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Effect of Cryopreservation Techniques on Aortic Valve Glycosaminoglycans

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Abstract: This study was designed to evaluate the effect of cryopreservation on the glycosaminoglycan (GAG) content of the aortic allografts. Twenty-one porcine aortic valves were obtained. Five aortic roots were immediately analyzed without cryopreservation, eight were cryopreserved in closed leaflet position, and eight in open leaflet position. The groups were compared in terms of GAG concentration and subclass proportion in three different zones including the aortic root wall, the commissures, and the leaflets. GAG content at the commissures was significantly lower in the closed leaflet group than in the other groups ($P = 0.001$). The electrophoretic analysis did not show any significant difference in the zonal distribution of GAG classes between groups. Quantitative analysis in various aortic valve zones suggests that cryopreservation can alter the GAG content. Cryopreservation of the aortic valve in an open leaflet position can preserve the matrix more efficiently and might prolong the durability of the aortic allograft. **Key Words:** Homograft—Aortic root—Cryopreservation—Glycosaminoglycans.

Aortic root replacement with cryopreserved aortic allograft is a well-known surgical therapy for patients with aortic valve disease. Although these valves are associated with excellent hemodynamic parameters and lower incidence of thromboembolic events (1), the main disadvantage in using them is their limited long-term durability especially in young recipients (2). Many factors have been implicated as the cause of the early allograft failure, including surgical techniques, ischemic times, cryopreservation methods, and the host immune response (3–5).

Recent studies have pointed out the possible role of the valvular matrix on accelerated degeneration of allograft valves (6). Glycosaminoglycans (GAGs), being a key component of the extracellular matrix, play a central role in management of valvular shear stress and the cyclic motion of the aortic valve (7–11). As many researchers demonstrated, loss of GAGs leads to valvular dysfunction and is a predisposing factor for calcification (9,12–14).

We hypothesize that cryopreservation technique can contribute to the early development of allograft failure by altering the GAG content of the aortic valve. Preliminary studies showed no significant differences in GAG content between cryopreserved and control aortic valves (14,15). However, GAG levels in different valvular zones were not evaluated. Moreover, the effect of different preservation techniques on GAG content needs to be further investigated. Therefore, we designed this study to evaluate the GAG content of three different areas of the aortic root cryopreserved with leaflets on either diastolic or systolic position.

MATERIAL AND METHODS

Harvesting and cryopreservation

Twenty-one porcine aortic roots were obtained from a local slaughterhouse. In order to minimize GAG loss during tissue processing, the aortic root

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was harvested immediately after the animal was sacrificed. The tissues were then placed in a cold balanced salt solution (Eurocollins at 4°C) and transported to the laboratory. Mean cold ischemia time was 51 ± 17 min. The valves were sized with a Hegar dilator, and all of them were 19–20 mm in diameter. Five aortic roots (Group 1, control) were immediately analyzed for GAG content. The other 16 aortic roots were cryopreserved.

Cryopreservation was performed at the Centro Cardiologico Monzino Regional Tissue Bank using the same exact methodology for human valve allografts. The explanted aortic roots were first incubated in the balanced salt solution containing antibiotics (RPMI 1640 with L-Glutamine tamponed until a pH of 7.2–7.4; cefoxitin 240 μg/mL, lincomycin 120 μg/mL, polymyxin B 100 μg/mL, vancomycin 50 μg/mL) at +4°C for 24 h and then placed into a hemofreeze bag containing a cryopreservation solution (100 mL of RPMI 1640 with L-Glutamine pH 7.2–7.4 with 10% dimethyl sulfoxide as cryoprotectant). Half of the aortic roots were stored with cusps in the closed position (Group 2, diastolic position, $n = 8$), whereas the remaining half were stored with cusps in the open position (Group 3, systolic position, $n = 8$). The diastolic position was secured by placing 6/0 polypropylene stitches into the ascending aorta and suspending the aortic root into the hemofreeze bag with the annulus in a downward position and the commissures in an upward position. For the systolic position, 6/0 polypropylene stitches were placed into the ventricular muscles below the aortic annulus and the aortic root was suspended into the hemofreeze bag, with the annulus in an upward position, leaving the commissures in a downward position. No stitches were passed through the leaflets. Diastolic and systolic positions were maintained only by gravity. No other diastolic load was applied to the closed leaflets in Group 2 (Fig. 1). The specimens were cooled in a controlled rate freezer (Kryo 10–16 series III, Planer, London, U.K.) that decreased the temperature at a rate of 1°C/min down to a temperature of –80°C and finally maintained in liquid nitrogen vapors (–180°C). After 48 h of storage, which was necessary to stabilize the effect of cryopreservation on extracellular matrix, the aortic roots were thawed, rinsed with culture medium (100 mL of RPMI 1640 with L-Glutamine pH 7.2–7.4) at 4°C, deprived of adventitial layer, and analyzed.

