 differed from the primary location of calcium deposition in the thoracic aorta treated with TGA suggesting a different underlying mechanism in calcification between GA and TGA crosslinking. In conclusion, TGA crosslinking in the allograft showed superior anti-calcification effect as compared to brief treatment by GA, although TGA crosslinking process was slow.

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Introduction

Glutaraldehyde (GA) has been widely used in the crosslinking treatment of heterograft bioprosthetic valves for the past 40 years [1, 2]. The GA crosslinking eliminates antigenicity and provides tensile strength and pliability. However, structural bioprosthetic valve deterioration may arise mostly due to histological calcification caused by the residue of unstable GA and phospholipids which react with the devitalized connective tissue cells even in an advanced GA fixation method that takes several days [3, 4]. A short time fixation of autologous pericardium on operation site is also a common practice in cardiovascular reconstruction. Such brief GA fixation, however, may cause functional disturbance due to serious calcification and loss of elasticity in the long term. Nonetheless, until now no well-defined guidelines for the on-site brief GA fixation have yet been established, and information regarding tissue strength, calcium contents and histological features of briefly treated tissues has been limited. Thus, an important purpose of this study is a search for a novel crosslinking agent that is superior to GA even in on-site brief crosslinking.

Epoxy compounds have been widely used as crosslinking agents, adhesion promoters, and stabilizers of textile and paper [5, 6]. Triglycidylamine (TGA; $C_9H_{15}NO_3$, MW 185.22) is a newly synthesized epoxy compound which has a large number of effective epoxy functional groups, and is thus expected to exhibit high reactivity (Fig. 1). A previous study reported increased calcification resistance in subdermal implants in a study using porcine aortic valve and bovine pericardium [7]. TGA-treated pericardium

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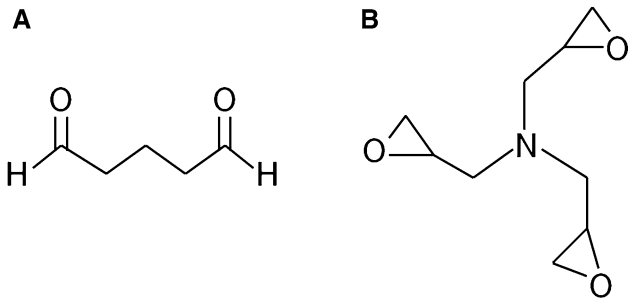


Fig. 1 A chemical structure of GA (**a**) and TGA (**b**). Whereas GA has a rigid and hydrophobic five-carbon chain, TGA has a flexible ether bond

demonstrated stable mechanical function in a mitral valvuloplasty model [8].

In this study, we aimed to explore the efficacy of TGA as a novel crosslinking reagent. We assessed the strength of crosslinked tissues using shrinkage temperature (Ts) measured by differential scanning calorimetry (DSC). We also conducted subcutaneous allografting of the crosslinked pericardium and thoracic aorta in rats, and verified the efficacy of TGA in terms of anti-calcification effect. Shrinkage temperature and anti-calcification property of TGA-crosslinked autologous tissues were compared with those of tissues treated by GA for a short period (10–30 min).

Materials and methods

TGA synthesis

TGA was synthesized as previously described [7]. In brief, aqueous ammonia was reacted with an excess of epichlorohydrin and catalytic amounts of ammonium triflate in isopropanol. The solvents were distilled off and the residual epichlorohydrin was removed. The resulting syrup consisting mostly of tris-(3-chloro-2-hydroxypropyl) amine was dissolved in a mixture of toluene, tetrahydrofuran and tert-butanol. An excess of 50 % aqueous NaOH solution was added. In 2 h, the epoxy ring closure was complete. Ice cold water was added to dissolve the precipitate of NaCl. The desiccant was filtered and vacuum-concentrated. The residue was vacuum-distilled to give pure TGA. A novel cold crystallization technique resulted in a coarse white water-soluble powder, preserved in dark place at room temperature.

