Trileaflet aortic valve reconstruction with a decellularized pericardial patch in a sheep model

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ABSTRACT

Background: The purpose of this study was to provide a preliminary assessment of the performance of a decellularized pericardial patch in a trileaflet aortic valve reconstruction in a long-term juvenile sheep model.

Methods: A sheep surgical model was used to perform a complete trileaflet reconstruction (Ozaki technique) of the aortic valve with 3 separate pericardial patches. Valve function was assessed 1 week, 3 months, and 6 months after surgery via transthoracic echocardiography. Calcification resistance and host cell infiltration of the pericardial material were assessed at 6 months after surgery.

Results: Three of 6 sheep with implanted pericardial neo-cusps survived until the planned time of sacrifice after surgery, whereas 3 animals had a successful implant but died shortly after the procedure as the result of a bad recovery from cardiopulmonary bypass. Echocardiography at 6 months revealed a high coaptation area with only minimal regurgitation. In all explanted leaflets, cusp tissue was soft. There was only minimal calcification in 8 of 9 leaflets.

Conclusions: Aortic valves reconstructed with a decellularized pericardial patch demonstrated adequate diastolic function with minimal regurgitation and resistance to calcification. Combining the Ozaki technique with this decellularized pericardial scaffold showed adequate hemodynamics, sustained mechanical integrity of the patch and limited calcification of the material. These results, together with earlier experimental and clinical data, indicate the potential of this material for aortic valve repair. (*J Thorac Cardiovasc Surg* 2016;152:1167-74)

Explanted CardioCel neo-cusps after 7 months in aortic position.

Central Message

A trileaflet aortic valve reconstruction in a pericardial scaffold in sheep revealed excellent results. Together with the existing clinical experience, a multicenter clinical trial will start.

Perspective

Aortic valves reconstructed with CardioCel demonstrated excellent function and very low calcification. The results support application of this pericardial scaffold in trileaflet aortic valve reconstruction. The unique combination of optimal hemodynamics of the reconstructed aortic valve, together with the decellularized character of the pericardial material, can offer benefits towards durability.

See Editorial Commentary page 1175.

Aortic valvular surgery is the most common valvular surgical intervention performed.¹ With the ideal replacement tissue yet to be found, several options, each with

their own advantages and disadvantages, are available to candidates for aortic valve repair. Prosthetic valves have been developed with xenograft tissues as leaflets that are surgically implantable and that also are deliverable via catheter. Although these technologies have been an improvement on previous technologies, the prosthetic valves still do not enable full motion of the aortic valve annulus and root and lead to suboptimal hemodynamics.

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Abbreviation and Acronym

TTE = transthoracic echocardiography

The development of stentless valves has alleviated this problem somewhat, but still a great increase in durability has not been reached. Other techniques seek to replace the leaflets all together with autologous pericardium and thus provide superior hemodynamics. In all these instances, limitations still exist in the tissue used to reconstruct the valve leaflets. Autologous pericardium is free from donor-derived pathogens and antigens but requires fixation with glutaraldehyde to avoid resorption and thickening and to improve durability.^{2,3} Homologous pericardium also requires fixation to overcome retraction and thickening.^{3,4} Xenogeneic pericardium, conventionally fixed with glutaraldehyde for sterilization and to reduce antigenicity, can be made readily available in unlimited supply but is prone to calcification leading to valve deterioration.^{5,6} For younger adults and pediatric patients, the inability of prosthetic valves and tissue heart valve substitutes to remodel and grow with the heart is a serious limitation.⁷ In addition, glutaraldehyde fixation presents problems with cytotoxicity.^{8,9} Thus, the search for a biocompatible material that provides patients with a lifelong solution is still ongoing.¹⁰

CardioCel (Admedus, Perth, Western Australia) is a pericardial scaffold manufactured from bovine spongiform encephalopathy-free pericardium. The manufacturing consists of several processes, which include steps to remove lipids, cells and cell remnants, nucleic acids (DNA, RNA) and α -Gal epitopes, resulting in a completely decellularized and α -Gal-free pericardial scaffold. In addition, cross-linking is achieved with a low and monomeric glutaraldehyde concentration to minimize glutaraldehyde cytotoxicity levels and to prevent the formation of long chains of polymerized glutaraldehyde. Cytotoxicity is further reduced by a proprietary anticalcification process and a nonglutaraldehyde sterilization and storage solution.

