

A New Model to Test the Calcification Characteristics of Bioprosthetic Heart Valves

Shigeyuki Ozaki, MD, PhD,¹ Paul Herijgers, MD, PhD,² and Willem Flameng, MD, PhD²

Background: Tissue degeneration and calcification are the two chief obstacles to the successful application of bioprosthetic heart valves. To enable the study of the durability of bioprosthetic heart valves and the efficacy of anti-calcification treatment, it has become necessary to develop animal models. The aim of this study is to validate a new model for implantation in the pulmonary position.

Methods: Three juvenile sheep underwent implantation of Carpentier-Edwards pericardial valves in the pulmonary position (experimental group). These three valves were compared with three Carpentier-Edwards pericardial valves in the aortic position in patients which had been explanted due to primary tissue failure (clinical group). The valves were analyzed.

Results: The findings of macroscopic, X-ray and light microscopic examination were very similar between the two groups. Scattered irregular calcification was seen near the commissures and at the base of the cusps in both groups. Quantitative calcium content analysis showed that calcification of the cusps had progressed to almost the same degree in both groups (experimental group, 3.7 ± 0.2 $\mu\text{g}/\text{mg}$ dry tissue; clinical group, 4.3 ± 0.3 ; $p > 0.05$). In the experimental group, calcification in the commissural area of the cusp was pronounced (6.5 ± 1.0). In the clinical group, calcification had also progressed in the commissural area of the cusp (6.0 ± 1.5), and extended to the base area of the cusp (6.6 ± 1.2).

Conclusions: This model is promising for preclinical evaluation of bioprosthetic heart valves. The degree of calcification is not significantly different between our experimental results after three months of implantation in sheep and clinical results after 10 years of implantation in elderly patients. However, the pattern of calcification is somewhat different between the two groups. (*Ann Thorac Cardiovasc Surg* 2004; 10: 23–8)

Key words: bioprostheses, large animal model, experimental, calcification

Introduction

Barnhart et al.¹⁾ demonstrated juvenile sheep to be a suitable experimental animal model for bioprosthetic valve implantation. In their study, a juvenile sheep is defined as younger than 5 months. They also showed the weight gain of the sheep. Juvenile sheep, aged 4 to 5 months, were used. They grow rapidly, increas-

ing in average weight from 29 kg at the time of valve implantation to 45 kg at the time of the study, four to seven months later. Their heart rate, cardiac output, and intracardiac pressures are essentially the same as those of healthy young humans. Also the size of their heart is suitable for implantation of bioprosthetic valves, and their vessels and body sizes are adequate for standard cardiopulmonary bypass techniques. They tolerate anesthesia and present no major difficulties in postoperative management. Most important, however, is that the bioprostheses implanted in these animals for several months show changes comparable to those that take several years to develop with bioprostheses implanted in humans.^{1,2)} This kind of accelerated degeneration clearly makes sheep very at-

From ¹Second Department of Surgery, National Defense Medical College, Tokorozawa, Japan, and ²University Hospital Leuven, Leuven, Belgium

Received February 26, 2003; accepted for publication July 7, 2003. Address reprint requests to Shigeyuki Ozaki, MD, PhD: Second Department of Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan.

tractive for studying long-term degenerative effects (the so called "chronic animal model").

Most frequently the implantation of bioprosthetic valves into the aorta,³⁾ left ventricle apico-aortic conduit,⁴⁾ mitral⁵⁾ or tricuspid position^{1,6)} of juvenile sheep is used to test the durability and calcification characteristics of bioprosthetic valves. Unfortunately, these models are expensive and complicated, because full extracorporeal circulation is necessary in most of them, or because serious blood loss occurs, resulting in a mortality rate of over 40%.⁴⁻⁶⁾ Therefore it is one of the goals of this study to develop an easier and less expensive model for in vivo valve testing.

Materials and Methods

All animals were cared for by a veterinarian in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication 85-23, revised 1985). The study was approved by the Ethics Committee of the Katholieke Universiteit Leuven. Juvenile sheep were bred specially for this study. The sheep weighed 51.4 ± 7.6 kg (mean \pm standard deviation; range, 40 to 63 kg) at implantation.

Valves studied

Carpentier-Edwards pericardial stented valves with well-known clinical behavior were selected for this study. Three Carpentier-Edwards pericardial valves were implanted in juvenile sheep in the pulmonary position (experimental group). These three valves were compared with three Carpentier-Edwards pericardial valves in the aortic position in patients, which were explanted because of primary tissue failure (clinical group). The mean patient age was 71 ± 3 years. The duration of implantation was 128 ± 15 months.

