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# Results of a Decellularized Porcine Heart Valve Implanted into the Juvenile Sheep Model

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## ABSTRACT

**Objective:** This study was performed to evaluate the possibility of creating a glutaraldehyde-free porcine xenograft to improve long-term durability.

**Methods:** A decellularized porcine pulmonary valve was implanted into the right ventricular outflow tract of 7 juvenile sheep. Valves were explanted after 3 months (n = 4) and 6 months (n = 3). Evaluation was performed by gross examination, radiography, histology (hematoxylin-eosin and Sirius red staining), and immunohistochemistry. Quantitative determination of calcium content was investigated by atomic absorption spectrometry.

**Results:** All animals showed fast recovery without complications. At explantation, all decellularized valves showed smooth and pliable leaflets without evidence of thrombosis. The valve wall was also smooth and pliable without hardness. Light microscopy showed a monolayer of host endothelial cells covering the inner surface of the heart valves and repopulation of host fibroblasts into the deeper layers. Sirius red staining enabled visualization of the production of new collagen. Radiographic results showed an absence of calcification, confirmed by the low calcium levels (1.08 ± 0.28 μg/g and 0.73 ± 0.31 μg/g at 3 and 6 months, respectively) revealed by atomic absorption spectrometry.

**Conclusions:** The results with the juvenile sheep model showed that decellularized heart valves are recellularized in vivo. Host endothelial cells form a monolayer on the inner surface of the valve matrix. Furthermore, host fibroblasts repopulate the valve matrix and produce collagen; thus, a remodeling potential can be expected.

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## INTRODUCTION

Viable heart valves have the potential for growth, remodeling, and regeneration, which will influence their long-term durability [Chambers 1997, Schoof 2000]. The currently available bioprostheses are nonviable heart valves. For many years, allografts had been thought to be viable; however, a histologic evaluation showed the absence of endothelial cells and only a few fibroblasts after years of implantation [Gall 1998]. The highly immunogenic character of endothelial cells could contribute to the structural degeneration of allografts [Mitchell 1998, O'Brien 1999]. Xenogeneic heart valves are normally cross-linked with glutaraldehyde to mask xenoantigens, a process that will prevent rejection and stabilize the connective tissue matrix to resist degeneration [Allen 1984, Elkins 2001a]. On the other hand, glutaraldehyde is toxic and prevents the repopulation of a heart valve. Avoiding treating a xenograft with glutaraldehyde could lead to a matrix with remodeling potential and long-term durability without tissue degeneration and calcification. The aim of this study was to investigate the in vivo behavior of xenogeneic pulmonary heart valves not treated with glutaraldehyde.

## MATERIALS AND METHODS

All experiments were performed in accordance with the *Principles of Laboratory Animal Care* prepared by the National Society of Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health [NIH 1985]. The study was approved by the ethics committee of the Pontificia Universidade Catolica do Parana.

### *Surgical Technique*

Implantation was performed as previously described [Dohmen 2003c]. In brief, a left mini-thoracotomy was performed at the third intercostal space. The pericardium was incised vertically to expose the heart. The main pulmonary artery was isolated, and the descending aorta was dissected. A 21F aortic cannula was inserted for arterial perfusion. The right atrium was cannulated for the venous return with a 34F cannula. Cardiopulmonary bypass was established with a

Biomédica ECO-01 (Braile Biomédica, Sao Jose do Rio Preto, Brazil) to an adjusted mean systemic blood pressure of 50 mm Hg. The native pulmonary artery was divided, and the native pulmonary valve leaflets were excised. The decellularized porcine valve was implanted as an interposition by using 2 running 5-0 polypropylene suture lines. Cardiopulmonary bypass was discontinued after the heart was contracting well. The mini-thoracotomy was closed in layers, and a chest drain was inserted.

### *Explantation*

Animals were observed carefully for external abnormalities, including palpable masses. The skin scar of each animal was also evaluated. The decellularized valves were explanted at 3 months (n = 4) and 6 months (n = 3). After 3 mg/kg heparin was administered intravenously, a high dose of potassium was infused, and the heart and lungs were removed en bloc.

### *Analysis*

**Gross Examination.** The explanted decellularized valves were inspected, and color photographs were taken before or after fixation. Leaflets were especially inspected for fenestrations, retraction, thrombotic material, and atheroma or calcification. Each valve was longitudinally transected through the commissure, and the transection included a short segment of the sheep's native pulmonary artery at both ends.

