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Time-Dependent Effect of Glutaraldehyde on the Tendency to Calcify of Both Autografts and Xenografts

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To determine mechanisms responsible for the reduced calcification in short-term glutaraldehyde (Glu)-treated autologous pericardial bioprostheses, we studied the time effect of Glu on subsequent calcification and differences in calcification of autograft and xenograft implants in a rat subcutaneous implantation model. In experiment 1, four groups of bovine pericardial pieces (1 cm²) were prepared: (A) fresh bovine pericardium without Glu, (B) with 15-minute Glu, (C) with 60-minute Glu, and (D) with 120-minute Glu. Seven young male Sprague-Dawley rats were used; each received four bovine pericardial pieces from group A, B, C, or D for subcutaneous implantation. Calcium content of the implants (µg/mg dry weight) 45 days later was 4.8 ± 2.9, 29.8 ± 13.6, 106.3 ± 13.7, and 176.3 ± 85.5 in groups A, B, C, and D, respectively (p < 0.05 between any two groups). Experiment 2 used 8 young male Sprague-Dawley rats from different mothers. Each received five subcutaneous skin implants. The five skin implants were prepared as follows: (1) fresh self skin, (2) self skin with 30-minute Glu, (3) self skin with 48-hour Glu, (4) fresh skin of others, and (5) skin of others with 48-hour Glu. After 45 days of implantation, the calcium content of the implants was 1.4 ± 1.1, 57.9 ± 35.4, 142.7 ± 61.4, 1.5 ± 1.1, and 94.9 ± 24.1 µg/mg dry weight in groups 1, 2, 3, and 5, respectively (p < 0.05 for 1 versus 2, 3, or 5; 2 versus 3, 4, or 5; 3 versus 4; and 4 versus 5). Neither self nor other skin implants without Glu fixation calcified; however, once treated with Glu, calcification developed in both types of implants. In this model, the extent of calcification of bovine pericardial pieces or skin implants was solely Glu fixation time-dependent. Autologous tissues treated with Glu behave biologically like Glu-treated xenogenic tissues; there was no difference between them regarding the degree of calcification that makes one preferable to the other. A short duration of Glu fixation of autologous tissue used in valve repair is important to avoid calcification.


For the past 20 years, glutaraldehyde (Glu)-treated xenograft pericardium has been used to fabricate bioprosthetic valves for cardiac valve replacement and as material for valve cusp extension or mitral valve chordae replacement. However, clinical application has been overshadowed by limitations in long-term durability, mainly due to pericardial tissue degeneration [1–4]. The search is ongoing for an ideal bioprosthesis valve substitute that is devoid of calcification. Recent reports by several authors have favored the use of autologous pericardium with short-term Glu treatment for valve cusp extension or repair and for whole valve replacement, because of its lack of calcification after implantation [5–10]. These findings have raised some interesting questions, such as whether low calcification can be attributed to the use of autologous tissue or to short-term Glu fixation. To answer this question, we studied the time effect of Glu fixation on calcification and the difference in calcification between autograft and xenograft implants in a rat subcutaneous implantation model.

Material and Methods

Material

Fresh bovine pericardium from ten calves was delivered on ice from the slaughterhouse by Jerry Spear Company (Quakertown, PA). The tissues were washed first with tap water and then with sterilized distilled water and were cut into 1-cm² pieces. Thirty-eight such pericardial pieces were prepared. Glutaraldehyde (25% in water) was obtained from Fisher Scientific Company (Fairlawn, NJ) and was diluted into 0.625% solution in 0.05 mol/L HEPES buffer.

Fifteen male Sprague-Dawley rats, weighing 75 to 100 g, were obtained from Taconic Farms (Tac: N(SD) FBR, Germantown, NY). They were fed laboratory chow (Purina Mills, St. Louis, MO) and received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication 85-23, revised 1985).

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Experimental Procedures
In the first experiment, we investigated the effect of Glu fixation time on xenograft pericardium. Bovine pericardial pieces were divided into four groups according to Glu fixation time: (A) fresh bovine pericardium without Glu, (B) 15-minute Glu, (C) 60-minute Glu, and (D) 120-minute Glu (n = 7 in each group). Seven rats were used. After anesthesia with sodium pentobarbital, each animal received four subcutaneous implants in the back (one each of A, B, C, and D). The implants were retrieved 45 days later. After gross examination, each specimen was cut in half, with one piece used for calcium measurement and the other used for histologic study.

