

# Biomechanical Characterization of Decellularized and Cross-Linked Bovine Pericardium

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**Background and aim of the study:** Although bovine pericardium has been used extensively in cardiothoracic surgery, its degeneration and calcification are important limiting factors in the continued use of this material. The study aims were to decellularize bovine pericardium and to compare the biomechanical properties of fresh and decellularized bovine pericardia to those treated with different concentrations of glutaraldehyde (GA).

**Methods:** An established protocol for decellularization using sodium dodecyl sulfate was used, and histological analysis performed to validate the adequacy of decellularization. Contact cytotoxicity was used to study the in-vitro biocompatibility of variously treated pericardia. Mechanical testing involved uniaxial testing to failure. Mechanical properties of the fresh and decellularized pericardia (untreated and treated with 0.5% and 0.05% GA) were compared.

**Results:** Histological analysis of decellularized

bovine pericardium did not show any remaining cells or cell fragments. The histoarchitecture of the collagen-elastin matrix appeared well preserved. Untreated decellularized pericardium was biocompatible in contact cytotoxicity tests with smooth muscle and fibroblast cells. The GA-treated tissue was cytotoxic. There were no significant differences in the mechanical properties of fresh and decellularized pericardia, but there was an overall tendency for GA-treated pericardia to be stiffer than their untreated counterparts.

**Conclusion:** An acellular matrix, cross-linked with a reduced concentration of GA, can be produced using bovine pericardium. This biomaterial has excellent biomechanical properties and, potentially, may be used in the manufacture of heart valves and pericardial patches for clinical application.

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The pericardium, a fibroserous sac which covers the mammalian heart and provides protection for the organ, contains pericardial fluid which acts as a lubricant during the pumping action of heart. The availability of this sheet of tissue first caught the attention of cardiac surgeons, who began to use it first to correct various cardiac defects, and later to create heart valves (1-3). Although, subsequently, uses of the pericardium expanded to other surgical specialties (4-6), the need for an alternative source of tissue was soon realized as access to human pericardial tissue is difficult and the supply limited. Bovine, equine and porcine pericardia

have been used both clinically and experimentally for many years, in addition to cadaveric human pericardium. Human and bovine pericardia have been used widely to repair congenital and acquired cardiac defects, and also to create artificial heart valves. In recent years there has been a progressive rise in the use of bovine pericardial heart valves (7), and today pericardial patches are used routinely and increasingly in a variety of complex operations to remedy congenital conditions.

When implanted in the human body, either as a patch or as a bioprosthetic heart valve, the pericardium undergoes degeneration and calcification and suffers wear and tear. Indeed, degeneration and calcification of the pericardium (especially in young patients) have been important limiting factors in its continued use (8,9). Currently, efforts are being directed towards producing a pericardial material which is more durable for various applications, including the creation of artificial valves. Consequently, different chemical treat-

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ments have been investigated in attempts to reduce the calcific degeneration of pericardial tissue once implanted in the human body (10-12).

The ideal heart valve substitute should combine satisfactory long-term durability, excellent biocompatibility, and hemodynamic performance resembling that of the natural heart valve. Although thromboembolic complications and anticoagulant-related hemorrhage with mechanical valves may impair the performance of these devices, the longevity of tissue heart valves is limited by deterioration resulting from both calcific and non-calcific processes, and this usually necessitates a difficult reoperation (13). The key differences between natural and substitute biological valves include the absence of endothelium and viable interstitial cells, a mechanically altered collagenous matrix, and residual fragments of non-viable donor interstitial cells which serve as preferred loci for calcification (14). Even autologous pericardial valves have shown a tendency for calcific degeneration (15-17).

In the present study, it was hypothesized that the decellularization of bovine pericardium would reduce the propensity for calcification *in vivo*. In addition, following decellularization and sterilization of the pericardium with peracetic acid, it would be possible to reduce the level of glutaraldehyde (GA) used to preserve the tissue (from 0.5% to 0.05%, v/v) and hence to generate a tissue with appropriate biological and biomechanical properties for preparing cardiovascular patches and cardiac valve prostheses.

Initially, the study aim was to validate a protocol for the decellularization of bovine pericardium, and subsequently to investigate the *in-vitro* cytotoxic properties of decellularized and variously cross-linked bovine pericardia.

In order to determine the biomechanical properties of the materials, the study was divided into two parts. In Part I, the mechanical properties of fresh and decellularized pericardia (untreated, or treated with 0.5% or 0.05% GA) were compared. In Part II, the mechanical properties of three groups each of fresh pericardia and decellularized pericardia were compared, either untreated, or treated with 0.5% or 0.05% GA.

## Materials and methods

### Tissue acquisition

Bovine hearts, along with the pericardial sac, were harvested and transported from the abattoir within 4 h of the animal's slaughter. The prepericardial fat was removed and the pericardium dissected free from its attachment at the base of the heart around the great vessels. Further dissection was carried out to clear the pleura, fat, and loose areolar tissue. The thickened pericardium near the attachments of the pericardio-

phrenic and the pericardiosternal ligaments was excised and discarded.

