Histological evaluation of explanted tissue engineered bovine pericardium (CardioCel®)

Sudesh Prabhu MCh1,2,3, Jane E Armes FRCPA2,3,4, Douglas Bell MBBS2, Robert Justo FRACP1,2,3, Prem Venugopal FRCS1, Tom Karl FRACS2,5, Nelson Alphonso FRACS1,2,3

1 Queensland Paediatric Cardiac Services, Lady Cilento Children’s Hospital, Brisbane, Australia
2 School of Medicine, University of Queensland, Brisbane, Australia
3 Mater Research Institute, University of Queensland, Brisbane, Australia
4 Department of Pathology, Mater Health Services, Brisbane, Australia
5 Cardiac Surgery, Johns Hopkins All Children’s Hospital, St. Petersburg, Florida, USA

Corresponding Author:
Nelson Alphonso
PO Box 3474, Level 7F, Clinical Directorate
Lady Cilento Children’s Hospital, 501 Stanley Street, Brisbane, QLD, 4101, Australia
Tel: +61 7 30683486 Fax: +61 7 30684229
E-mail: nelsonalphonso@mac.com
Category: Congenital heart disease

Word count: 3488

Declaration of conflict of interest: The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article

Funding: The authors received no financial support for the research, authorship and/or publication of this article
ABSTRACT

Objectives

CardioCel is bovine pericardium which is subjected to a novel anti-calcification tissue engineering process. We present the histopathological findings of human explants of CardioCel that were used in operations for congenital heart disease in children.

Methods

Six explants were identified from 140 patients undergoing CardioCel implants from October 2012 to March 2015. CardioCel explants were evaluated histologically using hematoxylin and eosin, Masson’s trichrome and immunohistochemical staining.

Results

A variable inflammatory response was seen in the surrounding native tissue, but not within the CardioCel graft in any of the explants. Neo-intimal layer of varying thickness developed on the visceral surface of 5 CardioCel explants with endothelialization of the longest duration explant. A granulation tissue layer developed on the parietal surface of the graft (consistently thicker than the neo-intima). Maintained collagen fibre architecture (laminated) and variable fibroblastic invasion (which increased with the age of the implant) were identified in all six cases. Scattered capillary vessels were noted in the majority of the explants with new collagen fibres in one, suggesting early remodeling. Calcium was seen in one explant at the interface of the graft and inflammatory response on its parietal surface.

Conclusions

Evidence of graft remodeling was noted in the majority of the explants without inflammatory cells or calcification within the explanted graft material. A noticeable feature was the differential thickness of the host reaction to the parietal compared with the visceral
surface of the graft. We will continue to evaluate CardioCel as a cardiovascular substitute for extracardiac and intracardiac reconstruction.

**Keywords:** remodeling; bovine pericardium; histopathology; CardioCel; congenital heart diseases

**Abstract word count:** 250
56 **Glossary of abbreviations**

57 ECM – extra cellular matrix

58 CHD – Congenital heart disease

59 IHC – immunohistochemistry

60 TAPVD – total anomalous pulmonary venous drainage

61 CT scan – computerized tomography

62 VSD – ventricular septal defect

63 IAA – interrupted aortic arch

64 TOF – tetralogy of Fallot
**PERSPECTIVE STATEMENT**

The inflammatory response to CardioCel is limited only to adjacent native tissue. Fibroblast ingrowth is a uniform characteristic. Neo-intimal formation occurs commonly. Early remodeling was demonstrated in the majority. Overall, the histological findings are encouraging and we will continue to evaluate CardioCel as a cardiovascular substitute for extracardiac and intracardiac reconstruction.

**CENTRAL MESSAGE**

CardioCel grafts show evidence of remodeling, neo-intima formation (visceral surface) and inflammatory response (parietal surface).

