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Institutional report - Valves

Inflammatory responses of tissue-engineered xenografts in a clinical scenario

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Abstract

Acellular tissue-engineered (ATE) xenografts and homografts are used in clinical cardiovascular surgery. The present study examined the specific role of carbohydrate antigen (α-Gal and T-antigen) in immune response after decellularisation in tissue-engineered xenografts (porcine pulmonary artery and bovine jugular vein). An enzyme-linked immunosorbent assay (ELISA) was used to ascertain whether implantation of bioprostheses, ATE xenografts and mechanical valve replacement result in augmentation of anti-α-Gal IgM antibodies within eight days of surgery (each group, $n=6$). Kinetics of host inflammatory response on surgically explanted ATE xenografts was also studied. Immunostaining for α-Gal and T-antigen detected the presence of them in the native tissue but they were absent in processed ATE xenografts from the same tissue. A significant increase in the concentration of anti-α-Gal IgM antibodies was observed in the serum of bioprosthetic valve recipients as compared to ATE xenograft recipients ($P<0.05$). Organised collagen, and decreased inflammatory response with increase in endothelisation and vascularisation was evident beyond one year of surgery as compared to early periods in ATE xenografts. This study demonstrates that decellularisation of xenografts and further processing of these tissues enabled reduction of inflammatory stimulus with autologous recellularisation with no calcification.

Keywords: Immune response; Bioprosthetic heart valves; Acellular tissue-engineered valved conduit; Decellularisation; Carbohydrate antigen

1. Introduction

Gold standard homografts traditionally have been the preferred valved conduits for right ventricular outflow tract reconstruction in complex cardiovascular malformations. In some cases, the non-availability of pulmonary homografts particularly in small sizes (10–18 mm) leads to the recourse of manipulating xenograft tissues of identical anatomical origin for such a repair [1]. Glutaraldehyde-fixed porcine pulmonary artery (PPA), valved bovine jugular vein (BJV) conduits, or conduits constructed from bovine pericardium have been clinically used over the years [2]. Detection of xenoreactive carbohydrate antigens [α-Gal and non-α-Gal Thomson Friedenreich (T)-antigen] from discordant species is therefore still relevant, as porcine heart valves and conduits and commercially available BJV conduits are frequently considered for clinical application which are not acellular [3, 4].

There is no scientific evidence to show that acellular tissue-engineered (ATE) xenografts are likely to experience a partial or complete autologous recellularisation and increase or decrease in levels of serum anti-α-Gal IgM antibodies if these xenografts are implanted in humans. This study, therefore, explores the difference in tissue reaction and inflammatory response between indigenously processed ATE xenogenic devices and the commercially available ones.

2. Materials and methods

This work was divided into three parts: (i) histological and immunohistochemical analysis of the native and ATE tissues (indigenously processed PPA and BJV conduits) for acellularity and presence or absence of α-Gal and T-antigen; (ii) change in anti-α-Gal IgM antibodies' levels in the serum of patients (under clinical trial) with ATE tissue replacements at different intervals in comparison to patients with bioprosthetic heart valves (BHVs); (iii) indigenously processed PPA and BJV conduits were implanted in patients aged between three months and 59 years for reconstruction of the right ventricular outflow tract. Histological and immunohistochemical analysis of the explanted ATE tissues (Table 1) were performed in seven of these patients. These three parts of investigation were independent of each other. The scoring criteria for histological and immunohistochemical studies are given in Table 2. The study was approved by Institutional Ethics Committee and conducted after informed consent of the patients.

2.1. Method I

2.1.1. Treatment of tissues

Native PPA conduits ($n=4$) and BJVs ($n=4$) were divided into three longitudinal sections. Two parts were left un-

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Table 1. Scoring criteria

Characteristic	Score
Cellular infiltration	
None	0
Weak	1
Moderate	2
Aggressive	3
Original ECM remaining	
<25%	0
25–40%	1
41–80%	2
>80%	3
Neovascularisation (blood vessels identified)	
None	0
<10	1
11–50	2
>50	3
Collagen/elastic fibers	
Totally disorganised	0
Slightly organised	1
Moderately organised	2
Well organised	3

ECM, extracellular matrix.

treated (native), while the third part was decellularised and processed further according to the proprietary protocols as described in our previous reports [5, 6].