### Sampling and treatment of pericardium: Part I

Three areas of suitable tissue were identified on the pericardium harvested from a single animal. Each of the three areas was cut into 12 strips each of 5×40 mm (Fig. 1). Six strips from each area were subjected to decellularization and six were retained as fresh tissue. Care was taken to ensure that matched samples of fresh and decellularized tissue were obtained in order to minimize the effects of regional variation in the mechanical properties of the tissue.

The six fresh and six decellularized strips from the first area were not subjected to further treatment. The six fresh and six decellularized strips from the second area were treated with 0.5% GA for 24 h at room temperature, while the six fresh and six decellularized strips from the third area were treated with 0.05% GA for 24 h at room temperature. All of the strips were then used for biomechanical testing.

In all six groups, additional pieces (three in each group) of pericardium of (5×5 mm) were obtained and treated alongside the six strips in the corresponding group. These pieces were used for contact cytotoxicity studies for each group, using porcine smooth muscle cells (SMC).

### Sampling and treatment of pericardium: Part II

Two areas of suitable tissue were identified on the pericardium harvested from a second animal. Each of the two areas was cut into 18 strips each of 5×40 mm

Fresh	Decellularised
Decellularised	Fresh
Fresh	Decellularised
Decellularised	Fresh
Fresh	Decellularised
Decellularised	Fresh

Figure 1: Chart indicating how pericardial strips were cut for fresh and decellularized groups. All boxes represents a strip of pericardium of size 5×40 mm.

Fresh untreated	Fresh + 0.5% glut	Fresh + 0.05% glut
Fresh + 0.5% glut	Fresh + 0.05% glut	Fresh untreated
Fresh + 0.05% glut	Fresh untreated	Fresh + 0.5% glut
Fresh untreated	Fresh + 0.5% glut	Fresh + 0.05% glut
Fresh + 0.5% glut	Fresh + 0.05% glut	Fresh untreated
Fresh + 0.05% glut	Fresh untreated	Fresh + 0.5% glut

Figure 2: Chart indicating how 18 pericardial strips were cut for Part II of the study from fresh pericardium.

Another 18 strips were cut from another area of pericardium which was decellularized. Each shaded box represents a strip of pericardium of size 5×40 mm. glut: Glutaraldehyde.

(Fig. 2). Eighteen strips from the first area were decellularized, and the other 18 were retained as fresh tissue. Care was taken to ensure that matched samples of fresh and decellularized tissue were obtained in order to minimize the effects of regional variation in the mechanical properties of the tissue.

Six fresh and six decellularized strips were not subjected to further treatment. Six fresh and six decellularized strips were treated with 0.5% GA for 24 h at room temperature, while the remaining six fresh and six decellularized strips were treated with 0.05% GA for 24 h at room temperature. Thus, a total of six groups was created.

In all six groups, additional pieces (three per group) of pericardium (5×5 mm) were obtained and treated alongside the six strips in the corresponding group. These pieces were used for contact cytotoxicity studies for each group, using human dermal fibroblasts (HDF).

#### Decellularization and cross-linking of pericardium

The method for decellularization as described by Booth et al. (18) was used, albeit with one modification, namely to use double the quantity of solutions during the process, but without altering reagent concentrations or their contact time. The thicker nature of bovine pericardium (and the anticipated increased cell load), compared to the porcine aortic valve leaflets used by Booth et al. was borne in mind.

The pericardial tissue was first washed three times in 200 ml wash buffer (phosphate-buffered saline (PBS) with aprotinin (0.1%, v/v; Bayer) and EDTA (0.01%, w/v; Sigma) at 4°C and shaken (320 rpm) for 30 min. The pericardium was then treated with 200 ml hypotonic buffer (2 M Tris-HCl, pH 8.0, with aprotinin

(0.1%, v/v) and EDTA (0.01%, w/v)) at 4°C and shaken (320 rpm) for 16 h. Treatment was then continued with 200 ml hypotonic buffer with sodium dodecyl sulfate (SDS) (2 M Tris-HCl, pH 8.0 with aprotinin (0.1%, v/v) and EDTA (0.01%, w/v) and SDS (0.1%, w/v)) at 20°C and shaken (320 rpm) for 24 h. The pericardium was washed three times with 200 ml PBS at 20°C for 30 min, and then treated with nuclease solution (2 M Tris-HCl, pH 7.5, 1 M MgCl<sub>2</sub>, bovine serum albumin (50 mg/l; Sigma), deoxyribonuclease (50 U/ml; Sigma) and ribonuclease (1 U/ml; Sigma)) at 37°C and shaken (160 rpm) for 3 h. This completed the decellularization process. Pericardium was then sterilized with 10 ml 0.1% (v/v) peracetic acid (Sigma) at 20°C for 3 h to prevent bacterial contamination. Any excess of peracetic acid was removed with three washes of 200 ml PBS at 20°C and shaken (320 rpm) for 30 min.

Samples of the decellularized pericardium was stored in a sterile container at -40°C until tested.

Appropriate pericardial strips from both the parts of the study were treated with 0.5% or 0.05% GA for a period of 24 h, and mechanical testing was carried out immediately after this to minimize the effects of ongoing cross-linking.