Tissue preparation and crosslinking

We obtained the remnant of fresh human pericardium which was harvested during surgery with the approval of our institutional ethics committee on human research (approval number H22-612). Written informed consent was obtained from each patient. Pericardium was rinsed in saline, then divided into 4 portions and fixed with GA and TGA as follows. We divided the samples into two groups to determine whether GA plays a role directly in the tissue calcification process: the GA group (0.6 % GA; diluted 8 % GA with distilled water) and the GA-c group (0.6 % GA following Carpentier's recipe; 8 % GA 26 mL, distilled water 700 mL, $MgCl_2$ 4 g, and 1 M HEPES 20 mL, pH 7.4) [3, 4]. In both GA-fixed groups, pericardium was treated at room temperature for 10, 20 or 30 min. Tissues fixed with TGA were treated with 100 mmol/L TGA in borate mannitol buffer (25 mmol/L sodium tetraborate decahydrate, pH 7.4) at 37 °C, for 1, 3, 6, 9, or 12 h.

DSC analysis

The temperature at which denaturation and shrinkage under constant load begins is termed "shrinkage temperature", an indicator of collagen crosslinking density and tissue strength [1, 7, 9]. DSC is a thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and a reference material (aluminum) is measured. When a solid sample melts, more heat energy is absorbed as compared to the reference material as a heat of fusion. In crystallization, heat is released as the exothermic reaction. By observing the difference in heat flow between the sample and reference material, DSC is able to measure the amount of heat absorbed or released during phase transitions [9]. In a DSC analyzer (X-DSC700, Seiko Instruments Inc., Japan), tissue samples (approximately 4–5 mg) were sealed in aluminum pans and heated at a rate of 10 °C/min from 40 to 100 °C, and then Ts was determined as the temperature measured at the endothermic peak.

Rat subdermal implants and tissue calcification measurement

The Committee on Animal Research at the University of Tsukuba approved the experimental protocols. The rats used were cared for according to the Guiding Principles based on the Helsinki Declaration. Male Wister rats were used ($n = 45$). The number of donor rat was 36 and recipient was nine. A piece of pericardium and thoracic aorta was excised from one donor rat. Total number of 36 pieces of pericardium and thoracic aorta were divided into four groups: GA, GA-c, TGA and untreated control ($n = 9$, each). One recipient rat accepted four pieces of pericardium and thoracic aorta from four different donors. Materials were fixed at room temperature for 10 min in the GA and GA-c groups, and at 37 °C for 12 h in the TGA

group. All materials and untreated control materials were implanted subdermally. After 21 days, on animal killing, all materials were removed and rinsed in saline. Each material (15–40 mg dry weight) was hydrolyzed in 1 mL of 6 N HCl at 70 °C for 4–6 h. The solution was filtered and diluted with distilled water in 0.01 N HCl. The calcium content of each material was measured based on the aliquot concentration of each hydrolysate using an inductively coupled plasma atomic emission spectrometry method (ICP-AES) by a spectrometer (ICP-8100, Shimazu, Japan). ICP-AES is a type of emission spectroscopy that uses inductively coupled plasma to produce excited atoms and ions [10].

Histological processing of explants

Rat subdermal explants embedded in paraffin were cut to 5 μm cross sections and stained with von Kossa stain. Serial sections were also immunostained using peroxidase methodology for either tenascin-C (TN-C) or matrix metalloproteinase (MMP)-9, using human TN-C monoclonal antibody (Cosmo Bio, Japan) and rabbit anti-MMP-9 monoclonal antibody (Abcam, Japan). Universal immuno-peroxidase polymer for rat (Nichirei, Japan) was used as secondary antibodies and peroxidase complex. The immune complexes were colored using diaminobenzidine solution (Simple Stain DAB solution, Nichirei, Japan).

Staining of von Kossa was scored visually, with a numerical rating of 1–5 assigned based on the following criteria: 1 = negative, 2 = rare detection, 3 = sparse but consistent, 4 = uniformly present, and 5 = intense and wide-spread staining.

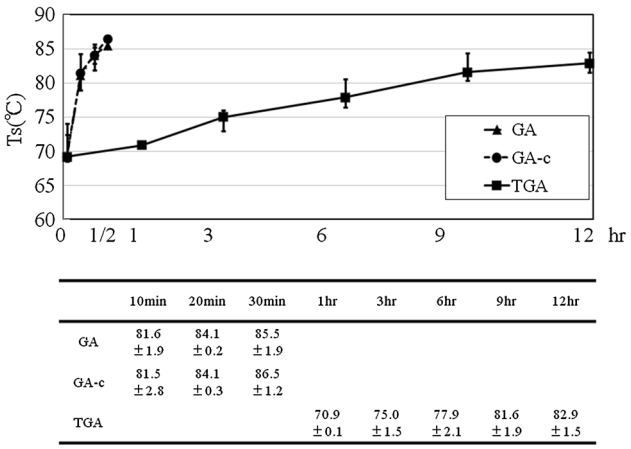
Data analyses

Quantitative results were expressed as mean ± standard error of the mean. Differences between each group were assessed using unpaired Student's *t* test. Data were termed significant when $p < 0.05$.