Central Figure

Remodelling of a CardioCel transannular patch graft after 502 days of implantation, seen as a fibroblastic infiltrate (†), surrounded by newly interposed, pale, eosinophilic collagen (*) between the brightly eosinophilic graft collagen layers. Neovascularisation (∇) is also demonstrated (H&E, original magnification x40).
INTRODUCTION

Glutaraldehyde preserved bovine pericardium was first used in 1977 to construct a prosthetic heart valve and has found widespread usage as a cardiovascular substitute for intracardiac and extracardiac repairs. Fresh (unfixed) bovine pericardial implants undergo inflammation and partial digestion without mineralization. Cross-linking collagen with glutaraldehyde stabilizes fibers, adding strength and durability, while reducing antigenicity. However, the limitations of most glutaraldehyde-preserved bovine pericardium are well known, and include degeneration and calcification which causes rigidity and shrinkage. Calcification of glutaraldehyde cross-linked bovine pericardium is predominantly promoted by the presence of cell remnants in the extracellular matrix (ECM), residual membrane-associated acidic phospholipid and non-viable interstitial cells rendered inactive by the glutaraldehyde treatment. Calcification has also been observed in the ECM proteins (collagen and elastin) and it has been suggested that collagen serves as a nucleation site for calcium phosphate, independent of devitalized cells. The amount of incorporated glutaraldehyde determines the degree of cross-linkage, with a high uptake associated with greater calcification. Antigenicity, immunological reactions and the inflammatory process may also contribute to calcification as evidenced by lower incidence of calcification in glutaraldehyde treated autologous tissue than heterologous tissues.

CardioCel is bovine pericardium which is subjected to an anti-calcification tissue engineering process (ADAPT TEP) developed by AdmedusRegen Pty Ltd (Perth, Western Australia). ADAPT TEP includes steps to reduce cytotoxicity by removing lipids, all cells and cell remnants, nucleic acids (DNA, RNA) and α-Gal (galactosyl) epitopes. Crosslinking to maintain strength and elasticity is achieved with a low concentration of monomeric glutaraldehyde. Detoxification is further promoted by non-glutaraldehyde sterilization and storage in a glutaraldehyde free solution.
Remodeling of an implantable biological scaffold with native tissue ingrowth, endothelialization and neovascularization in humans remains an elusive goal and such a scaffold would undoubtedly represent the ideal biological material for repair of congenital heart defects (CHD). Animal studies have demonstrated migration of autologous cells into a CardioCel scaffold with endothelialization and neovascularization. Remodeling has not been demonstrated in human implants. We present the histopathological and immunohistochemical (IHC) findings of 6 human explants of CardioCel patches that were used initially in operations for CHD in children.

**METHODS**

**Subjects**

Data for patients undergoing CardioCel implants from October 2012 to March 2015 was retrieved from our departmental database. Patients who had explantation of CardioCel (partial or complete) were identified. Inclusion criteria were (1) a congenital cardiac anomaly in patients aged 1 day to 18 years, (2) CardioCel used as a cardiovascular tissue substitute, (3) subsequent surgery (any indication) with explantation of CardioCel patch (partial or complete).

Clinical data were collected for all patients from electronic health records and paper charts. Patients were consented and ethics committee approval was obtained (HREC/15/QRCH/93).

**Histologic examination**

CardioCel explanted at re-operation was fixed in formalin. Hematoxylin and eosin (H&E) stain was used for assessment of grade and type of inflammation, type of inflammatory cells, including eosinophil response, degree of inflammatory cell infiltration into CardioCel collagen layers, neovascularization and neo-intima formation. Collagen characteristics were
studied using H&E and Masson’s trichrome stains. All specimens underwent immunohistochemical staining. Endothelial cells were identified using antibodies to CD34 and Factor-VIII. IHC was also used to discern the subtypes of inflammatory cells, which included T lymphocytes (CD3), helper T lymphocytes (CD4), suppressor T lymphocytes (CD8), B lymphocytes (CD20), plasma cells (CD79a), macrophages and histiocytes (CD68).

Unimplanted CardioCel was used as control and after fixation in formalin was studied using H&E and Masson’s trichrome stains.

For the purpose of description of the histological response to CardioCel, we divided the surgical specimen into 3 zones: (1) native tissue response on the parietal (rough/outer) surface of the graft, (2) reactive change within the CardioCel graft itself and (3) native tissue response on the visceral (smooth/inner) surface of the graft.