Implantation

The valves were implanted as described previously.^{7,8)} The animals were premedicated with ketamine (10-20 mg/kg intramuscularly). Anesthesia was induced and maintained with halothane and N₂O. Fentanyl (Janssen, Beerse, Belgium) was administered in boluses as necessary. After endotracheal intubation, mechanical ventilation was instituted. A left thoracotomy was performed through an incision at the second intercostal space. The main pulmonary artery was isolated. After administration of 3 mg/kg heparin (Rhone-Poulenc Rorer, Brussels, Belgium) intravenously, a pneumatic right ventricular assist sys-

tem (Medos-HIA VAD 54 ml ventricle, Medos-Helmholtz Institute, Aachen, Germany) was installed with the inflow cannula in the right atrium and the outflow cannula 1 cm before the pulmonary bifurcation. Proximal and distal clamping of the pulmonary artery were carried out. After longitudinal incision of the pulmonary artery, a stented valve was implanted into the pulmonary artery using continuous 4-0 prolene suture, 1 cm above the native pulmonary valve. After removal of the clamps, the native pulmonary valve was destroyed by tearing two cusps with a clamp introduced through a pursestring suture placed at the sinuses, and afterwards the Medos system was stopped. The chest was closed in layers with a chest drain in the left pleural space. After waking up, the animal was extubated and brought to the recovery room. Feeding was allowed immediately. Intravenous fluid administration was stopped after two hours. The chest drain was removed after six hours.

The animals received analgesics (piritramide, Dipidolor, Janssen, Beerse, Belgium) for the first two days on regular schemes and diuretics as necessary. Antibiotics (ampicillinum anhydricum, Albipen LA, Mycofarm, Mechelen, Belgium) and low molecular weight heparin (enoxaparine, 20 mg twice daily, Clexane, Rhone-Poulenc Rorer, Brussels, Belgium) were administered for six days. Afterwards, the sheep returned to the controlled animal facility where the general health of the sheep was checked daily.

Explantation and analysis

All valves were explanted after three months. Sheep were premedicated and anesthetized in the way described before. The left thoracotomy was reopened and the heart dissected free. The valve function was studied by echocardiography. Heparin 3 mg/kg was administered, and after exanguination, the valve was excised together with a proximal and distal part of the sheep pulmonary artery.

a) Macroscopical examination

Valves were grossly inspected and color pictures were taken. Afterwards the valve was longitudinally transected through the commissures. Each of the three specimen thus includes a pre- and postvalvular part of the sheep pulmonary artery, together with a part of the porcine aortic wall, and respectively a right coronary cusp (RCC), a non-coronary cusp (NCC), or a left coronary cusp (LCC). Color pictures were taken again.

b) X-ray assessment

X-ray examination (face, profile) was performed under mammography conditions to demonstrate and localize macroscopical calcifications.

c) Histology

For histology, a longitudinal transection of the specimen through the middle of the left coronary cusp was embedded in paraffin. Four μm thick sections were routinely stained with hematoxylin and eosin (HE), with Masson's trichrome stain for collagen, with von Giesson staining for elastin, with phosphotungstic-acid-hematoxylin (PTAH) for fibrin, and von Kossa staining for calcium.

d) Transmission electron microscopy

For transmission electron microscopy (TEM), the RCC was divided into a basal part, a middle part and a free edge of the valve. Also, a part of the aortic wall at the inflow and outflow side was taken. From each of these five parts, three to 10 samples (<1 mm in diameter) were embedded in Epon. One μm thick sections were stained with Toluidine blue and examined by light microscopy. From each of the three cusp fragments an area of the outflow side, the inflow side and the middle part was selected for TEM. By analogy, from the aortic inflow and outflow, also the intimal inner medial and outer medial wall and adventitia were thus sampled for TEM. Ultra-thin sections were cut, stained with uranylacetate and lead citrate. Sections were treated with 2% potassium pyroantimonate to demonstrate calcium. Grids were examined in a Philips CM10 electron microscope. Random photographs were taken.

e) Quantitative calcium determination

Half of every specimen (RCC, NCC, LCC) was used for quantitative calcium determination. The cusps were divided in three parts: the commissural area, basal part, and free edge. After lyophilization, the tissue was pulverized, and desiccated to constant weight in an oven. Pulverized tissue was diluted in 20% hydrochloric acid at a ratio of 10 mg dried tissue: 1 ml HCL. Calcium content was measured by flame atomic absorption spectrometry, and expressed as μg per mg of dry cuspal weight.

f) Data management and statistical analysis

Replicate data were calculated and expressed as mean \pm standard error of the mean (SEM). ANOVA was performed on calcium content data with valve type as independent factors and calcium content as dependent factor.

