Evaluation of Stentless Kangaroo Aortic Valves in the Mitral Position of Juvenile Sheep

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Bioprosthetic heart valve prostheses, manufactured from glutaraldehyde (GA)-preserved bovine or porcine valve tissue, are prone to calcification and structural degeneration (1,2) after implantation, and this ultimately affects the longevity of these bioprosthetic heart valves. The study aim was to assess the feasibility of a mitral model for stentless valves and to evaluate the calcification behavior of stentless glutaraldehyde-preserved kangaroo heart valves in the mitral position of a sheep model.

Methods: Medtronic Freestyle (n = 10) and kangaroo (n = 11) stentless aortic valves were implanted in the mitral position of juvenile sheep and retrieved after a maximum of 200 days. Retrieved stentless valves were examined for morphological changes and calcification of the valve tissue, using radiological screening, Von Kossa’s staining and atomic absorption spectrophotometry.

Results: Four sheep (40.0%) with Medtronic Freestyle and 10 sheep (90.9%) with kangaroo valves could be weaned from bypass and mechanical ventilation. Two animals (20.0%) with Medtronic Freestyle and six animals (54.5%) with kangaroo prostheses survived more than 30 days postoperatively. No significant difference (p >0.05) was seen between the calcification potential of Medtronic Freestyle valve leaflets (3.21 ± 1.67 µg/mg) after 93 days and kangaroo valve leaflets (2.39 ± 0.80 µg/mg) after 200 days.

Conclusion: The present results suggest that implantation of a stentless valve in the mitral position of sheep is possible, but technically difficult. The calcification potential of kangaroo valve tissue is comparable to that of Freestyle valve tissue in the mitral position of sheep.

Materials and methods

Valves

Freestyle stentless aortic valves (n = 10) which...
served as control valves (external diameter 21-27 mm) were obtained from Medtronic Inc. (Minnesota, USA).

Fresh Western Grey kangaroo (*Macropus fuliginosus*) hearts (n = 11) were supplied under law by members of the Professional Roo Shooters Association in Perth, Western Australia. Hearts were washed in 0.9% cold saline (4°C) and the aortic roots removed. After trimming to a complete conduit shape (total length 5 cm) and oversewing the coronary ostia, the valves (external diameter 18-24 mm) were fixed with 0.625% phosphate-buffered GA for seven days and stored at 4°C in the same solution until further use.

**Animals**

Surgical implantation was performed at the large animal facility of the University of Western Australia. The investigational protocol was approved by the Animal Ethics Committee of the University of Western Australia. All animals received humane care in compliance with the *Principles of Laboratory Care* (National Society for Medical Research) and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Twenty-one Dorset-Merino cross-bred sheep (*Ovis aries*), aged 3-4 months and body weight 20-26 kg, were used in the study.

**Surgical implantation**

A previously described method (14) was used for anesthesia and surgical preparation of animals for cardiopulmonary bypass.

The right femoral artery was cannulated for arterial inflow and venous return obtained by cannulation of the right atrial appendage. Cardiopulmonary bypass was initiated, the animal cooled to 28 ± 4°C, and the aorta cross-clamped. Cold, crystalloid cardioplegia (500 ml) was delivered into the aortic root for myocardial protection. The left atrial appendage was opened coronally across the dome and the edges retracted. The left ventricle was vented through the atrium across the mitral valve during infusion of the cardioplegic solution, and the thoracic cavity was flooded with CO2 until closure of the left atrial appendage. Animals were ventilated postoperatively and extubated within 2-3 h. All animals received broad-spectrum antibiotics, analgesia and subcutaneous heparin sulfate (2 × 1000 units/day) for three days postoperatively. All animals were maintained with standard husbandry techniques until death or sacrifice.

