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Enhancing anti-calcification efficacy in veterinary cardiovascular surgeries: evaluating short-term ethanol's role in glutaraldehyde fixed pericardial tissues in rats

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Abstract

Autologous pericardial tissues are utilized in veterinary cardiovascular surgeries due to their accessibility and effectiveness. To enhance handling and biomechanical properties, glutaraldehyde (GA) fixation is applied. However, GA fixation can induce calcification, leading to tissue failure. This study aimed to establish an optimal rapid anti-calcification protocol by integrating ethanol treatment with the proven effective GA concentration and fixation time, facilitating application from collection to utilization. Pericardia were fixed with 0.625% GA for 20 min and subjected to ethanol treatment for 0 (group A, control), 20 (group B), and 30 minutes (group C). The treated tissues underwent mechanical test and were implanted subcutaneously in 3-week-old male rats for 7 weeks before extraction, followed by calcium analysis and histological examination via hematoxylin and eosin staining. No significant differences in mechanical properties were observed among the groups. The ethanol-treated groups (groups B and C; $p < 0.05$) exhibited significantly lower calcium levels than control (group A). Microscopy confirmed collagen and elastic fibers preservation, without significant immune cell variance. However, higher fibrocyte presence was noted in the ethanol-treated groups. This study presents a rapid anti-calcification protocol combining ethanol treatment with optimal GA fixation, suitable for direct surgical use of autologous tissues. Further research is necessary for long-term efficacy evaluation.

Keywords: autologous pericardial tissue; glutaraldehyde; ethanol; veterinary cardiovascular surgery; dogs

Introduction

Autologous pericardium is widely utilized as a patch material, vascular conduit, and for valve reconstruction in both acquired and congenital cardiovascular surgeries due to its availability and non-prosthetic characteristics, which confer a superior immune response and long-term calcification resistance compared with xenografts or bioprosthesis [1–4]. In veterinary cardiac surgery, the clinical application of autologous pericardial patches has significantly advanced for correcting congenital heart defects or facilitating partial cardiac reconstruction [5,6]. However, untreated fresh autologous tissues tend to undergo immediate shrinkage and

significant recoil after excision, necessitating fixation with glutaraldehyde (GA) to enhance handling, tissue stability, tensile strength, and immune response by inducing cross-linking between collagen helices [2,3,7,8].

Nevertheless, GA-fixed autologous tissues are prone to calcification after long-term implantation, which is a major factor limiting their durability and occasionally leading to detrimental implant failure in the cardiovascular system [2,3,8,9]. The mechanism of tissue calcification is complex and involves various factors, including the persistence of unbound residual free aldehyde groups of GA, tissue phospholipids, and collagen structural calcification [3,8,10].

To address mineralization and potential tissue failure, several anti-calcification strategies have been proposed and developed for xenograft tissues or bioprosthetic valves [2,3]. However, conventional protocols often require several operational days before tissues are ready for use, thus limiting their immediate applicability from tissue collection to surgical implementation [3,11].

Numerous studies have investigated the optimal concentration and duration of GA fixation to minimize calcification and cytotoxicity [12–15]. Fixation with 0.6% GA for 20 min has been suggested as suitable for preserving extensibility, reducing antigenicity, and mitigating calcification [8]. Additionally, the efficacy of a 24-h ethanol pretreatment in preventing calcification induced by GA fixation has been demonstrated in both subdermal and circulatory implants [10]. Based on this research, a protocol has been proposed for rapidly treating ethanol to prevent calcification, particularly using the rat aorta model, which can be implemented during surgical procedures.

In this study, we hypothesized that a brief ethanol co-treatment following GA fixation of harvested canine pericardium could effectively prevent post-implantation tissue calcification. The objective of this report is to investigate the efficacy of an ethanol-based rapid anti-calcification protocol in canine autologous pericardium and establish a method applicable to veterinary cardiovascular surgery within surgical procedures.

Materials and Methods

Animals

Twelve-3-week-old male Sprague-Dawley rats were purchased from, Orient Bio (Orient Bio Inc., Korea), and acclimated for 1 week before *in vivo* implantation. This experiment was conducted with an approval from the Institutional Animal Care and Use Committee of the Clinical Research Institute, Chungnam National University (No. 202310A-CNU-187). Additionally, Fresh canine pericardia were acquired from the euthanized heart of a beagle approved for unrelated experiments (202203A-CNU-013).

