Elimination of α-Gal Xenoreactive Epitope: α-Galactosidase Treatment of Porcine Heart Valves

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Background and aim of the study: **Porcine heart valves are among the most widely used tissue valves in clinical heart valve implantation. However, immunologic responses have been implicated as potential causes of the limited durability of xenograft heart valves. The study aim was to determine the effectiveness of α-galactosidase treatment used to degrade the major xenoreactive antigens found in xenograft heart valves.**

Methods: **Fresh porcine heart valves and pericardium treated with α-galactosidase were studied to evaluate the xenoreactive galactose (α1,3) galactose (α-gal) antigen. Removal of the α-gal epitope from the porcine heart valve was monitored via 3,3′-diaminobenzidine staining intensity, while the removal of α-gal from *N*-glycans on porcine heart valves treated with recombinant α-galactosidase was determined either qualitatively or quantitatively by mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The porcine pericardium was used for monitoring the change in mechanical properties after α-galactosidase treatment. In addition, the biomechanical modification property of collagen fiber rearrangement on tissue was assessed using transmission electron microscopy (TEM).**

Results: **Following a 24-h incubation at pH 7.2, 4°C, employing 0.1 U/ml of *Bacteroides thetaiotaomicron*-derived recombinant α-galactosidase, the enzyme effectively removed the α-gal epitopes expressed on porcine heart valves. The identification type of α-gal *N*-glycan on fresh aortic valve, aortic wall, pulmonary valve, and pulmonary wall was 7.1%, 10.3%, 6% and 8%, respectively. In the presence of α-galactosidase treatment, α-gal-containing *N*-glycans were converted into α-gal-negative *N*-glycans. Likewise, α-gal-containing *N*-glycans were not detected when MALDI-TOF MS quantitative analysis was used. Furthermore, no significant difference was observed in the mechanical properties and findings from TEM in α-galactosidase-treated porcine pericardial tissue when compared to fresh porcine pericardium.**

Conclusion: **α-galactosidase can effectively remove the α-gal epitope from porcine heart valves and pericardium. This may possibly alleviate harmful xenoreactive immunologic responses by α-gal, without adversely affecting the biomechanical properties of the α-galactosidase-processed tissue.**

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In the field of living organ xenotransplantation, hyperacute rejection mediated by natural anti-α-Gal antibodies and the classically activated complement pathway have long been known to be a major barrier for the survival of xeno-organs in humans (1,2). Hyperacute rejection is triggered exclusively by pre-existing human xenoreactive natural antibodies (XNAs), which recognize Galα1-3Galβ1-4GlcNAc-R (α-gal epitope) that is present on the surface of mammalian cells and tissues (3-8).

In the past, biological heart valves have been widely used in patients who require heart valve replacement. However, the main concern following the implantation of a xenograft heart valve is not a catastrophic hyperacute rejection but rather a bioprosthetic valve dysfunction causing xenograft leaflet calcification. The major problems associated with bioprosthetic valve calcification are host-related factors, mechanical stress, chemical treatment, and the composition of the implant (9).

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However, several reports have suggested that the cause of degeneration of xenograft heart valve replacements may be mediated by role of the immune response, due in part (10-14) to α-gal xenoreactive antigens being expressed in valve cells and connective tissues (11,15). Previously, various attempts have been made to remove the harmful effects of xenoreactive major antigens, by utilizing different methods of tissue decellularization and preservation. Although this led to the xenoreactive antigenicity of tissue valve being attenuated or perhaps slightly reduced, the majority of tissue protein antigens remained, even after glutaraldehyde treatment (10,16).

Thus, the aims of the present study were to: (i) investigate the effectiveness of the recombinant *Bacteroides thetaiotaomicron* α-galactosidase treatment to remove the α-gal epitopes expressed on porcine heart valves by mass fingerprinting with a quantitative analysis tool employing matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and GSIB4 lectin-related histochemistry; and (ii) evaluate any biomechanical property modifications following the α-galactosidase treatment of pericardial tissue, as indicated by permeability, compliance, and strength testing, as preservation of the native ultrastructure of the extracellular matrix during tissue processing is desirable.

Materials and methods

Tissue preparation

Porcine aortic and pulmonary roots and porcine pericardium were obtained from a local slaughterhouse, stored at 4°C, and immediately transported to the laboratory. The tissues were washed several times in phosphate-buffered saline (PBS) containing antibiotics (penicillin 60 mg/l, streptomycin 100 mg/l; Sigma, St. Louis, MO, USA).