Results

Ts measurements by DSC analysis

The DSC results showed that brief treatment with GA increased the Ts of the pericardium, indicating GA's ability to rapidly crosslink the tissue. There was no significant difference in the Ts of pericardium between GA and GA-c groups for any treatment period. For TGA treatment, increases in the Ts of the pericardium were slow in comparison with those observed in the GA-crosslinked tissues; it took 9–12 h of reaction time to reach the equal level of Ts achieved by GA crosslinking (Fig. 2).



	10min	20min	30min	1hr	3hr	6hr	9hr	12hr
GA	81.6 ±1.9	84.1 ±0.2	85.5 ±1.9					
GA-c	81.5 ±2.8	84.1 ±0.3	86.5 ±1.2					
TGA				70.9 ±0.1	75.0 ±1.5	77.9 ±2.1	81.6 ±1.9	82.9 ±1.5

Fig. 2 Crosslinking strength of GA, GA-c and TGA groups. Shrinkage temperature (Ts) of human pericardium. 9–12 h of 37 °C treatment with TGA results in Ts equal to that of the GA and GA-c groups' 10 min-treatment at room temperature

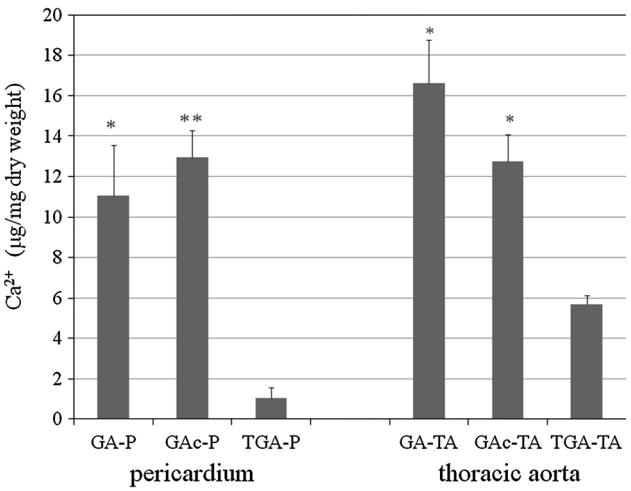


Fig. 3 Calcium content of 21-day rat subdermal explants, showing reduced calcification in both homologous pericardium and thoracic aorta treated with TGA as compared to GA or GA-c group. $*P < 0.005$ vs. TGA, $**P < 0.001$ vs. TGA-*P*: pericardium, -*TA*: thoracic aorta

Rat subdermal implants and tissue calcification measurement

There were no significant differences between the GA and GA-c groups in the calcium content (μg/mg, on a dry tissue weight basis) of the pericardium (11.0 ± 2.4 vs. 12.9 ± 1.3, $p = 0.52$) or thoracic aorta (16.6 ± 2.1 vs. 12.7 ± 1.3, $p = 0.14$). Calcium content was significantly lower in the