RESULTS

During the study period, 140 patients underwent various cardiovascular procedures in which CardioCel was used as a cardiovascular substitute. Fifteen patients (10.7%) underwent re-operations (excluding mediastinal exploration for bleeding). The CardioCel patch was explanted (1 partial and 5 complete) in 6 patients (4%). The indications for re-operation were related to the CardioCel patch in 2 patients and not related to CardioCel in the remaining 4 patients.

Subjects

Case 1 (10 days in situ): An 8 year old child presenting with severe mitral insufficiency underwent mitral valve repair. The posterior mitral leaflet (PML) was enlarged with a CardioCel patch. On the 8th postoperative day (POD), the patient was re-operated for severe mitral insufficiency and low cardiac output syndrome secondary to an atrial tachyarrhythmia
resistant to amiodarone and cardioversion. Mitral valve replacement with a 25mm bileaflet mechanical prosthesis with chordal preservation, de Vega tricuspid valve annuloplasty and radiofrequency bi-atrial maze were performed. The CardioCel patch used for augmentation of the PML was explanted.

Case 2 (67 days in situ): A 3-day old neonate with obstructed mixed total anomalous pulmonary venous drainage (TAPVD) underwent repair on day 3 of life with a Warden procedure, atrial septectomy and construction of an intra-atrial baffle using CardioCel. At 60 days follow up, echocardiography, cardiac catheterization and computerized tomography (CT scan) demonstrated severe obstruction within the intra-atrial baffle and suprasystemic right ventricular (RV) systolic pressures. At re-operation, the patch of CardioCel used to create the intra-atrial baffle was noted to be thickened and shrunken. The patch was completely explanted and replaced with glutaraldehyde treated homograft pericardium.

Case 3 (134 days in situ): A 3 day old neonate underwent a Yasui operation for type-B interrupted aortic arch (IAA) with ventricular septal defect (VSD) and subaortic obstruction. The IAA was repaired by direct anastomoses of the descending aorta to the ascending aorta posteriorly with anterior augmentation using a CardioCel patch. The child subsequently underwent 2 attempts at balloon angioplasty for severe stenosis of the arch anastomosis before undergoing re-operation at 5 months. There was a thick neo-intima related to the CardioCel patch. The CardioCel patch was explanted and replaced with pulmonary artery homograft. The neo-intima got separated and was not sent for histological examination.

Case 4 (272 days in situ): A 4 month old child with tetralogy of Fallot (TOF) underwent takedown of a Blalock-Taussig shunt and repair of TOF with augmentation of the anterior pulmonary valve leaflet using glutaraldehyde treated autologous pericardium. CardioCel was used to close the VSD and 2 separate additional patches were used to augment the left
pulmonary artery (LPA) and the transannular right ventricular outflow tract (RVOT). The patient was re-operated at 12 months for severe pulmonary regurgitation and severe tricuspid regurgitation. CT scan demonstrated focal narrowing of the origin of the LPA. Dense pericardial adhesions were noted between native pericardium and the transannular CardioCel patch (Figure 1A). The neo-pulmonary valve was stiff and immobile and completely covered with neo-intima on both surfaces. The neo-intima extended under the transannular CardioCel patch into the left pulmonary artery. The neo-intima was loosely adherent to the overlying CardioCel patch (Figure 1B). There was a shelf of neo-intima causing localized narrowing at the origin of the LPA where the two CardioCel patches used for the transannular repair and the LPA repair were sutured together. Both CardioCel patches and autologous pericardial neo-pulmonary valve were completely explanted.

Case 5 (428 days in situ): A child with common arterial trunk underwent repair on day 40 of life (detachment of branch pulmonary arteries [PA] from common arterial trunk, closure of VSD, closure of inter atrial communication and interposition of 12mm valved conduit from RV to PA). The child was re-operated at 12 months for severe distal RV-PA conduit obstruction. The PA bifurcation was reconstructed using a CardioCel patch and the RV-PA conduit was replaced with a 15mm pulmonary homograft using an anterior hood of CardioCel for the proximal anastomosis. Thirteen months later the patient presented with severe narrowing of the proximal segment of the pulmonary homograft without stenosis of the branch PAs. Balloon angioplasty caused a contained rupture necessitating reoperation. At reoperation there was no narrowing of the anastomosis between the homograft and the reconstructed PA bifurcation. The luminal surface of the CardioCel patch at the bifurcation was covered with neo-intima. The bifurcation was enlarged by resecting a portion of the CardioCel patch.
Case 6 (augmented anterior pulmonary valve leaflet in situ for 292 days, transannular patch [TAP] -peripheral section 502 days, central section 292 days): A 3 month old child underwent primary repair of TOF using CardioCel for the TAP and closure of the VSD. The child was reoperated 7 months later for a residual VSD and pulmonary insufficiency. CardioCel was used to augment the anterior pulmonary leaflet and the TAP centrally. The child underwent a second reoperation 9 months later for severe pulmonary and tricuspid insufficiency and origin stenosis of both branch PAs. The entire valve complex and TAP were explanted.