Preparation of recombinant α-galactosidase

The gene for the α-galactosidase from *B. thetaiotaomicron* (designated BtGal110B) was amplified from the corresponding genomic DNAs by polymerase chain reaction and introduced into the pET28a vector for expression of the His6-tagged proteins using appropriate restriction sites for protein expression in *Escherichia coli* Rosetta2 (DE3) (Novagen, Madison, WI, USA). *E. coli* was grown in Luria-Bertani (LB) medium with 34 μg/ml chloramphenicol, 30 μg/ml kanamycin, induced at A_{600} nm of ca. 0.6 with 1 mm isopropyl-1-thio-β-D-galactopyranoside. The harvested cell pellet was lysed in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole) using an Ultrasonicator (Misonix Inc., Farmingdale, NY , USA). The crude lysates were centrifuged for 20 min at 13,000 rpm, 4°C, and the expressed protein was then purified using a Nickel-NTA agarose column (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

α-galactosidase treatment

In order to remove the α-gal epitope, porcine heart tissues (aortic valve, pulmonary valve, aortic wall, pulmonary wall, cardiac muscle and pericardium) were incubated with 0.1 U/ml α-galactosidase in 50 mM Tris buffer, 150 mM NaCl, 0.05 % (w/v) ethylendiaminetetraacetic acid (EDTA), aprotinin 10 KIU/ml), neomycin 0.05 g/l, pH 7.2 at 4°C for 24 h.

As it was difficult to monitor the biomechanical testing of the porcine aortic valve, pulmonary valve, aortic wall, pulmonary wall, and cardiac muscle, the porcine pericardium was used to measure the mechanical properties after α-galactosidase treatment. Consequently, the porcine pericardium tissue was divided into two groups according to the method of treatment. Group 1 tissue samples were immediately rinsed with normal saline and freed from any adherent fat, while group 2 tissues were treated with α-galactosidase. Porcine pericardia for each analysis were obtained from the same animal (no difference between experimental groups) in order to avoid any clustering effects.

Histochemistry

The frozen tissue blocks were sectioned at 4 μm thickness, and the sections then incubated with 1 μg/ml biotin-GSIB4 lectin (Vector Labs, Burlingame, CA, USA). The tissues were washed with PBS and treated with 5 μg/ml avidin-horseradish peroxidase (Molecular Probes, Eugene, OR, US). α-Gal on the valves was visualized by using 3,3'-diaminobenzidine (DAB; Vector Labs) as a substrate. The DAB staining intensity of the valve tissues was monitored using light microscopy.

Preparation of *N*-glycans from porcine heart valves

For this preparation, a previously described protocol was followed with minor modifications (17). Freshly sliced porcine heart tissues (aortic valve, pulmonary valve, aortic wall, pulmonary wall, cardiac muscle and pericardium) were incubated with *N*-glycosidase F (Roche, Mannheim, Germany) for 16 h at 37°C. The *N*-glycans were then precipitated by adding four volumes of cold ethanol and incubated at -20°C for 2 h. The mixture was centrifuged at 16,500 × *g* for 15 min, and the supernatants were removed and dried in a centrifugal vacuum concentrator (Hanil Research and Development, Seoul, Korea). The dried *N*-glycans were reconstituted in 1 ml of water and further purified by solid-phase extraction using a porous graphitic carbon (PGC) cartridge (Alltech Associates, Deerfield,

IL, USA) as follows. The cartridge was washed with 30% (v/v) acetic acid (AA) and then equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile (ACN)/H_2O, and 0.1% (v/v) TFA in 5% (v/v) ACN. The *N*-glycan solution was applied to the PGC cartridge and washed with H_2O and 0.1% (v/v) TFA in 5% (v/v) ACN/H_2O. Finally the *N*-glycans were eluted with 0.1% (v/v) TFA in 50% (v/v) ACN/H_2O.

Identification of *N*-glycans by mass fingerprinting using MALDI- TOF MS

The *N*-glycans were permethylated as described previously (18) and then dissolved in 100% (v/v) methanol. An aliquot of the resultant *N*-glycan solution (1 μl) was mixed with an equal volume of 2,5-dihydroxybenzoic acid solution (30 mg/ml in 70% (v/v) ACN/H_2O) and spotted onto a plate. The relative percentage was expressed as the ratio of the intensity of each ion to the summed intensity of the total ions detected.

Permeability and compliance testing

Permeability variations were evaluated by measuring the leakage volume of the saline solution after applying a constant 100 mmHg pressure to the porcine pericardium (1 cm^2, n = 12) for 1 h. This demonstrated the difference in penetration and elongation caused by the degree of gap appearance in the collagen fiber bundle.

Compliance was evaluated by measuring the transformation volume of saline solution as the pressure was increased from 100 mmHg to 200 mmHg on one side of the pericardium (n = 12). The unit of compliance was set as μl/mmHg × cm^2, by dividing by the unit area. Compliance changes were used to evaluate the stiffening of the collagen fibers in the high-strain working region, where the elastic behavior is dominated by the fibers themselves (19).