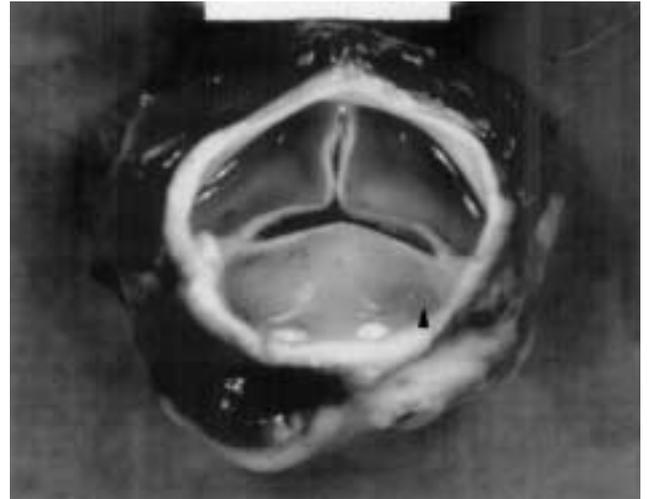


Fig. 1. A representative example of a valve explant in the experimental group. No overall calcification is seen. Slight fibrous sheathing is seen near the commissures and at the base of the cusps (arrowhead).

Posthoc testing with the Fisher-test was performed if ANOVA yielded $p < 0.05$.

Results

Survival rates

No sheep died during the observation period.

Examination of macroscopically identifiable features

The valves in the experimental group were satisfactorily pliable and showed no evidence of calcification or tearing (Fig. 1). However slight fibrous sheathing was seen near the commissures and at the base of the cusps. In the clinical group, some scattered calcification was seen near the commissures, but no fibrous reaction was seen comparable to that in the experimental group. One cusp was prolapsed towards the inflow side due to degenerative changes (Fig. 2).

X-ray examination

In the experimental group, scattered irregular calcification was seen near the commissures and at the base of the cusps (Fig. 3). In the clinical group, more extensive calcification was located near the commissures and at the base of the cusps (Fig. 4). The degree and pattern of calcification in the experimental group was very similar to those in the clinical group. However, the degree of calcification in the clinical group appeared to be more exten-



Fig. 2. A representative example of a valve explant in the clinical group. Some scattered calcification is seen near the commissures (arrow). In the clinical group, no fibrous reaction was seen comparable to that in the experimental group. One cusp was prolapsed towards the inflow side.

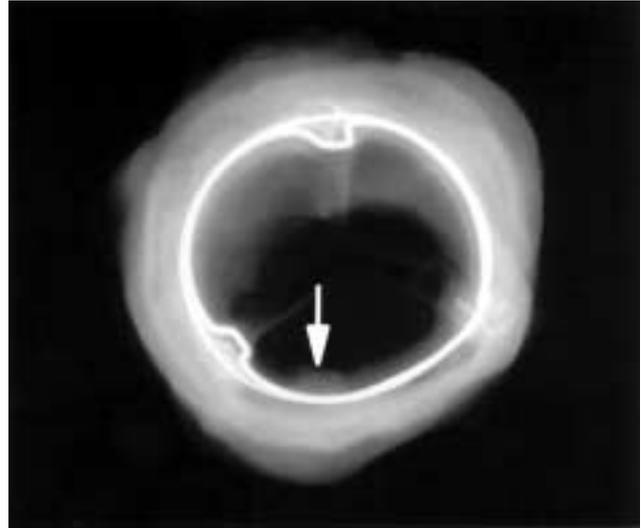


Fig. 3. A representative example of an X-ray taken from a valve in the experimental group. Scattered irregular calcification is seen near the commissures and at the base of the cusps (arrow).

sive than that in the experimental group.

Light microscopy

In the experimental group, nodule-type calcification was visible at the base of the cusps (Fig. 5). Thick fibrous sheathing was evident covering the inflow side of the cusp. In the clinical group, nodule-type calcification was also present at the base of the cusp (Fig. 6), closely resembling that in the experimental group. The degree and pattern of calcification in the experimental group were very similar to those in the clinical group. However, no fibrous reaction comparable to that seen in the experimental group was observed in the clinical group.

