

# Morphological Analyses of Ice-free and Frozen Cryopreserved Heart Valve Explants

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**Background and aim of the study:** The pathophysiology of allogeneic heart valve failure is not fully understood. It is hypothesized that the rapid deterioration seen in some allograft heart valve recipients is due to disruptive interstitial ice damage that occurs during cryopreservation by freezing.

**Methods:** The hypothesis was tested by comparing a standard commercial heart valve freezing and ice-free, vitrification cryopreservation methods with fresh controls in: (i) a subcutaneous, juvenile rat implant model of calcification; and (ii) a descending thoracic aorta implant rat model for histopathology. Calcium concentrations in one- to six-week explants were determined using atomic absorption spectroscopy; calcification rates were also determined.

**Results:** The calcification rate of frozen valves was

significantly greater ( $p < 0.01$ ) than that of vitrified valves in both syngeneic and allogeneic recipients, supporting prior observations that ice-free cryopreservation reduces allogeneic heart valve calcification. Cryopreservation by freezing and vitrification resulted in mild morphological changes in two- and four-week explants, a slight decrease in leaflet cellularity, and a more rapid onset of intimal hyperplasia than in fresh valve explants. The allograft explant groups exhibited similar changes, regardless of how the valves were processed.

**Conclusion:** These findings provide only weak support for the tested hypothesis, and further studies in a large animal model are warranted.

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Cryopreserved human heart valves are used in approximately 20% of the tissue heart valve procedures performed annually (1-4). These tissues often fail in young patients (5), particularly infants (6,7). The pathophysiology of allograft failure is not fully understood (8-10), but it has been hypothesized that the rapid deterioration seen in some allograft heart valve recipients is due to disruptive interstitial ice damage that occurs during cryopreservation and which leads subsequently to accelerated valve degeneration upon implantation.

An ice-free alternative to conventional cryopreservation has now been developed, termed cryopreservation by vitrification, to freeze tissues for long-term storage (11-13). The prevention of freezing requires

that the water in a tissue remains liquid during cooling. However, as cooling proceeds, the molecular motion in the liquid permeating the tissue decreases. Eventually, an 'arrested liquid' state known as a 'glass' is achieved if the solute concentration and cooling rate are sufficiently high. It is this conversion of a liquid into a glass that is called 'vitrification' (Greek *vitri* = glass).

In previous studies it was shown that extensive interstitial, extracellular ice formation occurs in 'conventionally' cryopreserved (frozen) heart valves (12). Since then, a commercial heart valve freezing method of cryopreservation and an ice-free, vitrification method of cryopreservation have been compared with fresh controls in a subcutaneous, juvenile rat implant model of calcification. Analysis of the tissues using atomic absorption spectroscopy (AAS) showed that vitrification resulted in a significant reduction in allogeneic heart valve calcification compared with frozen valves ( $p < 0.01$ ) (14). These studies of calcification were extended in the present studies, and morphological analyses of fresh and cryopreserved rat heart valve explants from the descending thoracic aorta of either syngeneic or allogeneic recipients were reported.

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## Materials and methods

Two implant models were employed in the present study. First, subcutaneous implantation studies of whole aortic heart valves were used to compare fresh control tissue samples with cryopreserved heart valves in a rat model of calcification (15). Second, morphological studies were performed using fresh control and cryopreserved heart valves implanted in the descending thoracic aorta (16). All animal care and handling complied with the *Principles of Laboratory Animal Care* as formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council.

Aortic heart valves from adult male Lewis or Brown Norway rats were implanted either into subdermal pouches in juvenile Lewis rats (3-week-old males, body weight 50-75g; n = 5-9 per group), or into the descending thoracic aorta of adult Lewis rats (n = 3-6 per group). The Brown Norway implants in Lewis rats are allogeneic, whereas the Lewis implants in Lewis rats are syngeneic (no immune response because the donor and recipient are genetically identical).

The subcutaneous valves were explanted after one to six weeks and analyzed for calcium deposition; the descending thoracic aorta grafts were explanted after two and four weeks for morphological examination.

### Heart valve preservation

#### Conventional cryopreservation

Conventional cryopreservation of valves by freezing was performed according to the steps disclosed in U.S. Patent 4,890,457. The heart valves were immersed in a cryoprotective solution of Dulbecco's modified Eagle's medium (DMEM) containing 10% dimethyl sulfoxide (DMSO) and 10% fetal bovine serum (FBS) at 4°C, for an incubation period of 15 min. The same pre-cooled

protective solution was added to the cryovial (Nunc) containing the heart valve. Samples were cooled slowly at a rate of 1°C per min to -80°C, and then placed in liquid nitrogen vapor at approximately -150°C for at least 24 h. Cryopreserved heart valves were re-warmed in two stages: an initial slow warming to -100°C, followed by rapid warming to melting. When re-warming had been completed, the cryopreservation solution was removed in a single step by placing the valve in a container of pre-chilled DMEM containing 10% FBS at 4°C.