#### Histological analysis

Two pieces (each 10×10 mm) were harvested from fresh pericardium, and six from different regions of decellularized pericardium during Part I of the study for histological analysis and validation of decellularization. These eight pieces were processed in an automatic tissue processor and then embedded in molten paraffin wax. Multiple sections (×6) of 5 μm thickness were obtained and stained using hematoxylin and eosin (H&E), Alcian blue, elastin Van Gieson and Hoechst DNA stains (19).

#### Contact cytotoxicity testing

The contact cytotoxicity assay described by Wilcox et al. (20) for biocompatibility testing of aortic valve leaflets was used for this part of study.

Two vials of porcine SMC at passage 5, and two vials of HDF (Cascade Biologics) at passage 6, were retrieved from liquid nitrogen storage. Cells were resurrected using culture medium (Dulbecco's MEM, 100 U/ml penicillin G sodium with 100 μm/ml streptomycin and 2 mM L-glutamine; all from Invitrogen) with 20% (v/v) fetal calf serum (FCS). Culture medium with 10% (v/v) FCS was then added to the cells and the suspension transferred to a 75-ml flask. The cells were incubated at 37°C in 5% (v/v) CO<sub>2</sub> in air. Cells were observed regularly for morphology, confluence, viability and bacterial contamination. The culture medium was changed every 48 h.

Three pieces of pericardium (5×5 mm) from each group were attached to the center of a well in a 12-well tissue culture plate using a drop of collagen gel (acid-solubilized rat tail collagen neutralized with 0.1 M NaOH). One drop of cyanoacrylate glue (VWR International Ltd., Poole, UK) was placed into three wells as a positive control, one drop of collagen gel was placed in three wells as a negative control for the gel, and three wells were left empty (no adhesive or tissue) to act as negative controls. Porcine SMC and HDF were seeded into the wells at a density that would allow the cells to achieve confluency on attachment. The plates were then incubated at 37°C in 5% (v/v) CO<sub>2</sub> in air for 48 h. The plates were viewed under the microscope to determine the confluency and morphology of the cells.

### Uniaxial testing

Uniaxial testing was used to compare the mechanical properties of all 12 groups. Strips of pericardium (5×40 mm) were cut as described earlier, and treated appropriately. All tests were performed on the tissue soaked in normal saline and at a room temperature of 20°C. The tissue was mounted on a holder using minimum handling. The length of tissue to be tested was maintained at 10 mm using a gauge in the middle of the holder and set at zero strain. The effective specimen size studied was 5×10 mm, giving an aspect ratio of 2:1. Korossis, in 2002, showed that this provides good reproducibility of results (14). The tissue holder was placed in the Shimadzu Autograph™ tensile testing machine, which was set to produce a specimen pre-loading of 0.02 N before beginning to acquire data. The specimen was extended to failure at the rate of 10 mm/min. The machine was stopped on occurrence of failure, as determined by the first significant decrease in load detectable during extension.

In order to minimize the effect of ongoing cross-linking by GA, specimens were loaded immediately after completion of 24-h treatment with GA.

### Statistical analysis

During testing, the load (N) imposed on the specimen, together with the specimen extension and time, were recorded for further analysis. The data were stored as dat files, and further processed using Application Software for Universal Testing Machines (Messphysik Laborgeräteges m.b.H.); the stress-strain curves were drawn from these data. The force and extension data acquired during tests were converted to stress (Y-axis, MPa) and strain (X-axis, %) respectively.

The stress-strain curves for each specimen thus obtained from the program were analyzed as shown in Figure 3, and used to obtain the following biomechanical parameters:

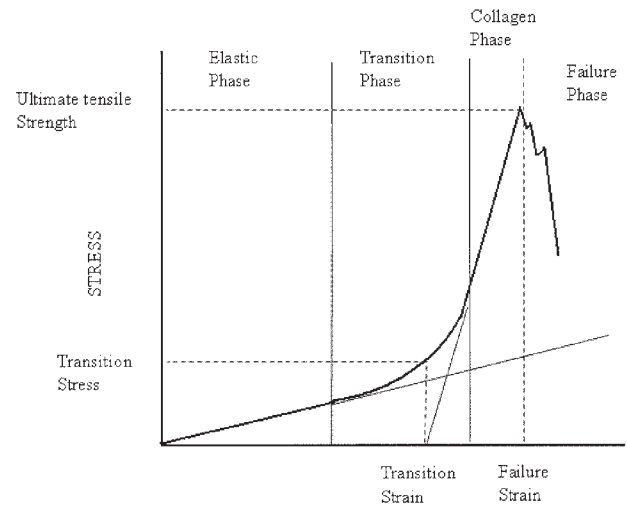


Figure 3: A typical stress-strain curve of pericardium subjected to uniaxial loading to failure showing failure test analysis parameters.

1. Elastic phase slope (EI-E)
2. Collagen phase slope (Coll-E)
3. Transition stress ( $\sigma_{\text{trans}}$ )
4. Transition strain ( $\epsilon_{\text{trans}}$ )
5. Ultimate tensile strength ( $\sigma_{\text{uts}}$ )
6. Failure strain ( $\epsilon_{\text{uts}}$ )

The analysis parameters from each group were averaged over the number of specimens in the group, and the results expressed as mean and 95% confidence interval (95% CI).