Tensile strength testing

Uniaxial testing was used to compare the mechanical properties of the differently treated tissues. Tissue strips (5 × 50 mm, 20 strips for each group) were cut in different directions to overcome any material anisotropy. The tissue thickness was measured at three points using a Mitutoyo thickness gauge (Quick-Mini 700-117; Mitutoyo, Japan). The tensile properties were evaluated using a tensile testing machine (K-ML-1000N; M-TEC, Republic of Korea) equipped with a digital force gauge (DS2-50N, IMADA, Japan) operating at an extension rate of 100 mm/min. The ultimate strength and strain at fracture were evaluated from the recorded stress-strain curves.

Transmission electron microscopy

The porcine pericardium specimen (n = 1) of about 1 mm^3 was fixed with 2.5% formaldehyde-2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.2, post-fixed with 2% OsO_4 in the same buffer, dehydrated with graded ethanol, and then embedded in Araldite/Epon. Thin sections were contrasted with uranyl acetate and lead citrate. Observations and photographic records were obtained using a JEM-1400 microscope (JEOL, Japan) operating at 80 KV.

Statistical analysis

A statistical analysis was performed with one-way analysis of variances (ANOVA) (SigmaStat 3.0; SPSS Inc., Chicago, IL, USA). Statistical differences were determined using either Student's *t*-test or a paired *t*-test. A p-value <0.05 was considered to be statistically significant.

Results

Staining of α-gal epitope on porcine heart valves by lectin histochemistry

GSIB4 is known to bind at the α-gal epitope (20-22); the signal of α-gal is shown as a brown coloration. After α-galactosidase treatment, α-gal intensity on the surface of the porcine heart valves was weakened (Fig. 1).

Detection of α-gal *N*-glycans by using MALDI-TOF MS

The changes of the peak, as qualitative analyses, are shown in Figure 2 and Table I, where N is the abbreviated form of the peak number. After α-galactosidase processing, the α-gal epitope of N1 and N3 (α-gal-containing *N*-glycan) were removed; consequently, the peak of N1 and N3 were converted into the peak containing the composition structure of N8 (α-gal-negative *N*-glycan). Similarly, the conversions of N2 to N7, of N4 to N9, of N5 to N9, and finally of N6 to N10, took place. As a result, six peaks were changed into four different peaks; their states before and after α-galactosidase treatment are linked with dotted lines in Figure 2. The non-labeled peaks were not α-gal-type glycans.

Quantitative evaluation of α-gal on the surface of porcine heart tissue

The types of α-gal-containing *N*-glycan on fresh aortic valve, pulmonary valve, aortic wall, pulmonary wall, pericardium and muscle were shown to be 7.1%, 10.3%, 6%, 8%, 29% and 20% (n = 1), respectively. The relative percentage was expressed as the ratio of the intensity of each ion to the summed intensity of the detected total ions. In addition, each sialic acid (*N*-glycolylneuraminic acid; Neu5Gc) glycan type from the porcine aortic valve, aortic wall, and pulmonary wall was higher (10.3%, 24.6%, and 2.6%, respectively) than the α-gal type (Fig. 3). Although α-gal-containing N-glycans were detected on the porcine aortic and pul-