Calcium content

Quantitative calcium content analysis showed that calcification of the cusps had progressed to almost the same degree in both groups (experimental group, $3.7 \pm 0.2 \mu\text{g}/\text{mg}$ dry tissue; clinical group, 4.3 ± 0.3 ; $p > 0.05$) (Fig. 7).

Furthermore, calcium content was analyzed in the three subsegments of the cusps (commissure, basal part and free edge). In the experimental group, calcification in the commissural area of the cusp was pronounced (6.5 ± 1.0). In the clinical group, calcification also progressed in the commissural area of the cusp (6.0 ± 1.5), and extended to

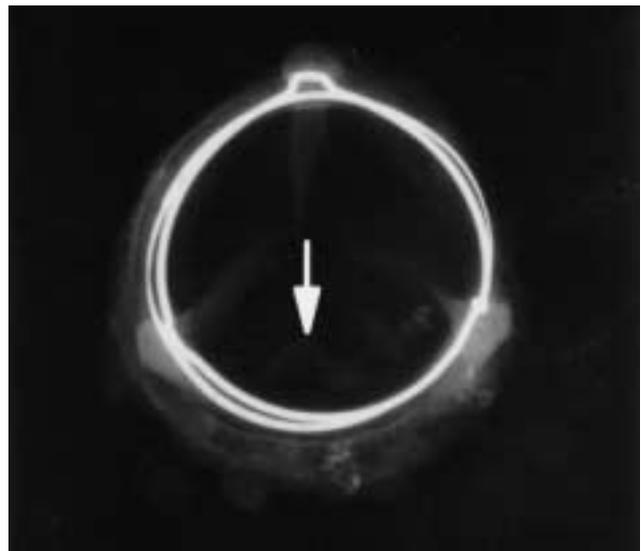


Fig. 4. A representative example of an X-ray taken from a valve in the clinical group. More extensive calcification is located near the commissures and at the base of the cusps (arrow).

the base area of the cusp (6.6 ± 1.2) (Fig. 8). However, there was no significant difference between the groups.

Discussion

The first aim of this work was to create an easier and less expensive model for studying bioprosthetic valves inside the circulatory system in juvenile sheep. This model was



Fig. 5. A representative light microscopic general view using the entire specimen of a valve in the experimental group is shown: von Kossa staining, magnification $\times 10$. The dark stained cusp (arrow) indicates nodule-type calcification.

designed to allow the scientific community to test more thoroughly new bioprostheses before the first clinical experiments, and thus avoiding potential clinical catastrophes.

The model presented here is clearly less expensive and easier to apply with lower mortality than the previously described models.^{1,4,9,10} This is probably caused by the less invasive procedure, which can be performed through a small thoracotomy of about 10 cm, with minimal blood loss, with minimal hemodilution due to the low priming volume of the right ventricular mechanical assist system, and which can be carried out while the lungs are being continuously ventilated and perfused.

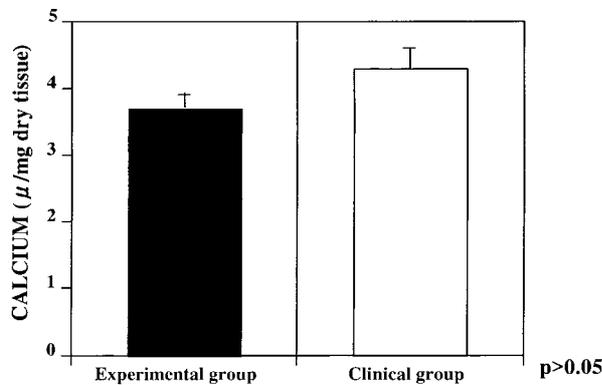


Fig. 7. Calcium content in the cusps is similar in both groups.



Fig. 6. A representative overview is shown of the appearance under light microscopy of a valve in the clinical group. Calcification is visible at the base of the cusp. A calcified nodule (arrow) is located (von Kossa staining, original magnification $\times 10$).