2.1.2. Histology and immunohistochemistry of the native and acellular tissue-engineered xenografts

Native and ATE tissues were fixed in 10% phosphate-buffered formalin or Carnoy's fixative (three parts 96% ethanol and one part acetic acid). Paraffin sections were cut to 5 μm thickness. Slides were stained with haematoxylin and eosin (H&E) for evaluation of cell removal and with Sirius Red and elastic Van Gieson (EVG) for visualisation of collagen and elastic fibers. Immunohistochemical analysis was performed for carbohydrate antigens, α-Gal [α-Gal epitope (M86), Alexis Corporation, Lausen, Switzerland] and 3C9, which recognises T-antigen (Galβ1–3GalNAc bound α1–3 to Ser/Thr), in the native and decellularised tissues. Bounded primary antibodies were visualised with horseradish peroxidase-conjugated mouse anti-human (Sigma, Saint Louis, MO, USA) antibody subsequently counterstained with haematoxylin.

2.2. Method II

2.2.1. Patients and clinical features

Twenty-four patients were divided into four groups, with each group consisting of six patients, and sera from these patients were taken for the study. The patients were divided into groups as follows: (1) with mechanical heart valve replacement (the control group, $n=6$); (2) with BHV replacement (experimental group, $n=6$); (3) replacement with ATE tissues [experimental group, i.e. with BJV implant, $n=6$) and (4) with PPA implant, $n=6$)]. Serum samples from the four groups of patients were obtained before surgery and eight days after the surgical intervention. The serum was stored at −80 °C. Bioprostheses were obtained from Edwards Lifesciences™, Irvine, CA, USA and mechanical valves were procured from Medtronic™ Inc, Minneapolis, MN, USA. Modified sandwich ELISA assay was performed to compare the incidence of naturally occurring cytotoxic anti-α-Gal IgM antibodies before the surgery and eight days after cardiac surgery, as previously described [7].

2.3. Method III

2.3.1. Histology and immunohistochemistry for explanted acellular tissue-engineered xenografts

From January 2005 through June 2009, 79 ATE BJV implants (age group, five months–45 years) and 90 ATE PPA implants (age group, three months–59 years) were performed in patients. The majority of the implant patients were below 10 years of age. Two ATE PPAs and five ATE BJVs were explanted for various reasons. Surgically explanted tissue was obtained from aneurysmally dilated conduits, small tissue bit from the ATE porcine graft during correction of residual ventricular septal defect (VSD) through the porcine graft and from deceased patients in whom death was unrelated to the conduit (Table 1). There was no his-

Table 2. Patient clinical information

Case no.	Implant material	Clinical reason for explants/death	Age at implant	Implant duration
1	PPA	Congestive heart failure, hepato-renal failure, death	23 years	14 months
2	PPA	Aneurysmally dilated conduit	Eight years	Four months
3	BJV	Piece of BJV Dilated conduit	Two years	20 months
4	BJV	Atrio–ventricular (A–V) canal defect with TOF with pulmonary artery stenosis tissue bit of BJV, as residual A–V valve regurgitation required reintervention	Two years and eight months	Four months
5	BJV	Thickened fibrosed contracted BJV required patch augmentation; in order to do so received a small sample during dissection	One year	14 months
6	BJV	Aneurysmally dilated conduit	Six months	Three months
7	BJV	Dilatation due to distal obstruction by peel formation, death	Three months	Four days

PPA, porcine pulmonary artery; BJV, bovine jugular vein; TOF, tetrology of fallot.