#### Vitrification

Ice-free cryopreservation by vitrification was achieved by gradually infiltrating the heart valves with an 8.4 M solution consisting of 3.1 M DMSO, 3.1 M formamide and 2.2 M 1,2-propanediol in Euro-Collins solution at 4°C (11). The valves were vitrified by cooling at 40°C per min to -100°C in a pre-cooled bath of 2-methylbutane followed by cooling at 4°C per min to -130°C, and final storage at -135°C. The valves were re-warmed using a two-step warming process (11).

#### Calcification assessment

Calcium-specific mineralization occurring in subcutaneous aortic valve grafts was quantified using AAS (17). The samples were extensively washed in distilled water (minimum five times, each for 5 min), air-dried for at least 12 h, and placed in individual tubes. The dry samples were then weighed, hydrolyzed in HCl, and analyzed by AAS after appropriate dilution. The results were corrected for dry weight and dilution factor, and expressed as mg Ca per gram tissue dry weight.

Table I: Explant histopathology; an overview.

Treatment group (n = 3-6 per group)	Explant time (weeks)	Leaflet hypo- cellularity	Intimal hyperplasia	Retrovalvular organizing thrombus	Inflammation
Fresh syngeneic	2	-	-	-	-
Fresh syngeneic	4	-	+	-	-
Frozen syngeneic	2	-/+	+	-/+	-
Frozen syngeneic	4	-/+	+	-	-
Vitrified syngeneic	2	+	+++	-	-
Vitrified syngeneic	4	+	+++	-	-
Fresh allogeneic	2	++++	-	+ / ++	+
Fresh allogeneic	4	++++	+	+++ / +++++	+ / +++
Frozen allogeneic	2	+++	- / +	- / +	++
Frozen allogeneic	4	+++ / +++++	+	-	++
Vitrified allogeneic	2	++++	- / +	+ / ++	++
Vitrified allogeneic	4	++++	- / +	+++	+

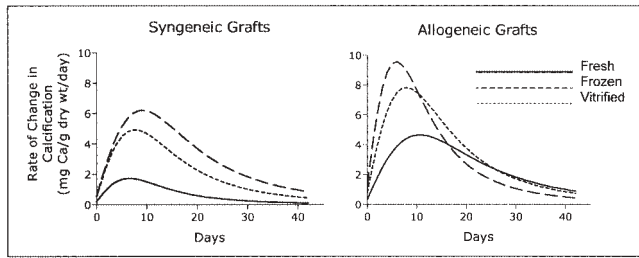


Figure 1: Rates of calcification were calculated using a non-linear logistic model. Analysis of the calcification data using the logistic model with  $n = 2$  showed high  $R^2$  values ranging from 0.61 to 0.76, except in the fresh syngeneic group ( $R^2 = 0.34$ ).

### Histopathology

Heart valve explants were fixed with 2% cacodylate-buffered glutaraldehyde solution for approximately 2 h, and then processed for light microscopy. Sections were stained with either hematoxylin and eosin or toluidine blue. Histopathology was assessed semi-qualitatively on images of heart valve leaflet and aortic wall sections. Leaflet hypoplasia, inflammation, intimal hyperplasia and retrovalvular organized thrombus were scored as negative (-), <25% (+), 25-50% (++) , 50-75% (+++) and >75% (++++) involvement.

### Statistical analyses

The rate of change in calcification was not constant; therefore a logistic equation,  $Y = M \frac{X^n}{X^n + K^n}$ , was used where  $X$  is the independent variable (time),  $Y$  is the dependent variable (calcium content), and  $M$ ,  $K$  and  $n$  are parameters chosen to fit the data to a non-linear curve. In this logistic model,  $M$  is a parameter which estimates the plateau or the maximum calcification level.  $K$  is a parameter which estimates the time it takes to reach 50% of maximum. For simplicity, the decision was made to fit the calcification data using  $n = 2$ , and allow the computer program (GraphPad

Software, 1999, San Diego California USA; www.graphpad.com) to determine values of  $M$  and  $K$  that would best fit the data (18). The GraphPad program achieved this via an iteration process so that the outcome variable ( $Y$  or calcium content) could be predicted from the independent variable ( $X$  or time) with the highest degree of accuracy.

## Results

### Tissue calcification

An analysis of calcification data using the logistic model with  $n = 2$  showed high  $R^2$  values ranging from 0.61 to 0.76 in all except one of the six groups. The exception was the fresh syngeneic group, where  $R^2 = 0.34$ . In general, it was not necessary to find a mathematical equation to describe the non-linear system perfectly. The purpose of a non-linear regression was to identify a relatively simple function that fitted the data reasonably well so that properties such as the plateau and the rate of change could be estimated and compared. The logistic model with  $n = 2$  appeared to satisfy that purpose well; hence no further regression models were tested. The rate of calcification was most rapid in valves cryopreserved by freezing, and was least in fresh controls (Fig. 1). Calcification rates in vitrified valves fell between those of fresh and frozen valves in both syngeneic and allogeneic comparisons (Fig. 1).