In the second experiment, the effect of Glu fixation time was examined in autograft and allograft skin implants. Eight rats from different mothers were used. Forty-eight hours before subcutaneous implantation, two pieces of 1 cm² full-thickness abdominal skin of each rat were excised and fixed with 0.625%/0 Glu. At the time of implantation, one extra piece was excised and rinsed in 0.625% Glu for 30 minutes. Each rat received five subcutaneous skin implants in the back (each 1 cm² in size). The five skin implants were prepared as follows: (1) fresh self abdominal skin, (2) self abdominal skin with 30-minute Glu, (3) self abdominal skin with 48-hour Glu, (4) fresh abdominal skin of others, and (5) abdominal skin of others with 48-hour Glu. After 45 days of implantation, the calcium content of the implants was measured.

Calcium Analysis
Calcium content of both bovine pericardium and rat skin implants was determined by atomic absorption spectrophotometry and was expressed as micrograms per milligram of dry tissue weight. The method has been described previously [11]. Briefly, after the specimens were dried at 95°C for 24 hours, they were ashed in a muffle furnace (Blue M Electric; Blue Island, IL) at 600°C for 10 hours. The ash was dissolved in 2 mL solution containing 1% lanthanum chloride in 5% hydrochloric acid. Samples that were clear were aspirated directly into a model 1290B Perkin Elmer Atomic Absorption Spectrophotometer (Perkin Elmer Inc., Norwalk, CT) for reading.

Histology
All specimens from bovine pericardial implants were sectioned into thin strips, embedded in paraffin after brief decalcification, and then stained with hematoxylin and eosin. Calcification was evaluated as nodular (destroying and expending the pericardium in discrete nodular shape) or as fiber type (linear deposition and/or replacement of collagen), and the severity was graded as none, mild, moderate, or severe depending on the extent of collagen destruction by calcium deposits. The extent of inflammatory reaction was also evaluated and graded as mild, moderate, or severe.

Data Analysis
Data were expressed as mean ± standard deviation. Statistical differences between any two groups in both experiments 1 and 2 were tested with Student's t test. The level of significance was set at p < 0.05. Linear regression analysis was performed in experiment 1 to evaluate the correlation between calcium content and Glu fixation time.

Fig 1. (A) Calcium content of bovine pericardial implants with different glutaraldehyde fixation times (mean ± standard deviation; 45 days implantation). (B) Relation between glutaraldehyde fixation time and calcification of bovine pericardial implants (linear regression; 45 days implantation).
Results

Experiment 1

After 45 days of implantation, the calcium content in bovine pericardial xenograft implants was $4.8 \pm 2.9$, $29.8 \pm 13.6$, $106.3 \pm 13.7$, and $176.3 \pm 85.5$ µg/mg dry weight in groups A, B, C, and D, respectively ($p < 0.05$ between any two groups, Student's t test) (Fig 1A). The linear regression of pooled data from groups A, B, C, and D showed a positive correlation between the Glu fixation time and the extent of implant calcification (Fig 1B). Mild calcification developed in implants fixed with Glu for as short as 15 minutes, and calcification increased with prolonged Glu fixation. Histologic findings showed that there was no calcification but there was marked inflammatory cell infiltration in group A (Fig 2A); the calcification in group B was mild and mostly fiber type, with some nodular-type changes (Fig 2B). In groups C and D, calcification was severe and mostly nodular type (Fig 3A, 3B). There was an inverse relation between the extent of calcification and the inflammatory reaction in the implants; the preservation of pericardial collagen against host digestion is positively related to Glu fixation time (Table 1).
Table 1. Histology of Bovine Pericardial Implants with Different Glutaraldehyde Fixation Time

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Calcification</th>
<th>Inflammation</th>
<th>Collagen Digestion</th>
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<tr>
<td>A</td>
<td>7</td>
<td>None</td>
<td>Severe</td>
<td>Severe</td>
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<tr>
<td>B</td>
<td>7</td>
<td>Mild</td>
<td>Moderate</td>
<td>Moderate</td>
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<tr>
<td>C</td>
<td>7</td>
<td>Moderate to severe</td>
<td>Mild to moderate</td>
<td>Mild to moderate</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>Severe</td>
<td>Mild</td>
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**Experiment 2**

Calcium content of the skin implants after 45 days of implantation was 1.4 ± 1.1, 57.9 ± 35.4, 142.7 ± 61.4, 1.5 ± 1.1, and 94.9 ± 24.1 μg/mg dry weight in groups 1, 2, 3, 4, and 5, respectively (p < 0.05 for 1 versus 2, 3, or 5; 2 versus 3, 4, or 5; 3 versus 4, and 4 versus 5; Student's t test) (Fig 4). There was no calcification in either self or other skin implants without Glu fixation; once the implants were fixed with Glu, calcification developed in both types. Calcium content also increased with Glu fixation time.

