Preparation and characterization of acellular porcine pericardium for cardiovascular surgery

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Abstract: The aim of this study was to fabricate and characterize acellular porcine pericardium that can be used to patch cardiovascular repair. Porcine pericardial tissues were treated with 10 mM Tris-HCl and sodium dodecyl sulfate (SDS) at different concentrations and time intervals. The optimal decellularization protocol was determined according to elimination of cellular components and DNA via histology and DNA quantification, respectively. There were no significant changes in stress and fracture strain after decellularization. Liquid extracts from the decellularized pericardium caused no cytotoxicity towards human fibroblasts, were capable of supporting an appropriate attachment of human endothelial progenitor cells, and did not cause a local inflammatory effect after 30 days of transplantation in mice.

Key words: Pericardium, decellularization, patch, cardiovascular, biocompatibility

1. Introduction
Cardiovascular diseases remain the leading cause of death for both men and women around the world. Such diseases killed approximately 17.5 million people in 2012 (Mozaffarian et al., 2012). For patients, taking medication can help alleviate symptoms, but there are several possible side effects associated with medicine. Based on the fact that it is more beneficial to treat the root cause by repairing injured tissues, many studies have focused on the manufacture of new patch materials with potential advantages for heart and vascular repairs. There are two main categories of materials that have been clinically used in cardiovascular patching applications, involving engineered tissues and synthetic materials. Composite grafts provide numerous benefits, including strength and durability (Venkatraman et al., 2008; Nelson et al., 2011) but carry risks of infection, thrombogenicity, calcification, and foreign body reaction as the well-known limitations (Kannan et al., 2005; Lam and Wu, 2012). The other potential choice for cardiovascular operations is xenogeneic tissues. In terms of cardiovascular patching, bovine pericardium, with the advantage of ready availability, excellent biocompatibility, and a low rate of infection, has been developed and tested in preclinical and clinical studies (Pires et al., 1999; Biasi et al., 2002; Kim et al., 2012). Porcine pericardial tissues with similar adequate biological and mechanical properties (Gauvin et al., 2013) have also recently been of great interest for cardiovascular transplantation. A previous report suggested that due to the structure and cross-linking of collagen bundles in porcine pericardium, it might be regarded as a suitable alternative to bovine pericardium for cardiovascular disease treatment (Gauvin et al., 2013). Studies have established that, in xenotransplantation, xenogeneic antigens may contribute to both in vitro compatibility problems and in vivo immune-mediated rejection of tissue (Auchincloss and Sachs, 1998; Cascalho and Platt, 2001; Byrne and McGregor, 2012). In order to overcome several host immune barriers, animal tissues such as porcine pericardia must undergo decellularization before application in patients. Numerous methods of decellularization, including physical, chemical, and enzymatic treatments, have been developed for the complete elimination of cellular components and the preservation of the pericardial extracellular matrix (ECM). However, it has been noted that almost all current decellularization processes for porcine pericardia have

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negative effects on the mechanical and biological properties of the ECM. In the present study, we aim to establish an efficient decellularization procedure for native porcine pericardia without causing alterations to the ECM structure and properties. The acellular pericardium is expected to be an ideal patch for cardiovascular tissue engineering.

2. Materials and methods
2.1. Decellularization of porcine pericardium
Porcine pericardial sacs collected at a local slaughterhouse were stored in cold 1X phosphate buffer saline (PBS, Gibco) and transported to the laboratory. They were dissected to remove external fat, washed thoroughly with PBS and sectioned into small strips (2 × 2 cm). Pericardial strips were treated in 10 mM Tris-HCl (Merck, USA) for 8 h and divided into 9 groups to be treated with sodium dodecyl sulfate (SDS, Sigma, USA) at different concentrations and time intervals (Table 1). Finally, all specimens were rinsed for 90 min in PBS to remove residual chemicals and cellular remnants. All steps were carried out at room temperature under continuous shaking at 80 rpm.

2.2. Histological analysis
Native and treated pericardial strips were fixed with 10% formalin at 4 °C for 24 h. They were dehydrated step-wise using ethanol, immersed in xylene, and embedded in paraffin. The paraffin sections were cut at 4 µm, deparaffinized, and stained with hematoxylin and eosin (H&E). Images were taken using an Olympus CKX-RCD microscope (Tokyo, Japan) equipped with a DP2-BSW microscope digital camera.

2.3. Evaluation of residual DNA
Pericardial strips were cut into small pieces and digested with 100 ng/mL Proteinase K (Sigma, USA) at 37 °C until no visible fragments remained. The digested samples were then centrifuged at 13,000 rpm for 30 min. Afterwards, the supernatants were purified with phenol–chloroform–isoamyl alcohol (25:24:1) followed by centrifugation at 13,000 rpm for 30 min. Finally, the DNA pellets were washed with 70% ethanol (Merck, USA) and dried by air. The DNA contents were quantified by measuring absorbance at 260/280 nm in a Beckman Coulter DTX 880 Multimode Detector spectrophotometer (Fullerton, CA, USA).

