**Introduction:** Gradual calcification of valve leaflets continues to be a common failure mode of bioprosthetic heart valves. Several factors have been demonstrated to play a role in leaflet calcification. These factors include (1) intrinsic leaflet tissue components, such as collagen, elastin, and lipids, (2) tissue fixation processes, and (3) valve functional dynamics. Addressing these factors in the design, processing, and manufacture of bioprosthetic heart valves are critical to achieving long-term structural integrity and robust hemodynamic performance.

This paper describes a novel process used in the preparation and preservation of pericardial tissue in the manufacture of bioprosthetic heart valves. The process addresses calcium mineralization in pericardial tissue from two distinct perspectives; residual glutaraldehyde reduction and phospholipid extraction. This paper presents biochemical and biomechanical data and analyses along with structural and functional data on pericardial tissue leaflets and whole valves prepared with the ThermaFix process.

**Key words:** Calcification; calcium; collagen; durability; fixation; glutaraldehyde; heart valve; pericardial tissue; phospholipid

---

**BACKGROUND**

Glutaraldehyde fixation is a tissue preservation process used in the manufacture of bioprosthetic heart valves. The objective of fixation is to produce stable, durable, and biologically inert tissue that is suitable for implantation. Collagen, a principle tissue protein, provides strength and resistance to breaking stresses in tissue and plays a critical role in its long-term durability. In 1982, Edwards Lifesciences developed a proprietary glutaraldehyde fixation process for bioprosthetic tissue. Edwards Neutralogic fixation utilizes a low pressure method of fixation that helps to preserve the natural elasticity of the collagen matrix. Neutralogic fixation allows glutaraldehyde to form crosslinks with collagen that result in durable fibrous tissue structures that have long-term performance properties that are well suited for bioprosthetic heart valve tissue.

As previously mentioned, calcification of heart valve leaflets is a primary contributor to structural valve deterioration. Calcification of pericardial tissue can result from a reaction between phospholipids in connective tissue and calcium in the circulatory system. Historically, tissue processing methods that reduce phospholipid levels in preserved tissue samples have been associated with reduced calcification levels in animal model studies. In 1985, Edwards Lifesciences announced the development of a unique tissue preparation technique for bioprosthetic heart valves. Edwards XenoLogiX treatment is a proprietary tissue preparation process which utilizes alcohol and surfactant to extract phospholipids from pericardial tissue after initial glutaraldehyde fixation and to address any glutaraldehyde-resistant organisms resulting in an 8 to 10 log reduction in microbial levels after terminal liquid sterilization. Data published by Cunanan et al., demonstrated that the XenoLogiX treatment removed greater than 90% of the phospholipids in both porcine leaflets and pericardial tissues.

As seen in Figure 1, Edwards Lifesciences pericardial valves prepared with the XenoLogiX treatment have maintained 81.5% actuarial freedom from structural valve deterioration for up to 20 years, in patients 65 years of age or older, with a cumulative risk of explant due to SVD of only 3.7% at 20 years. Further, these valves display exceptionally low incidence of endocarditis.
In 2004, Edwards Lifesciences announced the FDA approval of the Carpentier-Edwards ThermaFix process. The ThermaFix process is an innovative tissue preparation method suggested by Alain Carpentier, MD, PhD and Sophie Carpentier PhD, pioneers of tissue treatment methods for bioprosthetic heart valves. The ThermaFix process builds upon the phospholipid extraction technique of the XenoLogiX treatment and includes additional biochemical processes which further reduce potential calcium binding sites in pericardial tissue.

As previously mentioned, tissue preservation with glutaraldehyde is based on stable crosslink reactions between tissue collagen and glutaraldehyde. During the fixation process, amino groups intrinsic to collagen (lysine side chains) may form a small number of unstable bonds with glutaraldehyde\(^7\). Unstable collagen-glutaraldehyde bonds are not responsible for long-term crosslinking and can be easily hydrolyzed (broken)\(^1\). Once an unstable bond is broken, aldehyde groups can be regenerated on the glutaraldehyde molecule which can, in turn, oxidize into carboxylic acids that will have the affinity to bind with calcium in the circulatory system. The ThermaFix process creates conditions that facilitate the removal of the unstable glutaraldehyde moieties and prevents them from becoming potential calcium binding sites.

Neutralologic fixation, XenoLogiX treatment, and ThermaFix process are diagrammed in Figure 2.

As seen in Figure 2, each of the methods of tissue preservation begins with Neutralologic fixation using a 0.625% glutaraldehyde solution at low pressure.

