

Anti α -Gal Immune Response Following Porcine Bioprosthesis Implantation in Children

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Background and aim of the study: Porcine bioprostheses have been widely used in cardiac surgery in the treatment of valvular heart disease. However, in younger patients, their use has been limited by early failures known to be associated with an immune response and subsequent degeneration. The natural antibodies directed at Gal α 1, 3-Gal β 1-4GlcNAc-R (α -Gal), have been thought to initiate an immune response in humans transplanted with porcine organ xenografts. The study aim was to determine the anti α -Gal immune response following commercial porcine bioprosthesis implantation in children.

Methods: Between January 2008 and April 2008, 19 consecutive patients underwent pulmonary valve replacement (PVR) with a commercially available porcine bioprosthesis for an incompetent pulmonary valve with congenital heart diseases. The median age at surgery was 132 months (range: 14-330 months). Previous PVR with a porcine bioprosthesis had been performed in seven patients at a median of 44 months (range: 26-117 months) before surgery (re-PVR group). Sera were obtained sequentially five times: immediately before surgery, and at one day, one week, three weeks, and two months postopera-

tively. All serum samples were analyzed using an enzyme-linked immunosorbent assay to investigate the α -Gal immune response.

Results: There were no operative deaths or complications. There was no statistically significant difference between the titers of anti α -Gal antibodies of the PVR and re-PVR groups. The titer of anti α -Gal antibodies (IgM and IgG) was decreased on the first postoperative day, but increased in the first postoperative week, regardless of the isotype. Whilst the titer of the anti α -Gal IgM antibody began to decrease after three weeks postoperatively, the titer of anti α -Gal IgG antibody remained increased after two months.

Conclusion: The implantation of a porcine bioprosthesis elicits the increased formation of anti α -Gal antibodies during the early postoperative period in children, with different patterns between the two isotypes. The IgM antibody response was rapid and transient, while the IgG antibody response was longer and more delayed.

Xenoreactive natural antibodies directed at the Gal α 1, 3-Gal β 1-4GlcNAc-R (α -Gal), epitope are known to be a major barrier in xenotransplantation. The natural anti-pig antibodies in human serum react predominantly with the α -Gal epitope (1), and α -Gal-xenoreactive antibodies comprise at least 80-90% of anti-pig antibodies in humans (2). Approximately 1% of the circulating antibodies are

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designated to the α -Gal epitope in humans, and although anti α -Gal antibodies are produced throughout the patient's lifetime due to exposure to the gut flora (3), the titers of natural antibodies show individual differences (4). In xenotransplantation, anti α -Gal antibodies mediate the hyperacute rejection with complement activation, and delayed or chronic rejection with an antibody-dependent cellular cytotoxicity mechanism (3,5-7).

The α -Gal epitope is expressed on a variety of tissues in pigs, including the cardiac valve endothelium (1,8-10); recently, the presence of the α -Gal epitope was documented in commercial bioprosthetic valves treated with glutaraldehyde (11).

Many patients who undergo procedures on the right

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Table I: Patient preoperative characteristics.

Patient no.	Age (months)	Diagnosis*	NYHA class	Preoperative medication ⁺	Re-PVR	Re-PVR interval (months)	Type of bioprosthesis [‡]
1	191.3	TOF	2	-	No	-	H
2	13.7	PA, VSD	2	Rhonal	No	-	CE
3	107.6	Truncus	1	Rhonal	Yes	43.9	SJM
4	213.5	TGA, VSD, PS	1	-	No	-	SJM
5	163.9	Truncus	1	-	Yes	25.5	SJM
6	208.5	cc-TGA, VSD, PS	2	DGX, ADT, ENL	Yes	34.5	SJM
7	204.9	Truncus	2	DGX, ENL	Yes	101.9	SJM
8	132.3	TOF	1	-	No	-	SJM
9	281.7	PA, VSD	1	-	No	-	SJM
10	143.4	PA, VSD	2	-	No	-	CE
11	13.9	PA, VSD	2	-	No	-	CE
12	27.5	PA, VSD	2	Rhonal	No	-	CE
13	19.1	PA, VSD	2	-	No	-	CE
14	131.6	PA, VSD	1	-	Yes	116.5	CE
15	22.4	TOF	2	DGX, ADT, ENL, Viagra	No	-	CE
16	181.4	TOF	1	-	Yes	60.2	SJM
17	55.8	cc-TGA, VSD, PS	1	-	Yes	30.2	CE
18	13.5	AORPA	2	ADT, DCZ	No	-	CE
19	329.5	TOF	2	-	No	-	SJM

*Diagnosis: AORPA: Anomalous origin of right pulmonary artery; cc-TGA: Congenitally corrected TGA; PA: Pulmonary atresia; PS: Pulmonary stenosis; PVR: Pulmonary valve replacement; TGA: Transposition of the great arteries; TOF: Tetralogy of Fallot; VSD: Ventricular septal defect.