For each set of data from uniaxial testing, the mean, SD and 95% confidence limits were calculated. Statistical analysis of the data was carried out using Microsoft Excel™. The results between test groups were further analyzed by a one-way analysis of variance (ANOVA). The p-values from the ANOVA tables were used to determine the statistical significance of the difference between test groups at the 0.05 cut-off level (95% confidence level). A p-value <0.05 was considered to be statistically significant.

A different statistical method was used in Part II of the study as the comparison involved three groups. Data were analyzed by a one-way ANOVA using Microsoft Excel™, followed by calculation of the minimum significance difference (MSD,  $p < 0.05$ ) to determine individual differences among the three groups by the T-method (21). Error bars were plotted for each group mean using MSD. Mean values for which error bars did not overlap were significantly different ( $p < 0.05$ ).

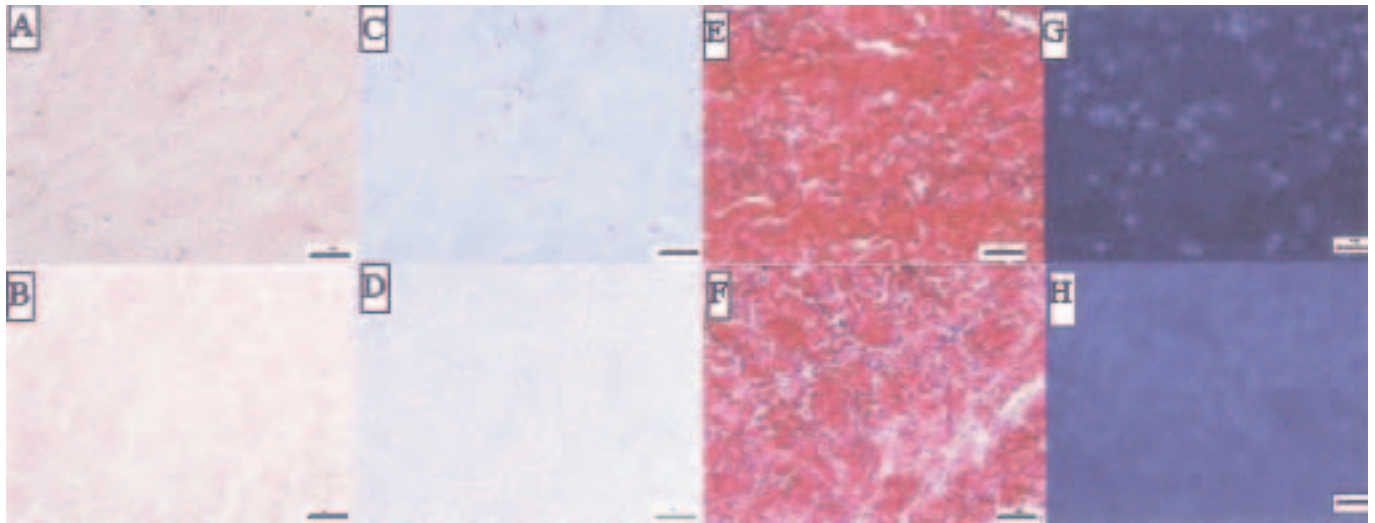


Figure 4: Histological analysis of fresh (upper row) and decellularized (lower row) pericardium (original magnification  $\times 200$ ; scale bar =  $50 \mu\text{m}$ ). A,B) Hematoxylin and eosin staining. C,D) Alcian blue staining. E,F) Elastin Van Gieson staining. G,H) Hoechst DNA staining.

## Results

### Histological analysis

Histological analysis (H&E, Alcian blue and elastin Van Gieson staining) of the decellularized bovine pericardium did not reveal any remaining cells or cell fragments (Fig. 4A-F). When analyzing with these three staining methods, the histoarchitecture of the collagen-elastin matrix appeared to be well preserved. DNA staining using Hoechst dye did not reveal any evidence of positive DNA staining in the decellularized specimens (Fig. 4G,H).

### Contact cytotoxicity studies

Microscopic examination of the contact cytotoxicity plates showed that the porcine SMC and HDF attached and grew well in contact with untreated, decellularized pericardium. There were no zones of lysis nor changes in cell morphology next to the decellularized pericardium (Fig. 5). The collagen also had no effect on the cells, but the pericardia treated with GA (0.5% and 0.05%) and the positive controls (cyanoacrylate glue) were clearly cytotoxic to SMC and HDF, as was the fresh, untreated pericardium.

### Biomechanical analysis

#### Part I

Comparison of fresh and decellularized pericardia showed (Table I) that there was no significant difference in the average thickness of pericardium between the two groups, nor in elastic phase slope, average collagen phase slope, average transition stress, average transition strain, ultimate tensile strength and average failure strain between the two groups.

Comparison of fresh and decellularized pericardium each treated with 0.5% GA showed there to be no significant difference in the average thickness of pericardium in both groups. Neither was there any significant difference in elastic phase slope, average collagen phase slope, average transition stress, average transition strain, ultimate tensile strength and average failure strain in both groups.