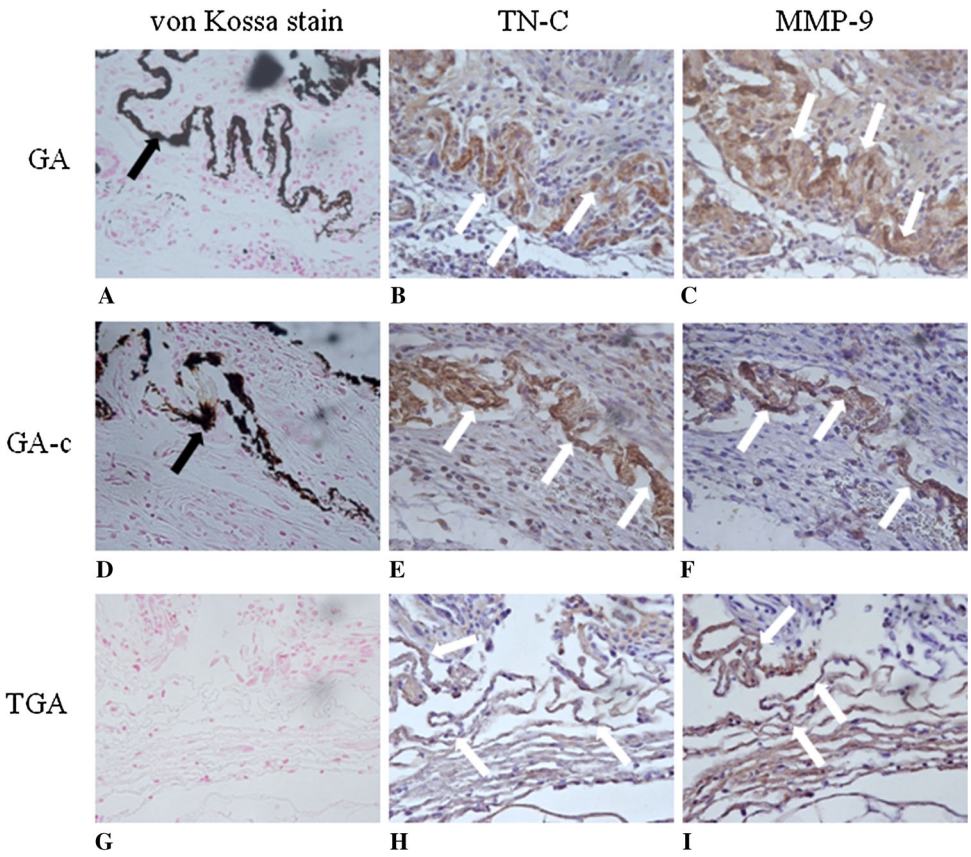


Fig. 4 Histochemistry of pericardium; von Kossa staining and immunohistochemistry of GA group (**a**, **b**, **c**), GA-c group (**d**, **e**, **f**) and TGA group (**g**, **h**, **i**). von Kossa staining of 21-day pericardium explants showing significant calcification (*black arrow*) in GA and GA-c groups (**a**, **d**), in contrast an absence of calcified staining in TGA group (**g**). Immunohistochemistry for TN-C and MMP-9 demonstrated lower expression in TGA group (*white arrow*). Magnification: ×400 (**a**–**i**)

TGA group than in both the GA and GA-c groups: the calcium content of the pericardium was 1.0 ± 0.5 ($p = 0.0036$ vs. GA group, and $p = 0.00036$ vs. GA-c group), and that of the thoracic aorta was 5.6 ± 0.4 ($p = 0.0013$ vs. GA group, and $p = 0.0011$ vs. GA-c group) (Fig. 3).

Histological processing of explants

Staining of von Kossa showed strong calcium deposition in the GA group from the tunica media to the adventitia, but not in the intima. In the TGA group, on the other hand, the degree of calcification was mild and the deposition was uniform from the tunica intima to the adventitia, although the accumulation was a little higher close to the adventitia. Moreover, in the GA group, strong calcification was noted in the smooth muscle layer, and some of the samples did not exhibit calcium deposition in the elastic lamina layers. Calcification observed in the TGA group was higher in the fenestrated elastic laminae than in the smooth muscle layer. Quantitatively, the overall calcium deposition in the pericardium was scored as 2.3 ± 0.3 in the GA group ($p = 0.00011$, vs. TGA), 1.4 ± 0.1 in the GA-c group ($p = 0.0091$, vs. TGA), and 1.0 ± 0.1 (nearly negative) in the TGA group, and that in the thoracic aorta was scored as 4.0 ± 0.1 in the GA group ($p = 0.00023$, vs. TGA), 4.0 ± 0.1 in the GA-c group ($p = 0.00026$, vs. TGA), and 2.4 ± 0.1 in the TGA group. Thus, the degree of calcification was significantly lower in the TGA-treated tissues compared with GA (Figs. 4, 5).

Immunohistochemical staining revealed that TN-C and MMP-9 were similarly expressed in the samples treated with GA and TGA. In the pericardium, both proteins were expressed diffusely along the pericardial tissue, and in the thoracic aorta they were strongly expressed in the cellular components located between fenestrated elastic laminae, especially in the layer rich in smooth muscle cells. The expression patterns of TN-C and MMP-9 were almost consistent with the calcification distribution in the pericardium and thoracic aorta in the GA and GA-c groups. In the TGA group, however, despite the similar expression of TN-C and MMP-9, calcification of the pericardium was significantly mild. Moreover, the expression site of TN-C and MMP-9 (mainly in the smooth muscle layer) differed from the primary location of calcium distribution (fenestrated elastic laminae) in the thoracic aorta treated with TGA (Figs. 4, 5).