**Implantation techniques**

A modification of the ‘top hat’ procedure described by Ross and Kabbani (15) was used to insert the stentless bioprostheses in the mitral position. Briefly, a donut-shaped kangaroo pericardial skirt (diameter 5.0 cm) fixed in 0.625% GA was used instead of Dacron (Fig. 1) to support the prosthesis on top of the mitral annulus. The native mitral leaflets and chordae were excised, and the length of the conduits was reduced to 1.5 mm above the commissural height. The outflow orifice of the prosthesis was sewn to the mitral annulus with continuous 5-0 Prolene sutures. The pericardial skirt was trimmed, and the edge sutured to the floor and walls of the left atrium circumferentially, in between the pulmonary vein orifices and the mitral annulus (Fig. 2). A false floor was therefore created in the left atrium to allow blood exiting the pulmonary veins readily to enter the prosthetic inflow. This created a small dead space inside the left atrium between the skirt and the prosthesis (Fig. 3). This arrangement appeared to be satisfactory only with trimmed kangaroo prostheses (profile height 16 ± 1.3 mm). The higher profile nature of trimmed Freestyle prostheses (profile height 21 ± 1.5 mm) resulted in obstruction of the left atrium, unacceptable hemodynamics and failure (death from acute pulmonary edema) when this method was applied (n = 3). Several modifications were employed to overcome this problem:

Left atrial patches (with GA-preserved bovine pericardium) both dorsal and anterior were employed (n = 2) to enlarge the atrial cavity.

The pericardial skirt was dispensed with, and the exterior of the conduit covered with kangaroo pericardium in order to minimize wastage of intra-atrial space and allow the trimmed prostheses to stand ‘free’
in the atrial cavity (n = 1). The atrium was still not large enough to accept this configuration, which also resulted in pulmonary edema from pulmonary venous obstruction.

An attempt was made to lift the floor of the left atrium up to the inflow orifice of the prosthesis (n = 2). This was done by dividing the left atrial wall medial, anterior and laterally several millimeters from the atrioventricular groove, and extending the incision posteriorly on either side as far as the interatrial septum and the coronary sinus. This allowed the atrial wall to hinge upward and to be sutured anteriorly to the inflow orifice of the prosthesis, using GA-preserved kangaroo pericardium to fill in the gaps medially and laterally. The left atrial roof remained contractile, and appeared to be effective in pumping blood into the inflow orifice of the prosthesis. Nevertheless, the left atrial obstruction remained and death still resulted from acute pulmonary edema.

Finally, the inflow orifice of the prosthesis was angled toward the orifices of the pulmonary veins by excising one of the prosthetic aortic sinuses and sewing this defect and the remainder of the outflow orifice to the mitral annulus. This caused the prosthesis to ‘lean’ toward the pulmonary vein inflow (n = 2). The stiffness of the GA-treated tissues allowed good maintenance of shape. The partly circumferentially divided atrial wall was then sewn to the inflow orifice of the prosthesis with gaps filled with pericardium, as in modification 3. This appeared to reduce flow obstruction, but pulmonary edema developed and resulted in death within 24 h.

**Hemodynamic evaluation**

Postoperatively, valve function was examined early (4-6 weeks), at medium term (12-15 weeks) and late (28-30 weeks) using external transthoracic echocardiography (Hewlett Packard Sonos 1000).

Valve function was assessed in terms of peak and mean transvalvular gradients (expressed in mmHg) as well as leaflet motion and valve regurgitation. Leaflet motion was categorized as either normal or absent. Valve regurgitation was categorized as either absent, mild, moderate or severe.

**Explant analysis**

Valves were retrieved postoperatively by elective sacrifice of the sheep at 200 days. Early explants were recovered from animals that died unexpectedly. Necropsies were performed on all animals, and the target organs (brain, myocardium, lungs, liver, spleen, kidneys) were examined histologically at the end of the study.
X-radiographic examination

All explanted bioprostheses were subjected to X-radiographic examination, using a mammographic film under mammography conditions from the outflow aspect.