Histological examination

CardioCel controls were uniformly 400 µm in thickness. Two distinct surfaces were identifiable; a smoother visceral surface and a rougher parietal surface. Native collagen fibres were arranged in layers and ‘ghost vessels’ (remnants of bovine blood vessels) were seen (Figure 2).

In our series, the parietal surface of each of the explanted CardioCel grafts was covered by a layer of granulation tissue showing neovascularization and containing fibroblasts and a mild to moderate inflammatory response (Figure 3). The thickness of this layer varied from 0.1mm (Case 1) through to approximately 1mm (e.g., Cases 2, 5 and 6) and did not appear to be temporally related. IHC showed that the infiltrating lymphocytes were predominantly T lymphocytes (CD3 positive) with a lesser B lymphocyte infiltrate (CD20 positive; Figure 3 - B1, B2), apart from Case 4 (which included a foreign body multinucleate giant cell response) where T and B lymphocytes were approximately equal in number. A semi-quantitative assessment revealed that there were either equal numbers of T helper and suppressor cells (CD4 and CD8 positive, respectively) or an excess of T helper cells compared with T suppressor cells. None of the cases had an excess of T suppressor cells. Plasma cells were
sparse in each case. Eosinophils were present in all cases, but the quantity varied (from 1/h high power field in Case 5 to >100/h high power field in Case 6) and the degree of eosinophil infiltrate did not appear to be temporally related.

The macrophage inflammatory component varied from mild to marked within the thickened surface layer. In Case 2 and Case 4, CD68-positive macrophages were arranged between the granulation tissue and the graft and in Case 4 there was a classical foreign body giant cell response (Figure 4A, 4B & 4C). This case was the only case in which dystrophic calcification was identified (Figure 4D). This calcification was closely aligned with the macrophages, between the granulation tissue layer and the CardioCel graft. Calcium was not seen within the graft material in any of the explants. There was no infiltration of any type of inflammatory cell into the collagen laminae of any of the CardioCel explants.

In all but one case (Case 1) there was an identifiable neo-intima on the visceral surface of the CardioCel patch (this was not sent for histology in Case 3). This layer was composed of neo-vascularised fibromyxoid substance with interposed fibroblasts (Figure 5A). There was minimal to no inflammation within this layer. The thickness of this layer was always less than that seen on the opposite, parietal surface of the graft and measured between 0.02mm (Case 4) and 0.4mm (Case 6). Using IHC, we were able to identify endothelial cells in the explant with the longest in situ implant duration (Case 6; Figure 5). CD34 and Factor VIII IHC positive endothelial cells were identified on the surface of the neo-intima covering the luminal surface (blood interface) of the TAP. Endothelial cells were also identified on both neo-intimal surfaces of the CardioCel patch which was used to augment the anterior leaflet of the pulmonary valve (explanted on day 292). In Case 1 (explanted on day 10), no neo-intima was identified. However, a single, discontinuous layer of cells were identified on the visceral surface.
In general, there was no patch breakdown and the architecture of laminated native collagen fibers was maintained within all 6 human explants. Neovascularization adjacent to the CardioCel patch was seen in all explants. Variable fibroblastic invasion was identified in all six cases (Figure 6), including Case 1, where the graft had been in place for only 10 days before explantation. This was associated with scattered capillary vessels (Figure 5). The degree and extent of fibroblast infiltration into the graft appeared, to increase with the age of the implant. Indeed, the longest implant (Case 6) had a fibroblastic infiltrate throughout all layers of the CardioCel graft (Figure 6). A function of fibroblasts is to produce collagen fibres and these were seen lying between the CardioCel laminated collagen bundles in association with the fibroblastic infiltrate in the longest-standing graft (Case 6) (Figure 5). This finding is consistent with early remodeling in the CardioCel patch.

