Adverse results of a decellularized tissue-engineered pulmonary valve in humans assessed with magnetic resonance imaging

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Abstract

OBJECTIVES: Matrix P® and Matrix P plus® tissue-engineered pulmonary valves (TEPV) were offered as an improvement for pulmonary valve replacement (PVR) because of recellularization by host cells. The high frequency of graft failure gave reason to evaluate the underlying morphological substrate using magnetic resonance imaging (MRI) and histology.

METHODS: Between June 2006 and August 2008, 17 Matrix P® and 10 Matrix P plus® TEPVs were implanted in 26 patients with a median age of 12.4 (range: 0.8–38.7, interquartile range: 6.1–18.1) years. The grafts were studied by MRI, and underwent histological examination when explantation was required.

RESULTS: Surgical (n = 13) or transcatheter (n = 1) TEPV replacement because of graft failure was needed in 14 cases (52%) 19 (0.5–53) months after implantation. MRI detected significant TEPV stenosis with mild insufficiency (Vmax = 3.7 ± standard deviation 0.5 m/s, regurgitant fraction (RGF) = 10 ± 3%) and stenosis with moderate-to-severe insufficiency (Vmax = 3.5 ± 0.8 m/s, RGF = 38 ± 10%) in 6 patients, respectively, and severe insufficiency (RGF = 40%) in 1 patient. In patients with graft failure, MRI showed hyperenhancement and TEPV wall thickening. Histology revealed severe inflammation, increased fibrous tissue and foreign-body reaction against valve leaflets and fascial tissue, while TEPV endothelialization was not detected in any case.

CONCLUSIONS: The high frequency of Matrix P® and Matrix P plus® graft failure can be related to inflammation and fibrosis revealed by MRI and histology. Our results do not support the use of these valves for PVR and suggest careful follow-up examinations, including MRI for early detection of graft inflammation and fibrosis.

Keywords: Tissue engineering  Pulmonary valve replacement  Immune response  Late gadolinium enhancement  Magnetic resonance imaging

INTRODUCTION

Pulmonary valve replacement (PVR) is often needed in patients with congenital heart defects. Various types of grafts for PVR exist, including synthetic-, homograft- and xenograft-based prostheses, which, however, show variable disadvantages. Biological valves have still dissatisfying life time due to graft failure and outgrowth in children [1, 2], while mechanical heart valves require anticoagulation with the inherent risk of bleeding and potential thromboembolic events, but have the advantage of long-term durability [3].

Decellularized tissue-engineered heart valves (TEPV) have recently been developed to overcome some of these disadvantages. It has been shown that reduction in cellularity enables host recellularization of the implanted valve, which may contribute to a normal valve function [4, 5]. Furthermore, it has been proposed that antigen reduction by decellularization of allograft heart valves may have a favourable effect on long-term durability by mitigating host immune response [4].

In this study, we present our clinical results as well as magnetic resonance imaging (MRI) and histological findings in a recent cohort of patients treated with a decellularized xenograft, Matrix P® or Matrix P plus® TEPV, for right ventricular outflow tract reconstruction.

MATERIALS AND METHODS

Patients

Between June 2006 and August 2008, 26 patients underwent PVR using a decellularized TEPV (Matrix P® or Matrix P plus®, AutoTissue GmbH, Berlin, Germany) at the University Hospital of Schleswig-Holstein, Kiel, Germany. Patient and surgical characteristics are...
presented in Table 1. One patient received two consecutive Matrix P plus® conduits after graft failure.

Data collection

After TEPV implantation, patients were seen at regular intervals (first visit 1 week after discharge, then every 6 months) in our outpatient department or by their referring cardiologists. We retrospectively reviewed hospital records, surgical reports and MRI, echocardiographic and angiocardiographic studies of all patients, as well as clinical follow-up reports from the patient’s cardiologists. Histological examinations were performed in explanted xenografts.

The day of TEPV implantation was considered as the starting point of xenograft survival. Xenograft failure was defined as any graft-related conditions leading to graft replacement. One patient died of unknown reasons and was censored at the time of death.

The median follow-up time was 46 (range: 0.04–73) months.