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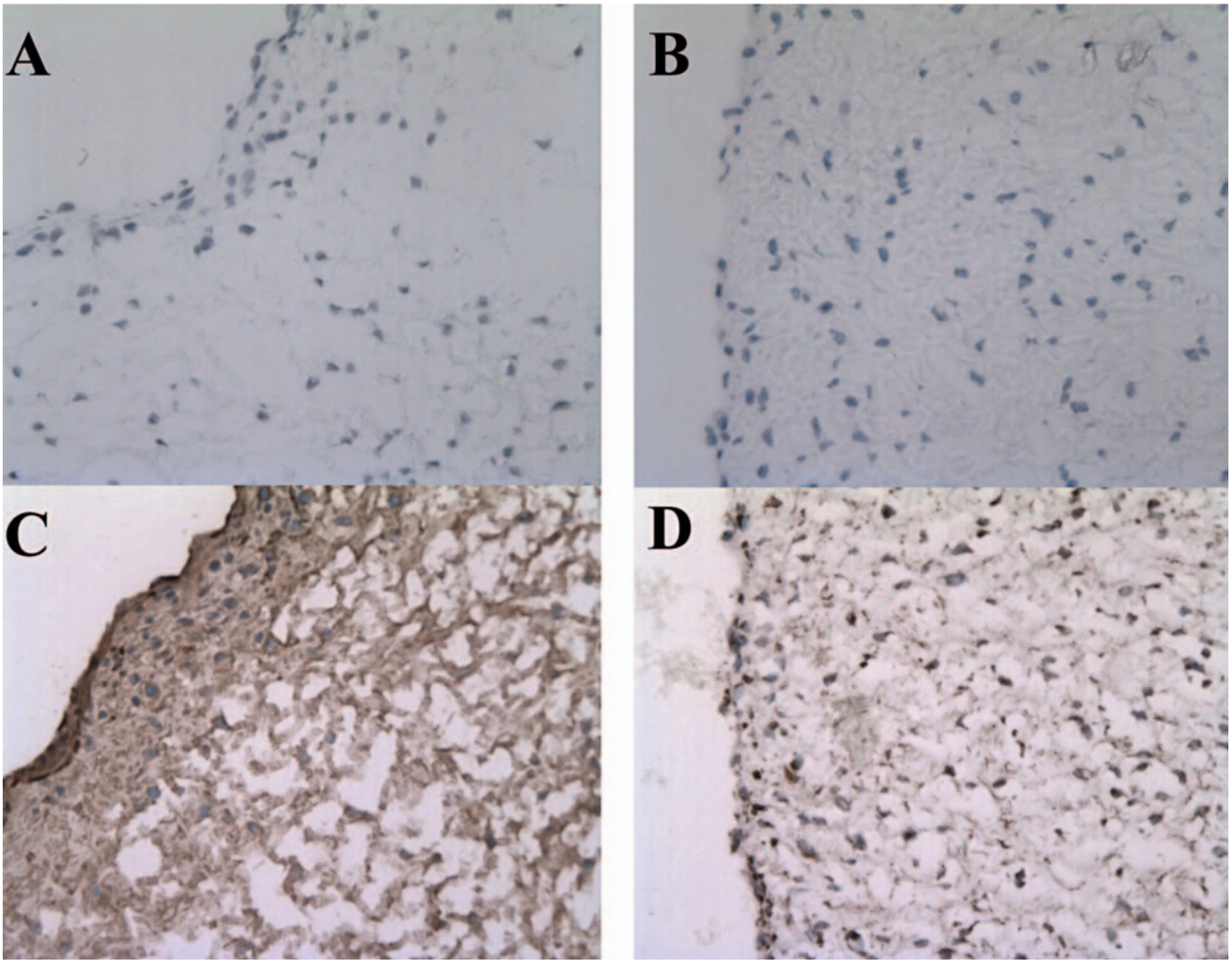


Figure 1: Staining of the α-*gal epitope on porcine heart valve using lectin histochemistry. The porcine heart valves were either treated with* α-*galactosidase (D) or were untreated (C). (A) and (B) are negative controls of (C) and (D), respectively.*

monary valve, aortic and pulmonary wall, muscle, and pericardium before enzyme treatment, α-gal-containing N-glycans were not detected after treatment with α-galactosidase (Tables II-IV).

Permeability and compliance testing

The effects of α-galactosidase on the tissue were monitored by measuring the leakage volume and compliance. The leakage volume and compliance of the α-galactosidase-treated tissue (group 2) were similar to that of group 1 ($p > 0.05$). This indicated that elongation of the collagen bundle and the extensibility of the tissue were not affected by α-galactosidase treatment (Table V).

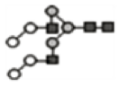
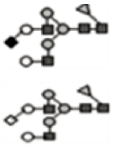
Tensile strength testing

The tensile strength of the α-galactosidase-treated tissues (group 2) did not differ from that of group 1 ($p > 0.05$); this also indicated that the α-galactosidase treatment had not adversely affected the strength of the tissue (Table VI).

Transmission electron microscopy

The gap between the collagen fiber bundle of fresh porcine pericardium (Fig. 4A) and the α-galactosidase-treated tissue (Fig. 4B) displayed a similar collagen pattern. In general, measurement of the gap from the collagen bundle permits a comparison to be made of the collagen shape and size. In order to analyze the tendency for gap formation, two areas from the same pericardial portion with the same distribution of collagen bundles were taken as specimens after enzymatic processing.

Table I: Identification of α-gal-containing and removed N-glycans on porcine heart valves before and after treatment with α-galactosidase.