**Radiography.** Face radiographic examination was performed under mammographic conditions to localize calcifications.

**Atomic Absorption Spectrometry.** Atomic absorption spectrophotometry was used to quantify calcium depositions, and the data were expressed as micrograms per gram dry tissue weight. For this investigation, a piece of each decellularized heart valve was divided into 2 parts, the valve cusp and the conduit wall. The tissue was prepared in 25% nitric acid for 4 hours at 70°C and immersed afterwards in 0.2 N HCl.

**Histologic Evaluation.** The specimens were examined with light microscopy and immunohistochemistry.

*Light Microscopy.* Specimens taken from the leaflet of each valve included a piece of the native pulmonary artery at both sides. Afterwards, these specimens were embedded in paraffin. Longitudinal sections 4 μm thick were stained routinely with hematoxylin-eosin and Sirius red.

*Immunohistochemistry.* Immunohistochemical staining was performed with factor VIII-related antigen (DakoCytomation, Hamburg, Germany). Several regions were investigated because the shear stresses at the leaflets were different from the rest of the valve. Representative samples of each region, including the inflow part and outflow part of the valve wall, were used as controls.

## RESULTS

All 7 sheep recovered soon after surgery without showing any complications during the follow-up period.

### *Gross Examination*

The decellularized heart valves showed no hematomas, vegetations, or thrombotic material after 3 and 6 months of implantation. The leaflets showed no tears, perforations, cusp deformations, retractions, or hardness. There was no evidence of embolism in the lungs of any of the juvenile sheep. The valves in toto gave the impression of extreme pliability, comparable with that of the preimplantation condition, without any loss in strength. During explantation, only minimal evidence of inflammatory reaction was observed. The differentiation of structures in the chest was extremely easy, even after 3 months of implantation (Figure 1).

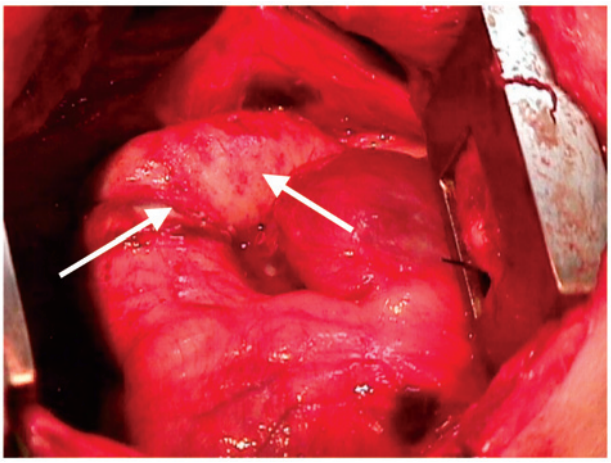

Figure 1. A decellularized heart valve 3 months after implantation into the right ventricular outflow tract of juvenile sheep. Notice the absence of extensive adhesions and inflammatory reactions at this time.

### *Radiographic Results*

No calcification was seen in the radiographs of any of the decellularized heart valves after 3 and 6 months of implantation (Figure 2).

### *Atomic Absorption Spectrometry*

The different explanted valve specimens showed similar calcium content values. The mean (±SD) calcium content for the decellularized valves in toto was 1.08 ± 0.28 μg/g after 3 months of implantation and 0.73 ± 0.31 μg/g at 6 months. After the wall was separated from the cusp, the mean calcium content of the leaflets was 1.13 ± 0.33 μg/g at 3 months and 0.99 ± 0.05 μg/g at 6 months. The mean calcium content of the valve wall was 1.02 ± 0.34 μg/g at 3 months and 0.48 ± 0.13 μg/g at 6 months.