Tissue preparation and experimental design

An overview of the experimental process, from pericardium preparation to application, is illustrated in Fig. 1. Following extraction of the canine heart, it was promptly rinsed with cold saline, then the pericardium was gathered and trimmed using scalpel and Metzenbaum scissors on a sterilized surgical table (Fig. 2). The connective tissues and fatty tissues adhering to the
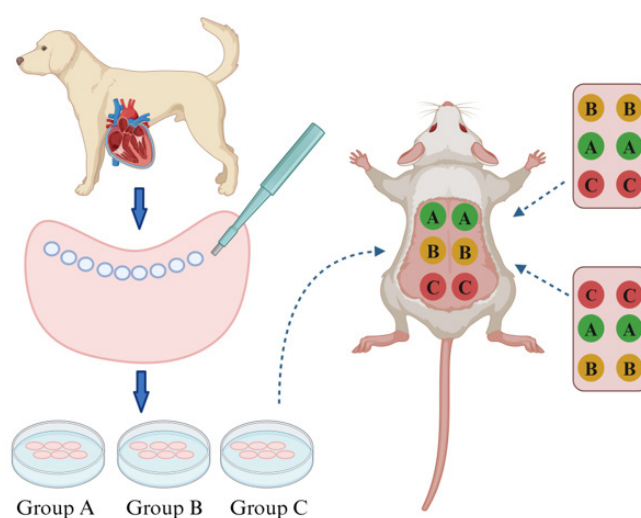

Fig. 1. A schematic overview of the experimental procedure. After isolating the pericardium from the canine heart, a 6 mm biopsy punch is used to generate a pericardial patch. Subsequently, each group undergoes treatment with glutaraldehyde and ethanol, followed by implantation into the dorsal subcutaneous tissue of rats, with a distinct sequence tailored to each rat. A, group A; B, group B; C, group C. Illustrated by the author.
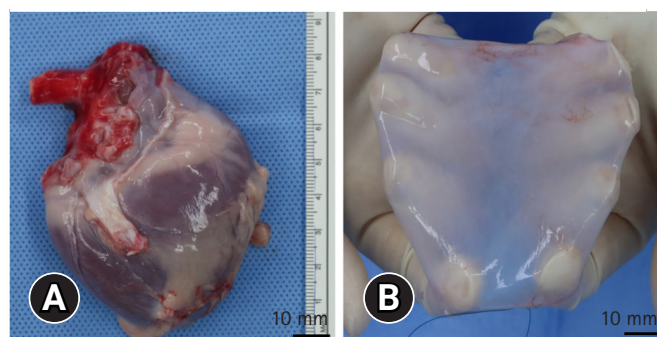

Fig. 2. The extracted heart from a canine. (A) Naive heart before trimming (scale bar = 10 mm). (B) Pericardium post-trimming following separation from the heart, with removal of fat and soft tissue (scale bar = 10 mm).

pericardium must be removed as much as possible. To fashion pericardial patches for subcutaneous implantation in rats, the trimmed pericardium was sectioned using a 6 mm biopsy punch. A total of 90 circular patches, each measuring 6 mm, were derived from the canine pericardium. Treatments were administered to 3 groups, each comprising 30 patches. For mechanical assessment, another trimmed pericardium was sliced into 5 mm × 10 mm pieces using scalpel, resulting in the preparation of 15 strips, which were then divided into 3 groups consisting of 5 strips each. For tissue fixation and decalcification, we utilized 0.625% GA in 0.1 M phosphate buffer (GA [0.625%], phosphate buffer 49.7%, distilled water 49.7%, pH 7.4; Electron Microscopy Sciences, USA) and 80% ethanol (80 mL of 99.9% ethanol [Merck, Germany] with 20 mL of distilled water) [2].

Tissues in group A were exposed to GA for 20 minutes; group B tissues underwent GA exposure for 20 minutes followed by post-treatment with 80% ethanol for 20 minutes; group C tissues were exposed to GA for 20 minutes followed by post-treatment with 80% ethanol for 30 minutes. Samples from each group were fixed with the GA solution for 20 minutes and rinsed with normal saline before undergoing ethanol treatment and subsequent implantation. All procedures were conducted under sterile conditions at room temperature.