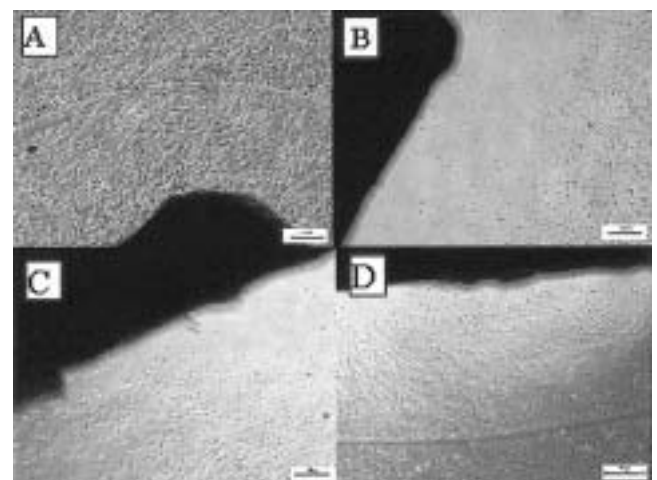


Figure 5: Contact cytotoxicity of pericardial tissues. A) Confluent porcine smooth muscle cells (SMC) in contact with decellularized pericardium. B) Zone of inhibition of porcine SMC in the vicinity of decellularized pericardium treated with 0.5% GA. C) Zone of inhibition of porcine SMC in the vicinity of decellularized pericardium treated with 0.05% GA. D) Zone of inhibition of porcine SMC in the vicinity of fresh untreated pericardium. Unstained specimen; original magnification  $\times 40$ ; scale bar =  $250 \mu\text{m}$ .

Table I: Uniaxial testing of data from Part I of the study.

Parameter	Untreated pericardium		0.05% GA-treated pericardium		0.5% GA-treated pericardium	
	Fresh	Decellularized	Fresh	Decellularized	Fresh	Decellularized
Elastic phase slope E (MPa)	0.366±0.161	0.368±0.181	0.657±0.304	0.290±0.066	0.307±0.305	0.424±0.399
Collagen phase slope Coll-E (GPa)	0.099±0.038	0.092±0.037	0.033±0.022	0.033±0.021	0.077±0.023	0.049±0.029
Transition stress $\sigma_{trans}$ (MPa)	1.715±0.828	1.653±0.541	0.957±0.536	0.777±0.316	1.792±0.499	1.468±0.726
Transition strain $\epsilon_{trans}$ (%)	22.517±6.084	18.154±12.927	25.558±6.080	27.668±6.498	35.257±6.060	28.857±8.994
Ultimate tensile strength $\sigma_{tis}$ (MPa)	13.862±6.655	14.342±4.437	8.134±5.019	7.107±3.437	9.077±4.466	6.151±3.103
Failure strain $\epsilon_{tis}$ (%)	36.228±9.018	31.769±18.950	52.293±6.106	52.218±9.661	47.985±2.689	42.340±10.913
Thickness (mm)	0.456±0.025	0.445±0.033	0.691±0.132	0.642±0.117	0.791±0.106	0.776±0.166

Values are mean ± SD.

\*Significant difference, p <0.05.

GA: Glutaraldehyde.

Table II: Uniaxial testing of data from Part II of the study.

Parameter	Fresh pericardium		Decellularized pericardium	
	Untreated	0.05% GA-treated	Untreated	0.05% GA-treated
Elastic phase slope E (MPa)	1.946±2.443	1.107±0.384	0.153±0.172*	0.786±0.453*
Collagen phase slope Coll-E (GPa)	0.040±0.015	0.052±0.013	0.023±0.016	0.028±0.009
Transition stress $\sigma_{trans}$ (MPa)	0.921±0.395	1.322±0.455	0.998±0.727	1.691±0.714
Transition strain $\epsilon_{trans}$ (%)	14.963±7.691	20.643±5.713	28.337±13.613	38.960±7.273
Ultimate tensile strength $\sigma_{tis}$ (MPa)	11.139±3.258	16.205±4.169	9.483±7.171	13.082±4.537
Failure strain $\epsilon_{tis}$ (%)	47.446±6.756	55.057±7.085	67.972±17.291*	89.830±12.227
Thickness (mm)	0.389±0.021*	0.533±0.067*	0.335±0.049*	0.475±0.085*

Values are mean ± SD.

\*Significant difference, p <0.05.

GA: glutaraldehyde; MSD: Minimum significant difference

Comparison of fresh and decellularized pericardium, each treated with 0.05% GA showed that the elastic phase slope in the fresh group was significantly higher than that in the decellularized group. However, there was no significant difference in any of the other parameters in the two groups.

### **Part II**

Comparison of variously treated fresh pericardia (Table II) showed that there was no significant difference in average elastic phase slope, collagen phase slope, average transition stress, average transition strain, ultimate tensile strength and average failure strain between the three groups.

The average thickness in the fresh, 0.5% GA-treated group was significantly higher than in the fresh, untreated group. The average thickness of the fresh 0.05% GA-treated group was also significantly higher than that of the fresh, untreated group.