⁺Preoperative medication: ADT: Aldactone; DCZ: Diclozide; DGX: Digoxin; ENL: Enalapril.

[‡]Valve: CE: Carpentier-Edwards Bioprosthetic Valved Conduit Model 4300 (Edwards Lifesciences); H: Hancock® II Aortic, Medtronic™; SJM: Epic™ SUPRA valve Aortic, St. Jude Medical, Inc.

ventricular outflow tract require pulmonary valve replacement (PVR), with or without a conduit. Although, in most centers (including that of the present authors) the prosthesis of choice for PVR is of porcine origin, such bioprostheses have a limited durability due to valve degeneration that occurs more frequently in younger patients (8,9,12-14). The degeneration of bioprostheses is multifactorial, and includes immunologic reactions, foreign body reactions, blood-surface interactions, chemical factors, infection, mechanical factors, material fatigue, and surgical factors (15). Although the mechanism leading to premature degeneration of the implanted bioprosthesis is not yet fully understood, the immune response has been considered to play an important role as an initial trigger of the degeneration process (16), and α -Gal has been noted as the major epitope.

The aim of the present study was to: (i) investigate whether bioprosthesis implantation elicits an

increased formation of anti α -Gal antibodies; and (ii) monitor perioperative changes in the titer of the anti α -Gal antibodies (IgM and IgG) over time.

Clinical material and methods

Patients

Between January 2008 and April 2008, 19 consecutive patients underwent PVR with porcine bioprostheses produced by three manufacturers: the Epic™ SUPRA valve aortic (St. Jude Medical, Inc.; the HANCOCK® II aortic (Medtronic); and the Carpentier-Edwards Bioprosthetic valved conduit Model 4300 (Edwards Lifesciences). The patients' diagnoses were tetralogy of Fallot or variants thereof in 12 cases, truncus arteriosus in three, congenitally corrected transposition of the great arteries with pulmonary stenosis in two complete transposition of the great arteries with pulmonary stenosis in one, and anomalous origin or right

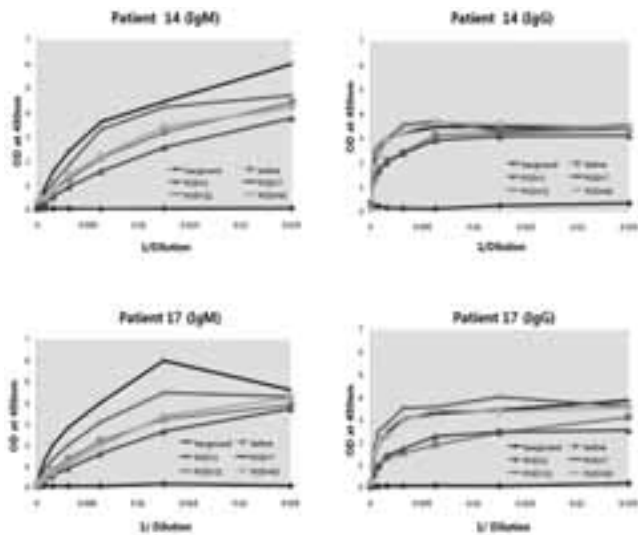


Figure 1: Changes in anti α -Gal activity in patient #14 (PVR group) and patient #17 (re-PVR group). PVR: Pulmonary valve replacement; OD: Optical density; POD: Postoperative day.

pulmonary artery from the ascending aorta each in one case. A previous PVR with a porcine bioprosthesis had been performed in seven patients (the re-PVR group) at a median of 44 months (range: 26 to 117 months) before surgery, while the remaining 12 patients underwent PVR for the first time (the PVR group). The preoperative characteristics of the patients are listed in Table I. The median age at surgery was 132 months (range: 14 to 330 months).

Postoperatively, all patients followed a similar medication protocol. Serum samples were obtained five times from each patient: immediately before surgery, at postoperative day 1, during the first and third postoperative weeks, and during the second postoperative month. All sera were stored in EDTA tubes and analyzed within one day of being obtained.

The study was approved by the institutional review board/ethical committee of the authors' institution, and informed consent was obtained from each of the patients. The study was conducted in accordance with the Helsinki declaration of 1975.