Surgical technique of pulmonary valve replacement

By use of cardiopulmonary bypass and mild hypothermia (35°C), the TEPV was implanted between the right ventricle and the pulmonary artery in all cases. The proximal anastomoses were performed with 5/0 or 6/0 polypropylene suture. The TEPV was implanted between the right ventricle and the pulmonary artery. The scan parameters were as follows: field of view (FOV) 270 × 270 mm; voxel size 1.64 × 1.4 × 7 mm; repetition time/echo time (TR/TE), 4.4/2.7 ms; flip angle, 15°. Axial, right ventricular outflow tract and right ventricular long axis views were acquired. The images were analysed with dedicated software (Extended MR WorkSpace, version 2.6.3.2 HF3 2010, Philips Medical Systems, Netherlands). Seventeen patients had a comprehensive follow-up MRI at a median time of 11.7 (range: 2.0–34.2, interquartile range: 4.9–18.4) months after PVR.

For conduit visualization and measurement as well as assessment of right ventricular size and function retrospectively, gated cine images were obtained using a gradient-echo cine sequence with the following sequence parameters: voxel size, 1.88 × 1.94 × 5 mm; repetition time/echo time (TR/TE), 4.4/2.7 ms; flip angle, 15°. Axial, right ventricular outflow tract and right ventricular long axis views were acquired. The images were analysed with dedicated software (Extended MR WorkSpace, version 2.6.3.2 HF3 2010, Philips Medical Systems, Netherlands).

Phase-contrast cine imaging was used to measure pulmonary blood flow, with a slice plane perpendicular to the main pulmonary artery. The scan parameters were as follows: field of view (FOV) 270 × 270 mm; voxel size 1.64 × 1.4 × 7 mm; TR/TE 15/2.5 ms; flip angle 30°; maximum velocity encoding 400 cm/s. Flow software (Extended MR WorkSpace, version 2.6.3.2 HF3 2010, Philips Medical Systems, Netherlands) was used to calculate pulmonary systolic forward flow and regurgitant fraction (RGF).

For tissue characterization, late gadolinium enhancement (LGE) imaging 10–15 min after contrast injection (Magnevist, Bayer Pharma AG, Berlin, Germany; 0.1 mmol/kg) using an ECG triggered 3D inversion recovery sequence (FOV 300 × 178 × 80 mm; voxel size 1.17 × 1.27 × 10 mm; TR/TE 3.7/1.83 ms; flip angle 15°) was applied in the same axial and right ventricular outflow tract planes that were used for cine imaging. Furthermore, T1- and T2-weighted turbo spin echo sequences were performed before and after administration of contrast agent (Scan parameter: (i) T1-weighted turbo spin echo sequence: FOV 340 mm; TR/TE

<table>
<thead>
<tr>
<th>Table 1: Patient and surgical characteristics</th>
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<tr>
<td>Parameter</td>
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<tr>
<td>Age, years, median (range, IQR)</td>
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<tr>
<td>Female/male, n</td>
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<td>Type of CHD, n (%)</td>
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<td>Pulmonary valve regurgitation, n (%)</td>
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<td>Homograft or conduit replacement, n (%)</td>
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<td>Ross procedure, n (%)</td>
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<td>Pulmonary stenosis, n (%)</td>
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<td>Biventricular surgical correction, n (%)</td>
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<td>TEPV type, n (%)</td>
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<td>TEPV size, mm, mode (range)</td>
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</table>

CHD: congenital heart disease; IQR: interquartile range; TEPV: tissue-engineered pulmonary valve.

Echocardiography

Transthoracic echocardiography was performed using a GE Vivid 7 Dimension ultrasound imaging system (GE Healthcare, Munich, Germany) equipped with a multifrequency MHz transducer. With the patient in the lateral supine position, pulmonary Doppler recordings were obtained from the parasternal short axis with the cursor placed at the level of the pulmonary annulus. All echocardiograms were stored digitally and therefore were available for offline analysis.

Right heart catheterization

In 12 patients, right heart catheterization was performed at a median time of 13.9 (range: 0.5–32.3, interquartile range: 6.5–27.6) months postoperatively to investigate the haemodynamic relevance of a TEPV stenosis, insufficientity or the combination of both. All data were recorded digitally for later analysis.

Magnetic resonance imaging

MRI studies were performed with a 1.5-T scanner (Achieva, Philips Medical Systems, Netherlands). Seventeen patients had a comprehensive follow-up MRI at a median time of 11.7 (range: 2.0–34.2, interquartile range: 4.9–18.4) months after PVR.