DISCUSSION

CardioCel is in an early phase of evaluation as a cardiovascular substitute in humans (implantation began in 2008). The Queensland Paediatric Cardiac Service (QPCS) began using CardioCel in October, 2012.

In a study from Melbourne, Brizard et al \(^7\) implanted CardioCel in a subcutaneous pocket created in the dorsal area of 6-week old male Albino Wistar rats. The rats were sacrificed at 8 (n=5) and 16 (n=5) weeks. The CardioCel patches demonstrated improved biostability (tested using shrinkage temperature) and durability (tested using resistance to enzymatic degradation), as well as reduced calcification compared with bovine pericardium fixed with 0.6% glutaraldehyde, cryopreserved human pericardium rapidly fixed with 0.6% glutaraldehyde and ADAPT treated cryopreserved human pericardium. Histology at 8 weeks (n=5) demonstrated host fibroblasts in the collagen matrix. By 16 weeks (n=5) there was an increased number of host fibroblasts and the presence of functioning neo-capillaries on the
edges of the explant without visible calcification. In a second study by the same investigators CardioCel was used for reconstruction of mitral valve (PML was replaced by CardioCel) and pulmonary valve (one of the leaflets was replaced by CardioCel) in a juvenile sheep model. After 7 months of implantation there was a continuous endothelial lining visible on the blood interface. A few neo-capillaries, myofibroblasts, monocytes, and cells with smooth muscle cell phenotype were visible within the CardioCel. There was no calcium deposition or thickening of the implant. More recently CardioCel was used for complete trileaflet replacement of the aortic valve in a surgical sheep model. At 6 months 8 of 9 leaflets were intact and pliable with diminished mobility and macroscopic calcification of only one cusp. Histology identified microscopic calcification of 2 additional cusps. The original CardioCel structure was well preserved. Large portions of the leaflets were covered with a neo-intima on both surfaces with endothelialization of many parts of the neo-intima. There was infiltration of new fibroblasts into the CardioCel at the base of the cusps. There were macrophages within the neo-intima but no inflammatory cells were demonstrated within the CardioCel.

A South African single center, prospective, non-randomized clinical study with 30 pediatric patients with various cardiac defects, demonstrated that there were no implant related events leading to death or morbidity in the 30-day post-operative period. Echocardiography assessment at 6 and 12 months showed intact anatomic and hemodynamically stable repairs, and no visible calcification. The authors concluded that CardioCel can be safely and efficaciously used as a cardiovascular substitute for surgical repair of both simple and complex congenital cardiac defects. In our series of 140 patients, 6 (4.2%) had CardioCel explanted at subsequent reoperation. CardioCel appeared to perform satisfactorily from an anatomic and hemodynamic standpoint in the majority of patients. In 2 (cases 2, 3) CardioCel was mainly responsible for the indication for reoperation and in this
sense these 2 patients can be considered to represent failures of the prosthesis. Both patients were neonates. We have used CardioCel in 15 additional neonates (20 implants) [arch repair (n=7), PA reconstruction (n=5), VSD patch (n=5), intra-ventricular baffle in DORV (n=1), ASD patch in arterial switch operation (n=1) and aorto-pulmonary window (n=1)]. The overall failure probability is comparable to other prosthetic materials.

In our study there was no inflammatory infiltrate into any of the grafts even after 502 days of implantation. The inflammatory response appeared to be confined to the granulation tissue which developed on the graft’s parietal surface; the thickness of which was variable and exceeded the thickness of the CardioCel patch in all cases except Case 1. The thickness of this layer did not appear to be temporally related though measurement of thickness can be affected by tangential embedding during sectioning. In 5 of our cases there was an identifiable neo-intima on the visceral surface of the graft. The neo-intima was uniformly thinner than the granulation tissue layer on the opposite surface and there was minimal to no inflammation within this layer. This demonstrates that there is a graft dependent different host response on its parietal compared with its visceral surface regardless of the site of implantation, intra-cardiac or extra-cardiac position or exposure to systemic or pulmonary pressures. When used as an intra-cardiac baffle in neonates, it is therefore possible that this layer could contribute to luminal compromise as seen in one of our cases (Cases 2). We recommend that CardioCel is implanted with the visceral surface designated as the luminal surface wherever possible.