Operative technique

In all patients, PVR was performed using moderate hypothermic cardiopulmonary bypass (CPB) and left atrial venting through the right upper pulmonary vein, with or without myocardial ischemia, under ventricular fibrillation for isolated PVR ($n = 10$) and after release of the aortic clamp in patients with additional intracardiac lesions ($n = 9$).

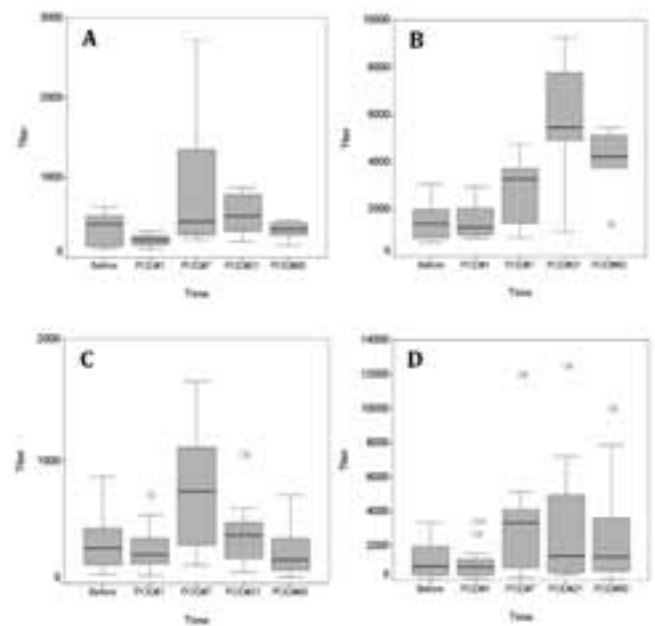


Figure 2: Changes in the anti α -Gal antibody titer in the individual groups. A) Re-PVR IgM titer. B) Re-PVR IgG titer. C) PVR IgM titer. D) PVR IgG titer. PVR: Pulmonary valve replacement; POD: Postoperative day.

ELISA

An ELISA analysis was used to determine the activity of IgM and IgG isotypes of the anti α -Gal antibodies. Bovine serum albumin (BSA) containing synthesized α -Gal (α -Gal-BSA), which was prepared by conjugation of α -Gal linker type 1 (Genkem, Seoul, Korea) and bovine serum albumin (Armresco, Solon, OH, USA) with 1-ethyl-3-[3-dimethylamino-propyl]carbodiimide hydrochloride (Pierce, USA) in conjugation buffer (MES buffered saline pack; Pierce), was used as a solid-phase antigen. Microtiter plates were coated with 100 μ l per well of α -Gal-BSA in PBS buffer (pH 7.4) (at a concentration of 1 μ g/ml for the IgM isotype, or 2.5 μ g/ml for the IgG isotype), and incubated for 1 h at 37°C. The plates were then washed with deionized water. Aliquots of the patient's serum (100 μ l per well) were added to the α -Gal-BSA-immobilized wells at a serial two-fold dilution, from 1:40 to 1: 2,560 in BSA-Triton X-100 (pH 7.4, PBS, 3% BSA, 0.01% Triton X-100). The plates were then incubated for 1 h at 37°C. Donkey anti-human IgG and IgM antibodies (Jackson, Human Research Laboratories Inc.) were used as a secondary antibody at a dilution of 1:15,000 for IgM and 1:10,000 for IgG in BSA-Triton X-100. The optical density (OD) was measured at 450 nm using the Thermo Electron-Lab Systems (Multiskan EX).

Table II: Anti α -Gal titers in the two groups.*

Isotype	Day	re-PVR group (n = 7)	PVR group (n = 12)	p-value
IgM	Before	350.4 \pm 225.5	315.2 \pm 223.7	0.745
	POD 1	213.0 \pm 80.9	288.5 \pm 190.3	0.337
	POD 7	947.4 \pm 936.7	732.7 \pm 482.4	0.515
	POD 21	2081.5 \pm 4222.3	401.4 \pm 260.6	0.178
	POD 60	1318.9 \pm 2646.5	250.3 \pm 195.6	0.173
IgG	Before	1342.0 \pm 979.0	1159.9 \pm 1063.4	0.716
	POD 1	1331.2 \pm 917.2	1066.7 \pm 1022.0	0.580
	POD 7	2540.8 \pm 1618.4	3282.1 \pm 3241.6	0.582
	POD 21	4880.1 \pm 3265.9	3142.5 \pm 3768.2	0.324
	POD 60	4020.5 \pm 1447.2	2664.7 \pm 3268.5	0.352

Values are mean \pm SD.