For conduit visualization and measurement as well as assessment of right ventricular size and function retrospectively, gated cine images were obtained using a gradient-echo cine sequence with the following sequence parameters: voxel size, 1.88 × 1.94 × 5 mm; repetition time/echo time (TR/TE), 4.4/2.7 ms; flip angle, 15°. Axial, right ventricular outflow tract and right ventricular long axis views were acquired. The images were analysed with dedicated software (Extended MR WorkSpace, version 2.6.3.2 HF3 2010, Philips Medical Systems, Netherlands).
Histology

In 10 of 12 patients in whom conduit explantation was necessary, a detailed histological examination of the conduit was done. They were fixed in 4% neutral buffered formaldehyde, and the macroscopic findings were documented. Afterwards, longitudinal sections of the conduit were taken, paraffin-embedded and tissue sections were stained histochemically for haematoxylin and eosin (H&E), Massons trichrome and Elastica van Gieson according to standard protocols. Additionally, immunohistochemical stains were performed for macrophages (CD68, 1:100, Neomarkers, Thermo Fisher Scientific, Inc., Kalamazoo, MI, USA), endothelial cells (CD34, 1:700, Beckman-Coulter, Krefeld, Germany) as well as B- and T-cells (CD20, 1:20, Clone JH1; CD4, 1:25, Novocastra, Leica Biosystems GmbH, Nussloch, Germany; CD8, 1:200, Dako, Glostrup, Denmark), using an automated system with DAB chromogen detection (Menarini Diagnostics, Berlin, Germany). Sections were analysed quantitatively (thickness of conduit in mm) and semiquantitatively for extent of inflammation, thrombus formation and presence of recellularization of graft tissue. An Axioscope microscope with AxiosCam MRc digital camera and computer-assisted morphometry unit (Zeiss, Axiosvision, Rel. 3.0, Oberkochen, Germany) were used for analysis and micrographs. Semiquantitative grading included a four-tiered system for leaflet thickening (0=absent, 1=slightly, 2=moderate, 3=heavy) and for fibrin content, inflammation, foreign-body reaction (against leaflets, fascial tissue and avital arteries) as well as endothelialization (for all parameters: 0=absent, 1=focal, 2=confluent but not continuous, 3=continuous). A two-tiered system was used for presence of avital porcine arteries of the graft wall (0=absent, 1=present).

Statistical analysis

Analysis used the Medcalc® statistical software (version 12.2.1.0, Ostend, Belgium). For continuous variables, the number of patients, mean value with standard deviation, median, interquartile range and minimum and maximum are reported, as appropriate. For categorical variables, the number and percentage of patients are provided. The freedom from TEPV failure was estimated by means of the Kaplan–Meier method.

RESULTS

Patients

Follow-up was complete in all the 26 patients. One patient with pulmonary atresia and severe pulmonary hypertension underwent replacement of a pulmonary homograft with a Matrix P plus® because of bacterial endocarditis. Shortly after surgery, the patient developed severe Matrix P plus® insufficiency and the valve had to be replaced by a pulmonary homograft 2 weeks later. The patient died due to pulmonary bleeding after this procedure. Another patient died 2 years after TEPV implantation unexpectedly at home for unknown reasons; no autopsy was performed.

Thirteen patients (50%) underwent a reoperation and 1 (4%) a transcatheter PVR (Melody®) due to failure of a Matrix P® (n = 5) and Matrix P plus® (n = 9) graft, respectively. The replacement was needed for stenosis (n = 9; 64%) and a combination of insufficiency and stenosis (n = 4; 29%). The remaining 14th patient received the graft because of a homograft endocarditis and needed reoperation 2 weeks later because of acute insufficiency of the TEPV. The median time to TEPV failure was 19 (range: 0.5–53, interquartile range: 10–30) months. The 2-year freedom rate from xenograft failure was 67% (95% confidence interval: 49–85%).

Echocardiography and right heart catheterization

Echocardiographic examination at the time of graft explantation (n = 13) or transcatheter pulmonary valve implantation (n = 1) showed a peak Doppler gradient across the xenograft of 73 ± (standard deviation) 32 mmHg (peak velocity 4.2 ± 1.0 m/s). Moderate or severe pulmonary insufficiency was present in four patients. In 9 patients cardiac catheterization prior to surgery was done. The peak systolic gradient across the right ventricular tract was 56 ± 27 (range: 30–116, interquartile range: 40–64) mmHg and the peak systolic right ventricular pressure was 78 ± 30 (range: 41–134, interquartile range: 50–113) mmHg.