	Peak Number	(M+Na)+		Composition	Change
		observed	calculated		
α–gal containing *N*-glycan	N1	2448.3	2448.2		
	N2	2478.3	2478.2		
	N3	2652.4	2652.3		
	N4	2809.4	2809.4		
	N5	2839.5	2839.5		
	N6	3101.5	3101.6		
α–gal negative *N*-glycan	N7	2244.2	2244.1		N2 → N7
	N8	2070.1	2070.0		N1,N3 → N8
	N9	2605.4	2605.3		N4 → v9 N5 → N9
	N10	2652.4	2652.3		N6 → N10

Symbol of glycan included in composition;

- GlcNac, *N*-acetylglucosamine
- Man, mannose
- Gal, galactose
- Fuc, fucose
- NeuAc, *N*-acetylneuraminic acid
- NeuGc, *N*-glycolylneuraminic acid

N; Abbreviation of number

(M+Na)+ observed; Detected mass at MRLDI detector

(M+Na)+ calculated; Calculated mass of composition

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Discussion

Although, currently, commercial porcine heart valves are widely implanted into patients with diseased heart valves, degeneration of the implanted heart valves due to their limited durability remains a major obstacle. Factors other than an immune reaction - such as chemical interaction, infections, mechanical stress, material fatigue and surgical factors - contribute to the degeneration of bioprostheses (23). Among these factors, the presence of the α-gal epitope on porcine cells or tissues may play a key role in inducing an early xenoimmune inflammatory response. It has been reported that the fixation of porcine heart valves with glutaraldehyde alone does not significantly prolong survival of the valve, due to the remaining antigenicity of the xenograft (24). The α-gal epitope is known to remain on commercial porcine heart valves following treatment with glutaraldehyde (25,26). In fact, the decellularization procedure using formaldehyde was initially introduced to resolve or at least attenuate the immune problem of the α-gal remnant on porcine heart valves. However, even with the decellularization treatment, α-gal antigens still appear to remain on the connective tissues and to trigger a xenoreactive immune inflammatory response by the remaining α-gal, and these are thought to play an important role as an initial trigger of the degeneration process (10,27). Whilst the exact molecular mechanisms of degeneration remain unproven, in clinical situations following xenograft heart valve implantation the α-gal immune responses are rapidly increased in the serum of patients with xenograft-related heart diseases (14,28,29). Thus, elimination of the α-gal epitope on implantable xenograft heart valves may increase the durability of the valves.