2.4. Mechanical testing
Strips of pericardium (5 × 2 cm) were prepared and treated with Tris-HCl and SDS as described earlier. The strips were mounted into holders and mechanically examined for tensile strength and fracture behavior by extending to fracture at an extension rate of 50 mm/min as described previously (Sung et al., 1999). The mechanical testing was conducted in an EZ50 universal testing machine (Lloyd Instruments, UK) equipped with NEXYGENPlus material test and data analysis software. The native tissues were used as a control group.

2.5. Extract cytotoxicity assay
The cytotoxicity of decellularized pericardia (dP) was analyzed using an extract test in accordance with ISO 10993/5. Human skin fibroblasts were used for the cytotoxicity assay. The cells were grown in culture medium (Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12, Sigma, USA). Following ISO 10993/5, fibroblasts were harvested at passage 4 and replated onto a 96-well plate (4 × 10^3 cell per well). The cells were cultured for 24 h. Meanwhile, the dP was incubated in a culture medium (6 cm² per mL) at 37 °C for 24 h. After incubation, the culture medium was separated and used as a full liquid extract. In the cytotoxicity assay, the fibroblasts were incubated with the liquid extract of the dP at 37 °C for 24 h. The culture medium alone and a liquid extract of latex gloves (Baek et al., 2005) were used as blank and positive controls, respectively. After incubation for 24 h, the liquid extracts were replaced with culture medium containing 0.5 mg/mL streptomycin (Sigma, USA), and 100 U/mL penicillin (Sigma, USA). Following ISO 10993/5, fibroblasts were harvested at passage 4 and replated onto a 96-well plate. Absorbance at 575 nm was determined using a spectrophotometer (Perkin, USA). Relative growth rate (RGR) was calculated on OD575 values according to the ISO 10993/5 protocol as RGR (%) = (ODtest group /OD blank group) × 100. If the RGR value decreased by more than 30%, the liquid extract would be considered to cause a cytotoxic effect (ISO 10993/5).

2.6. In vitro cell attachment assay
Decellularized pericardia were cut into square strips (1 × 1 cm) and individually placed into the wells of agarose-coated 4-well plates. The coatings were prepared by adding 200 µL of 1% sterile molten agarose to each well. Human endothelial progenitor cells (hEPCs) were then seeded at a density of 10^4 cells per pericardium and allowed to adhere for 1 h. The attachment and morphology of the hEPCs

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**Table 1.** Experimental sample groups treated with defined concentrations of SDS for defined incubation periods.

<table>
<thead>
<tr>
<th>SDS concentration</th>
<th>Incubation time</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>0.1%</td>
<td></td>
<td>0.1–6</td>
<td>0.1–12</td>
<td>0.1–24</td>
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<tr>
<td>0.3%</td>
<td></td>
<td>0.3–6</td>
<td>0.3–12</td>
<td>0.3–24</td>
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<tr>
<td>0.5%</td>
<td></td>
<td>0.5–6</td>
<td>0.5–12</td>
<td>0.5–24</td>
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the pericardial tissues were observed at 24 h by scanning electron microscope (SEM).

2.7. Mouse subcutaneous implantation

The experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication No. 85–23 Rev. 1985). The animal experiment protocol was approved by the Animal Care and Use Committee of the University of Science in Ho Chi Minh City, Vietnam (Registration No. 03/15-010-00). In order to observe the biocompatibility of the decellularized pericardia in vivo, dP was implanted into the dorsum of mice (Mus musculus var. albino, male, 6–8 weeks old, 25–30 g). Nine mice were divided into three groups including one test group (dP) and two control groups, one using native pericardia (nP) and the other using skin incision without implantation. Each pericardium sample was prepared as approximately 0.5 × 0.5 cm² and sterilized with a dose of 25 kGy gamma irradiation. Subcutaneous transplantation was carried out under aseptic conditions. After 30 days, the mice were euthanized. The implanted samples were obtained and fixed in 10% formalin for histology (H&E staining).

2.8. Statistical analysis

All quantitative data are expressed as means ± standard deviations. Statistical analyses were conducted using Prism 5 (GraphPad Software, Inc., San Diego, CA). Student’s t test was used to analyze the differences between groups. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Morphology and histology

Pericardial strips were treated in 10 mM Tris-HCl and SDS at different concentrations and time intervals (Table 1). The pericardium is a tough and flexible membrane that surrounds the heart. The pericardial strips treated with 0.1% SDS had a well-preserved appearance (Figure 1A), whereas treating with 0.3% and 0.5% SDS made them stiff and swollen compared with native pericardium, and this was clearly observed for all indicated time intervals (Figure 1B). Therefore, 0.1% SDS, rather than 0.3% or 0.5% SDS, is suitable to apply for decellularization of pericardium.