Histological examination

Representative tissue samples (leaflet and aortic wall) of unimplanted Freestyle and kangaroo stentless valves were fixed in 4% formaldehyde, processed and stained with hematoxylin and eosin for control microscopic evaluation.

Two tissue samples (the non-coronary valve leaflet and a mid-section of the supracommissural aortic wall) of each explanted valve were fixed in 4% formaldehyde and stained with Von Kossa to examine the presence and distribution of calcification in the valve tissue.

Extractable calcium content

Tissue samples (the left and right coronary valve leaflets and a mid-section of the supracommissural aortic wall) of the retrieved valves were dried in an oven at 70°C for 48 h. The calcium content of the dried samples was extracted into 50% nitric acid, and the extractable calcium content (expressed as µg calcium per mg tissue) was measured using atomic absorption spectrophotometry.

Statistical analysis

All numeric data were presented as mean ± SEM. The statistical analysis included a variance check (ANOVA), paired t-test and pairwise comparison of 95% confidence intervals. A p-value <0.05 was considered to be statistically significant.

Results

Hemodynamic evaluation

Transthoracic echocardiography results (Table I) revealed visible leaflet motion in the Freestyle valves (at 4 and 12 weeks) and kangaroo valves (at 4, 15 and 30 weeks). Transvalvular pressure gradients showed a significantly lower mean gradient (2.32 ± 0.58 mmHg) in kangaroo valves compared to the two Freestyle valves (4.0 ± 2.50 mmHg) at four weeks. Transvalvular pressure gradients in kangaroo valves remained low at 12-15 weeks (3.64 ± 0.92 mmHg) and at 28-30 weeks (4.20 ± 1.40 mmHg). The mean external diameters of the Freestyle and kangaroo valves were 23 mm and 20 mm, respectively.

Post-mortem observations

Post-mortem observations of early deaths (within 24 h) revealed severe pulmonary edema in the Freestyle group. Preoperative and postoperative hematological data were normal in both groups of animals. Histopathological findings showed no abnormalities in the respective target organ tissues in either group after a maximum survival of 93 days with the single Freestyle valve, and with all six of the kangaroo valves after 200 days.

Table I: Transthoracic echocardiography.

<table>
<thead>
<tr>
<th>Postoperative time/ Parameter</th>
<th>Freestyle (n = 10)</th>
<th>Kangaroo (n = 11)</th>
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<tbody>
<tr>
<td>4 weeks</td>
<td></td>
<td></td>
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<tr>
<td>Leaflet motion</td>
<td>Visible</td>
<td>Visible</td>
</tr>
<tr>
<td>Regurgitation</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Mean gradient (mmHg)</td>
<td>4.0 ± 2.5</td>
<td>2.32 ± 0.58</td>
</tr>
<tr>
<td>Peak gradient (mmHg)</td>
<td>6.2 ± 1.8</td>
<td>4.1 ± 1.22</td>
</tr>
<tr>
<td>12-15 weeks</td>
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<td></td>
</tr>
<tr>
<td>Leaflet motion</td>
<td>Visible</td>
<td>Visible</td>
</tr>
<tr>
<td>Regurgitation</td>
<td>Mild</td>
<td>Absent</td>
</tr>
<tr>
<td>Mean gradient (mmHg)</td>
<td>12.4*</td>
<td>3.64 ± 0.92</td>
</tr>
<tr>
<td>Peak gradient (mmHg)</td>
<td>16.6*</td>
<td>5.20 ± 1.22</td>
</tr>
<tr>
<td>28-30 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaflet motion</td>
<td>-</td>
<td>Visible</td>
</tr>
<tr>
<td>Regurgitation</td>
<td>-</td>
<td>Absent</td>
</tr>
<tr>
<td>Mean gradient (mmHg)</td>
<td>-</td>
<td>4.20 ± 1.40</td>
</tr>
<tr>
<td>Peak gradient (mmHg)</td>
<td>-</td>
<td>7.50 ± 2.30</td>
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</table>

*Only one Freestyle valve.
Gross examination
At 31 days, the explanted Freestyle valve revealed soft, flexible cusps without signs of visible macroscopic calcification or valvular abnormalities. At 93 days, the single explanted Freestyle valve (Fig. 4A) had a retracted leaflet which was partially covered with a fibrous sheath of tissue (pannus). Both of these Freestyle survivors died early in chronic pulmonary edema.