### *Histology*

**Light Microscopy.** There was no fibrous tissue overgrowth of the wall or at the leaflets in any of the examined heart valves at both 3 and 6 months. Only minimal inflammatory reaction was observed on the outside. On the luminal side of the valve matrix, autologous endothelial cells were visible after 3 months. The ingrowth of the endothelial cells started at both anastomoses. From there, endothelial cells grew to the middle of the leaflet by 3 months after implantation (Figure 3). The invasion of fibroblasts was similar to that of endothelial cells, although a delay was seen because fibroblasts did not migrate in the tissue as far as the endothelial cells during the same period. At 6 months of implantation, we

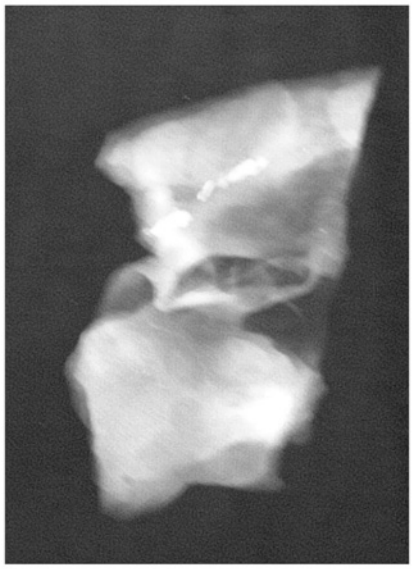

Figure 2. Radiograph of an explanted heart valve after 6 months. No calcifications can be visualized in the leaflets and valve wall.

observed a confluent monolayer of endothelial cells that reached the free edge of the heart valve cusps (Figure 4).

The ingrowth of fibroblasts also increased during this period. After 6 months of implantation, the fibroblasts had almost completely invaded the leaflets. Host fibroblasts, however, had not yet reached the free edge of the leaflets by this time.

Staining with Sirius red confirmed the production of collagen within the matrix. The intensity of the production of new collagen in the valve wall was similar to that observed in the valve leaflets (Figure 5). The production of collagen seemed to be influenced by the availability of endothelial cells. At 6 months, the production intensity of new collagen had increased; however, 6 months was too early to see new collagen at the whole valve.

**Immunohistochemistry.** Immunohistochemical staining of the cells covering the luminal side of the valve was positive for factor VIII, showing that they constituted a monolayer of endothelial cells (Figure 6).

## DISCUSSION

Cryopreserved allografts are the most favorable bioprosthesis because long-term follow-up has shown excellent hemodynamic results [O'Brien 2001]; however, structural deterioration of the tissue has been shown [Dohmen 2003a]. The mechanism of cryopreserved allograft degeneration is controversial, but several studies have strongly suggested an immunogenic humoral response [Rajani 1998, Shaddy 2002]. Dignan et al [2003] showed that elevation in class II human leukocyte antigen (HLA) levels was responsible for the deterioration in valve structure, which was recognized by hemodynamic changes during echocardiographic evaluation. Furthermore, this study showed that elevation in class I HLA levels did not affect allograft degeneration.

Hawkins et al [2000] showed that tissue decellularization was able to decrease the levels of class I and class II HLA antibodies in the valves. Postoperative antibody levels were a maximum of 10% higher than preoperative levels. Cryopreserved allografts used to reconstruct the right ventricular outflow tract normally show increases in the levels of class I and class II HLA antibodies to a maximum of 81% and 59%, respectively. The effect of the increased HLA level was a statistically nonsignificant increase in the average peak gradient at the decellularized heart valve (13 ± 15 mm Hg) and at the cryopreserved allograft (24 ± 18 mm Hg).

Bechtel et al [2003] were not able to show any short-term benefit of valve decellularization on hemodynamic parameters because the flow velocity seen with the SynerGraft (Cryolife, Kennesaw, GA, USA) was similar to that of cryopreserved allografts. These workers also observed a decrease in the levels of HLA antibodies in their patients.

In our study, we completely decellularized a pulmonary heart valve without interfering with the integrity of the