Comparison of variously treated decellularized pericardia (Table II) revealed that the elastic phase slope in the decellularized, 0.5% GA-treated group was significantly higher than that in the decellularized, untreated group. The elastic phase slope in the decellularized, 0.05% GA-treated group was also significantly higher than that in the decellularized, untreated group. Based on elastic phase slope, GA-treated decellularized pericardia were stiffer than untreated decellularized pericardium.

The average failure strain in the decellularized, 0.5% GA-treated group was significantly higher than that in the decellularized, untreated group. However, there was no significant difference in average failure strain between the decellularized, untreated group and decellularized, 0.05% GA-treated group and between decellularized, 0.5% and 0.05% GA-treated groups.

The average thickness of the decellularized, 0.5% and 0.05% GA-treated groups was significantly higher than in the decellularized untreated group. There was no significant difference in average collagen phase slope, average transition stress, average transition strain and ultimate tensile strength between the three groups.

## **Discussion**

The aims of the present study were to validate a protocol for the decellularization of bovine pericardium, and to study the biomechanical properties of fresh and decellularized bovine pericardia and pericardia treated with different concentrations of GA. Ideally, the decellularization process should leave no cells or cell fragments in the matrix scaffold, as evidence exists that these components (viable or dead cells) are associated with inflammation and calcification which, ultimately,

may lead to calcific tissue degeneration and limited tissue valve longevity (22). It was also vital that the cell extraction process preserved the extracellular matrix.

Histological analysis (using H&E, Alcian blue and elastin Van Gieson staining) of the decellularized bovine pericardium did not show the presence of any remaining cells or cell fragments. The histoarchitecture of the collagen-elastin matrix appeared to be well preserved. Analysis with Hoechst dye did not show any evidence of positive DNA staining in the decellularized specimens. The decellularization protocol described by Booth et al. (18) for porcine aortic valve leaflets was thus validated for bovine pericardium.

Previously, Courtman et al. (11) showed that using SDS as the decellularization agent with bovine pericardium caused extensive swelling and decreased the thermal stability of collagen. These authors also found that there was no effective post treatment to remove the residual SDS, and subsequently described a four-step detergent and enzymatic process using Triton X-100 instead of SDS. However, it must be noted that Courtman et al. used 1% (w/v) SDS, while in the present study a 0.1% (w/v) solution of SDS was used, and this may explain the different outcomes of decellularization in the two studies. The protocol of Courtman et al. was more recently validated by Chang et al. (12) for bovine pericardium.

Contact cytotoxicity tests were performed to assess the cytotoxic properties of the decellularized pericardium, including any residual effects of SDS and of a reduced concentration of GA. Previously, it has been reported that SDS was toxic and led to cell lysis within 24 h. Rieder et al. (23) used 0.1% SDS for decellularization of porcine aortic and pulmonary roots, and demonstrated a massive cell lysis of human saphenous vein endothelial cells within 24 h of incubation with the treated tissue. However Wilcox et al. (20) showed that porcine aortic wall decellularized with 0.1% SDS did not cause any substantial cytotoxicity, as evidenced by contact and extract cytotoxicity assays performed with porcine fibroblasts and SMC. In the present study, the porcine SMC and HDF attached and grew well in contact with untreated, decellularized pericardium, and there were no zones of lysis nor changes in cell morphology adjacent to the decellularized pericardium. These results compared well with those described by Wilcox et al. (20). A direct comparison of the present study findings with those of Rieder et al. (23) cannot be made due to differences in methodologies (e.g., washing procedures) and the cells used. It is also unclear in the report by Rieder et al. whether protease inhibitors (e.g., aprotinin and EDTA, as used in the present study) were utilized and, if not, whether this might account for the different results.

The pericardia treated with GA (0.5% and 0.05%) and the positive controls (cyanoacrylate glue) were clearly cytotoxic to SMC and fibroblasts. The fresh, untreated pericardium, which was not treated with peracetic acid, was also cytotoxic to SMC and fibroblasts. Toxicity of the fresh tissue may be explained by deep-freezing (at -40°C) and thawing of the pericardial samples, which led to the release of metabolites by viable cells into the culture medium, or the release of degradative products (e.g., lysosomal enzymes) from non-viable cells. Reducing the concentration of GA to 0.05% did not have any beneficial effect on the cytotoxic properties of pericardium treated with GA.

The cytotoxicity of GA-treated tissue has been previously recorded. Glutaraldehyde is a five-carbon bifunctional aldehyde, and not only the linear monomer of GA but also the dehydrated forms of monomeric GA, monomeric and polymeric hemiacetals and aldol condensation products such as polymeric aldehydes, have been shown to leach slowly from the GA-treated tissue, leading to cytotoxicity (24).

All decellularized tissues (untreated, and those treated with 0.5% or 0.05% GA) were treated with peracetic acid, which is known to be an effective sterilant for skin allografts (25,26) and acellular xenogeneic tissues (27). The adequacy of peracetic acid treatment to sterilize decellularized bovine pericardium would require extensive microbiological evaluation prior to clinical use, although it is important to recognize that peracetic acid offers an alternative means of sterilizing acellular xenogeneic tissues, which are not treated with a sterilizing dose of GA.