The electively explanted stentless kangaroo valves (n = 6) at 200 days revealed soft, flexible leaflets without signs of visible macroscopic calcification or valvular abnormalities (Fig. 4B). Both inner and outer aortic wall were fully covered by host pannus tissue, the cusps being spared.

The aortic wall portion of both the Freestyle valve and the kangaroo valves showed extensive calcification after 93 and 200 days, respectively.

X-radiographic examination
Radiographic examination showed moderate calcific deposits in the commissural areas and leaflets of the explanted Freestyle prosthesis (Fig. 5A) after 93 days. No visible calcification was seen in the leaflet areas of any of the explanted kangaroo valves (Fig. 5B) after 200 days. The aortic wall tissue of both valve groups showed signs of dense, extensive transmural calcification.

Explant analysis
Explant analysis (Table II) indicated that 20% of the animals with Freestyle valve implants and 54% with kangaroo valve implants survived the early perioperative period of 30 days. Surgical survival with Freestyle valves (n = 2) was achieved with enlargement of the left atrium (modification 1) and ‘lifting the left atrial floor’ (modification 3). Survival with kangaroo valves (n = 6) was achieved by creating a ‘false atrial floor’ with kangaroo pericardium to direct flow from the pulmonary veins toward the inflow of the prosthesis. This approach was not successful with the porcine prostheses.

Early deaths (n = 8) with Freestyle valves occurred within 12 h postoperatively, and were caused by respiratory insufficiency and uncontrollable surgical bleeding due to high left atrial pressure. Early deaths (n = 5) with kangaroo valves occurred within 12-18 h postoperatively, and were caused either by a suspected protamine reaction (n = 1) or by uncontrollable surgical bleeding (n = 2). Two animals died because of unex-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Freestyle valves</th>
<th>Kangaroo valves</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of implants</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Extubated</td>
<td>n = 4 (40.0)</td>
<td>n = 10 (90.9)</td>
</tr>
<tr>
<td>Survival (&gt;30 days)</td>
<td>n = 2 (20.0)</td>
<td>n = 6 (54.5)</td>
</tr>
<tr>
<td>Mortality (&lt;30 days)</td>
<td>n = 8 (80.0)</td>
<td>n = 5 (45.5)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
pected non-surgery-related events (one sheep was strangled in the postoperative pen during the night, and another died from accidental early extubation). No animals receiving kangaroo valves died with pulmonary edema.

**Histological findings**
Explanted leaflets of the Freestyle and kangaroo valves, when stained with Von Kossa, indicated no visible calcification after 93 days and 200 days, respectively. The aortic wall in both the explanted Freestyle valve (93 days) and kangaroo valves (200 days) showed extensive transmural calcification.

**Extractable calcium content**
The extractable calcium content of the Freestyle valve leaflets (Table III) was slightly higher (3.21 ± 1.67 µg/mg) after 93 days compared to kangaroo valve leaflets (2.39 ± 0.80 µg/mg) after 200 days. Aortic wall tissues of both the single Freestyle (127.5 µg/mg) and kangaroo (110.35 ± 15.67 µg/mg) valve explants (Table III) showed high levels of calcium. However, no statistical difference (p > 0.05; Table III) was seen between these two types of valve tissues, even though the in-vivo duration of the kangaroo valves was more than twice that of Freestyle valves leaflets.

**Discussion**
The clinical use of heart valve bioprostheses requires effective in-vitro and in-vivo testing prior to clinical implantation in humans. Hemodynamic performance, durability and calcification potential must meet international standards before clinical trials can commence (16).