*Titer defined as the dilution at 1.0 of the OD.

PVR: Pulmonary valve replacement; POD: Postoperative day.

Statistical analysis

Background OD-values were subtracted from the test OD-values. The titer was defined as the dilution at 1.0 of the OD. All data were presented as mean \pm SD, or median plus range, using Microsoft Excel 2007. Comparisons were performed using either Student's *t*-test or a paired *t*-test. A *p*-value <0.05 was considered to be statistically significant.

Results

Clinical results

No early deaths occurred among the patients. The median CPB time was 167 min (range: 94 to 392 min), while in nine patients aortic cross-clamping was required for additional intracardiac procedures. The median myocardial ischemic time was 108 min (range: 55 to 140 min). In all patients, the preoperative preparation, anesthetic management and operative strategy were similar and the hospital course was uneventful, and all were discharged within 10 days after surgery, without complications. Treatment with an angiotensin-converting enzyme inhibitor was required during the early postoperative period in eight patients, and anti-coagulation for six months in all patients. The median duration of follow up was 11 months (range: 8 to 14 months). All patients were in NYHA functional class 1, and none of the patients was administered any cardiac medication (except warfarin) at the last follow up examination.

ELISA: Anti α -Gal antibody titer

Results from random samples (patient #14 of the PVR group and patient #17 of the re-PVR group) are shown in Figure 1. In all cases, the OD decreased

immediately postoperatively (at one day) and then increased after one week, when compared to the pre-operative OD, regardless of the isotype. The ODs of the IgG isotype remained elevated at the end of the study (at 60 days after surgery), but those of the IgM isotype began to decrease at three weeks postoperatively.

Although the postoperative titer seemed to be higher in the re-PVR group (Table II; Fig. 2), there was no significant difference between the two groups (PVR and re-PVR) with regards to the anti α -Gal IgM and IgG titers (Table II). The change in titer of the anti α -Gal antibodies with time was similar in each patient (see Fig. 1). The immediate postoperative (one-day) titer appeared to decrease, though not statistically significantly (Table III; Fig. 3). At one week after surgery the titer increased significantly, up to seven-fold in IgM and 32-fold in IgG (Table III; Fig. 3). The IgM titer began to decrease at three weeks after surgery, but that of IgG increased from three weeks and was maintained until the end of the study (at two months after surgery) (Table III; Fig. 3).

Discussion

As α -Gal is a major barrier for xenotransplantation, much effort has been expended to avoid the immune response directed against it, including immunoglobulin or enzymatic treatment and genetic manipulation (α 1,3-galactosyltransferase knock-out) (17-19). Unfortunately, the α 1,3-galactosyltransferase knock-out pig is not yet available within a clinical setting, and other methods such as enzymatic removal of the α -Gal epitope have caused only a delay in the rejection. The major antibodies that are thought to initiate the hyperacute rejection of porcine xeno-organs include IgM

Table III: Anti α -Gal titer in all patients (versus preoperative titer).*

Isotype	Day	Titer	p-value
IgM	Before	328.1 \pm 218.7	-
	POD 1	260.7 \pm 160.4	0.189
	POD 7	811.8 \pm 667.9	0.001
	POD 21	1020.4 \pm 2584.1	0.245
	POD 60	644.0 \pm 1624.4	0.388
IgG	Before	1227.0 \pm 1009.3	-
	POD 1	1164.2 \pm 967.4	0.637
	POD 7	3009.0 \pm 2725.7	0.006
	POD 21	3782.6 \pm 3602.0	0.002
	POD 60	3116.7 \pm 2821.6	0.004

Values are mean \pm SD.

*Titer defined as the dilution at 1.0 of the OD.

PVR: Pulmonary valve replacement;

POD: Postoperative day.

xenoantibodies; however, IgG xenoantibodies can also mediate complement activation as well as antibody-dependent cellular cytotoxicity mechanisms (6).

In cardiac surgery, non-viable porcine bioprosthetic valve implantation has been performed for four decades, and today the main concern of cardiac surgeons following the implantation of a bioprosthesis is not a catastrophic hyperacute rejection but rather a late degeneration of the implanted valve, which often requires reoperation.

Although the currently available porcine bioprosthetic valves include a variety of design, manufacturing, and treatment options, the vast majority are glutaraldehyde-treated. It is generally accepted that bioprosthetic valve degeneration is due to calcification of the valve and tissue disruption or tearing. Glutaraldehyde fixation has been introduced to reduce valve antigenicity and to improve its mechanical strength (20). However, the failure of glutaraldehyde-treated porcine xenografts in clinical series has been reported, while others have shown that glutaraldehyde treatment does not remove xenograft antigenicity. Thus, one of the major causes of bioprosthesis degeneration is an immune reaction (21-24).