Mechanical test

A uniaxial mechanical test was performed to compare the differences in mechanical properties among the groups. The prepared tissue strips (5 mm × 10 mm, with 5 pericardial strips per group, totaling 15 strips) were cut in various directions to account for material anisotropy [16]. Electronic calipers (Digital Caliper; Mitutoyo, Japan) were used to measure tissue length, width, and thickness. Tensile properties were evaluated using a dynamic mechanical analyzer (DMA 850; TA Instruments Co., Ltd., USA) equipped with 2 tensile clamps: a film tension clamp. All samples were tested at room temperature (25°C) with an extension rate of 100.0 mm/min and a preload force of 0.01 N. Tensile strength and elongation at breakage were analyzed from the recorded stress (MPa) – strain (%) curves using TRIOS Software ver. 5.1.0 (TA Instruments, USA).

Rat subcutaneous implantation

Twelve 3-week-old male Sprague-Dawley rats (Orient Bio Inc.) were utilized. After anesthetization with isoflurane inhalation, the dorsal hair of each rat was shaved. Meloxicam 1 mg/kg (Metacam, 5 mg/mL; Boehringer Ingelheim, Germany) was subcutaneously administered before surgery for anti-inflammatory and analgesic effects [17]. A skin incision was made along

the dorsal midline to create subcutaneous pouches for patch implantation (Fig. 3A) [18]. Prepared pericardial patches (6 mm) were implanted in pairs in each group (6 patches in each one) in the following order: group ABC, BAC, CBA (4 rats per group). After suturing with 6-0 blue nylon, the incision line was closed using 5-0 blue nylon (Fig. 3B–E).

The rats were euthanized by inhalation of a high concentration of isoflurane after 7 weeks. The implanted patches were harvested, and surrounding adherent tissues were dissected after rinsing with normal saline (Fig. 3F). One portion of each rat sample was placed in 1 × phosphate buffered saline (PBS) for calcium analysis, while the other portion was immersed in 10% buffered formalin for microscopic examination.

Microscopic examinations (post-implantation)

The implanted tissues were fixed in 10% formalin and embedded in paraffin wax blocks for sectioning into 5 μm-thick sections. Hematoxylin and eosin (H&E) staining was performed on the prepared slides for histological analysis using bright-field microscopy. The stained slides were scanned using a digital pathology scanner (KF-FL-005; KFBIO, China). The magnification of the scanned histological images was adjusted to 400 × using the K-Viewer software ver. 1.7.1.1, (KFBIO), enabling observation of immune cells and fibroblasts. To assess the degree of infiltration, the number of immune cells and fibrocytes in each group was counted by microscopic examination of 40 representative fields per group; the observed cell counts were subjected to statistical analysis.

Calcium quantitative analysis

Harvested tissue samples (11 samples per group, totaling 33 samples) were rinsed with normal saline and immersed in 1 × PBS. The calcium content of these samples was assessed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES; OPTIMA 7300 DV, Perkin-Elmer, USA), which analyzes the specific wavelengths and light intensities of elements emitted by ionized plasma. Prior to analysis, the samples underwent a pretreatment stage involving the addition of nitric acid for digestion, converting them into aerosols using an ICP sample preparation system (UltraWAVE; Milestone Inc., USA). Calcium content was measured in milligrams per liter (mg/L).