The subcutaneous rat model (11) is only effective in monitoring the calcification behavior of valvular tissues. The juvenile sheep model is effective in measuring both calcification potential and hemodynamic performance of stented bioprostheses in the mitral position (14), which offers more rigorous pressure gradients. The testing of stentless aortic bioprostheses in the aortic position of juvenile sheep (12,13) is possible, but also technically difficult.

To date, bioprostheses have been mainly constructed

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Freestyle (n = 10)</th>
<th>Kangaroo (n = 11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaflet</td>
<td>3.21 ± 1.67</td>
<td>2.39 ± 0.80</td>
<td>0.851</td>
</tr>
<tr>
<td>Aortic wall</td>
<td>127.5</td>
<td>110.35 ± 15.67</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean ± SEM.

*Only one aortic wall sample available with single Freestyle valve.*

Figure 5: Representative radiographs of explanted valves demonstrating the presence of calcification in the leaflets of both (A) Freestyle (at 93 days) and the absence of calcification in the leaflets of (B) kangaroo stentless valves (at 200 days). Both valves demonstrate extensive wall calcification.
from porcine, bovine or human tissues. During the mid-1980s, Weinholdt et al. demonstrated the anatomical advantages of stented kangaroo aortic valves over stented porcine aortic valves during in-vitro accelerated fatigue testing (17), as well as in the tricuspid position of sheep (18). Studies conducted by the present authors’ group have shown that GA-preserved kangaroo valve leaflets have a significantly lower calcification potential in the rat model (19), as well as in the descending aortic position of juvenile sheep (20).

In the present study, the feasibility of testing stentless aortic bioprostheses in the mitral position of juvenile sheep was examined, and the calcification behavior of stentless GA-preserved kangaroo aortic valves in this high-pressure position quantified.

The reduced transvalvular pressure gradient of kangaroo valves compared to Freestyle valves is best explained in terms of the reduced profile and more flexible leaflets of kangaroo valves (17,19). These observations were confirmed by post-mortem findings after early deaths with Freestyle valves. Enlarging the atrial cavity by various plasty techniques, minimizing intra-atrial dead space, streamlining intra-atrial flow patterns by pericardial baffling and positioning, and maintaining effective atrial systole failed to overcome the physical disadvantages of the Freestyle porcine prosthesis.

The reduced qualitative and quantitative calcification of explanted Freestyle leaflets may be explained in terms of the antimineralization protection obtained by amino-oleic acid treatment, as well as the short period of exposure to the circulation. The reduced calcification of kangaroo leaflets after full-term exposure in the sheep model is in accordance with previous findings by Weinholdt et al. (18), as well as by the present authors’ group (19,20), which explained the low calcification potential in terms of unique leaflet morphology. Briefly, kangaroo valve leaflets have a low glycosaminoglycan content, almost no spongiosal layer, and more densely packed elastic fibers (tunica elastica) compared to porcine valve tissue.

The severely calcified aortic wall portion in both Freestyle and kangaroo valves can be related to the different calcification mechanisms of leaflet and wall tissue, respectively (21).

Study limitations

One limitation of the present study was the small number of survivors with Freestyle valves and the short postoperative exposure of these valves compared to the kangaroo valves, which complicated the statistical analysis. Identically positioned samples were taken from all explanted valves, and the non-coronary leaflets of the two Freestyle valves (surviving 31 and 93 days) were not macroscopically calcified. The fellow right and left leaflets of the one Freestyle valve (after 93 days) were substantially calcified.

In conclusion, testing stentless valves in the mitral position of juvenile sheep is possible with a low-profile prosthesis such as the kangaroo stentless valve. The results of the present study clearly demonstrated that stentless kangaroo valve tissue without antimineralization measures has a calcification potential comparable to that of stentless porcine valve tissue processed with anticalcification measures. The study results further demonstrated the superior performance and durability of kangaroo valves in a high-pressure environment.

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