Table 3. Histological scoring of the acellular tissue-engineered xenografts

Cases	Collagen I/ECM	Collagen III	α SMA	vWF	T-antigen	CD11b	CD3	Extent of neovascularisation
TE PPA								
Case 1	3	1	2	2	0	1	0	3
Case 2	2	1	2	1	0	2	1	1
TE BJV								
Case 3	2	2	2	2	0	1	0	2
Case 4	1	2	2	1	0	1	1	0
Case 5	2	2	2	1	0	1	0	2
Case 6	2	1	1	1	0	1	1	1
Case 7	1	1	2	1	0	2	1	0

ECM, extracellular matrix; PPA, porcine pulmonary artery; BJV, bovine jugular vein; TE, tissue-engineered; SMA, smooth muscle cell actin; vWF, von Willebrand factor.

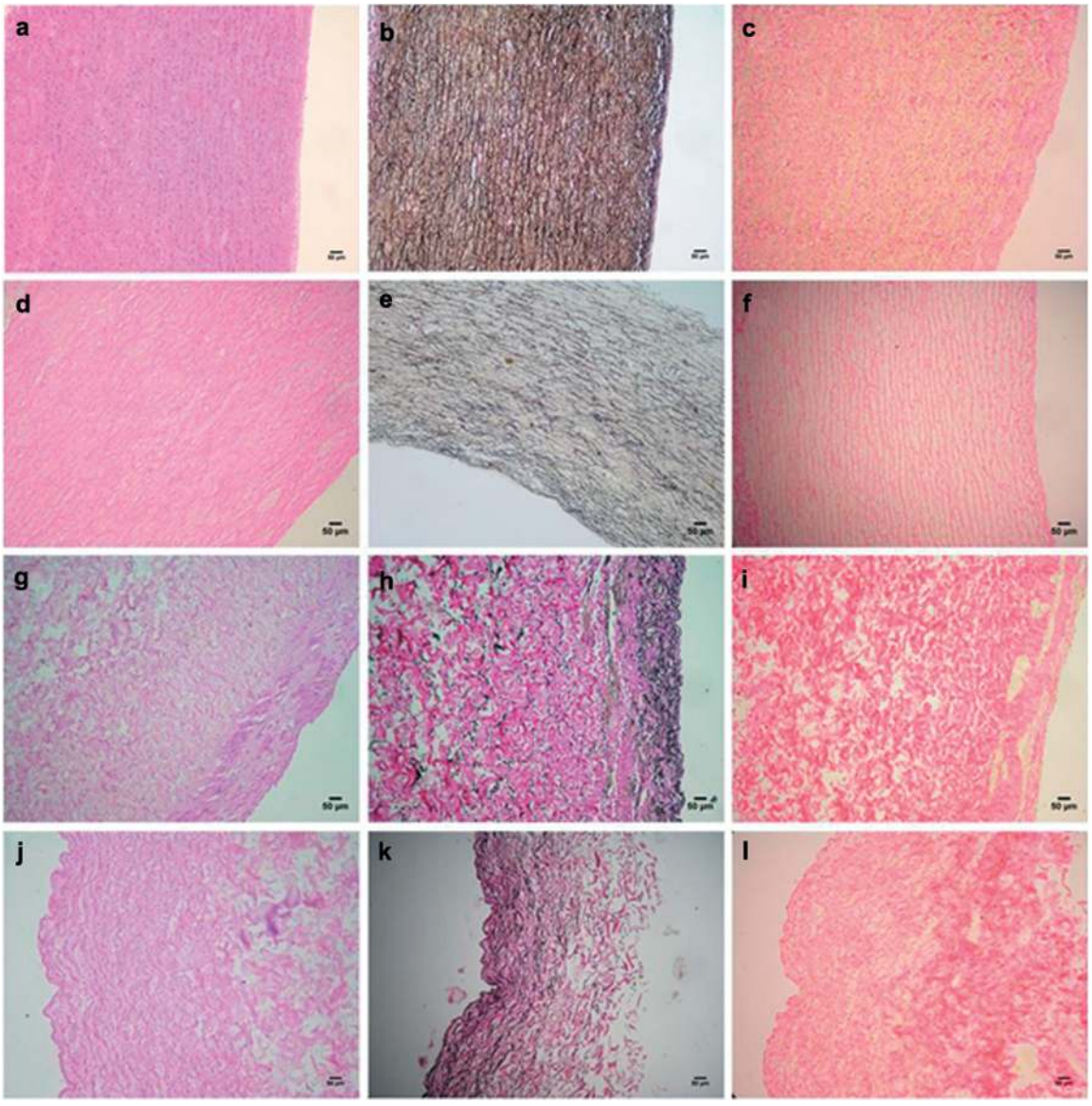

Fig. 1. Histological characterisation of native porcine pulmonary artery (a–c) and bovine jugular vein (g–i) and tissue-engineered porcine pulmonary artery (d–f) and bovine jugular vein (j–l). Sections were stained with H&E (a, d, g, j), EVG (b, e, h, k), Sirius Red (c, f, i, l). H&E, haematoxylin and eosin stain; EVG, elastic Van Gieson. Tissue-engineered porcine pulmonary artery and bovine jugular vein demonstrated efficient preservation of extracellular matrix structures with mild distortion of elastic fibres (e, f) and collagen (k, l) with no detectable nuclear staining (d, j). Bars: 50 μm.

tory of infective endocarditis in any of the explanted tissue. This study includes evaluation of the explanted ATE xenografts to ascertain how the host tissue reacts to these grafts (Table 3).

Standard histological sections were stained with H&E, Sirius Red and EVG to study cellular infiltration, vascularity, and immune response; collagen and elastic fibers distribution, respectively and also stained for Von Kossa (to detect calcification). Serial sections (5 μm) were stained with primary antibodies specific for α SMA (smooth muscle cell actin) (Sigma, Saint Louis, MO, USA), Collagen I (Sigma, Saint Louis, MO, USA), Collagen III (Sigma, Saint Louis, MO, USA), von Willebrand factor (vWF, endothelial cell marker) (Dako, Glastrup, Denmark), CD3 (T-lymphocyte marker) (Sigma, Saint Louis, MO, USA), CD11b (neutrophils, monocytes, macrophages, dendritic cells, CD8-T cells and natural killer cells) (Santacruz, CA, USA). Bounded primary antibodies were visualised with horseradish