The immune mechanism has been considered to play an important role as an initial trigger of the degeneration process, and the α -Gal epitope is the major antigen. In the same context, the younger the patient's age, the more frequently will valve degeneration occur, which suggests a more active and robust immunologic response to xenoantigens (8,9,14). Simionescu (25) noted that excessive wear and tear, and a higher calcium turnover secondary to growth, is responsible for early calcification in children; however, even after the adolescent period (young adulthood, when a high calcium

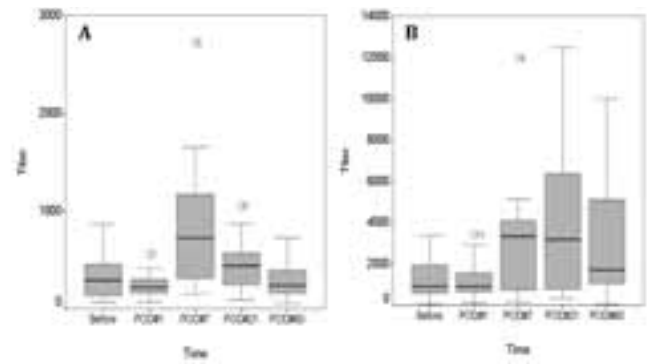


Figure 3: Changes in the anti α -Gal antibody titer in all patients. A) Changes in IgM titer. B) Changes in IgG titer. POD: Postoperative day.

turnover would not be expected) these patients still develop calcification soon after transplantation along with valve destruction. This suggests that another process is involved, which may be an immune reaction.

Manji et al. (24) reported that significant inflammation of the xenografts, as well as a significant humoral response to the xenografts, had occurred within a short period of time, and that the calcification correlated with the amount of inflammation in a young animal model. Konakci and colleagues (16) noted that degeneration of the bioprosthetic valve begins with the penetration of immunoglobulins into the valve-matrix, followed by the subsequent deposition of macrophages onto the valve surface; the process is then completed with collagen breakdown and calcification. The same group (16) reported that the α -Gal epitope still existed on the commercial porcine bioprosthetic valve, thus confirming the findings of Kasimir et al. (11), and that the bioprosthesis implantation elicited a specific humorally-mediated immune response directed against α -Gal. Several in vivo studies have reported that the titer of anti α -Gal IgM or IgG antibodies was increased, as measured using ELISA (5,26).

In the present study, it was observed that the titer of anti α -Gal antibodies changed with different patterns between the two isotypes. At one week after surgery, the anti α -Gal antibody titer increased, regardless of the isotype. Whilst the anti α -Gal IgM antibody titer began to decrease after three weeks, that of IgG was maintained over two months after surgery. The immediate postoperative (day 1) titers showed a tendency to decrease, which may be ascribed to the binding of circulating anti α -Gal antibody to the α -Gal isotope on the valve and/or a dilutional effect of CPB or intravascular volume infusion.

There was no statistically significant difference in the anti α -Gal antibody titer between the PVR and re-PVR groups. Before surgery, the anti α -Gal antibody titer

was similar between the two groups, which suggested that the antigenicity of the α -Gal epitope on a previously implanted valve may have decreased and almost disappeared with time. However, the mean postoperative titer (on days 21 and 60) seemed to be higher in the re-PVR group (though not statistically significantly), such that the possibility of an influence due to previous exposure on the immune response to the second exposure, perhaps by host sensitization, may not be excluded.

Study limitations

The primary limitation of the study was the small patient numbers, and the wide range of age and body weight. The study was also limited by the use of different types of bioprosthesis with different anticalcification treatments. Despite these limitations, the implantation of a porcine bioprosthesis was found to elicit the anti α -Gal immune response in each patient and isotype, with similar pattern.

In conclusion, porcine bioprosthesis implantation elicits an increased production of anti α -Gal antibodies in humans, albeit with different patterns between the two isotypes (IgM and IgG). Notably, the IgM antibody response was more rapid and transient, while that of IgG was longer and more delayed. The most important requisites for the ideal valve substitute in cardiac surgery are reliability and durability. Although an immunologic response to the xenoantigen other than α -Gal (non-gal antigen) has been reported (27-29), the α -Gal epitope is a major antigen that must be overcome in pig-to-human transplantation, including bioprosthesis implantation. For this reason, further studies are required to either avoid or minimize the immune response to the α -Gal epitope, including decellularization as well as enzymatic or genetic manipulations.

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