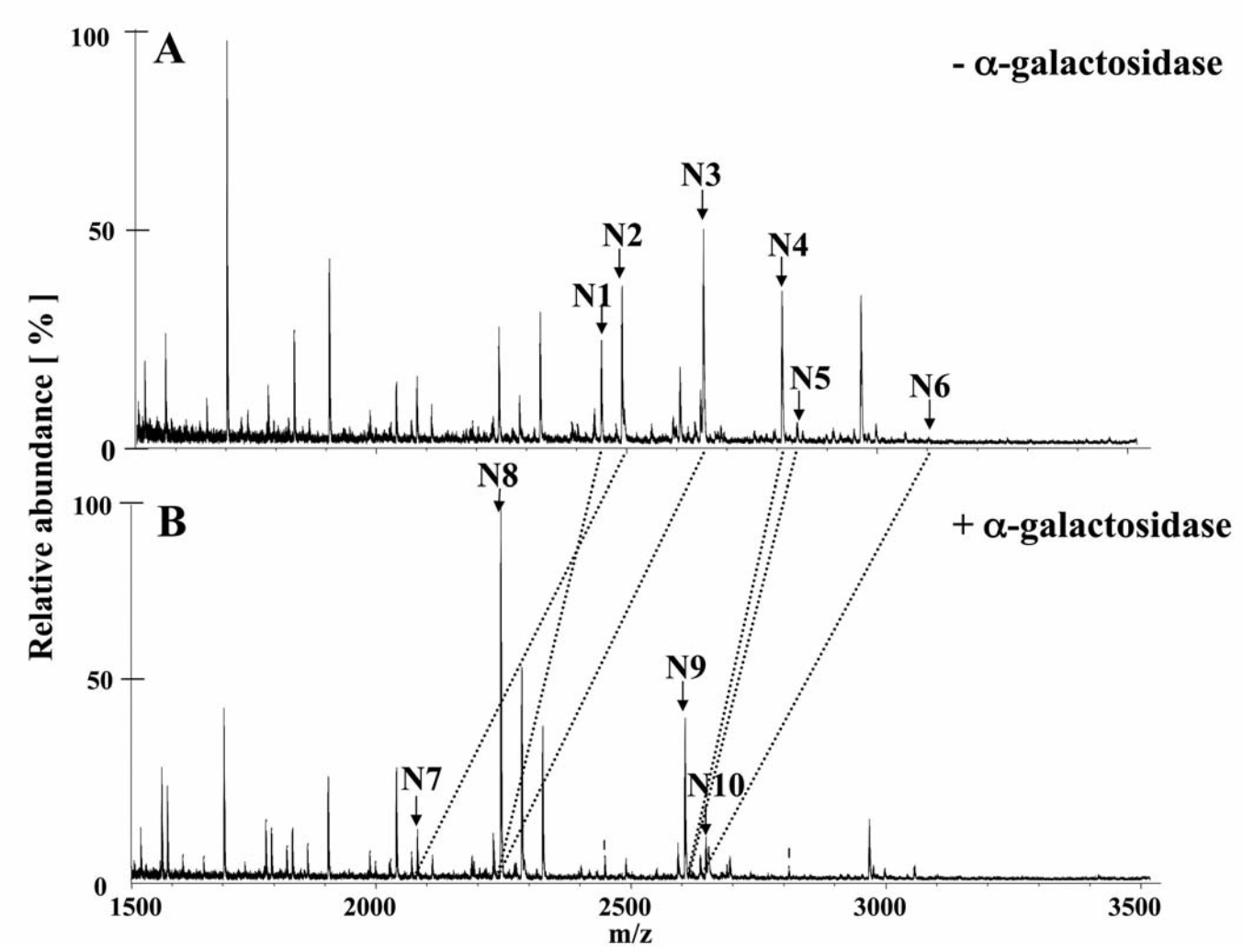

Figure 2: α-*Galactosidase-mediated remov*al of α-*gal from N-glycans on the porcine heart valve, monitored using MALDI-TOF MS. The valves were incubated in either the presence (B) or absence (A) of* α-*galactosidase. In the presence of* α-*galactosidase treatment, the peak of* α-*gal containing N-glycans (N1-N6) was converted into the peak of* α-*gal negative N-glycans (N7-N10). The unlabeled peaks were not* α-*gal-type glycans.*

Table II: Change in degree of α-gal-containing N-glycan on porcine heart tissue before and after treatment with α-galactosidase.

Tissue	Peak number	[M+Na]+ observed	α-Galactosidase-negative (%)	α-Galactosidase-positive (%)
Aortic valve	N43	1999	0.9	ND
	N73	2448	3.1	ND
	N82	2652	2.1	ND
Aortic wall	N35	1892	1.3	ND
	N73	2448	3.6	ND
Muscle	N72	2433.9	8.5	ND
	N82	2651.9	9.2	ND

[M+Na]+ observed: Detected mass at MRLDI detector.
%: The relative percentage was the ratio of the intensity of each ion to the summed intensity of the total ions detected.
ND: Not detected.

The use of α-galactosidase to remove α-gal in the xenograft prior to implantation, as well as attempts to minimize the cellular immune response, have previously been investigated. Luo et al. (30) reported that green coffee bean α-galactosidase was effective in removing the terminal α-gal from porcine vein graft cells and delayed onset of the hyperacute rejection of these veins when transplanted into monkeys. Watier et al. (31) also showed that removal of the terminal α-gal residues from xenogeneic porcine endothelial cells by green coffee bean α-galactosidase decreased any complement-mediated cytotoxicity. In the present study, it was postulated that the use of α-galactosidase may remove the α-gal antigens more effectively in xenograft heart valve tissues. Consequently, the effectiveness of removal of α-gal from porcine heart valves was evaluated by using recombinant α-galactosidase. The enzyme activity was investigated both qualitatively and quantitatively by using histochemistry and MALDI-TOF MS. Glycome profiling methodologies of cells and tissue using MS analysis as a tool have been developed and optimized to identify *N*- and *O*- glycans as well as glycosphingolipid glycan from a variety of species (32,33). Protein glycosylation has long been recognized as a very common post-translational modification whereby carbohydrates are typically linked either to serine or threonine residues (*O*-linked glycosylation) or to asparagine residues (*N*-linked glycosylation). Protein glycosylation - and in particular *N*-linked glycosylation - is prevalent in proteins destined for extracellular environments (34). Hence, the *N*-glycan of protein was analyzed by using *N*-glycosidase F. The data acquired confirmed that, in the presence of α-galactosidase, α-gal-negative *N*-glycans were observed, whereas α-gal-containing *N*-glycans were not detected after α-galactosidase treatment. According to the observation of type identification of *N*-glycans on porcine heart tissue, each sialic acid (Neu5Gc) glycan type of porcine aortic valve, aortic wall, and pulmonary wall was higher (10.3%, 24.6%, and 2.6%, respectively) than was the α-gal type. This might play a potential role in the antibody-mediated rejection of the pig xenograft as a non-gal xenoreactive antigen in non-human primates. The identification of specific non-α-gal antigens in pigs, against which primates have natural antibodies, might allow gene-knockout and thus further reduce the effect of the primate humoral immune response. In a recent study