The XenoLogiX treatment includes the use of alcohol and surfactant in a multi-step process. The objectives of the XenoLogiX treatment are to chemically extract phospholipids from the cellular components of pericardial tissue and to inactivate glut-resistant micro-organisms. Extraction of the lipids in pericardial tissue reduces potential sites for calcification initiation.

The ThermaFix process includes the phospholipid extraction as seen with the XenoLogiX treatment in Figure 2, but includes the addition of a mild heat treatment step that is unique to the ThermaFix process. The objective of the heat treatment step is to remove unstable glutaraldehyde moieties which are usually present in glutaraldehyde-fixed tissue. This step targets the unstable Schiff bond formation that can exist between the lysine side chains and glutaraldehyde. Removing unstable glutaraldehyde bonds from preserved tissue circumvents potential oxidation of the glutaraldehyde moieties into carboxylic acids which can serve as potential binding sites for calcium. As outlined in Figure 2, the ThermaFix process removes calcium binding sites from preserved tissue by; (1) using a mild heat treatment step to subtract unstable glutaraldehyde moieties after glutaraldehyde fixation, and (2) using a wash of alcohol and surfactant to extract naturally occurring phospholipids from the cellular components of pericardial tissue.

**EXPERIMENTAL METHODS**

*In vitro* and *in vivo* studies were performed on bovine pericardial leaflet tissues and intact valves that were preserved using the three tissue treatment methods shown in Figure 2. Samples were subjected to calcification, biochemical, biomechanical, and histological analysis. Studies were performed utilizing small and large animal models. All animal model studies were conducted in compliance with the National Institute of Health, “Guide for the Care and Use of Laboratory Animals.”
Calcification Analysis

Calcification potential of the prepared pericardial tissue samples was assessed using three animal models; rat subcutaneous implant, rabbit intramuscular, and juvenile sheep. Calcification analysis was performed using atomic absorption spectroscopy (AAS).

The rat subcutaneous leaflet implant study was performed on 12-day old Wistar rats (Charles River Laboratories) for 120 days. Four subcutaneous pockets were made parallel to and on either side of the dorsal midline. An 8 mm leaflet tissue disc from each tissue process group was implanted in each pocket. A total of 150 discs per test group and 40 discs per control group treated with glut-only were implanted. At 120 days, the leaflet discs were retrieved for calcium analysis.

The rabbit intramuscular leaflet implant study was conducted on 8-week old, female New Zealand White rabbits (Western Oregon Rabbitry) for 28 days. Three pocket incisions parallel to the midline were made at the cranial, midsection and caudal regions on the dorsal surface. An 8 mm leaflet tissue disc from each tissue process group was implanted in each pocket. A total of 62 discs per test group and 20 discs per control group treated with glut-only were implanted; six samples were implanted per animal. At 28 days, the leaflet discs were retrieved for calcium analysis.

A juvenile sheep study was performed using implanted heart valves to assess valve hemodynamic performance, biocompatibility, and leaflet calcification. The juvenile sheep study was performed using two types of Carpentier-Edwards PERIMOUNT heart valves; models 6900P and 6900PTFX. Six of each valve type was implanted in the mitral position in three to six-month old Suffolk/Suffolk-crossbred sheep for 20 weeks. Hemodynamic examinations were performed at 60 days using transthoracic echocardiography and Doppler. At 20 the time of implant, valve hemodynamics was evaluated with contrast angiography and direct pressure measurements. At explant, valves were visually assessed and scored for leaflet calcification. Segments of the valves were harvested for calcium analysis and the remainder of each valve was submitted for histopathological analysis.

Chemical Analysis

Chemical analysis was used to compare pericardial tissue samples prepared with Neutralogic fixation and the ThermaFix process. Chemical analysis assessed residual glutaraldehyde levels in the prepared tissue by quantifying tissue acid content. Tissue acid content was quantified using reverse phase high performance liquid chromatography (HPLC). Tissue samples were subjected to a mild acid hydrolysis to remove residual, unstable forms of glutaraldehyde. A small amount of hydrolysate was then tagged to specifically identify carboxylic acid levels using HPLC methods.

Biochemical Analysis

Biochemical analysis evaluated pericardial tissue moisture content, shrinkage, and free amine content for all three tissue groups. These tests reveal the degree of hydration, degree of cross-linking, and collagen-specific stability.

Leaflet samples discs were weighed before and after lyophilization to determine moisture content.