H&E staining revealed the underlying structure of the native pericardial tissue, in which extracellular fibers were stained pink, and nuclei were stained purple, indicating the remaining cells (Figure 2A, arrows). Strips treated with 0.1% SDS showed no cells, and the three treated groups, 0.1–6 (Figure 2B), 0.1–12 (Figure 2C) and 0.1–24 (Figure 2D), did not differ microscopically. Moreover, the structure of the extracellular fibers of the treated samples retained a lamellar arrangement, similar to native pericardium.

3.2. DNA quantification

DNA quantification was done to determine the level of elimination of cellular debris from the decellularized samples. Native pericardial tissue with complete cellular contents had a DNA amount as high as 445.3 ± 17.4 ng per mg dry weight (Table 2). The DNA contents of the pericardia specimens treated with 0.1% SDS for 12 and 24 h were significantly reduced to less than 50 ng per mg dry weight (Figure 3). Taken together, 0.1% SDS applied for 12 h was the most effective approach for decellularization of porcine pericardia.

3.3. Mechanical properties

Native and decellularized pericardial samples (0.1–12) were tested for maximal stress (Figure 4) and fracture strain (Figure 5). The mean maximal stress and fracture strain of the decellularized samples were not different from those of native pericardial samples (P > 0.05). This suggested that the decellularization process had a minimal effect on the mechanical properties of the pericardial samples.

Figure 1. Representative macroscopic appearance of treated pericardial strips. 1A. Pericardial strips treated with 0.1% SDS. 1B. Pericardial strips treated with either 0.3% or 0.5% SDS.
3.4. Cytotoxicity assay

The morphology of the human fibroblasts after being treated with liquid extracts is shown in Figure 6. The fibroblasts have elongated shapes (Figure 6A), which remained unchanged after 24 h of incubation in culture medium (Figure 6B). The liquid extract from the decellularized samples had no effect on the cells, as indicated by their normal cell morphology after incubation for 24 h (Figure 6C) and their RGR value, which was found to be as high as the RGR value of the group treated with the culture medium (Table 3). On the other hand, as expected, the liquid extract from latex gloves (as the positive control) caused cell cytotoxicity after incubation for 24 h. The cells were dead and exhibited cellular shrinkage after treatment (Figure 6D) as well as having an RGR value reduced more than 50% (Table 3).

![Figure 2. H&E staining of pericardial samples. 2A. Native samples. 2B. 0.1% SDS at 6 h incubation time. 2C. 0.1% SDS at 12 h. 2D. 0.1% SDS at 24 h. Cell configuration in native tissue (black arrow). 40× magnification.](image)

<table>
<thead>
<tr>
<th>DNA (ng/mg dry weight)</th>
<th>0.1–6</th>
<th>0.1–12</th>
<th>0.1–24</th>
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<tr>
<td>Native pericardia</td>
<td>445.30 ± 17.410</td>
<td>120.00 ± 5.003</td>
<td>40.63 ± 4.677</td>
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3.5. Cell attachment

hEPCs were used to examine cell attachment support of the decellularized pericardium. The hEPCs were seeded onto decellularized pericardial samples and visualized using SEM. The SEM images revealed the morphology of the hEPCs on the decellularized pericardium. The hEPCs attached to the pericardial matrix and spread into polygon-rounded appearance after being cultured for 48 h (Figure 7, arrows).

3.6. Subcutaneous implantation of porcine pericardia in mice

After the surgery, all mice recovered well with no signs of distress or disease, and no deaths occurred. All of the implanted decellularized (dP) and native pericardia (nP) were explanted at the end of 30 days of study. It was observed that the pericardia from both groups had been surrounded by a thin fibrous capsule. H&E staining showed the fibrous capsule and vascular proliferation surrounding the nP; moreover, local inflammation was recognized via infiltration of the inflammatory cells (Figure 8A, red arrows). The explanted dP also showed fibrous and vascular proliferation, but no inflammation was detected (Figure 8B).

4. Discussion

Pericardial specimens were treated in Tris-HCl for 12 h, followed by submersion in SDS solution at three concentrations (0.1%, 0.3%, and 0.5%) for three time intervals (6, 12, and 24 h). The histological structures as well as the physical and biological properties of the treated samples were examined to determine the optimal experimental group for the production of tissue-engineered pericardia for use in cardiovascular patching applications.