Improved biostability and durability and reduced cytotoxicity and calcification potential were demonstrated compared with autologous pericardium.¹⁰⁻¹⁴ This pericardial scaffold has demonstrated comparable strength and durability to conventional fixed bovine pericardium, with less potential for calcification and lower capacity to evoke an immune response in a rat subcutaneous model.¹³ A comparison of the performance of CardioCel versus conventionally fixed autologous pericardium in a juvenile sheep model of mitral and pulmonary valve repair showed that the mechanical properties of CardioCel were preserved at 8 months follow-up, with evidence of a more controlled healing process and a resistance to calcification.¹⁴

CardioCel has shown adaptive growth potential in pre-clinical models. Host cell infiltration of the scaffold has been demonstrated in a rat subcutaneous model and in jugular vein implants in juvenile sheep.^{11,12} Development of neocapillaries within the scaffold also has been observed in a rat subcutaneous model.¹¹

Congenital cardiac defects including valve reconstruction have been corrected in pediatric and adult patients with this material.¹⁵⁻¹⁷ The scaffold performed well at 6-48 months' follow-up, with no signs of device calcification, infection, thromboembolic events, or device failure based on echocardiographic and magnetic resonance imaging data.¹⁵⁻¹⁷ Although the longest-term follow-up data are now out to 7 years and longer-term data still are required, clinical experience with CardioCel thus far is promising.

Previously, the pulmonary and mitral valves of juvenile sheep have been repaired successfully with CardioCel, but the performance of this material in the aortic valve position has not been studied in sheep. The aim of this study was to develop a juvenile sheep model whereby the performance of this scaffold could be evaluated in trileaflet repair of the aortic valve. The handling properties, valve function, calcification, and recellularization potential of aortic valve cusps reconstructed with CardioCel are described.

MATERIALS AND METHODS**Animals**

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 Institutes of Health (NIH publication 85-23, revised 1985). The study was approved by the Ethics Committee of the Katholieke Universiteit Leuven. Six female juvenile sheep, between 6 and 8 months of age and weighing between 37 and 45 kg, were obtained from the Zoötechnische Center of the Katholieke Universiteit Leuven and were quarantined at the animal facility before undergoing surgery.

Patch Description

An aortic valve reconstruction was performed with CardioCel pericardial patches.¹³⁻¹⁵ The manufacturing of this patch involves several processes: complete decellularization and removal of the α -Gal epitope is performed, cross-linking is achieved with a low monomeric glutaraldehyde concentration, and cytotoxicity is further reduced by a proprietary anticalcification process.¹⁰⁻¹⁴

Surgical Procedures

The animals were operated on under general anesthesia. After premedication with ketamine (10-20 mg/kg body weight, intramuscular), anesthesia was induced with increasing concentrations of isoflurane (2.5%; Isoba, Schering-Plough Animal Health, Middlesex, United Kingdom) in oxygen and was maintained with halothane and N₂O. After endotracheal intubation, mechanical ventilation (11-13/min; tidal volume 0.7 times body weight; positive end-expiratory pressure at 4 cm water after chest opening) was started. A large bore oro-gastric tube was placed in the rumen and allowed to drain by gravity to prevent rumenal distention. Electrocardiogram limb leads were

connected and monitored. A maintenance intravenous drip of Ringer solution was started, and gentamicin and penicillin were administered.

The animal was placed on the operating table in a right lateral recumbent position and surgically scrubbed and draped to expose the distal left cervical region. A 20GA arterial pressure monitoring line was placed in the right central ear artery. The left carotid artery and the jugular vein were exposed and freed from surrounding tissue before a left thoracotomy was performed in the third intercostal space. The ribs were retracted for adequate exposure of the heart and the pericardium was incised in a T-shaped fashion anterior of the phrenic nerve.