Part I of the present study involved uniaxial testing of comparable samples from fresh and decellularized groups, and did not reveal any significant difference in their biomechanical properties. This compared well with the findings of Courtman et al. (11), who used 1% SDS. These authors had used similar testing methods but a different agent (Triton X-100) for the decellularization of bovine pericardium. They also used 4 mm-wide pericardial strips and employed preconditioning of the pericardium immediately before testing. Subsequently, the decellularized bovine pericardium was found to be significantly thicker than fresh bovine pericardium; this increase in thickness was considered to be due to an increase in the water content of the decellularized pericardium. In the present study, however, there was no difference in the thickness of fresh and decellularized pericardia in comparable samples, and this may be due to the different concentrations of SDS used in each study.

In the present study there were no significant differences in the biomechanical properties of fresh and decellularized pericardia when both were treated with 0.5% GA for 24 h. Fresh pericardium treated with

0.05% GA was stiffer than the decellularized pericardium treated with 0.05% GA, based on a higher elastic phase slope. None of the other mechanical properties showed any significant differences between treatments with 0.5% and 0.05% GA.

In Part II of the study, a comparison was made, first among the three groups of fresh pericardia and then among the three groups of decellularized pericardia. This part of the study aimed to analyze the effects of fixation with a lower GA concentration (0.05%) compared to conventionally treated (0.5% GA) pericardia and untreated pericardia.

Fresh pericardia treated with 0.5% or 0.05% GA were both significantly thicker than untreated fresh pericardium. Decellularized pericardia treated with 0.5% and 0.05% GA were also significantly thicker than untreated decellularized pericardium. Both the pericardia (fresh and decellularized) treated with GA felt stiffer during handling. It is possible that, during the thickness measurements, the jaws of the thickness gauge encountered more resistance (compared to those not treated with GA), and that this led to higher readings of thickness.

The elastic phase slope was significantly higher in both GA-treated decellularized groups compared to the untreated decellularized group.

There was no significant difference in the mechanical properties of all three groups of fresh pericardia. Cross-linking with two different concentrations of GA did not significantly alter the biomechanical properties of the fresh pericardium in comparable samples. There was a trend, however, for fresh, cross-linked pericardium to be stiffer (based on increased collagen slope) and stronger than fresh, untreated fresh pericardium, though this difference did not reach statistical significance.

Similarly, there was a trend for decellularized, cross-linked pericardium to be stiffer (based on increased collagen slope) than decellularized, untreated pericardium but, again, this difference was not significant ( $p > 0.05$ ). The decellularized pericardium treated with 0.5% GA was stronger than the untreated decellularized pericardium, based on failure strain. The decellularized pericardium treated with 0.05% GA tended to be stronger than the untreated decellularized pericardium (also based on failure strain), but the difference was not statistically significant.

Overall, there was a tendency for the cross-linked pericardium to be stiffer and stronger than the untreated comparable samples. However, a ten-fold reduction in the concentration of GA did not cause any significant difference in the elasticity and the strength of the pericardia, both fresh and decellularized.

Vincentelli et al. (28) reported the mechanical properties of human pericardium after brief treatment with

GA, over contact periods of 5, 10, 30, and 60 min, and 6 months. These authors concluded that brief immersion of human pericardial tissue in 0.625% GA reduced the tissue's stiffness and improved its durability for use in cardiac surgery, but they did not investigate the effects of GA treatment over a 24-h period.

The results of the present study have underlined the advantages of different biomaterials that may have potential for clinical use, but require further investigation. The three types of decellularized bovine pericardium - untreated, and those treated with 0.5% and 0.05% GA - may prove to be superior biomaterials compared to the commercially available fresh bovine pericardium treated with 0.5% GA, as they are devoid of any cells or cell debris which might be responsible for calcification. These biomaterials will, however, require further - notably immunohistochemical - investigations in both small- and large-animal models in order to determine the presence and/or identity of any residual cellular material.

*In conclusion*, the results of the present study have shown that an acellular matrix, cross-linked with a reduced concentration of GA, can be prepared from bovine pericardium. Decellularized bovine pericardium which is cross-linked with 0.05% GA has excellent biomechanical properties, and the potential to be a viable alternative for clinical use in heart valves and pericardial patches. Moreover, the properties of this material are comparable to those of both untreated and 0.5% GA-treated decellularized bovine pericardium.