Statistical analysis

The collected data are presented as means and standard deviations for mechanical properties, calcium levels, and post-implantation cell infiltration counts. Bar graphs illustrate the range of values in each group. Group comparisons were performed
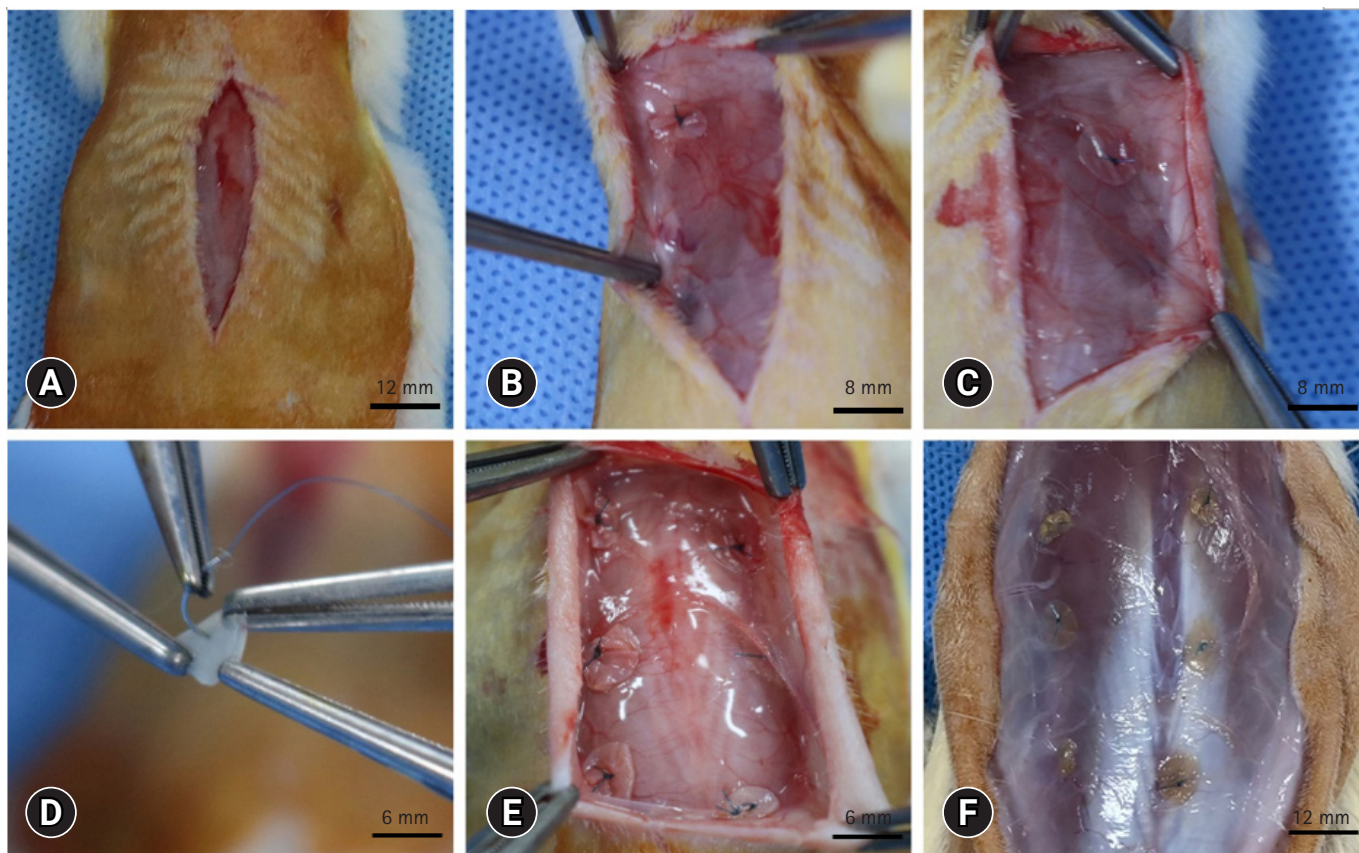
Fig. 3. Following a midline incision on the rat's back, 6 pericardial patches (2 patches per group) were implanted into the subcutaneous tissue, with 3 on each side (A-E). Pericardial patches were secured using 6-0 non-absorbable polyamide sutures (D). Observations were made at 7th week post-implantation, and samples were collected following euthanasia (F). Scale bars: (A, F) 12 mm, (B, C) 8 mm, (D, E) 6 mm.

using non-parametric statistics, specifically the Kruskal–Wallis and Mann-Whitney tests, particularly for the mechanical test and calcium analysis, due to the small sample sizes. Statistical significance was established at a p-value < 0.05. *Post-hoc* tests were employed to assess differences in dependent variables among the 3 experimental groups using Bonferroni's method. 40 slides were evaluated for cell count, meeting the assumption of normality, and additional normality tests using the Kolmogorov-Smirnov test were performed. One-way analysis of variance (ANOVA) was utilized to examine differences within the groups, followed by *post-hoc* Tukey's tests to identify specific groups with significant differences. Statistical analyses were performed using IBM SPSS Statistics ver. 26.0 (IBM Corp., USA).