In the study on juvenile sheep cited above, no histological evidence of calcification was noted after 210 days. In the study from South Africa, post-operative echocardiography at 6 and 12 months did not show calcification; however echocardiography will not identify the presence of microscopic calcium deposit. We observed focal calcification in 1 of our patients.
(case 4, 272 days Figure 4C). The calcification was aligned with macrophages and situated between the granulation tissue layer covering the parietal surface and the CardioCel graft used as TAP. As this reaction was present only on the external surface it is possible that it represents a more generalized post-operative pericarditis than a foreign body reaction to CardioCel itself (12, 13). The calcification did not have any functional consequence as seen in the aortic valve leaflet in the sheep model 9. Calcification was not demonstrated in any of the other 5 implants, 2 of which were in situ for longer periods of time (428 days and 502 days).

CardioCel is intended for use as a biological scaffold. Its durability and strength as compared to autologous pericardium has been previously demonstrated in animal studies 7. Previous human and animal studies 8, 9, 10 have demonstrated its capacity to provide structural integrity when used for cardiac repairs. Evidence of early remodeling has been demonstrated in animal studies 7, 8, 9 but has never been previously demonstrated in humans. Development of a neo-intima is important for the remodeling process. A recognizable layer of neo-intima was seen on the surface of all our explants, excepting Case 1, which had only been in situ for 10 days. In one case the neo-intima was peeled off at the time of explantation and could not be demonstrated on histology. In the juvenile sheep model cited above, there was a continuous endothelial lining visible on the external blood interface after 7 months 8. In the surgical sheep model many parts of the neo-intima covering the CardioCel valve leaflets were endothelialized by 6 months 9. We demonstrated endothelial cell formation on one implant with the longest duration of implantation (16 months). Endothelial cells are very fragile and can be easily disrupted during histopathological sampling. It is possible that the development of an endothelial cell lining in humans is temporally related to the number days in situ and to the site of implantation with quicker endothelialization in valve reconstruction. Both animal models cited above demonstrated the development of neo-capillaries on the edges with
migration of the animal’s own fibroblasts into the CardioCel scaffold\textsuperscript{7, 9}. All our explants demonstrated fibroblastic infiltration associated with neovascularization that appeared to be temporally related. In the longest implant (Case 6) there was a fibroblastic infiltrate throughout all layers of the CardioCel graft (Figure 6) and new collagen fibres were seen lying between the laminated collagen bundles of CardioCel. This finding is consistent with early remodeling in the CardioCel patch.

Complete remodeling into a three-layered vessel wall, valve or inter-atrial septum was not seen in our series. It is possible that this is a time-related phenomenon that can be only be proved or disproved from further explants.

**CONCLUSION**

We demonstrate that the CardioCel graft incites a thick granulation tissue response on its parietal surface and a thinner, neo-intima on its visceral surface, a finding which may be of importance when considering the site of implant of a CardioCel graft. The inflammatory response to CardioCel is limited to the granulation tissue response and there was no inflammatory cell infiltration into the graft collagen lamina. Calcification did not occur in the CardioCel patch. Temporally related fibroblast ingrowth into the graft was shown to be a uniform characteristic. This feature, together with the development of a neo-intimal layer indicates that early remodeling was occurring in our explanted CardioCel grafts.