The heart was suspended in a pericardial cradle by suspension of the pericardial flaps. After administration of heparin (3 mg/kg), the carotid artery and the jugular vein were cannulated with an arterial 16-Fr cannula (DLP, Medtronic Inc, Minn) and a 21- to 23-Fr venous cannula (HLS, Maquet Inc, NJ) and connected to the heart–lung machine. Extracorporeal circulation was started and maintained at an adequate flow rate without cooling. A left ventricular vent was inserted through the left atrial appendage. The aorta was then cross-clamped and a single-shot of cold, crystalloid cardioplegia was administered through an aortic root needle. The native aortic valve leaflets were excised. Sizing and preparation of the new aortic valve leaflets with the CardioCel patch was done according to the technique described by Ozaki and colleagues.^{18,19} All neo-cusps were sutured with a continuous running Prolene 4/0 suture, with additional reinforcements at the commissures. After complete reconstruction of the valve, the aorta was closed, the cross-clamp opened, and the heart defibrillated if needed. The heart–lung machine was stopped when hemodynamics were stable. The cannulas were then removed and the vessels ligated. Careful hemostasis was performed while we closed the wound in the neck. The chest was closed in layers, and a chest drain placed in the left pleural space. Immediate echocardiographic control was performed with sterile epi-aortic echocardiography. The animal was weaned from the ventilator as soon as there was spontaneous respiration with adequate tidal volumes and stable hemodynamics. In Video 1, we illustrate the technical details of the surgical procedure in a clinical case. Informed consent was obtained from the patient to share these images.

Postoperative Care and Echocardiography

After 1 week, transthoracic echocardiography (TTE) was performed with the sheep under mild sedation. The general health of the animals

VIDEO 1. Clinical video illustrating a trileaflet aortic valve reconstruction in a patient requiring aortic valve replacement. The compiled narrated video fragment nicely illustrates the sizing tools, the Ozaki template for the construction of the 3 pericardial cusps, the use of CardioCel as source material for the valve reconstruction, the additional stitch used for the construction of the commissures, and the final result on transesophageal echocardiography. Video available at [http://www.jtcvsonline.org/article/S0022-5223\(16\)30462-7/addons](http://www.jtcvsonline.org/article/S0022-5223(16)30462-7/addons).

VIDEO 2. Gathered echo samples from the animals that died short after the procedure. Valve implantation as such was successful in all animals, with good valve opening and no leakage. Video available at [http://www.jtcvsonline.org/article/S0022-5223\(16\)30462-7/addons](http://www.jtcvsonline.org/article/S0022-5223(16)30462-7/addons).

was checked daily. At 3 and 6 months postoperatively, a new TTE evaluation was performed. The following parameters were obtained: peak and mean gradients, grade of regurgitation, cusp mobility, and left ventricular function. The echocardiographic examinations that we perform in our animals are performed with the sheep in right recumbent position. Perfect alignment with the blood flow can be challenging, leading to possible underestimation of the gradients across the valve. Planned explant was set at 6 months.

Explant Procedures

The weight and condition of the animals was documented before being prepared and anesthetized as described previously. Heparin (3 mg/kg) was administered intravenously and the animal was euthanized with an overdose of a Euthasol solution (Virbac USA, Fort Worth, Tex) intravenously then the heart was removed. The implanted valve tissue was excised and analyzed.

VIDEO 3. Gathered echo samples from the echocardiograms made at 6 months follow-up in the remaining long-term survivors. The large coaptation area is visible, with absent or trace regurgitation. Video available at [http://www.jtcvsonline.org/article/S0022-5223\(16\)30462-7/addons](http://www.jtcvsonline.org/article/S0022-5223(16)30462-7/addons).

TABLE 1. Transvalvular gradients 1 week, 3 months, and 6 months after trileaflet aortic valve reconstruction (mean $\pm$ standard deviation, n = 3)

	Peak gradient, mm Hg	Mean gradient, mm Hg
1 wk	11.3 $\pm$ 5.2	5.9 $\pm$ 2.3
3 mo	6.6 $\pm$ 2.8	3.7 $\pm$ 1.3
6 mo	13.2 $\pm$ 6.4	7.0 $\pm$ 3.4

Macroscopic Examination

Complete autopsies for each animal were conducted by a qualified pathologist to assess for any major embolism. Explants were inspected and examined for vegetations, cuspal hematoma, thrombosis, tears or perforations, tissue overgrowth, paravalvular leak, visible calcification, and cusp retraction. Macroscopical photographs of the valves were taken. After examination, the valves were then transected longitudinally through the commissures.