peroxidase-conjugated mouse anti-human (Sigma, Saint Louis, MO, USA) or rabbit anti-human (Dako, Glastrup, Denmark) antibody subsequently counterstained with haematoxylin. The ATE tissues were evaluated by histological methods with a scoring parameter for the presence of angiogenesis, cellular infiltration, host neo-extracellular matrix (ECM) deposition and connective tissue organisation (Table 2).

2.4. Statistical analysis

Statistical analysis was performed using SPSS software (SPSS for Windows Version 16; SPSS Inc, Chicago, IL, USA). Due to the reasonably small sample size and non-parametric distribution of the data, the Mann–Whitney *U*-test was used to calculate the significance of the data and a *P*-value of 0.05 was considered to be statistically significant.

3. Results

3.1. Removal of cellular components (*Method I*)

Histological analysis of ATE xenograft (H&E) showed no evidence of cells in the tissue sections (Fig. 1d and j). Examination of the sections stained for elastic fibers and collagen (EVG and Sirius Red) showed that the major histoarchitecture of the ECM (collagen and elastic fibres) is retained with mild distortion in the ATE PPA (Fig. 1e and f) and BJV (Fig. 1k and l) as compared to, respective native tissues (Fig. 1b,c,h and i). Immunostaining of tissue sections with an antibody against porcine or bovine for α-Gal and T-antigen revealed the presence of cellular structures in the native tissue (Fig. 2a and c) but no detectable staining in ATE xenografts (Fig. 2b and d).

3.2. Serum anti-α-Gal IgM antibodies (*Method II*)

Bioprosthetic valve recipients displayed a significantly increased mean ± standard error of mean (S.E.M.) at an optical density (OD) value of 405 nm in the concentration of anti-α-Gal IgM antibodies in the serum (26.08 ± 5.80%) as compared to the ATE PPA (−3.93 ± 1.12%), ATE BJV (−5.93 ± 1.11%) ($P < 0.05$). The recipients of mechanical prostheses (control group) showed no percentage increase in their serum, for anti-α-Gal IgM antibodies (−10.13 ± 5.15%) (Fig. 2e). There was no significant difference between ATE PPA and ATE BJV in concentration of anti-α-Gal IgM antibodies in the serum.