**ACKNOWLEDGEMENT**

We thank Janelle Johnson, for her help with data management.
REFERENCES

1. Ionescu MI, Tandon AP, Mary DA, Abid A. Heart valve replacement with the

2. Strange G, Brizard CP, Karl TR, Neethling L. An evaluation of Admedus’ tissue
   engineering process treated (ADAPT) bovine pericardium patch (CardioCel) for the

3. Golomb G, Schoen FJ, Smith MS, Linden J, Dixon M, Levy RJ. The role of
   glutaraldehyde-induced cross-links in calcification of bovine pericardium used in


   calcification of glutaraldehyde-preserved bovine pericardium. *J Cardiovasc Surg
   (Torino)* 2006; 47: 711-718

6. Neethling WM, Yadav S, Hodge AJ, Glancy R. Enhanced biostability and
   biocompatibility of decellularized bovine pericardium, crosslinked with an ultra-low
   concentration monomeric aldehyde and treated with ADAPT. *J Heart Valve Dis* 2008;
   17: 456-463

7. Neethling WML, Brizard CP, Firth L, Glancy R. Biostability, durability and
   calcification of cryopreserved human pericardium after rapid glutaraldehyde
   stabilization versus multistep ADAPT treatment in a subcutaneous rat model. *Eur J
   Cardiothorac Surg* 2014; 45: e110-e117

8. Brizard CP, Brink J, Horton SB, Neethling WML et al. New engineering treatment of
   bovine pericardium confers outstanding resistance to calcification in mitral and


FIGURE LEGENDS

Figure 1: Intraoperative photographs (case 4): A - Fairly dense pericardial adhesions between native pericardium and the transannular CardioCel patch. B - Loosely adherent neo-intima on the luminal surface of the transannular CardioCel patch.

Figure 2: Control: Non-implanted CardioCel graft showing laminated collagen fibres. One surface is rough (upper) i.e. parietal, whilst the opposite is smooth, i.e. visceral (A: H&E, original magnification x20). Non-implanted CardioCel graft in which residual bovine vascular outlines can be identified (B: Masson’s Trichrome, original magnification x 20). A smooth (lower, visceral) and rough (upper, parietal) surface are again noted (* Remnants of bovine blood vessels - ghost vessels).

Figure 3: A. Photomicrographs of all 6 patients (H&E, original magnification x 10) demonstrating response to the parietal (rough) surface in the form of granulation tissue of variable thickness (arrow) which contained an inflammatory infiltrate, predominantly T lymphocytes, with a lesser population of B lymphocytes (B1- T lymphocytes demonstrated by CD3 IHC and B2 - B lymphocytes demonstrated by CD20 IHC, original magnifications x 20). Neo-intima (*) formation on the visceral (smooth) surface can be seen in cases 2, 4, 5 and 6. No discernible neo-intima was seen in case 1 and in case 3 the neo-intima was separated intraoperatively and not sent for histology.

Figure 4: Case 4, transannular patch A and B: Macrophages arranged between the granulation tissue and the graft, with a classical foreign body giant cell response (* ; H&E, original magnification x 20). C: Histiocytes, including multi-nucleated foreign body giant cells, expressing the macrophage marker CD68 (anti-CD68 IHC, original magnification x 40). D: Dystrophic calcification (arrows) was closely aligned with the macrophages, between the granulation tissue layer and CardioCel.

Figure 5: Remodeling (case 6, 502 days)
A: CardioCel transannular patch graft with neo-intima (lower) on the visceral side of the graft and a thicker granulation tissue layer, including an inflammatory cell infiltrate, on parietal surface of the graft (H&E, original magnification x10). B: Remodeling of the graft seen as fibroblastic infiltrate (†), surrounded by new pale, eosinophilic collagen (* between the brightly eosinophilic graft collagen layers. Neovascularisation (∇) is also demonstrated.
(H&E, original magnification x40). C: Endothelium (↑) extending into the graft (Factor VIII IHC, original magnification x 10). D: Neo-intima which has formed on the visceral surface of the graft showing a surface layer of endothelial cells (↑) (CD34 IHC, original magnification x 20). E: Native pulmonary valve leaflet composed of fibromyxoid tissue with interspersed fibroblasts and overlying endothelial cells. There is no inflammatory cell infiltrate nor neovascularisation (H&E, original magnification x 10).

Figure 6: Fibroblastic infiltration Photomicrographs of all 6 patients (H&E, original magnification x 20) demonstrating unchanged thickness of CardioCel patches and temporally related fibroblast infiltration (arrow). Infiltration of fibroblasts seen as early as 10 days (case 1); and the longest implant (506 days, case 6) demonstrating fibroblastic infiltration throughout the layers of the CardioCel patch.

Page 22 of 22