Shrinkage was assessed by measuring tissue strain using a differential transducer to record corresponding changes in tissue length with tissue temperature. Shrinkage temperature was recorded when tissue length decreased by one percent.

Free amine content of the pericardial tissue samples was measured using a modified Ninhydrin assay by a spectrophotometric method.

Histological Analysis

Histological analysis assessed collagen and elastin morphology in the tissue samples prepared with the XenoLogiX treatment and the ThermaFix process. Analysis included the assessment of intact collagen bundles and crimping patterns that are associated with tissue stability. Tissue leaflets were embedded, sectioned, and stained with Hematoxylin and Eosin (H&E), as well as, Movat Pentachrome. H&E was used to assess tissue morphology, while Movat Pentachrome was used to assess the connective tissue components of collagen, elastin, and non-collagenous structures.

Biomechanical Tissue Analysis

Biomechanical tissue analysis included uniaxial, biaxial, and flexure testing for leaflet tissue samples prepared with the XenoLogiX treatment and the ThermaFix process. Uniaxial tension testing is a measure of the overall strength of the tissue and is related to durability. Yield stress and yield strain were compared in the uniaxial testing.

Biaxial tension testing is a two-way tissue stretch test and is an indicator of the anisotropic behavior of the tissue. Biaxial testing is capable of detecting differences in mechanical behavior through linear and areal stretches. Linear and areal stretches were calculated by measuring load and deflection along the radial and circumferential axes of the tissue. These axes in the tissue correspond to similar axes in the leaflets of a functioning valve and data from the tests are associated with leaflet coaptation and to the stress in the closed valve.
Flexure testing is an extremely sensitive measure of the bending behavior of the pericardial tissue. Flexure tests are capable of detecting subtle differences in mechanical behavior as it relates to valve opening and durability. In this test a force is applied to the strip of tissue to cause it to bend. The radius of the bend is related to the bending moment resulting from the applied force with flexure assessed by measuring the change in position of the tissue edge under an applied bending load. The change in edge position is plotted with the slope of the curve representing the flexural rigidity of the tissue or instantaneous effective modulus.

**Functional Valve Analysis**

Functional valve performance was assessed using standard hydrodynamic tests and accelerated wear tests using intact valves prepared with the XenoLogiX treatment and the ThermaFix process.

Analysis for hydrodynamic performance included testing for pulsatile flow pressure gradient, pulsatile flow regurgitation, and pressure drop at high cardiac output. Valves tested in the XenoLogiX treatment group included aortic model 2800, sizes 19 and 25, and mitral model 6900P, size 31. Valves tested in the ThermaFix process group included aortic model 2800, sizes 19, 21, 23, 25, and mitral model 6900P, sizes 25, 27, 29, and 31.

In pulsatile flow testing, the effective orifice area (EOA) and transvalvular pressure gradients are measured.

Regurgitation is a measure of closing and leakage volumes through the valve leaflets during diastole. Regurgitation volume (in milliliters) was measured at various cardiac outputs.

Pressure drops at high cardiac output was assessed in tests specially designed to drive fluid through a tissue valve in a steady continuous manner. This test measures differential pressure across the valve leaflets in the open position at different system flow rates.

Accelerated wear testing (AWT) involves opening and closing a bioprosthetic heart valve at high rates. The established criterion for valve failure is leaflet incompetence that results in excessive backflow leakage. Valve durability was assessed at 1000 to 1200 CPM for 200 million cycles (the equivalent of 5 years) at peak pressure differentials appropriate for the aortic and mitral valve positions. Valves were videotaped to document proper opening and closing throughout the test. At 40, 100, 160, and 200 million cycles, valves were removed from the testers and visually assessed for wear and backflow leakage. At 200 million cycles, all valves were radiographically assessed to confirm the structural integrity of the wire form.

**RESULTS**

**Calcification Analysis Results**

Results obtained from the rat subcutaneous leaflet study showed that the calcium content of the ThermaFix process (45 ± 90 µg Ca/mg dry wt) was 44% lower than the XenoLogiX treatment (80 ± 110 µg Ca/mg dry wt.).

Results obtained from the rabbit intramuscular leaflet study demonstrated that the ThermaFix process (59 ± 90 µg Ca/mg dry wt) was 35% lower than the XenoLogiX treatment (59 ± 48 µg Ca/mg dry wt.).

Results from the explanted whole valves in the juvenile sheep study demonstrated that the calcium content in the valve leaflets of the ThermaFix process valves was 75% lower than the XenoLogiX treatment valves.