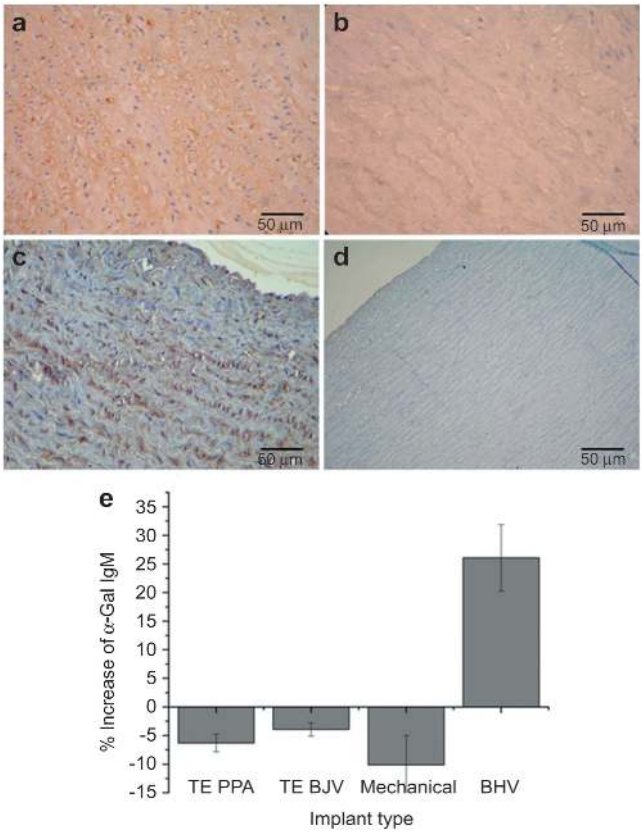

Fig. 2. Immunohistochemical characterisation of native (a, c) and tissue-engineered (b, d) porcine pulmonary artery. Immunohistochemical analysis of porcine pulmonary artery for α-Gal (a, b) and T-antigen (c, d). No detectable immunostaining for α-Gal (b) and T-antigen (d) was observed in tissue-engineered porcine pulmonary artery. Bars: 50 μm. (e) A significant increase in mean ± S.E.M. at OD value 405 nm in the concentration of anti-α-Gal IgM observed in commercial BHV recipients when compared with recipients of mechanical prostheses patients and tissue-engineered PPA and BJV ($P \leq 0.05$). BHV, bioprosthetic heart valve; PPA, porcine pulmonary artery; BJV, bovine jugular vein; TE, tissue-engineered. Bars: 50 μm.

3.3. Explanted tissue-engineered xenograft (*Method III*)

The mononuclear cells infiltration was seen extending to adventitial surface through a cellular fibrocollagenous wall (Figs. 3a and 4a,k). EVG/Sirius Red staining along with immunostaining for Collagen I and Collagen III showed well-organised elastic fibres and collagen in case one (Fig. 3b–e) and case five (Fig. 4b–e) as compared to case four (Fig. 4l) (Table 3). Vasculature in the graft was assessed by staining of endothelial cells (vWF, Figs. 3f and 4f) and smooth muscle (α SMA, Figs. 3g and 4g). Sparse small vessels were detected in the matrix throughout (Figs. 3f,g and 4f,g). There was no calcification observed in explanted tissues (data not shown).