Results

Mechanical test

The collected pericardium was fixed using a GA solution and cut in various directions to a size of 5 × 10 mm, considering the difference in tensile strength along different orientations [19]. The average tensile strength in groups A, B, and C was 19.9 ± 5.5 MPa, 17.6 ± 6.8 MPa, and 15.2 ± 7.6 MPa, respectively (Fig. 4A). The average elongation at breakage for groups A, B, and C was 41.3% ± 11.6%, 46.6% ± 9.6%, and 41.1% ± 4.7%, respectively (Fig. 4B). There were no significant differences between the groups regarding the presence or absence of ethanol treatment and treatment duration (20 or 30 minutes) on tensile strength and elongation at breakage (p = 0.527 and p = 0.533, respectively).

Calcium analysis

Quantitative calcium analysis of implant calcification in groups A, B, and C revealed concentrations of 319.6 mg/L (4.18– 832.9 mg/L), 84.9 mg/L (0.3–485.8 mg/L), and 23.5 mg/L (0.3–179.5 mg/L), respectively. A significant difference in calcium concentration was observed between the group treated solely with GA and the group treated with ethanol. The calcium
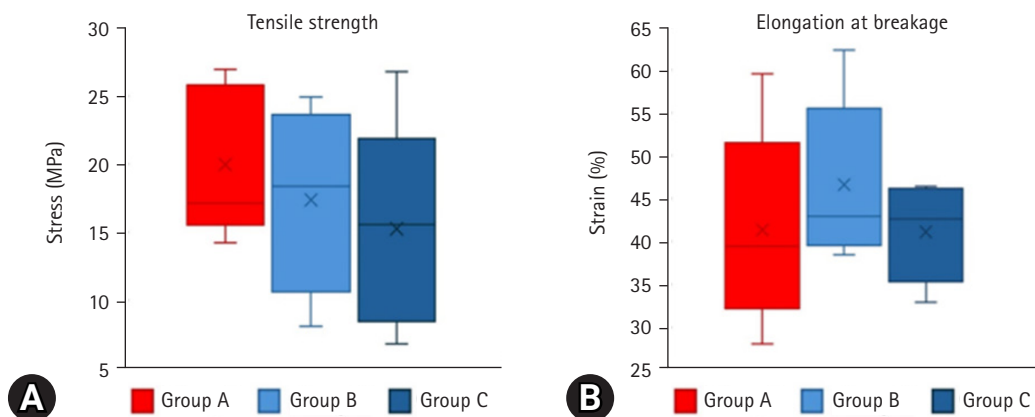
Fig. 4. Tensile strength (A) and elongation at breakage (B) of pericardium treated with glutaraldehyde (GA) alone or GA and ethanol (groups A, B, and C). All data on chart represent the mean and standard deviation. Average tensile strength is 19.9 ± 5.5 MPa, 17.6 ± 6.8 MPa, and 15.2 ± 7.6 MPa, respectively. No significant differences between groups ($p = 0.527$, $p > 0.05$). Average elongation at breakage is $41.3\% \pm 11.6\%$, $46.6\% \pm 9.6\%$, and $41.1\% \pm 4.7\%$, respectively. No significant differences are observed between the groups ($p = 0.533$, $p > 0.05$).

concentration in group A treated with GA alone was significantly higher than that in the ethanol post-treatment groups ($p < 0.05$). Although some differences were noted between ethanol treatments for 20 and 30 minutes (groups B and C), they were not statistically significant ($p > 0.05$, 0.332). Thus, ethanol treatment effectively mitigated calcification following implantation (Fig. 5).