**Comment**

**Calcification Determined by Glutaraldehyde, Not by Tissue Antigenicity**

The use of fresh autologous pericardium as cardiac valve repair or replacement material was abandoned because of the problems encountered in both experimental and clinical studies in the early years. The most common failure patterns of autologous pericardium after implantation include tissue retraction and thickening, fibrosis and loss of pliability, degeneration and endocarditis, and sometimes hyalinization and cartilage formation [12-15]. In the past 20 years, although the use of Glu-treated bovine pericardium for valve repair or replacement did not present the above-mentioned failure patterns, calcification-induced valve or patch failure was prominent [16, 17].

Recently, renewed interest in the use of short-term Glu-treated autologous pericardium was encouraged by the observation that such pericardium appeared not to calcify or shrink after implantation. Although mechanisms were not clear and little basic research was conducted, several groups claimed that the lack of calcification in such pericardium is due to its lack of antigenicity [5-10]. We previously studied the relation between the host immune reaction and pericardial implant calcification and found no relation between calcification and antigenicity of the pericardial implants [18]. In the current study, we demonstrated substantial calcification when xenograft pericardium was exposed to Glu for as short as 15 minutes, and calcification increased with Glu fixation time (Fig 1). A similar degree of calcification was noted in Glu-treated skin implants, regardless of whether it was an autograft or allograft (Fig 4). There was calcification in all autograft, allograft, or xenograft implants as long as they were exposed to Glu for 15 minutes, and this increased proportionally with Glu fixation time. Histologic study supported the biochemical findings and also showed a Glu time-dependent calcification pattern in the implants (Table 1; Figs 2, 3).

Although Glu fixation could not totally eliminate antigenicity of xenograft pericardium by making it “dead,” it certainly changed the biologic properties of xenograft pericardium by cross-linking protein chains in collagen and dramatically reducing their antigenicity [18, 19]. Even as little as 15 minutes of Glu fixation can make up to 80% of the total cross-linking that was achieved in 2 weeks (measured by shrinkage temperature in another study from this laboratory). However, this same effect of Glu when used in treating autologous pericardium may also change the biologic identities of autologous pericardium by changing its surface antigen, because antigen is composed of proteins. As a result, after Glu treatment, autologous pericardium may be recognized as a "foreign body" by the host and induce certain inflammatory reactions. This may explain the results of our previous study: after 60-minute Glu fixation, both xenograft pericardial and autologous pericardial implants in hearts behaved grossly and histologically the same, and both induced inflammatory infiltration and calcification. Our study in the rat model demonstrated similar findings. Thus, the lack of calcification observed in short-term Glu-fixed autologous pericardium is due to the short time of Glu treatment, not to the lack of antigenicity.

![Fig 4. Calcium content of rat skin graft implants of different groups (mean ± standard deviation; 45 days implantation). 1 = fresh self skin; 2 = self skin with 30-minute glutaraldehyde; 3 = self skin with 48-hour glutaraldehyde; 4 = fresh skin of others; 5 = skin of others with 48-hour glutaraldehyde.](image)
**Lack of Calcification Versus Preservation of Tissue Strength**

Fresh autologous pericardium used in valve repair or replacement underwent an excessive healing process, resulting in dense fibrosis and loss of function as valve tissue. Calcification was occasionally seen. The scar tissue formation was much less evident in Glu-fixed xenograft material. Glutaraldehyde fixation appears to slow down the excessive scar formation, but at the potential cost of more calcification. The longer the Glu fixation time, the more stable is the implanted pericardium and the more resistant to host destruction (see Table 1). However, prolonged Glu fixation can induce calcification in pericardial implants, which eventually impairs their durability. The ideal pericardial implant should induce minimal calcification while preserving maximal tissue stability after long-term implantation. One of the methods that will achieve this purpose is to adjust the Glu fixation time carefully, as this is the only factor that determines both tissue calcification and stability. The studies of our group and others recommend that short-term Glu treatment for autologous pericardium be not less than 15 minutes to preserve basic tissue stability and strength, and no more than 60 minutes to avoid excessive calcification. If Glu fixation is too short, eg, less than 10 minutes, elongation of tissue and reduction of strength have been observed in a thoracic aortic patch [20]. Adequate fixation time is extremely important in the application of valve repair or replacement to avoid any prolapse and regurgitation. When Glu fixation time reached 60 minutes, excessive calcification of implants was observed in both big and small animal models.

In conclusion, the degree of calcification of bovine pericardium or skin implants was solely related to Glu fixation time in studies with a fixed concentration of Glu. Calcification is not causally related to antigenicity of the implants. Autologous tissues treated with Glu behave biologically like Glu-treated xenogenic ones; there was no difference between them regarding calcification tendency that makes one preferable to the other.

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**References**

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