Discussion

In efforts to prevent calcification of GA-treated bioprosthetic valves, Carpentier et al. reported that a 6-day-long thermal fixation decreases unstable GA and phospholipids

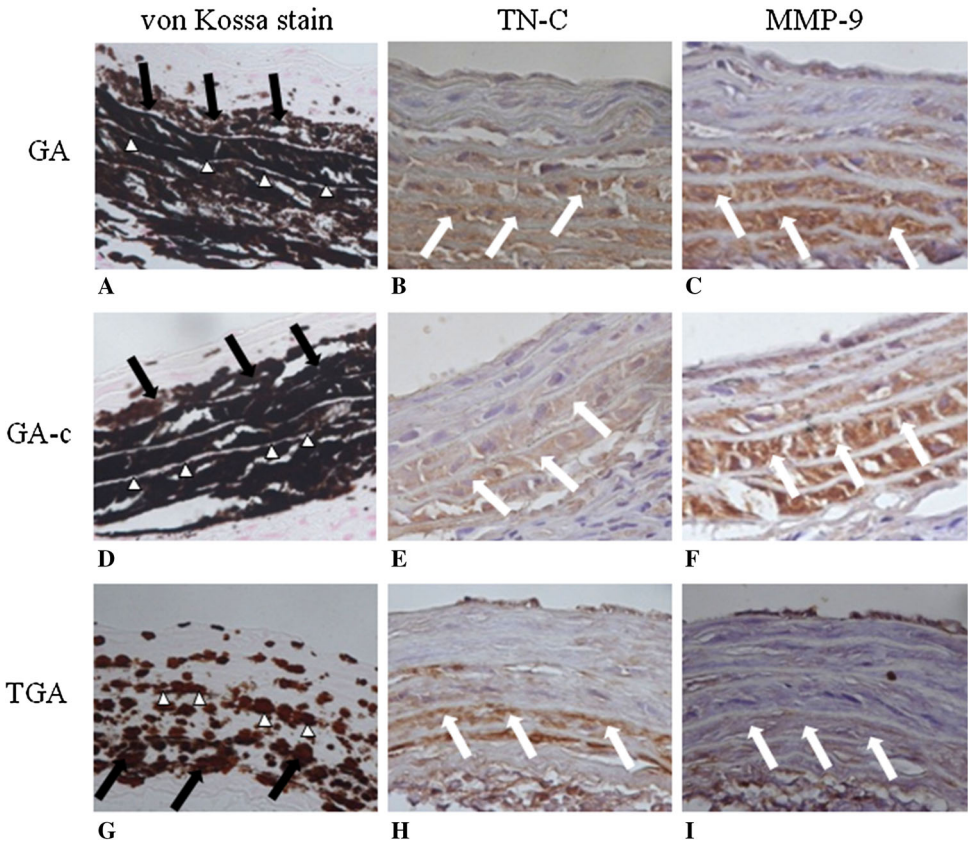


Fig. 5 Histochemistry of thoracic aorta; von Kossa staining and Immunohistochemistry of GA group (**a**, **b**, **c**), GA-c group (**d**, **e**, **f**) and TGA group (**g**, **h**, **i**). von Kossa staining of 21-day thoracic aorta explants showing less calcified tissue in TGA group (**g**) compared to GA and GA-c groups (**a**, **d**) (*black arrow*). Calcium deposition was observed in the layer containing smooth muscle cells separated by fenestrated elastic laminae (*white arrow head*) in GA and GA-c groups, while in the layer corresponding to fenestrated elastic laminae (*white arrow head*) in TGA group. Immunohistochemistry for TN-C and MMP-9 demonstrated lower expression in TGA group (*white arrow*). Magnification: ×400 (**a–i**)

inside bovine pericardium, and therefore inhibits calcification with better elasticity [3, 4]. An additional treatment with ethanol, which was reported by Vyavahare et al. [1], has also been in practical use. These treatments have been effective in enhancing the durability of bioprostheses; however, even with such a long time fixation, complete inhibition of calcification has not yet been achieved.