H&E staining as well as DNA determination demonstrated that the decellularization procedure using 0.1% SDS for 12 h was effective at removing cellular components while still preserving the ECM architecture (Figures 2–4). It has been reported that the decellularization of porcine aortic valve leaflets and bovine pericardium using 0.1% SDS for 24 h resulted in the complete removal of cellular contents (Booth et al., 2002; Gonçalves et al., 2005). In one study done by Liao et al. (2008), after treatment with different processes using Triton X-100, SDS, sodium deoxycholate, MEGA 10, TnBP, CHAPS, and Tween 20, over a range of concentrations, analysis showed that there were no cell nuclei present in the tissues after 24 h of treatment with SDS (0.03%–1%). This study suggested
that protocols utilizing SDS were successful for leaflet decellularization, and these methods are being further studied to produce tissue-engineered solutions for clinical implantation. Furthermore, the combination of 0.1% SDS and a hypotonic Tris buffer (10 mM Tris, pH 8.0) resulted in acellular tissue as well as preservation of the structural and mechanical properties of porcine aortic heart valve leaflets. The total time consumption for this established protocol was 48 h. In our study, the optimal procedure for porcine pericardial tissues was identified with SDS at the same concentration (0.1%), but with a shorter time period (12 h).

Previous studies on aortic valves have considered SDS decellularization to be a gentle treatment to maintain the major structural components of elastin and collagen (Grauss et al., 2003; Liao et al., 2007). On the other hand, several reports have demonstrated SDS to cause strong structural alterations (Kasimir et al., 2003). It was also noted that this ionic detergent tends to cause a decrease in glycosaminoglycan (GAG) concentration and a loss of collagen integrity (Funamoto et al., 2010; Crapo et al., 2011; Dong et al., 2013). This controversial finding seems to be a consequence of the different incubation times and SDS concentrations used in the decellularization process.

**Table 3.** OD575 values and relative growth rates (%) of the three experimental groups. All values are expressed as mean ± SD (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Culture medium</th>
<th>Pericardium extracts</th>
<th>Latex extracts</th>
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<tr>
<td>OD575</td>
<td>0.27 ± 0.01</td>
<td>0.33 ± 0.03</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>RGR (%)</td>
<td>100</td>
<td>122.00 ± 0.16</td>
<td>46.13 ± 0.05</td>
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**Figure 6.** Morphological appearance of human fibroblast cells in a cytotoxicity assay. 6A. Cell morphology after 24 h reseeded. 6B. Cell morphology when incubated for 24 h in the culture medium. 6C. Cell morphology when incubated for 24 h in the sample extract. 6D. Cell morphology when incubated for 24 h in the latex extract. 10× magnification.
Processes to decellularize bovine pericardia or aortic valves using 0.1% SDS for 24 h and 48 h, as described in previous studies (Booth et al., 2002; Gonçalves et al., 2005; Liao et al., 2008) showed no negative effects on ECM integrity. In contrast, higher concentrations of SDS (higher than 0.5%) were reported to have adverse effects when treating tissue for a period of time (Samouillan et al., 1999; Zhou et al., 2010; Dong et al., 2013). In our study, we found that using 0.1% SDS for 12 h was effective in reducing the cellular components of porcine pericardia without any negative effects on the structure of the pericardial collagen fibrils. Furthermore, the average ultimate tensile strength and percent deformation of the decellularized pericardia showed that mechanical properties of the studied tissues were well maintained. These results are in accordance with previously reported data (Booth et al., 2002; Gonçalves et al., 2005; Liao et al., 2008).

The most important feature of decellularized tissue for clinical applications is biocompatibility. Before doing transplantation in humans, the safety of the implant materials must be considered seriously. Multiple studies have established that residual SDS in the tissue causes cytotoxicity (Bodnar et al., 1986; Rieder et al., 2004; Mirsadraee et al., 2007). However, it was noted in our previous study that no cytotoxic effects were observed among normal cells treated with the decellularized pericardial extract. The relative growth rate, at 122.00 ± 0.16% (higher than the 70% recommended by ISO 10995/5), also suggested that the studied tissue might be beneficial to cell proliferation. Another interesting result was the degree of cell adhesion to treated pericardia, which indicated that these pericardia had good biocompatibility and could be used as in vivo biological patches. For the in vivo study, the biocompatibility of cellular and acellular porcine pericardia was evaluated subcutaneously in mouse models. By and large, the ECM grafts exhibited high stability and compatibility with the surrounding tissues. On the basis of our results, we suggest that porcine pericardial tissue decellularized with 0.1% SDS for 12 h could be a potential material for further cardiovascular patching applications.

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**Figure 7.** SEM examination of the attachment of hEPCs (arrows) on acellular pericardia. Scale bar corresponds to 10 µm.

**Figure 8.** H&E staining. 8A. Native pericardium (nP). 8B. Decellularized pericardium (dP) implanted subcutaneously into mice after 30 days. F = fibrous capsule, red arrows = immune cells. 40× magnification.
References