Post-implantation histological test

Under bright-field microscopy, collagen and elastic fibers were well-preserved in all groups. In the medial portion of the patch, immune cells, including monocytes, lymphocytes, and macrophages, were similar in all groups, with relatively low observed numbers. However, a higher proportion of fibrocytes was noted in the ethanol-treated groups (groups B and C), particularly in the medial portion of the patch (Fig. 6A–C). Upon direct counting of fibrocytes in 40 representative field at 400x magnification for each group, a higher number of fibrocytes was observed in groups B and C compared with group A (Fig. 6D). Fibrocyte counts in each group followed a normal distribution (Kolmogorov-Smirnov test, $p > 0.05$). ANOVA confirmed differences among the groups ($p = 0.029$); *post-hoc* Tukey's tests revealed significant differences between groups A, B, and C (group A and B; $p = 0.044$, group A and C; $p = 0.03$). However, no significant difference was observed between groups B and C ($p > 0.05$, 0.066).

Discussion

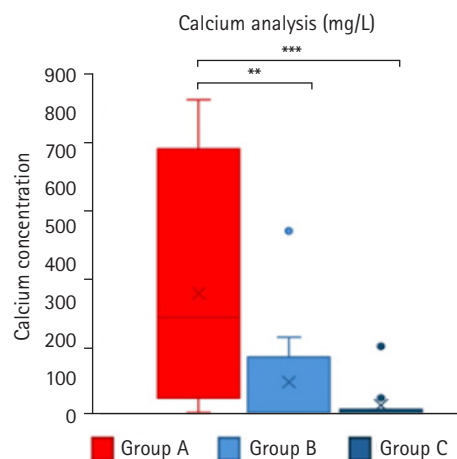GA-fixed autologous pericardium is extensively employed as

Fig. 5. Quantitative calcium analysis (mg/L) in explanted implantations. Group A, B, and C exhibit calcium concentrations of 319.6 mg/L (range, 4.18–832.9 mg/L), 84.9 mg/L (range, 0.3–485.8 mg/L), and 23.5 mg/L (range, 0.3–179.5 mg/L), respectively. All data on chart represent the mean and standard deviation. Significantly distinct calcium concentrations are observed between group A and B, as well as between group A and C (group A vs. group B, $p = 0.008$ and group A vs. group C, $p = 0.001$). **$p < 0.01$, ***$p < 0.001$.

a substitute for defects or reconstructions in various congenital or acquired cardiovascular surgeries in veterinary medicine due to its non-bioprosthetic nature [20]. Particularly in congenital heart defect cases, where pediatric patients' growth and prolonged implant duration are factors, ideal implants must possess mechanical strength and flexibility. Furthermore, to prevent long-term dystrophic calcification post-implantation, which is primarily associated with implant failure, various anti-calcification protocols have been introduced to address recognized cal-
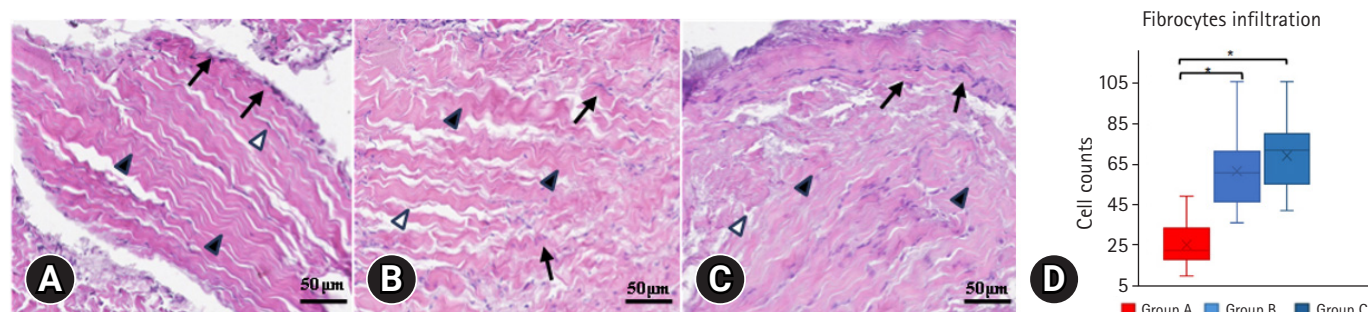
Fig. 6. Bright-field microscopy images of extracted pericardial patches at 7 weeks post-implantation (A, group A; B, group B; C, group C). Elastic fibers and collagens are preserved in all groups, with similar numbers of observed immune cells across all groups, indicating low levels. Ethanol-treated groups (groups B and C) exhibit a higher proportion of fibrocytes. The count of fibrocytes in a 400 × field of view is presented (D, 40 representative sections per group; *$p < 0.05$). All data on chart represent the mean and standard deviation. Significantly higher fibrocyte counts are noted in groups B and C compared with group A ($p < 0.001$). However, no significant difference was observed between groups B and C ($p = 0.066$, $p > 0.05$). Black arrowheads, elastic fibers; white arrowheads, collagen fibers; arrows, fibrocytes. (A–C) Hematoxylin and eosin stain, scale bars = 50 μm.