**Chemical Analysis Results**

HPLC analysis of the tissue samples demonstrated a 40% reduction in carboxylic acid levels found in the tissues prepared with the ThermaFix process (0.71± 0.26 nmoles/mg dry tissue) compared to glutaraldehyde fixation (1.16± 0.26 nmoles/mg dry tissue).

**Biochemical Analysis Results**

The results of moisture content, tissue shrinkage and ninhydrin levels are summarized in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>XenoLogiX Treatment</th>
<th>ThermaFix Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture Content (%)</td>
<td>78.57±1.02</td>
<td>76.56±1.07</td>
</tr>
<tr>
<td>Tissue Shrinkage (°C)</td>
<td>83.41±0.40</td>
<td>83.79±0.22</td>
</tr>
<tr>
<td>Ninhydrin Level</td>
<td>0.403±0.09</td>
<td>0.244±0.06</td>
</tr>
<tr>
<td></td>
<td>(moles NH₂/mole of collagen)</td>
<td></td>
</tr>
</tbody>
</table>

No statistically significant differences were demonstrated between pericardial tissues prepared with the ThermaFix process and pericardial tissues prepared with the XenoLogiX treatment in moisture content, shrinkage, and free amine levels.

**Histology Analysis Results**

Histological assessment demonstrated intact bundle morphology and a distinct collagen crimping patterns in pericardial tissue samples prepared with the XenoLogiX treatment and the ThermaFix process. Similar collagen bundle and crimping patterns were observed with H&E staining. The presence of elastin fibers was variable and multidirectional in both groups. These results show no appreciable differences in the microstructures of the pericardial tissue prepared with the XenoLogiX treatment or the ThermaFix process.
Biomechanical Tissue Analysis Results

Uniaxial Testing: Uniaxial tissue testing was performed using one-way stretch tests for yield stress and yield strain for tissue test groups prepared with XenoLogiX treatment and the ThermaFix process.

There was no statistical difference (p=0.643) in yield stress between the XenoLogiX treatment and ThermaFix process tissue test groups. There was no statistically significant difference (p=0.772) in yield strain between the XenoLogiX treatment and ThermaFix process tissue test groups. Uniaxial testing demonstrated that the heat treatment step of the ThermaFix process had no statistical impact on the uniaxial tension properties of pericardial tissue.

Table 2: Summary of Biaxial Testing Data for Pericardial Tissue

| λC (XenoLogiX) | 1.057±0.007 | 1.053±0.009 |
| λC (ThermaFix) | 1.057±0.007 | 1.053±0.009 |

Biaxial Testing: The results of Table 2 provide a summary of the biaxial data for the pericardial tissue test groups prepared with the XenoLogiX treatment and ThermaFix process.

Biaxial testing demonstrated that the heat treatment step of the ThermaFix process had no statistically significant impact on the biaxial tension properties of the pericardial tissue. Additionally, radial and circumferential stretches indicate that the pericardial tissues prepared with the ThermaFix process and XenoLogix treatment behave in a generally isotropic manner.

Flexure Testing: Flexure testing results are presented as the instantaneous effective modulus, or Ebar, for all test groups. Ebar provides a comparative measure of tissue flexural stiffness or tissue bending compliance. The data demonstrate no statistical difference (p=0.901) in the flexural properties between pericardial tissue prepared with the XenoLogiX treatment and pericardial tissue prepared with the ThermaFix process.

Functional Valve Analysis Results

Hydrodynamic performance was assessed by measuring pulsatile flow pressure gradient, pulsatile flow regurgitation, and steady flow pressure drop at high cardiac output.

Pulsatile Flow Pressure Gradient: Table 3 summarizes the pressure gradients and EOAs at a cardiac output of 5 liters/minute for 19mm, 25mm and 31mm pericardial tissue valves prepared using the XenoLogiX treatment and the ThermaFix process.

Table 3: Pulsatile Flow Pressure Drop (ΔP) and Effective Orifice Area (E.O.A.)

<table>
<thead>
<tr>
<th>Valve Size</th>
<th>Cardiac Output = 5L/min &amp; Heart Rate = 70 bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P (mm Hg)</td>
</tr>
<tr>
<td></td>
<td>XenoLogiX</td>
</tr>
<tr>
<td>19 mm</td>
<td>25.1</td>
</tr>
<tr>
<td>25 mm</td>
<td>-</td>
</tr>
<tr>
<td>31 mm</td>
<td>1.6</td>
</tr>
</tbody>
</table>

The in vitro results demonstrate that pericardial tissue valves prepared with the heat treatment step of the ThermaFix process performed in the same manner as pericardial valves prepared with the XenoLogiX treatment.