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#### References

1. Bender HJ, Stewart J, Merrill W, Hammon JJ, Graham TJ. Ten years' experience with the Senning operation for transposition of the great arteries: Physiological results and late follow-up. *Ann Thorac Surg* 1989;47:218-223
2. Okita Y, Miki S, Ueda Y, et al. Early and late results of repair of tetralogy of Fallot with subarterial ventricular septal defect: A comparative evaluation of tetralogy with perimembranous ventricular septal defect. *J Thorac Cardiovasc Surg* 1995;110:180-185
3. Ionescu M, Smith D, Hasan S, Chidambaram M, Tandon A. Clinical durability of the pericardial xenograft valve: Ten years' experience with mitral replacement. *Ann Thorac Surg* 1982;34:265-277
4. Tuli B, Parmar T, Lal M. The role of homologous pericardium in myringoplasty. *Indian J Otol* 1998;4:91-92
5. Martucci RC, Calado AA, Zerati M, Muller MA. Pubovaginal sling with bovine pericardium for treatment of stress urinary incontinence. *Braz J Urol* 2000;26:208-214
6. Caroli E, Rocchi G, Salvati M, Delfini R. Duraplasty: Our current experience. *Surg Neurol* 2004;61:55-59; discussion 59
7. Oswal D. Biomechanical characterisation of decellularised and cross linked bovine pericardium. MSc Thesis. The School of Mechanical Engineering, University of Leeds, UK, 2005:9 ([www.leeds.ac.uk/library](http://www.leeds.ac.uk/library))
8. Desai N, Christakis G. Stented mechanical/bio-prosthetic aortic valve replacement, in Cohn L, Edmunds LJ (ed.), *Cardiac Surgery in the Adult* McGraw-Hill, New York, 2003:834-839
9. Yankah CA, Schubel J, Buz S, Siniawski H, Hetzer R. Seventeen-year clinical results of 1,037 Mitroflow pericardial heart valve prostheses in the aortic position. *J Heart Valve Dis* 2005;14:172-179; discussion 179-180
10. Simmons D, Kearney J. Evaluation of collagen cross linking techniques for the stabilization of tissue matrices. *Biotechnol Appl Biochem* 1993;17:23-29
11. Courtman D, Pereira C, Kashef V, McComb D. Development of a pericardial acellular matrix biomaterial: Biomechanical and mechanical effects of cell extraction. *J Biomed Mater Res* 1994;28:655-666
12. Chang Y, Lee M, Liang H, Hsu C, Sung H. Acellular bovine pericardium with distinct porous structures fixed with genipin as an extracellular matrix. *Tissue Eng* 2004;10:881-892
13. Oswal D, Woo E, Kay P, McLenachan J. Haemodynamic performance of a 16 mm CarboMedics aortic prosthesis. *Eur J Cardiothorac Surg* 1997;11:1183-1184
14. Korossis S. Biomechanics and hydrodynamics of decellularised aortic valves for tissue engineering. MSc Thesis. The School of Mechanical Engineering, University of Leeds, Leeds, UK, 2002:114-120 ([www.leeds.ac.uk/library](http://www.leeds.ac.uk/library))
15. Duran C. Aortic valve replacement with freehand autologous pericardium. *J Thorac Cardiovasc Surg* 1995;110:511-516
16. Dahm M, Prufer D, Mayer E, Groh E, Choi Y, Oelert H. Early failure of autologous pericardium aortic heart valve (ATCV) prosthesis. *J Heart Valve Dis* 1998;7:30-33
17. Gross C, Simon P, Grabenwöger M, et al. Midterm results after aortic valve replacement with the autologous tissue cardiac valve. *Eur J Cardiothorac Surg* 1999;16:533-539
18. Booth C, Wilcox H, Korossis S, et al. Tissue engineering of cardiac valve prostheses. I. Development and histological characterisation of an acellular porcine scaffold. *J Heart Valve Dis* 2002;11:457-462

19. Bancroft JD, Stevens A (ed.), *Theory and Practice of Histological Techniques*. Churchill Livingstone, 1996
20. Wilcox H, Korossis S, Booth C, et al. Biocompatibility and recellularization potential of an acellular porcine heart valve matrix. *J Heart Valve Dis* 2005;14:228-237
21. Sokal R, Rohlf F. Single classification analysis of variance, in *Biometry*. W. H. Freeman & Co., 1981:208-270
22. Schoen F, Levy R. Tissue heart valves: Current challenges and future research perspectives. *J Biomed Mater Res* 1999;47:439-465
23. Rieder E, Kasimir M, Silberhumer G, et al. Decellularization protocols of porcine heart valves differ importantly in efficiency of cell removal and susceptibility of the matrix to recellularization with human vascular cells. *J Thorac Cardiovasc Surg* 2004;127:399-405
24. Schmidt C, Baier J. Acellular vascular tissues: Natural biomaterials for tissue repair and tissue engineering. *Biomaterials* 2000;21:2215-2231
25. Huang Q, Dawson R, Pegg D, Kearney J, Macneil S. Use of peracetic acid to sterilize human donor skin for production of acellular dermal matrices for clinical use. *Wound Repair Regeneration* 2004;12:276-287
26. Lomas RJ, Cruse-Sawyer JE, Simpson C, Ingham E, Bojar R, Kearney JN. Assessment of the biological properties of human split skin allografts disinfected with peracetic acid and preserved in glycerol. *Burns* 2003;29:515-525
27. Hodde J, Hiles M. Virus safety of a porcine-derived medical device: Evaluation of a viral inactivation method. *Biotechnol Bioeng* 2002;79:211-216
28. Vincentelli A, Zegdi R, Prat A, et al. Mechanical modifications to human pericardium after a brief immersion in 0.625% glutaraldehyde. *J Heart Valve Dis* 1998;7:24-29