Radiographic Assessment of Valve Tissue

Radiography was performed under mammography conditions (soft-tissue radiography, Faxitron) to assess for gross calcification.

Histology

Longitudinal samples from the free edge to the base were dissected from the middle part of each leaflet and from one commissure. The remains of the leaflet were saved for calcium content assessment. Five-micrometer thick cross sections were prepared from the samples, embedded in paraffin, and stained with hematoxylin and eosin, Masson trichrome, Von Giesson, phosphotungstic-acid-hematoxylin, Von Kossa, and picro-sirius red (Abcam, Cambridge, Mass). With light microscopy, evaluation of the histologic integrity of the tissue, the localization and the extent of calcification, the presence of an inflammatory response in the tissue, and the extent of fibrous sheeting or pannus over the valve tissue was assessed.

Calcium Content Analysis

Calcium content of the leaflets was quantified spectrophotometrically with a Calcium colorimetric Kit (Chema Diagnostica, Monsano AN, Italy) and expressed as microgram (μ g) calcium per milligram (mg) of dry tissue weight.

Statistical Analysis

No formal statistical analyses were performed as this was an observational pilot study. All values are represented as means $\pm$ standard deviation, or as proportions, as appropriate.

RESULTS

Surgical Procedure and Early Deaths

Six sheep (37–45 kg) underwent the procedure. Average cross-clamp time and cardiopulmonary bypass time was 86 minutes and 105 minutes, respectively. Cusp sizes varied between 17 and 21 mm in all animals. All animals had competent valve function before bypass removal, and all animals survived the procedure (Video 2). There were 3 deaths within 24 hours postoperatively. One animal was impossible to defibrillate and had to be reclamped and given 500 mL of cardioplegia in the root.

FIGURE 1. Representative 6-month transthoracic echocardiogram image. Large coaptation of aortic neo-cusps is clearly visible.

Slow spontaneous heart rhythm resumed. The animal died 5 hours postoperatively as the result of poor recovery from surgery. This animal was 37 kg. The reconstructed aortic valve was functional and intact and the death was a result of the experimental method and not related to the CardioCel tissue. One animal (41 kg) was weak after surgery with a very slow recovery and died 24 hours postoperatively. The other animal (of 37 kg) appeared to recover from surgery but also died suddenly 24 hours after surgery. In both, autopsy found fully functional valve leaflets and deaths attributed to difficulty of aortic valve surgeries in small young sheep.

Functional Valve Assessment in Long-Term Survivors

The 3 surviving animals were all 44 kg or heavier. Sacrifice was planned for 6 months after surgery. All following results are related to these 3 long-term survivors.

TTE revealed good aortic valve function in all 3 animals 1 week, 3 months, and 6 months after surgery. Mean transvalvular gradients were low and stable across all measurements (Table 1). There was no paravalvular leakage and only trace valvular leakage (aortic regurgitation $<1/4$ in all animals). Cusp motion appeared pliable with large coaptation area (Figure 1). On the 6-month echocardiograms, the coaptation length reached an average of 1.2 ± 0.2 cm in the 3 long-term survivors (Video 3). Only 1 cusp (of 9) appeared less pliable at the 6 month measurement.

Macroscopic and Radiographic Examination

Short after the final TTE examination at 6 months, 1 animal developed an irregular heart rate (most likely atrial fibrillation), subsequently suffered from anuria, and died shortly after. Autopsy revealed several renal infarctions,

FIGURE 2. Macroscopic and radiographic examination of CardioCel leaflets. Left panels, Macroscopic examination. Note that all 3 leaflets in left upper panel are macroscopically intact and very pliable despite the early death of this animal with thromboembolic damage (atrial fibrillation). Only 1 leaflet had a region of stiffened tissue suspect for calcification (lower left panel). Right panels, radiographs of separated leaflets. One leaflet had a clear large calcification (right lower panel).

most likely originating from a left atrial thrombus. The implanted neo-cusps were all pliable and clear from any thrombotic attachment.

All 9 leaflets from the 3 long-term surviving animals were intact and pliable and showed no signs of detachment, thrombosis, tissue failure, or endocarditis (Figure 2, upper

panels). Only 1 leaflet showed signs of calcification within the commissural area (Figure 2).