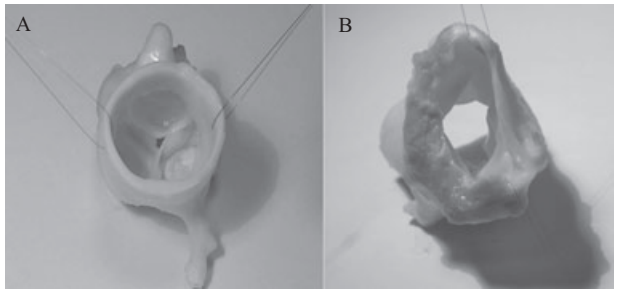

FIG. 1. The diastolic and systolic positions were secured by placing 6/0 polypropylene stitches into the ascending aorta (A) and into the ventricular muscles below the aortic annulus (B), respectively. Diastolic and systolic positions were maintained only by gravity.

Tissue analysis

Three zones were cut using a surgical blade and separately processed: the aortic root wall, the commissure, and the leaflet. After peeling and segregation of the three different types of tissue, their wet weight was measured. Tissues were fixed with 20 mL volume of acetone at 4°C for 24 h, delipidated with 20 mL volume of chloroform : methanol solution (2:1, vol/vol) at 4°C for 24 h, and dried at 60°C. The mean final weight (dry defatted tissue [DDT] weight) of the aortic wall, commissure, and the leaflet was 32.67 ± 4.97, 23.58 ± 44, and 8.60 ± 1.61% of the wet weight, respectively.

Extraction of total GAGs

DDTs (100 mg) were rehydrated for 24 h at 4°C in 0.1 M sodium acetate, pH 6.0, containing 5 mM cysteine and 5 mM ethylenediaminetetraacetic acid. Papain (0.3 U/mg of DDT) was then added to the mixture, which was incubated at 56°C for 24 h under mild agitation. The digest was clarified by centrifugation ($9000 \times g$ for 20 min at +4°C) and the residue was washed.

Purification of GAGs

Digest supernatant and the washing were combined and loaded on a (diethylamino)ethyl-cellulose column (0.7 × 6 cm, 2.3 mL), equilibrated with 50 mM sodium acetate, pH 6.0. The column was then washed with 50 mL of the same buffer and eluted with a two-step salt gradient (0.55 and 1.0 M NaCl). Fractions of 1 mL were collected. These were assayed for hexuronate content by the method of Bitter and Muir, using glucuronolactone as a standard (16).

Fractions containing GAGs were pooled and precipitated using 4 cc volume of absolute ethanol. The mixture was left overnight at 4°C and the precipitate was separated by centrifugation, washed twice with ethanol and diethyl ether, and then dried.

Acetate cellulose electrophoresis

GAG composition was determined by discontinuous electrophoresis according to Cappelletti et al. (17). Identification of GAG was performed by treating aliquots of the samples with specific eliminases, as previously described (18). The specificity and the efficiency of enzyme treatments were checked with the standard GAGs under the same experimental conditions. The study was approved by our institutional ethics committee.

Statistical analysis

Data were reported as mean ± standard deviation or total numbers and relative frequencies. For comparison between two groups, Student's *t*-test was performed. When we compared more than two groups, repeated measures analysis of variance (ANOVA) was performed. If statistically significant, Student's *t*-test was then performed, with Bonferroni's method used to correct for multiple comparisons. A value of $P < 0.05$ was considered statistically significant. The analyses were performed with Microsoft SPSS 11.0 software.

RESULTS

Control cusps ($n = 5$) contained 5.1 ± 1.1 μg hexuronate/mg DDT in the leaflet, 2.4 ± 0.7 μg hexuronate/mg DDT at the commissure, and 3.9 ± 0.5 μg hexuronate/mg DDT in the aortic wall. Aortic roots cryopreserved in systolic position ($n = 8$) contained 4.6 ± 1.8 μg hexuronate/mg DDT in the leaflet, 2.4 ± 0.5 μg hexuronate/mg DDT at the commissure, and 3.8 ± 0.6 μg hexuronate/mg DDT in the aortic wall. No significant differences were found between the control and open leaflet groups ($P > 0.05$ at the commissure, in the leaflet, and in the aortic wall).