The explants that were more than 14 months old post-surgery contained weak to no staining for CD3 (Figs. 3j and 4j) and CD11b immune-positive cells (Figs. 3i and 4i), although moderate detectable staining was evident in most explants that were four days to four months old

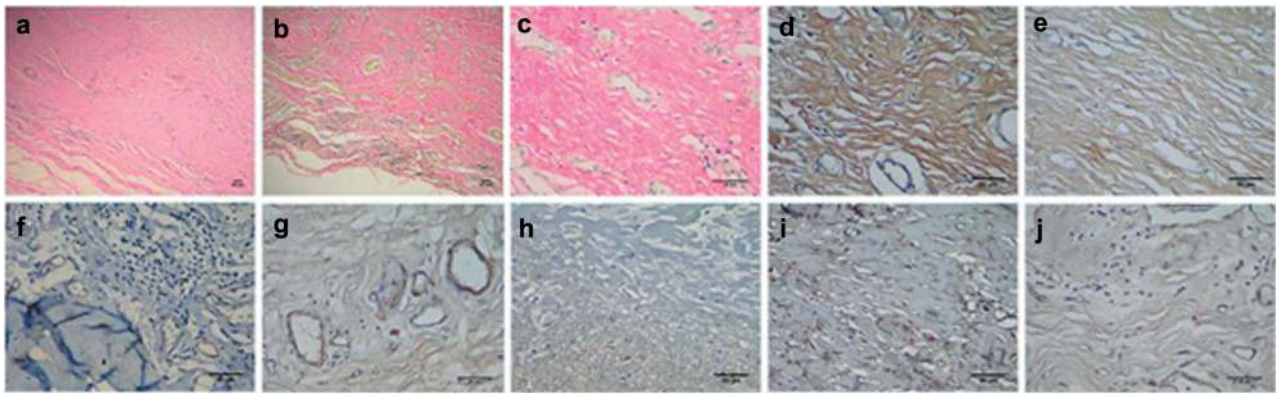

Fig. 3. Histological and immunohistochemical staining of explanted tissue-engineered porcine pulmonary artery (case one) for H&E (a), EVG (b), Sirius Red (c), Collagen type I (d), Collagen type III (e), von Willebrand factor (f), α SMA (g), T-antigen (h), CD11b (i) and CD3 (j). Autologous recellularisation (a), increase in collagen staining (c–e), with neovascularisation [vWF (f) and α SMA (g)] and mild to no detectable immunostaining for CD11b (i) and CD3 (j) was observed. Bars: 50 μm.

(Fig. 4n and o) (Table 3). There was no acute or chronic immune rejection observed for any of the implanted ATE xenografts (according to the signs, symptoms and the clinical parameters, data not shown).

4. Discussion

Tissue-engineered heart valves and vascular structures engineered from a xenogenic or an allogenic source eventually covered with autologous endothelial cells are considered to overcome shortcomings of cryo-preserved homografts and have already been successfully implanted in humans and animals [8, 9]. Stable cross-links in cellular and decellularised matrix proteins are considered to reduce immunogenicity [10], but glutaraldehyde treatment for cross-linking has been demonstrated to increase calcification by fixing cellular debris in place with reduced performance and longevity [2, 11].

However, the role of carbohydrate antigen in mediating immune responses to xenogenic ECM is not well-understood. The present study investigated the presence or absence of the carbohydrate epitope in the ATE tissues (i.e. PPA and BJV) and their effects on implantation. The analysis of increase or decrease in levels of cytotoxic (anti-α-Gal IgM) antibodies in cellular and decellularised tissues implanted in patients are less studied. This study showed that implantation of cellular bioprostheses elicits a specific humoral-mediated immune response directed against α-Gal, which is minimal in ATE recipients.

The findings from the present study are consistent with the observed clinical success of xenogenic ECM scaffolds for a variety of applications [12]. All ATE tissues were well-