Magnetic resonance imaging

MRI detected a severe TEPV stenosis (peak velocity: 3.7 ± 0.5 m/s) with mild insufficiency (RGF: 10 ± 3%) in 6 patients. While a stenosis (peak velocity: 3.5 ± 0.8 m/s) with moderate-to-severe insufficiency (RGF: 38 ± 10%) was found in 6 patients. Another patient developed isolated severe insufficiency (RGF: 40%). The patient, who underwent two implantations of a TEPV, again developed graft stenosis and mild insufficiency documented by MRI (peak velocity: 4 m/s, RGF: 11%) after the second operation. Only in 4 patients, the conduit showed no relevant stenosis or insufficiency (Fig. 2). LGE- and post-contrast T1-weighted black blood imaging detected severe hyperenhancement of the entire conduit wall and the surrounding tissue in all cases with a TEPV stenosis (Figs 3–5).
Histology

The 10 evaluated explanted grafts (eight Matrix P®, two Matrix P plus®) showed inflammation and increased fibrous tissue content of their wall and leaflets (Table 2, Fig. 6). Irrespective of time since implantation, they showed mostly a heavy granulomatous and partly foreign-body inflammatory reaction at the interface to porcine leaflets, fascial or vascular tissue. All but one graft contained an avital porcine artery (Table 2, Fig. 6). No graft (including all parts) revealed re-endothelialization or other types of recellularization (Table 2, Fig. 6).

DISCUSSION

This study confirms the recently reported disappointing clinical results of Matrix P® and Matrix P plus® TEPV [6–8] and adds new insights into the aetiology and pathophysiology of graft failure. On follow-up examinations, MRI showed significant hyperenhancement and abnormal wall thickening in all failing xenografts. This correlated with extensive inflammation and increased fibrosis tissue content as detected by histological examinations of explanted conduits. Additionally, no re-endothelialization of wall and leaflets was detected in any of the explanted TEPVs.
Clinical aspects of TEPV failure

The inventors of TEPVs announced to provide a valve that has reduced immunogenicity, allows repopulation with viable autologous vascular cells [4, 5, 9] and therefore may have a longer conduit durability. The present study, however, documents the adverse results of Matrix P® and Matrix P plus® grafts which have been processed with a patented special procedure (AutoTissue GmbH, Berlin, Germany). Xenograft failure requiring reoperation or transcatheter PVR occurred in 14 cases (52%) <2 years after implantation due to TEPV stenosis, insufficiency or both. Two recent studies reported similar clinical results using the same graft types [6, 7]. Rüffer et al. [6] found a rate of 38% reoperations for Matrix P plus® failure 2–15 months after implantation in a group of 16 patients needing PVR. Perri et al. [7] reported Matrix P® or Matrix P plus® failure in the pulmonary position in 33 of 93 patients during a median follow-up period of 12 months (2 days to 51 months); the 2-year freedom from xenograft failure was 60%. In both studies, the most common reason for failure was xenograft obstruction [6, 7]. This was true also in our cohort; however, 6 of these 12 cases showed also relevant regurgitation.

In contrast to these findings, da Costa et al. [10] showed a normal function of decellularized pulmonary allografts of the same provider (AutoTissue GmbH) in 11 patients during a follow-up period of 18 months after Ross operation. The different results compared with our study may be explained by the different origin of the valve. da Costa et al. used a decellularized allograft, whereas in our study xenografts were implanted. Another explanation for the contrasting results may be the shorter follow-up period, besides the smaller size of the patient cohort. In another study, reporting with a different TEPV type (CryoValve SG), only 19 of 342 patients developed valve-related failure during a mean follow-up period of 4 years [11]. Apart from the dissimilar origin of the valve (allograft), the differences to our study may be related to a different decellularization process of the manufacturer. Compared with right ventricular outflow tract reconstruction with other xenograft or allograft types which were not decellularized [12, 13], our data demonstrate that the durability of Matrix P® or Matrix P plus® TEPV is much lower [14, 15]; thus, the negative results of our and previous studies [6, 7] may be due to the decellularization process itself. Another reported cause for graft failure may be an unfavourable haemodynamic situation, such as a high pulmonary vascular

Table 2: Histology of explanted tissue-engineered pulmonary valves

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Maximal wall thickness (mm)</th>
<th>Leaflet thickening a</th>
<th>Fibrosis b</th>
<th>Inflammation b</th>
<th>Foreign-body reaction in the leaflets b</th>
<th>Foreign-body reaction in fascial tissue b</th>
<th>Avital artery c</th>
<th>Foreign-body reaction against porcine artery b</th>
<th>Endothelialization b</th>
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<tr>
<td>1</td>
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a Grading of leaflet thickening: grade 0=absent, grade 1=slightly, grade 2=moderate, grade 3=heavily.

b Points allocated for various grades were as follows: grade 0=absent, grade 1=focal, grade 2=confluent, grade 3=continuous.

c Grading of avital artery: grade 0=absent, grade 1=present.