Brief fixation of autologous pericardium for about 3–10 min at low GA concentrations on surgical site is also a common practice. This quick GA crosslinking without thermal fixation, however, may cause not only functional disturbances due to serious calcification, but also hardening of autologous pericardium and loss of elasticity in the long term. In addition, because of the hydrophobicity and persistent toxicity of unstable GA, host cells do not infiltrate the implanted tissue, resulting in decreased biocompatibility [5]. Nonetheless, on-site fixation of pericardium is still indispensable for various surgical procedures and the fixation needs to be completed within no more than 30 min otherwise it prolongs operation time unnecessarily.

The possible use of epoxy compounds as crosslinking agents for bioprostheses has been studied, and the compounds with high water solubility have been the focus [6, 11]. Whereas GA has a rigid and hydrophobic five-carbon chain which makes GA-treated bioprostheses less pliable and hydrophobic, epoxy compounds have a flexible ether bond in each molecule and therefore epoxy compound-treated tissues are considered to maintain better pliability (Fig. 1). Epoxy compounds form crosslinks with amino, carboxyl, and hydroxyl groups, and create hydroxyl group-bearing molecules after the reaction, making the products more hydrophilic (Fig. 6). Also, TGA crosslinking has been demonstrated to result in a change in collagen structure that could interfere with collagenase-substrate interactions. Moreover, epoxy crosslinking is considered to involve reactions with sulfur-containing amino acids besides common amino acid groups such as the lysine amino side chain in collagen. These connective tissue matrices generated from TGA-amino acid reactions have a complex and firm structure involving collagen–collagen and collagen-protein bonds [7].

TGA is an epoxy compound originally synthesized by Connolly et al. [7] in crosslinking experiments. They reported superior anti-calcification effects, maintenance of pliability, and favorable mechanical hemodynamics. Our DSC results, however, showed that increases in the Ts of the pericardium were markedly slow in the TGA group. It took 9–12 h to attain equivalent levels of strength to brief GA treatment. Since crosslinking reactions of epoxy compounds begin with an epoxy ring opening and such ring-opening reactions are slow processes, this may explain why TGA treatment required the longer reaction time than

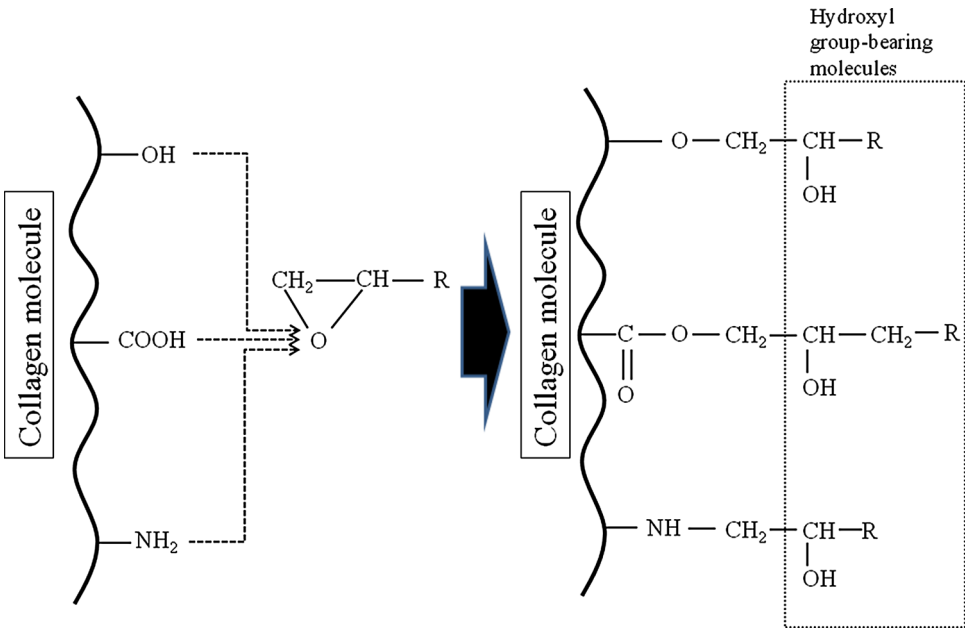


Fig. 6 Epoxy group forms crosslinks with amino (-NH_2), carboxyl (-COOH), and hydroxyl (-OH) groups of collagen, and then creates hydroxyl group-bearing molecules, making the crosslinked products more hydrophilic. R denotes the epoxy-bearing substances