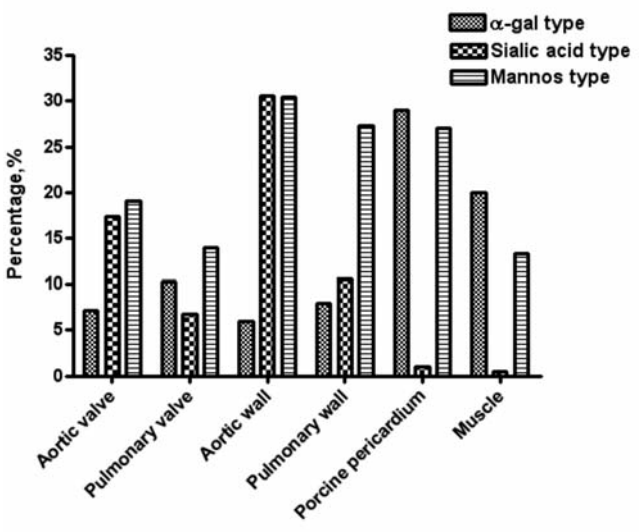

Figure 3: Type identification of N-glycans on porcine heart tissue. α-Gal levels on fresh aortic valve, pulmonary valve, aortic wall, pulmonary wall, pericardium and muscle were 7.1%, 10.3%, 6%, 8%, 29% and 20% (n = 1), respectively. Sialic acid-type levels were 17.4%, 6.7%, 30.6%, 10.6%, 1% and 0.5%, respectively, and mannos-type levels were 19%, 14%, 30.5%, 27.3%, 27% and 13.3%, respectively.

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Table III: Change in degree of α-gal-containing N-glycan on porcine heart tissue before and after treatment with α-galactosidase.

Tissue	Peak number	[M+Na]+ observed	α-Galactosidase-negative (%)	α-Galactosidase-positive (%)
Pulmonary valve	N28	1780	4.0	ND
	N41	1999	1.5	ND
	N67	2326	0.4	ND
	N73	2448	2.0	ND
	N82	2652	2.4	ND
Pulmonary wall	N42	1987.8	1.6	ND
	N82	2652.1	5.3	ND

For the key to structural symbols, see Table II.

conducted by Yeh et al. (35), the details of potential carbohydrate antigens including Neu5Gc, Forssman, β-LacNAc, and α-LacNAc were discussed. All non-human primates also express Neu5Gc. Human serum demonstrated binding to Neu5Gc, but at a much lower level than to α-gal, whereas baboon and pig IgM and IgG showed minimal or no binding to Neu5Gc. The pig is Forssman-negative, and this carbohydrate does not play a role in the rejection of pig grafts in primates. Another possible non-α-gal carbohydrate is β-LacNAc, an oligosaccharide that is currently attracting interest because it is exposed when the α-gal epitope is removed. Both, IgM and IgG binding to α-LacNAc is minimal in human and baboon serum. The binding to α-LacNAc was also investigated because of its similar structure to αLacNAc (where the α-linkage replaces a β-linkage). The antibodies that bound α-LacNAc were cross-reactive with anti-α-gal antibodies. Furthermore, αLacNAc has not yet been found in a naturally occurring form, and has been obtained only via a synthetic route.

Although in the past it has been regarded as normal to find similar scaffold mechanical changes after enzyme treatment, in the present study no significant difference was observed in the mechanical properties of α-galactosidase-treated tissue. Moreover, the shape