With this model, valves are implanted in a right-sided pulmonary position with significantly lower closing pressure and flow velocities over the tested valve than in the left-sided position. The frequency of valve opening is certainly equal and the range of excursion of the cusps is expected to be nearly the same. The different hemodynamic load might alter the rate and pattern of calcification and degeneration of the bioprostheses. If the results

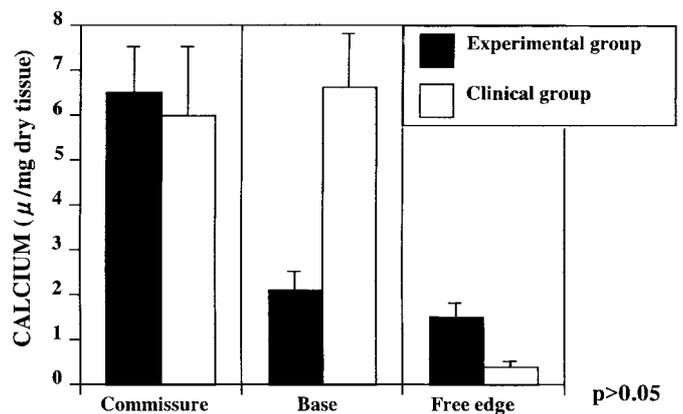


Fig. 8. Calcium content analysis of subsegments of the experimental and the clinical groups. In both groups, calcification is pronounced in the commissural area of the cusp. Furthermore, calcification also extended to the base area of the cusp in the clinical group. However, there was no significant difference between the groups.

of the rat subcutaneous model are looked at, calcification is induced without hemodynamic stress. In the light of this evidence, contact with calcium-containing fluid might trigger calcification and mechanical stress might induce further calcification. Thiene et al.,⁶⁾ however, were unable to find a significantly different rate of calcification when bioprostheses were implanted in the tricuspid or mitral position. Changes in bioprosthetic valves implanted in the tricuspid position in juvenile sheep are clinically and pathologically very similar to those that occur in human beings.

In our analysis, we were able to identify some differences between the experimental group and the clinical group. The calcium content was almost the same between groups, however, the pattern of calcification was different. In the experimental group, calcification was mainly located in the commissure area. In the other group, calcification was also pronounced in the commissure part, but extended to the basal area. This fact suggests that contact with blood may initiate calcification and mechanical stress might accelerate further calcification. Another difference is the fibrous overgrowth which was seen in our model, as well as in the tricuspid position,⁶⁾ which was absent in clinical cases.

We conclude that 1) this model is promising for pre-clinical evaluation of bioprosthetic heart valves; 2) the degree of calcification is not significantly different between our experimental results after three months of implantation in sheep and clinical results after 10 years of implantation in elderly patients; 3) however, the pattern of calcification is somewhat different between the groups, and fibrous overgrowth is only seen in the experimental group.

References

1. Barnhart GR, Jones M, Ishihara T, Rose DM, Chavez AM, Ferrans VJ. Degeneration and calcification of bioprosthetic cardiac valves. Bioprosthetic tricuspid valve implantation in sheep. *Am J Pathol* 1982; **106**: 136–9.
2. David TE, Ropchan GC, Butany JW. Aortic valve replacement with stentless porcine bioprostheses. *J Card Surg* 1988; **3**: 501–5.
3. Bortolotti U, Milano A, Tartarini G, et al. Hemodynamic performance of the Edwards Prima stentless valve. *J Heart Valve Dis* 1997; **6**: 134–7.
4. Gott JP, Girardot MN, Girardot JM, et al. Refinement of the alpha aminooleic acid bioprosthetic valve anticalcification technique. *Ann Thorac Surg* 1997; **64**: 50–8.
5. Tamura K, Jones M, Yamada I, Ferrans VJ. A comparison of failure modes of glutaraldehyde-treated versus antibiotic-preserved mitral valve allografts implanted in sheep. *J Thorac Cardiovasc Surg* 1995; **110**: 224–38.
6. Thiene G, Laborde F, Valente M, et al. Morphological survey of a new pericardial valve prosthesis (Pericarbon): long-term animal experimental model. *Eur J Cardiothorac Surg* 1989; **3**: 65–74. Erratum in: *Eur J Cardiothorac Surg* 1989; **3**: 281.
7. Herijgers P, Ozaki S, Verbeken E, et al. Calcification characteristics of porcine stentless valves in juvenile sheep. *Eur J Cardiothorac Surg* 1999; **15**: 134–42.
8. Ozaki S, Herijgers P, Verbeken E, et al. Are stentless aortic heterografts suitable for right ventricular outflow tract reconstruction? An experimental study in juvenile sheep. *Semin Thorac Cardiovasc Surg* 1999; **11** (Suppl): 176–82.
9. Ali ML, Kumar SP, Bjornstad K, Duran CM. The sheep as an animal model for heart valve research. *Cardiovasc Surg* 1996; **4**: 543–9.
10. Hazekamp MG, Goffin YA, Huysmans HA. The value of the stentless biovalve prosthesis. An experimental study. *Eur J Cardiothorac Surg* 1993; **7**: 514–9.