cification mechanisms that are still under investigation [21,22]. Commonly recognized mechanisms include inadequate GA concentrations, cytotoxic effects caused by free aldehyde groups in GA, tissue cholesterol, phospholipids, collagen structure, and long-term immune responses to antigenicity.

In this study, our aim was to demonstrate the efficacy of rapid ethanol anti-calcification treatment following GA crosslinking of autologous tissues. Based on our results, post-implantation tissue calcification was significantly lower in the groups treated with ethanol for 20 or 30 minutes compared with those treated solely with GA for 20 minutes. These anti- calcification outcomes were akin to a recent study on rapid anti-calcification therapy in aortic tissue, which involved long-term pretreatment with ethanol for 24 hours, evaluated using an aortic valve bioprosthesis and cusps [10,23]. In a previous investigation, pretreatment of a bioprosthesis with 80% ethanol effectively inhibited implant calcification by removing cholesterol phospholipids from the implant and altering collagen composition. Although our study did not directly elucidate the short-term anti-calcification mechanism, we hypothesized that short-term ethanol treatment would yield a similar effect as the observed mechanism induced by 24 hours of ethanol treatment in previous studies. Another recent study introduced a rapid anti-calcification method using ethanol after 3 min of GA fixation [2]. Significant reduction in calcification was noted after 10 min of ethanol therapy. However, the optimal processing time remains unclear. In our study, in terms of post-implantation tissues calcium concentration, there was statistically significant difference among groups A, B, and C. Although groups B and C, differing in ethanol treatment duration (20 and 30 minutes), did not exhibit a statistically significant difference, there was a tendency

toward reduced calcium concentration in group C compared with group B. Considering evidence from previous studies (24-hour ethanol treatment) and recent findings showing significant reduction in calcification concentration after 10 minutes of ethanol treatment, our overall analysis supports a 30-minute ethanol treatment as producing significant anti-calcification effects induced by GA. Therefore, in veterinary medicine, particularly for cardiopulmonary surgeries using cardiopulmonary bypass with an aortic cross-clamping time of 90 minutes [24], it is anticipated that a total fixation time of 60 min, including 20 minutes of GA and 30 minutes of ethanol, would be suitable for efficiently utilizing pericardium collected within the surgical field as an autologous defect patch.

Immunological responses and inflammatory reactions play significant roles in implant calcification and dystopic failure following prolonged implantation. Autograft tissue exhibits superiority over xenograft tissue or synthetic materials in terms of antigenicity and immune response. In a previous study utilizing decellularized porcine aortic wall patches in rat subcutaneous tissue, notably lower inflammatory reactions involving macrophages and monocytes, alongside increased fibrocyte infiltration, were observed during the tissue healing process or inflammatory responses [18]. Although this study did not explicitly investigate the specific mechanism leading to fibroblast infiltration, the observed gradual decrease in inflammatory cells and fibroblasts suggests that recellularization and tissue healing may contribute to fibroblast infiltration, possibly attributed to the lack of cytotoxicity in the decellularization process. Moreover, given the established efficacy of 70% ethanol decellularization in tissues, it is expected that the impact of 80% ethanol treatment in this study on tissue recovery post-implantation will be

akin to that observed in previous studies [18,25]. In this study, no significant differences were noted in the number of inflammatory cells among the 3 groups. Since GA diminishes antigenicity, the observed inflammatory cells typically consist of monocytes, lymphocytes, or macrophages that do not exhibit significant distinctions [26]. However, due to the limitations of H&E staining alone in accurately distinguishing immune cells, it appears necessary to conduct additional immunostaining in the future to differentiate each cell type.