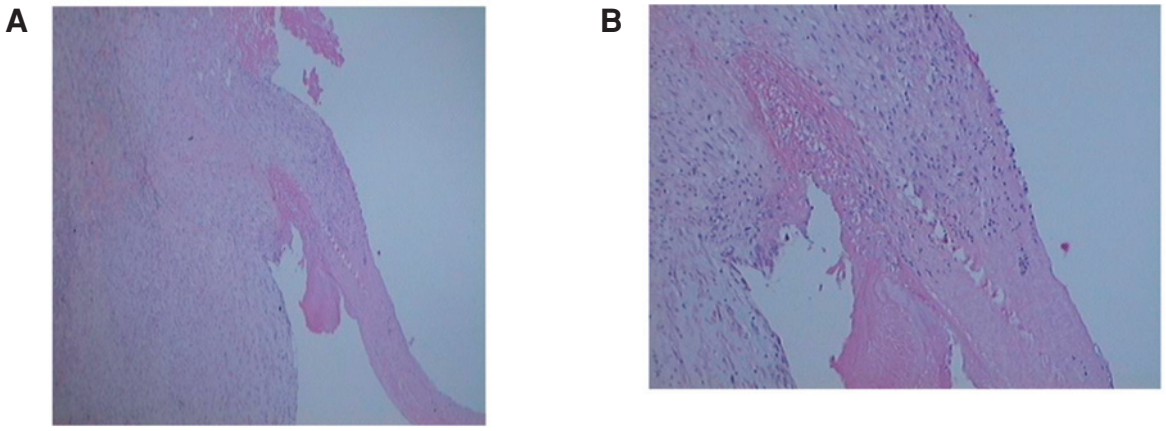


Figure 3. A sample of a decellularized heart valve at 3 months showing recellularization of the valve. Not only are fibroblasts growing into the matrix but endothelial cells are also overgrowing the inner surface of the matrix. A, Overview of the recellularized valve. B, A close-up of the invasion of host interstitial cells into the decellularized valve matrix.

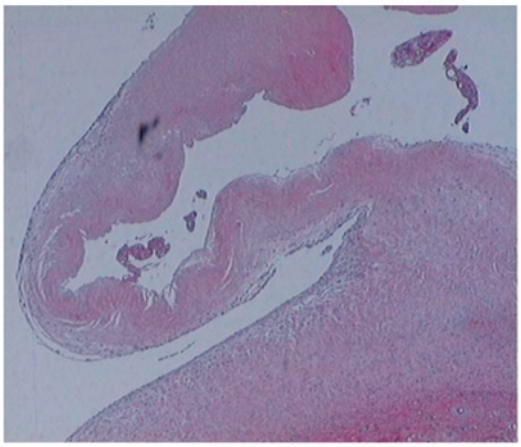

Figure 4. A sample at 6 months of implantation shows a progression of valve infiltration by host fibroblasts. Endothelial cells showed an overgrowth on the matrix as well as on the cusps at both sides.

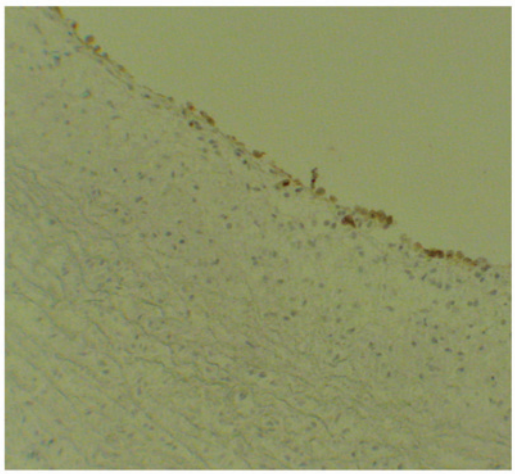

Figure 6. Factor VIII staining confirms that the monolayer on the inner surface consists of endothelial cells.

matrix. This method was thought to overcome humoral and cellular rejection (C. von Glehn, personal communication, 2004) and improve the long-term function of the valve [Dohmen 2003b, da Costa 2004].

In the juvenile sheep model, any tissue reactions would have been seen very early; however, we were unable to see any calcification or structural deterioration in these heart valves. At explantation, only minimal inflammatory reaction could be seen, and so tissue identification was easy. Decellularization creates the potential for tissue repopulation.

Several articles have been published about the decellularization of heart valves and the in vivo recellularization of these heart valves [Elkins 2001b, Leyh 2003]; however, the mechanism of recellularization is not really known. Elkins et al [2001b] have described several stages of repopulation in a decellularized heart valve.