Pulsatile Flow Regurgitation: Table 4 summarizes the comparative data of closing volume and percent regurgitation measured in 19mm and 31mm pericardial tissue valves prepared using the XenoLogiX treatment and the ThermaFix process.

Table 4: Pulsatile Flow Measurement; Closing Volume and Percent Regurgitation

<table>
<thead>
<tr>
<th>Valve Size</th>
<th>Closing Volume (ml)</th>
<th>% Regurgitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XenoLogiX</td>
<td>ThermaFix</td>
</tr>
<tr>
<td>19 mm</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>25 mm</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>31 mm</td>
<td>2.6</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The percent regurgitation values presented in Table 4 represent the sum of the closing volume and any see page through the valve assembly.

The in vitro results demonstrate that pericardial tissue valves prepared using the ThermaFix process and XenoLogix treatment demonstrate no regurgitation through the valve.

Pressure Drop at High Cardiac Output: Table 5 provides a comparative assessment of steady flow pressure drops between valves prepared using the XenoLogiX treatment and the ThermaFix process.

Table 5: Steady Flow Pressure Drop at Different Cardiac Outputs (C.O.)

<table>
<thead>
<tr>
<th>Valve Size</th>
<th>C.O. = 5 L/min ΔP (mm Hg)</th>
<th>C.O. = 30 L/min ΔP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XenoLogiX</td>
<td>ThermaFix</td>
</tr>
<tr>
<td>19 mm</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>25 mm</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>31 mm</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The in vitro results obtained by measuring pressure drops at high cardiac outputs demonstrate that pericardial tissue valves prepared using the ThermaFix process consistently
Accelerated Wear Testing (AWT)

Pre-test evaluation of the control and test valves indicated that valve leakage performance was within accepted specification ranges for Carpentier-Edwards PERIMOUNT valves. Steady backflow leak testing under a hydrostatic pressure of 100 mmHg after 40M, 100M, 160M, and 200M cycles resulted in all valves exhibiting leakage levels within ISO 5840:2005 performance specifications. All valves were photographed and x-rayed after 200M cycles of AWT. Radiographic inspection confirmed the structural integrity of the valve wireforms. Additionally, no evidence of leaflet tissue tearing, abrasion, or abnormal wear was observed in the tested valves. All valves functioned competently at the end of 200M cycles of AWT.

The results of the accelerated wear testing on Carpentier-Edwards PERIMOUNT valves prepared with the heat treatment step of the ThermaFix process demonstrated that valve function was within performance specifications and no evidence of tissue degradation or abnormal wear was seen in the valve leaflets.

CONCLUSION

Phospholipids and residual glutaraldehyde-derived moieties have the potential to serve as binding sites for calcium in pericardial tissue. The ThermaFix process is a novel tissue preparation method that combines the elimination of unstable glutaraldehyde moieties with the extraction of phospholipids in pericardial tissue.

Data from multiple animal model studies show that the ThermaFix process is an effective method of extracting potential calcium binding sites in pericardial tissue; showing significant reduction in leaflet calcium content compared to Neutralogic fixation alone and to XenoLogix treated tissue. Pericardial tissue treated with the ThermaFix process maintains functional and structural integrity as assessed by a variety of biomechanical, histological, biomechanical and whole valve functional tests, indicating that the ThermaFix process has no adverse effects on tissue stability, tissue durability and long-term performance.

Specific findings:

1. The ThermaFix process demonstrated a statistically significant reduction in calcification in small animal models when compared to tissue prepared with the XenoLogix treatment.

2. Pericardial tissue prepared with the ThermaFix process had significantly reduced tissue acid levels indicating fewer potential binding sites for systemic calcium when compared to tissue prepared with the XenoLogix treatment.

3. Histological analysis demonstrated that the ThermaFix process maintains microstructural integrity of the collagen and elastin structures in pericardial tissue.

4. Functional analysis of whole valves prepared with the ThermaFix process confirmed valve function is maintained at ISO performance specifications.

REFERENCES:

10. Edwards internal study RD 127 and R&D 128.
12. Edwards internal study RD 795.
15. Edwards internal study RD 3878, Cardiovascular Implants – Cardiac Valve Prostheses, 4 ed.