According to a report, 0.625% GA fixation resulted in 35 times more calcification in the heterogeneous group compared with the autologous group, suggesting that the tissue origin also influences the extent of calcification post-implantation [27]. Although the experiments were conducted under conditions simulating the collection of autologous pericardium in actual surgeries, the experimental groups were subcutaneously implanted in rats; hence, complete autologous tissue implantation was not achieved. Consequently, it is anticipated that if transplanted under actual autologous conditions during a patient's operation, a lower immune response would occur.

In the preparation of autologous pericardium for cardiovascular surgery, GA solution has been employed as a cross-linking agent for collagenous tissues to enhance handling and biomechanical stability while reducing antigenicity [28]. Inadequately low concentrations of GA can result in residual implant antigenicity, leading to calcification, whereas elevated concentrations induce calcification due to GA's cytotoxicity and calcium-binding characteristics. Additionally, a study recommended limiting GA fixation time to less than 60 minutes to mitigate excessive inflammation and calcification [29]. Hence, achieving an appropriate concentration and fixation time of GA solution is crucial for effective tissue treatment due to its cytotoxic and calcifying properties. Building upon prior research investigating optimal GA concentration and fixation time concerning material characteristics and pericardial tissue calcification, we employed a fixation method involving immersion in a 0.625% GA solution for 20 min, deemed suitable for autologous pericardium fixation [8]. This approach was selected to evaluate the anti-calcification effects of ethanol in autologous pericardium fixation, adapting a methodology previously used in surgeries where only GA fixation was applied [7].

Regarding the measurement of mechanical properties such as tensile strength and elongation at break percentage, no significant difference was observed between the groups with or without ethanol. In one study comparing mechanical properties between ethanol dehydration and GA fixation groups, no significant differences were found in macroscopic evaluation, surgical

handling during suturing, and uniaxial tensile strength [20]. This suggests that short-term ethanol treatment is a viable option for reconstruction materials, yielding mechanical characteristics akin to those of the GA crosslinking method. In another study, mechanical properties of fresh bovine pericardium, GA-treated pericardium post-treated with glycine, and pericardium fixed with GA and an organic solvent (ethanol 65% + octanol 5%) were found to be similar [15]. This indicates that anti-calcification therapy, involving tissue phospholipid extraction and removal of free aldehyde groups, does not compromise the structural integrity of pericardial tissue [2,16,23]. In our study, preservation of collagen structures and elastic fibers in all groups was confirmed through histological examination, with similarities in mechanical properties observed via a uniaxial mechanical test. Combining our study's results with those of previous reports, concurrent treatment with ethanol and GA effectively preserved the mechanical properties of the pericardium, ensuring the conservation of structural components.

The tissue processing method proposed in this study offers a practical approach directly applicable in surgical settings; however, it comes with certain limitations. First, although this study yielded significant results under xenograft conditions, it may not precisely replicate autologous tissue application. Nevertheless, our results suggest that implementing the proposed protocol for autologous pericardium implantation would likely result in superior outcomes concerning immune responses and calcification. Second, the small sample size constrained the statistical analysis, limiting the ability to compare tissue changes and cell infiltration during short- and long-term post-implantation periods. Additionally, the use of subdermal implantation in rats limits the assessment of tissue durability and mechanical changes relevant to actual cardiovascular tissue application. Future studies should investigate actual autologous conditions to evaluate factors significantly impacting tissue durability after implantation, particularly tissue calcification. Long-term follow-up with a larger sample size is essential to assess the extent of calcification and tissue changes in various experiments, including immunohistochemical staining, based on the protocol's background.

This study proposes the incorporation of short-term ethanol treatment, alongside the optimal concentration and fixation duration of GA established in various studies, to mitigate calcification induced by GA. The devised protocol is tailored for implementation within the surgical setting for cardiovascular procedures, facilitating both rapid crosslinking and anti- calcification treatment within a concise timeframe. The protocol encompasses a swift anti-calcification approach that entails harvesting

autologous tissue from the patient, subjecting it to fixation, and proceeding with implantation. Further investigations are warranted to authenticate the applicability of this protocol under authentic autologous conditions and to evaluate the extent of calcification and tissue alterations during long-term follow-up.

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Author's Contributions

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Data Availability Statements

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

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