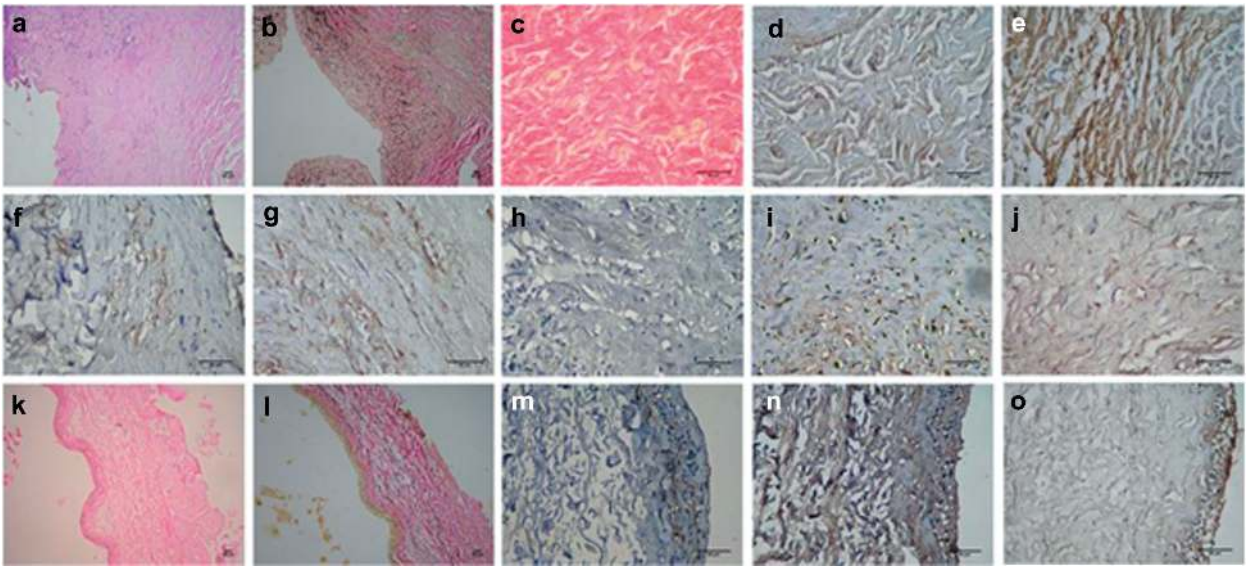

Fig. 4. Histological and immunohistochemical staining of explanted tissue-engineered bovine jugular vein (cases four and five) for H&E (a, k), EVG (b, l), Sirius Red (c), Collagen type I (d), Collagen type III (e), von Willebrand factor (f, m), α SMA (g), T-antigen (h), CD11b (i, n) and CD3 (j, o). Sections of immunohistochemical staining of bovine jugular vein for cases five (a–j) and four (k–o). Increase in autologous recellularisation, increase in collagen staining, neovascularisation (vWF and α SMA) and mild to no detectable immunostaining for CD11b and CD3 were observed in case five (implant duration 14 months) as compared to case four (implant duration four months). Bars: 50 μm.

tolerated by the recipients. It has been found that the collagen organization of the implanted ATE tissues increases in course of time.

Antibody reaction with α-Gal and T-antigen in tissues, is associated with haemolysis; and hyperacute and acute humoral xenograft rejection followed by reduced in vivo survival had already been reported [3, 4], which was not present in any of our ATE xenografts. In the present study, ATE tissue recipients displayed significant decrease in serum levels of anti-α-Gal IgM antibodies as compared to commercial bioprosthetic tissues without implant failure. The host histological response to engrafted tissue regardless of species of origin had shown initial normal innate response (acute wound healing response) which had subsided subsequently. Early biopsy specimens were characterised by an irregularly dispersed infiltration of mononuclear cells peaking from day four to four months of implantation. Host remodelling of the tissue was well advanced and the tissue was no longer distinguishable from the native tissue with decreased inflammatory response which was evident beyond one year of surgery compared to early periods.

Notably, a recent study on the implantation of Food and Drug Administration (FDA)-approved engineered heart valves (decellularised porcine valves) has failed, leading to the death of three out of four patients [13]. The published data from Vienna by Simon et al. proved no sign of graft reseeding, even one year after implantation [13]. In this study, inflammatory stimulus was effectively reduced with increase in autologous recellularisation (endothelialisation) and supplemental neovascularisation with absence of calcification in explanted ATE tissues. Abating chronic inflammation and absence of calcification are the result of a considerable decrease in antigenicity due to decellularisation, and non-use of glutaraldehyde cross-linking. These findings are consistent with previous studies of xenogenic and homograft ECM implanted in humans and primates, in which recellularisation was observed [10, 14, 15]. The limitations of this study are the small number of explants and the restriction of the data from one health centre only.

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