Aortic roots cryopreserved in diastolic position ($n = 8$) contained 6.0 ± 2.3 μg hexuronate/mg DDT in the leaflet ($P > 0.05$ diastolic position vs. control group, and vs. systolic position), 1.7 ± 0.3 μg hexuronate/mg DDT at commissure, and 3.8 ± 0.7 μg hexuronate/mg DDT in the aortic wall. No significant differences in GAG total content were found between closed leaflet, open leaflet, and control groups in the leaflet and in the aortic root wall ($P > 0.05$). GAG analysis at the commissure showed a significant decrease in GAG content in the closed leaflet group compared with both the control and the open leaflet groups ($P = 0.001$ by repeated measures ANOVA) (Table 1, Fig. 2).

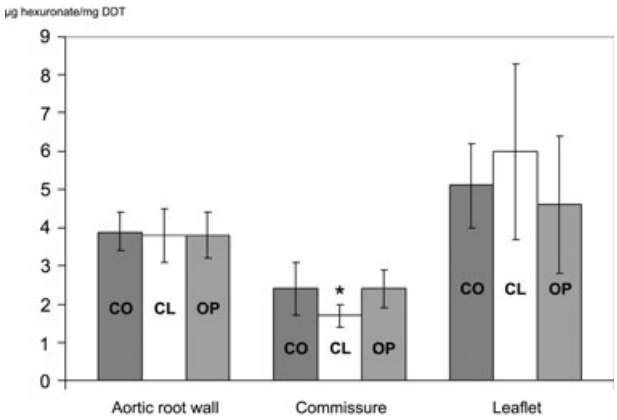

FIG. 2. Total aortic, leaflet, and leaflet flexion zone GAG content (μg hexuronate/mg DDT).
*GAG analysis at the commissure showed a significant decrease in GAG content in the closed leaflet group compared with both the control and the open leaflet groups ($P = 0.001$ by repeated measures ANOVA). Vertical bars indicate standard deviation (SD).
CO, control group; CL, closed leaflet group; OP, open leaflet group.

Analysis of concentration of different GAG classes revealed no significant differences in composition among the three groups (control, open leaflets, and closed leaflets) both in the leaflet and at the commissure and in the aortic wall ($P > 0.05$ by repeated measures ANOVA) in all groups (Table 2).

Our data suggest that cryopreservation of aortic valve with cusps in diastolic position produces a significant reduction in total GAG quantity at the commissure, although the relative percentage of single GAG classes remains unchanged.

DISCUSSION

Over the last 10 years, studies about homograft degeneration have focused on viability and host immune response (3–5), with less attention to the

TABLE 1. *Total GAG content (μg hexuronate/mg DDT) in aortic wall, at commissure, and in leaflet*

	Control group	Closed leaflet group	Open leaflet group	*P* value
Aortic root wall	3.9 ± 0.5	3.8 ± 0.7	3.8 ± 0.6	0.327
Commissure	2.4 ± 0.7	1.7 + 0.3†‡	2.4 + 0.5	0.001*
Leaflet	5.1 ± 1.1	6.0 + 2.3	4.6 + 1.8	0.593

*$P = 0.001$ by repeated measures ANOVA.
†$P = 0.011$ versus control group.
‡$P = 0.027$ versus open leaflet group.

TABLE 2. *GAG subclass proportions in different zones (%)*

	Control group	Closed leaflet group	Open leaflet group	*P* value
Aortic root wall				
HS	29.1 ± 3.6	27.9 ± 4.1	27.7 ± 4.3	0.341
HA	4.3 ± 3.1	4.3 ± 1.5	4.5 ± 4.8	0.290
DS	26.9 ± 5.1	27.5 ± 5.9	27.8 ± 5.7	0.338
CS	39.1 ± 4.1	41.2 ± 2.5	39.9 ± 4.3	0.135
Commissure				
HS	13.5 ± 1.9	12.3 ± 2.0	10.3 ± 1.5	0.117
HA	19.9 ± 3.2	16.9 ± 5.4	17.2 ± 3.9	0.291
DS	17.3 ± 3.4	15.3 ± 4.3	18.6 ± 2.7	0.198
CS	24.7 ± 2.8	22.0 ± 5.8	24.1 ± 3.2	0.252
CuS	27.9 ± 2.4	33.6 ± 4.2	29.8 ± 1.6	0.131
Leaflet				
HS	1.2 ± 0.9	1.1 ± 1.0	1.5 ± 1.0	0.288
HA	37.4 ± 3.2	31.3 ± 4.2	35.9 ± 3.3	0.101
DS	10.5 ± 3.0	11.0 ± 2.6	12.0 ± 2.4	0.473
CS	15.7 ± 2.9	15.3 ± 4.0	13.4 ± 4.4	0.294
CuS	38.7 ± 3.3	41.3 ± 3.1	37.2 ± 4.8	0.148

$P > 0.05$ by repeated measures ANOVA.
CS, chondroitin sulfate; CuS, chondroitin unsulfate; DS, dermatan sulfate; HA, hyaluronic acid; HS, heparan sulfate.

damages caused by cryopreservation technique on extracellular matrix. Shon et al. analyzed the effect of cryopreservation on proteoglycans without finding significant alterations, but GAG levels in different valvular zones were not evaluated (14,15). Considering different areas of the aortic root, we pointed out that a decrease in GAG content was already evident at the commissures before implantation when homograft was cryopreserved with leaflets in diastolic position.