We believe that this study makes possible a description of the repopulation pattern of decellularized heart valves, and our explanation has been confirmed by light microscopical evaluation. First of all, the endothelial cells start growing from the distal and proximal anastomoses onto the decellularized valve matrix. The process appears to take 3 months until the base of the leaflets are totally covered by endothelial cells. Furthermore, islands of endothelial cells sometimes are seen at the more distal parts of the leaflets. This observation can be explained by circulating endothelial cells that are able to bind to the valve matrix. Similarly, interstitial cells such as fibroblasts start invading the decellularized heart valve from the native pulmonary wall as well as from the right ventricle. This process, which also progresses with time, seems to require approximately 6 months, until all layers of the valve wall are finally infiltrated. This study indicates that the overgrowth of the inner surface of the heart valve is apparently completed earlier than the infiltration of the wall by fibroblasts.

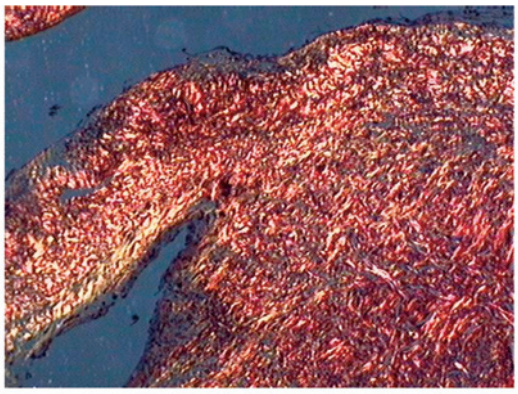

Figure 5. Sirius red staining shows new collagen in the wall as well as in the leaflets of the decellularized heart valve.

In addition, the availability of endothelial cells appears to be of great importance to the production of collagen by fibroblasts. Endothelial cells have been shown to have a nourishing effect on fibroblasts. Zilla et al [1993] described the importance of the endothelium as well as the effects of endothelial cells on fibroblasts; however, the complete mechanisms are not yet known.

In this study, we were also able to confirm the findings of other groups that the seeding of decellularized heart valves in vitro to overcome platelet and fibrin deposition on the collagen surface does not appear to be necessary. Although we know that endothelial cells will increase the patency rate in small-diameter vessels [Konertz 2001], there seems to be no need to seed heart valves with endothelial cells to overcome thromboembolic complications.

In conclusion, decellularized heart valves can apparently be implanted safely without thromboembolic risks. In the juvenile sheep model, decellularized heart valves appear be recellularized in vivo. Furthermore, valves repopulated with host fibroblasts are able to produce collagen, and these cells may regenerate the valve matrix.

## ACKNOWLEDGMENT

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## REVIEW AND COMMENTARY

***Editorial Reviewer MB134 writes:***

a) Has any attempt been made to implant the decellularized valve in the aortic position or in a position such as the one Hufnagel used (heterotopic aortic) to determine if the rate of calcification or host endothelial covering is different under systemic arterial pressure?

b) This is not a cross-species model. Clinical bioprosthetic devices are xenografts, whereas this experiment is an allograft (same species). How does that affect the presence or absence of a humoral or cellular rejection mechanism impairing repopulation of endothelial and matrix cells in a potential human application?

***Authors' Response by Dr. Pascal M. Dohmen:***

a) I fully agree with the reviewer that there could be a difference in calcification between the left and right sides. Thiene et al (Eur J Cardiothorac Surg 1989;3:65-74) has shown in the juvenile sheep model that if a pericardial valve has been implanted into the mitral or tricuspid position there will be no difference in calcification intensity. Therefore, many groups use the right-side model (Duran, Flameng, Elkins, Haverich, and so on).

Of course, it is important to know not only the rate of calcification but also the hemodynamic behavior of the valve, which is different on the left and right sides. The use of left-side implantation is more demanding and more expensive. Therefore, many groups have used the right side.

Our reason for using the right side in the juvenile sheep model was that this valve was designed to reconstruct the right ventricular outflow tract. This type of valve was not developed to be implanted into the left side, replacing the aortic valve.

During a more recent study, we implanted a modified decellularized valve into the aortic position. We were able to explant these heart valves after 3 months and saw a similar behavior in this study (after small modification, however) compared with the right-side model. During these implantations, we used the orthotopic implantation after we had experienced some problems in previous work with the Hufnagel implantation technique (paralysis, smaller-diameter valve implantation, and so forth).

b) In responding to this question, I have to apologize to the reader because we seem not to have been clear enough that the implanted heart valves were xenografts. We implanted decellularized porcine (pig) pulmonary valves into the juvenile sheep, a model we believe is a cross-species model. We did not use decellularized ovine (sheep) valves during these experiments.