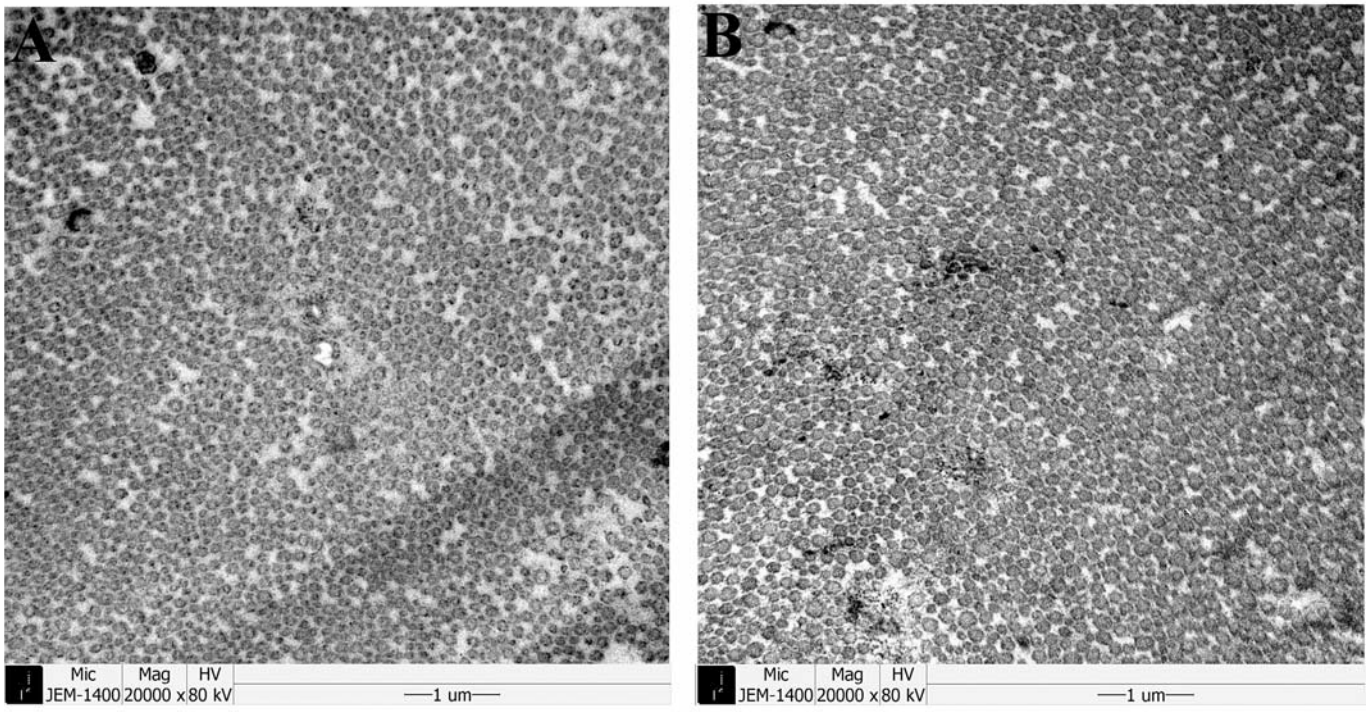

Figure 4: Transmission electron microscopy assessment of the porcine pericardium before and after treatment with α-galactosidase. A) Before enzyme treatment. B) After enzyme treatment.

Table IV: Change in degree of α-gal-containing N-glycan on porcine heart tissue before and after treatment with α-galactosidase.

Tissue	Peak number	[M+Na]+ observed	α-Galactosidase-negative (%)	α-Galactosidase-positive (%)
Pericardium	N35	1892	3.0	ND
	N43	1999	2.4	ND
	N59	2219	0.7	ND
	N65	2274	0.6	ND
	N73	2448	10.1	ND
	N74	2464	0.9	ND
	N75	2479	0.3	ND
	N82	2652	10.8	ND
	N83	2669	0.2	ND

For the key to structural symbols, see Table II.

of the collagen fiber arranged in bundles (as viewed with transmission electron microscopy; TEM) was not affected by α-galactosidase.

However, the incomplete removal of the α-gal of porcine heart valve scaffold and matrix, which were not accessible to α-galactosidase, might induce a residual xenoreactive immune response. Consequently, future studies will require the in vitro performance and in vivo implantation of non-human primate to monitor the xenoreactive immune response. This may provide evidence of a positive relationship between the durability (a reduction in calcification) of heart valves and the degree of xeno-specific immune response by removing the α-gal epitope of the xenograft heart valve using an α-galactosidase treatment.

In conclusion, α–gal on xenograft heart valves and tissues can be removed almost totally with α-galactosidase treatment, without affecting the mechanical properties of the tissues. An absence of changes in the mechanical properties was confirmed by compliance, permeability and tensile strength testing, as well as by TEM imaging following enzyme treatment. Clearly, the application of α-galactosidase to xenograft heart valves and tissues appears to offer a reliable means of reducing the initiation of the xenograft immune response, resulting in a prolonged durability of the valves and tissues.

Acknowledgements

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Table V: Permeability and compliance of the porcine pericardium.

Group	Permeability (ml/h × cm^2)	Compliance; 100-200 mmHg (µl/mmHg × cm^2)
1	2.31 ± 1.61*	0.29 ± 0.12*
2	2.22 ± 1.47*	0.32 ± 0.13*

Values are mean ± SD of samples for treatment (n = 12).
Group 1: Fresh porcine pericardium; Group 2: Porcine pericardium treated with α-galactosidase.
*p > 0.05, group 1 versus group 2.

Table VI: Tensile strength of the porcine pericardium.

Group	Sample size (n)	Thickness (mm)	Ultimate strength (MPa)	Strain at fracture (%)
1	20	0.07 ± 0.01 (0.05~0.09)	31.53±9.50* (13.71~50.40)	27.82±3.91 (23.33~33.33)
2	20	0.09±0.03 (0.05~0.13)	31.31±11.58* (12.00~68.57)	23.43±3.61 (20.07~30.00)

Values are mean ± SD of samples for treatment.
Group 1: Fresh porcine pericardium; Group 2: Porcine pericardium treated with α-galactosidase.
*p > 0.05, group 1 versus group 2.

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