Figure 5: Matrix P plus® TEPV 2.3 years after PVR. (A) Axial T2-weighted black blood image before administration of contrast agent. Severe xenograft wall thickening. (B) Hyperenhancement of the TEPV (T1-weighted black blood image after contrast).
resistance or pulmonary artery pressure [16]. In our series, only 1 patient showed severe pulmonary hypertension, which may have contributed to severe TEPV insufficiency shortly after implantation. The surgical technique of PVR can also be a factor that influences TEPV durability. However, all patients were operated by 2 experienced cardiac surgeons, using the same technique for correct sizing and trimming of the TEPV.

Possible reasons for TEPV failure: MRI and histological findings

This paper reports for the first time MRI data of anatomical changes of failing TEPV grafts which showed significant graft hyperenhancement and thickening of the wall shown by LGE-imaging (Figs 3 and 4). This technique was originally used to assess myocardial fibrosis [17], but it can also identify inflammatory processes as myo- and pericarditis or vascular disease [18, 19]. Furthermore, LGE-MRI can detect non-viable tissue of vascular walls [20, 21]. The histological examinations of all the 10 explanted xenografts prove that the described MRI findings result from inflammatory processes leading to increased fibrous tissue content as well as to foreign-body reaction against leaflets and fascial tissue. Histology further discovered avital porcine arteries in almost all explants which cause significant foreign-body reaction. These findings indicate that instead of the expected reduced immune response, decellularized TEPVs induce an ongoing immunological and inflammatory process leading to clinical graft failure. Our findings are in accordance with earlier observations of inflammatory infiltrates and increased fibrous tissue in the wall of explanted Matrix P® and Matrix P plus® xenografts [6–8]. Similarly, an inflammatory response...
with severe foreign-body type reaction was described in 4 paediatric patients with the SYNERGRAFT™ TEPV [22]. Also an in vitro study by Rieder et al. [23] found that the porcine valves are not weakly immunogenic, but cause a substantial mononcytic migratory response, whereas human decellularized heart valves do not exhibit a similar antigenic behaviour. Bastian et al. [24] showed that contact of decellularized porcine pulmonary conduits with human plasma leads to IgG deposition and activation of the classical complement pathway with consequent activation of polymorphonuclear leucocyte.

Besides immune-mediated inflammatory reactions, thrombus formation can lead to early valve dysfunction [25]. However, histology of explanted grafts in this study did only show microscopic thrombotic material, but the amount was not sufficient to cause valve dysfunction. Our MRI and histological data suggest that the main reason for early Matrix P® and Matrix P plus® TEPV failure is related to immunological factors.

It was suggested that decellularization of allograft heart valves will allow host recellularization [5, 6]. Cicha et al. found an endothelialization of Matrix P plus® valve leaflets, whereas other parts of the valve showed only a low recellularization [8]. In contrast, histology of explanted TEPVs in this series showed no endothelialization of the entire xenograft independent of the time interval since implantation.

Limitations

As soon as unfavourable results after PVR with Matrix P® and Matrix P plus® TEPVs were found, we immediately stopped their use. Therefore, the number of patients is lower than in previous studies with other xenograft or allograft types.

CONCLUSION

Based on our MRI and histological findings, Matrix P® and Matrix P plus® TEPVs showed an abnormal immunologic response leading to significant and long-standing xenograft inflammation, which seems to be the most important reason for graft stenosis and failure. The asserted endothelialization of the valve leaflets could not be confirmed by histology.

As a consequence of the disappointing results early after surgery, the use of these grafts can no longer be recommended. All patients with Matrix P and Matrix P plus TEPVs are in need of careful follow-up examinations, including especially MRI for early detection of LGE as a marker of inflammation and fibrosis.

ACKNOWLEDGEMENTS

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REFERENCES