For all 3 animals, radiographs were taken of explanted tissue with the 3 leaflets separated from the aorta (Figure 2, lower panels). Six of the 9 leaflets were completely free from calcifications. Two leaflets, each from different animals, had 1 small density in 1 commissural area. In the third sheep, 1 of the 3 leaflets clearly displayed a large commissural calcification which was also visible macroscopically (Figure 2). This leaflet also corresponded to the one with slightly less mobility on TTE.

Calcium Content

The median calcium content of the 9 leaflets from the 3 long-term surviving sheep was 1.62 $\mu\text{g}/\text{mg}$ (interquartile range 1.314–2.462). All values were low except one sample, which corresponded with the calcified area seen on the radiograph (Table 2; sheep 4240).

TABLE 2. Calcium content in the commissural area of 9 aortic leaflets after 204 (sheep 4232) and 223 days (sheep 4144 and 4240)

Sheep number	Calcium content, $\mu\text{g}/\text{mg}$
4232	4.923
	1.620
	2.462
4144	1.186
	1.454
	1.964
4240	1.314
	0.982
	84.014

FIGURE 3. Hematoxylin and eosin–stained samples of commissural (A–C) and mid-portion (D) areas of CardioCel leaflets. Samples are oriented with the aortic side on top. The original CardioCel structure is preserved and covered with a newly formed layer on both the aortic and ventricular sides in commissural samples and mainly on the aortic side in mid-portion samples. Greater magnification images of the base of the cusps show infiltration of cells into the CardioCel structure (C). Magnification $\times 50$ for A, B, and D; $\times 100$ for C.

Histology

No abnormalities were observed in the organ samples from the 2 sheep that survived until planned sacrifice. Organ samples from the sheep that died before planned sacrifice (at 6 months) revealed clear organ damage from a systemic thromboembolic event, with mainly embolic damage to kidneys.

Hematoxylin and eosin staining showed that the original CardioCel structure was well preserved with no sign of mechanical tissue failure (Figure 3). Large portions of the leaflets were covered with newly formed layer of tissue on both the aortic and ventricular sides in commissural areas and mainly on the aortic side in mid-portion areas. Other parts of the material were not covered by pannus. There were no signs of major calcification or of major inflammation or infection. Only small areas of calcification were seen near suture line regions.

DISCUSSION

The purpose of this study was to provide a preliminary assessment of the performance of a decellularized pericardial scaffold (CardioCel) used in complete trileaflet aortic valve reconstruction with a long-term juvenile sheep model. Previously, the pulmonary and mitral valves have been

repaired successfully with this pericardial scaffold in a juvenile sheep model, but experimental valve repair in the aortic position has not been performed.¹⁴ This study shows that trileaflet aortic valve reconstruction is an appropriate application of this material.

The pericardial material had excellent handling characteristics, was easy to suture, and had good suture retention. Macroscopic examination showed that the integrity and stability of the material was preserved after long-term implantation of 6 months in aortic position. The mechanical properties of the scaffold have been investigated previously.^{12,13}

Calcification leading to device failure is a major drawback of cardiac valve bioprostheses.^{5,6} The decellularized pericardial scaffold studied here is produced with a multistep, anticalcification manufacturing process that has been shown to confer long-term calcium resistance to patches implanted in the jugular veins of juvenile sheep or used to repair pulmonary and mitral leaflets, as well as in a rat subcutaneous model.^{11,13,14} Similarly, we report here that aortic valve leaflets reconstructed with this scaffold are resistant to calcification, even in commissural areas of the leaflets which are most at risk for calcification. The median calcium content in the 9 leaflets after long-term implantation is very low

(1.62 $\mu\text{g}/\text{mg}$). CardioCel patches used to repair mitral and pulmonary valves had an even lower median calcium content 8 months after implantation compared with the calcium content observed in this study (1.62 $\mu\text{g}/\text{mg}$ vs 0.46 $\mu\text{g}/\text{mg}$ in the mitral patches and 0.24 $\mu\text{g}/\text{mg}$ in pulmonary patches).¹⁴ This difference is attributable on one hand to the difference in implant position (aortic vs pulmonary) and on the other hand to the complete trileaflet reconstruction model used in this study versus the unicuspal repair model in the previous mitral study. Compared with currently used biological valves, the calcification levels reached in this study are exceptionally low. Perimount valves for example easily reach up to 7 $\mu\text{g}/\text{mg}$ after a long-term orthotopic implant period in our sheep models.²⁰