that of GA crosslinking. This issue is a major flaw of TGA crosslinking process and therefore increasing the reaction rate in TGA treatment would be one major goal to enhance the clinical relevance of TGA crosslinking. Similar to other epoxy compounds, it is recognized that reaction rate in TGA treatment is accelerated by high temperature over 100 °C (personal communication). However, considering the denaturation of proteins, a reaction temperature range between about 20 to 40 °C should be appropriate for crosslinking treatment of biological materials. In the present experiment, we applied TGA only at a concentration of 100 mmol/L in accordance with the investigation by Connolly and colleagues [12]. Using higher TGA concentrations may be another option to reduce the treatment time and further investigations are needed. From another aspect to accelerate the TGA reaction, tertiary amines and imidazoles may be useful as catalysts for ring-opening reactions [11]. While tertiary amines are highly cytotoxic and inapplicable to bioprostheses, imidazole-containing compounds are used as antifungal drugs in clinical settings and therefore are likely to become a useful option.

Elastin is an extracellular matrix protein present in various tissues, including arterial walls and heart valves. Elastin calcification occurs in arteriosclerosis or valvular heart disease other than structural bioprosthetic valve deterioration [13]. The expression of the MMP family proteins is known to be involved in elastin calcification. MMPs are mainly produced by smooth muscle cells and macrophages, and their activity is inhibited by tissue inhibitors of metalloproteinases (TIMP). Various studies have revealed that the histological calcification induced by GA treatment is caused by an imbalance of MMP-9 and TIMP, resulting in a significant increase in MMP-9 activity that promotes elastin degradation, thereby increasing the levels of elastin peptides. The elastin peptides stimulate migration of fibroblasts, smooth muscle cells, and monocytes, and these components activate MMP-9 in a positive feedback [13]. It has been reported that TN-C is often co-expressed with MMPs in a variety of tissues, and is involved in the activation of cytokines and the promotion of calcium binding. In addition, TN-C has been reported to increase the expression of MMP family members [14].

There were no significant differences in Ts, the degree of calcium deposition, and expression patterns of TN-C and MMP-9 between the GA group and the GA-c group. In both the GA and TGA crosslinking treatments, TN-C and MMP-9 were observed in the smooth muscle layer of the thoracic aortic media, indicating that the extracellular matrix degenerating enzyme of the MMP family is produced in smooth muscle cells and extracellular matrix glycoprotein TN-C is involved in the calcification process. However, in the TGA-treated thoracic aorta, the expression sites of TN-C and MMP-9 were not consistent with the calcium deposition sites, whereas the expression sites of both proteins well coincided with the calcium deposition sites in the GA-treated aorta. The reason for this phenomenon is unclear, but it may suggest the existence of a different mechanism from the emergence of TN-C and MMP-9 to calcium deposition between GA and TGA crosslinking in the aortic wall. It is also unclear why the degree of calcium deposition was different between the pericardium and the thoracic aorta in the TGA treatment. Whereas the aortic wall consists of smooth muscle cells, fibroblasts and plasma cells in addition to elastin and

collagen, the pericardium mostly involves mesothelium, collagen and elastin, and lacks smooth muscle layer which is the main expression site of TN-C and MMP-9 in the TGA-treated aorta. This fact may be a clue to solve a riddle in the difference of calcium deposition between the two different tissues. Difference in the histological background may vary the expression or activity of TN-C and MMP-9, or may produce different signal transmission between cells in the activation of cytokines and further promotion of calcium binding process.

There are several limitations. The most significant of which concerns the usage of allograft in the rat tissue implant experiments. In addition, it is a subdermal implantation for only 21 days. Therefore, the present study may not be clinically relevant and further chronic animal studies which implant autograft need to be carried out.

Conclusions

TGA crosslinking showed superior anti-calcification effect as compared to GA. The expression site of TN-C and MMP-9 in the TGA group differed from the primary location of calcium deposition. When the underlying mechanism of such a TGA's feature in calcification process is elucidated, efficacy and clinical relevance of TGA as a promising crosslinking agent may be more emphasized even in brief on-site fixation.

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Conflict of interest The authors declare that there is no conflict of interest in respect of this study.

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