This outcome can represent an ultrastructural basis of allograft mechanical degeneration, as GAGs are essential for the correct modulation of valvular stresses and impaired extracellular matrix makes the valve prone to damages from repetitive stresses (7,12). GAGs are grossly hydrated and act as a semifluid gel that confers sliding properties to different collagen layers, permitting bending movement without degeneration (19). Membrane, shearing, and tensile stresses are modulated by GAGs, which actively change their distribution according to different stresses (9). Decreased GAG contents at commissures, the cusp's areas subjected to maximal stresses, do not confer normal shearing properties to the homograft leaflets and do not modulate bending correctly stresses, promoting damage from mechanical stresses (6). Moreover, structural changes are accelerated by other factors after implantation, such as decreased cellular viability, immune responses, and no matrix replacement (3,8,9,20–22).

The relationship between damaged matrix, mechanical stresses, and valvular degeneration is confirmed by studies on aortic bioprostheses. Mechanical stresses produce nonhomogeneous structural changes in the xenograft valvular tissue (12,13). At the initial stage of bioprostheses degeneration, the pattern of matrix degeneration was demonstrated similar to the pattern of mechanical stresses, with the first mechanical damages localized in the area of leaflet attachment that is the site of greatest flexion in the aortic valve (12). It suggests that, also in all bioprostheses, mechanical stress initiates the cusp's degeneration and the degeneration could be faster if the GAG matrix is impaired.

Even the subsequent calcification is related in part to GAG loss. The highly negative charge of GAGs is hypothesized to attract calcium, thereby preventing calcium phosphate nucleation (13). Thus, GAG loss from the cuspal matrix can contribute to calcification. Moreover, GAG decrease impairs normal stress modulation, leading to the breakdown of collagen and exposition of calcium-binding sites on collagen (12). Calcium phosphate deposition is directly related to unfavorable mechanical stresses of the leaflets, and there is a correlation between sites of high mechanical stresses and subsequent calcification (13).

In our study, changes in commissural GAG content were found on leaflets in diastolic position, which is the usual technique of valve banking. The reason for commissural GAG loss after cryopreservation is still unclear, but it could be related to valvular stresses. At the commissures, the GAG honeycomb-like architecture is less thick, highly hydrated, and different in GAG classes composition, conferring more fluidity to the gel-like matrix and permitting a better management of bending stresses (19). The demonstration of commissural GAG decrease only in dias-

tolic position leads to the hypothesis that closed position creates a particular stress condition that makes GAGs more susceptible to damages related to cryopreservation. This hypothesis is confirmed by evidence of no significant GAG alteration in aortic leaflets and aortic wall, as stress-independent GAG damages should cause a homogeneous GAG loss in all the samples considered. Preserving the homograft with leaflets in open position, we observed that cryopreservation did not cause a significant GAG decrease at commissures. This technique preserved the matrix more efficiently and might prolong the durability of the aortic allograft.

The mechanisms of cryopreservation-induced matrix degeneration is far from clarified. Conventional cryopreservation was demonstrated to induce extensive extracellular ice formation (23), and the presence of ice in valve tissues causes a significant distortion of extracellular matrix, leading to degeneration and calcification (6,24). We can hypothesize an effect of icing on GAGs in areas subjected to particular stresses with direct structural damages or damages related to an increase in the tension forces due to fluid content expansion.

Limitations of this study

Our study was focused on damages of cryopreservation on GAGs before homograft implantation. We did not evaluate if these different cryopreservation techniques influenced homograft degeneration after implantation. After implantation, many mechanisms can affect GAGs, including mechanical stresses, inflammatory response, decrease in GAG synthesis, and active GAG replacement. In vitro fatigue studies are also needed to understand if leaflet mechanical properties are different between open and closed leaflets groups. Moreover, we only considered GAGs without evaluating the effects of cryopreservation on all the other valvular components. Other studies are required to clarify the effects of cryopreservation in diastolic and systolic positions on all valve components.

CONCLUSION

This study demonstrated that cryopreservation of aortic valve with leaflets in diastolic position causes total GAG loss at the commissures, the areas subjected to maximal mechanical stresses and the starting point of allograft degeneration and calcification. Cryopreservation of the aortic valve in an open leaflet position can preserve the matrix more efficiently and might prolong the durability of the aortic allograft.

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