Echocardiography data showed that our reconstructed valves functioned well in the aortic position. Perfect alignment of the Doppler signal to blood flow, however, is difficult to obtain in these animals, which prohibited us from obtaining detailed hemodynamic measurements across the valve. Although 3 animals died within 24 hours postoperatively for reasons related to the demanding surgical procedure, none of the early deaths were related to any failure of the pericardial patch material. The early postoperative mortality rate in this experimental model reflects the delicacy of this complex surgical procedure in smaller sheep. The sheep model is used extensively for the assessment of heart valve bioprostheses and was an appropriately selected animal model in this setting; unfortunately, however, deaths are not uncommon in the development of sheep models of aortic valve surgery.^{21,22}

The 3 long-term surviving animals remained in perfect clinical shape and showed no signs of aortic valve insufficiency up to 6 months after surgery. We were able to demonstrate a large coaptation height in the reconstructed valves. This is one of the key aspects of the technique, as described by Ozaki and colleagues^{18,19} in aortic valve reconstruction. This large coaptation area may have a positive impact on tissue durability, because the stress on the leaflets at valve closure is distributed over a large amount of tissue.

Valve repair with materials that can remodel and grow with the patient would overcome the limitations of valve replacement in the relatively younger adult and pediatric population.²³ CardioCel is designed as a scaffold material intended to provide structural integrity at the time of repair with a capacity for remodeling by permitting autologous recellularization and development of blood supply over time.¹⁰ In this and previous studies, CardioCel has shown potential to allow infiltration of host cells into the scaffold^{12,14}; however, this phenomenon was limited to the base of the cusps. We were not able to demonstrate remodeling within the pericardial scaffold as a whole.

Valve repair is now a favored strategy for mitral and tricuspid valve disease. Valve repair in the aortic position is a more technically demanding procedure; hence, aortic valve replacement remains more common.^{24,25} Valve repair is preferable to replacement for preservation of the natural architecture of the annulus and valve interaction and negates the need for lifelong anticoagulant therapy. Repair of the aortic valve with the trileaflet reconstruction strategy with glutaraldehyde-fixed autologous pericardium in patients with various aortic valve diseases has produced good postoperative results in up to 3 years' follow-up.^{18,19} This procedure has meanwhile been performed with CardioCel in pediatric and adult patients with excellent early results.^{16,17} CardioCel may offer a more durable alternative to glutaraldehyde-fixed autologous pericardium for candidates for aortic valve repair, without the problems with calcification and cytotoxicity presented by xenogeneic pericardium. Obviously, long-term follow-up data and more rigorous clinical testing are needed to support the application of CardioCel in trileaflet repair.

Recently, Mazzitelli and colleagues¹⁷ described their initial results in using this material for complete aortic valve reconstruction in pediatric patients. On the basis of the growing clinical experience together with the promising results from this experimental evaluation, a worldwide, multicenter, prospective clinical trial has been initiated. The first 19 patients already have been included and successfully operated with this technique and material. The unique combination of optimal hemodynamics of the reconstructed aortic valve, with a large coaptation height, together with the decellularized character of this pericardial material, potentially can offer significant benefits towards long-term durability.

Limitations

This work collects the data from a pilot study in young sheep. Mortality was high, given the technical complexity of the performed procedures. Echocardiographic examination of the aortic valve in sheep can be challenging. Therefore, it is possible that the obtained gradients across the valve are underestimated. We did not perform detailed immunohistochemical examinations or cell phenotyping or counting. Despite these limitations, 3 long-term surviving animals provided valuable data to assess the long-term behavior of CardioCel in aortic position.

Conflict of Interest Statement

W. Neethling is a consultant to Admedus. D. Rhodes serves as Chief Scientific Officer of Admedus and G. Strange is the CEO of the Pulmonary Hypertensions Society, Director of Mozaic Solutions and serves as the Medical Director for Admedus. All other authors have nothing to disclose with regard to commercial support.

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