### Tissue morphology

In the morphological studies, the fresh syngeneic leaflets showed minimal changes, with a low level of intimal hyperplasia being observed at four weeks

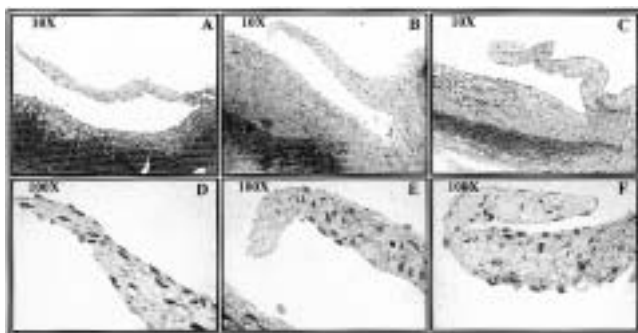


Figure 2: Representative photomicrographs of syngeneic rat valve graft 14-day explants. A, D) Fresh grafts; B, E) frozen grafts; C, F) vitrified grafts. Original magnifications are shown on each figure.

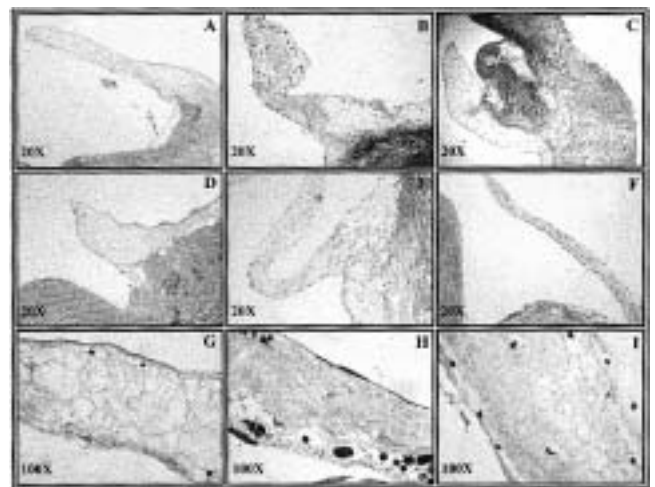


Figure 3: Representative photomicrographs of allogeneic rat valve graft 14-day explants. A, D, G) Fresh grafts; B, E, H) frozen grafts; C, F, I) vitrified grafts. Original magnifications are shown on each figure.

(Table I). Cryopreservation by freezing and vitrification resulted in mild changes, a slight decrease in leaflet cellularity, and a more rapid onset of intimal hyperplasia (Table I; Fig. 2). Vitrified syngeneic explants demonstrated more intimal hyperplasia than either fresh or frozen explants.

An initial phase of hypercellularity due to an inflammatory response followed by hypocellularity in the subsequent healing phase was anticipated in the allogeneic leaflets. However, the latter were generally hypocellular, devoid of myofibroblasts, and many contained variable amounts of acute inflammatory cells. It is possible that early hypercellularity was missed in the allogeneic leaflets because the first explant time point (2 weeks) occurred during the healing phase of the process. A single example of hypercellularity, inflammation combined with leaflet thickening due to fibrosis, was observed in the frozen allogeneic, two-week group.

Typically, the arterial component of the allografts exhibited mild (+) to medium (++)-grade diffuse acute inflammation (Table I). Leaflet degeneration was due to thickening at the base of the leaflets secondary to either intimal hyperplasia in the graft arterial wall or to retrovalvular organizing thrombus in the valve sinuses (Table I). These two phenomena appeared to lead to progressive leaflet degeneration and incorporation into the graft wall.

## Discussion

The cryopreservation process is another factor that may induce tissue damage during heart valve storage, whether or not a freezing method with associated ice formation or an ice-free vitrification method is employed. The syngeneic histopathological observations in the heart valve explants generally supported those of Legare et al. (16), who recently provided evidence that the cryopreservation of aortic valves resulted in increased structural deterioration. However, the changes presently observed in cryopreserved syngeneic valves were less severe than those indicated by Legare et al. (16), with only one case of retrovalvular thrombus formation with leaflet incorporation being observed. The only consistent difference seen between frozen and vitrified cryopreserved syngeneic heart valves was a higher level of intimal hyperplasia in the vitrified valves (Table I). The fresh, frozen and vitrified allogeneic leaflets were hypocellular and showed significant leaflet thickening and progressive leaflet degeneration due to a combination of intimal hyperplasia and retrovalvular thrombus organization (Table I; Fig. 3). Marked diminution of the cellular component in explanted human valves with progressive severe loss of any normal layered structure and connective

tissue cells has previously been reported (8). The allogeneic frozen cryopreserved explants contained less retrovalvular thrombus formation than the fresh and vitrified allogeneic explants. The changes observed in the allogeneic rat heart valves were most likely due to immunological incompatibility of the graft and the recipient, as was previously suggested in both rats (19) and human infants (6,7).

Previously, the ice-free cryopreservation method demonstrated a significant reduction in allogeneic heart valve calcification at three weeks after implantation ( $p < 0.01$ ) (12). Additional study time points were required to generate the calcification rate analyses presented in the present study. The rate of calcification was least in fresh tissue, intermediate in vitrified tissue, and greatest in frozen tissue (Fig. 1), suggesting that the method of cryopreservation can indeed influence the rate of calcification.

It has been proposed previously that the rapid deterioration (including calcification) observed in some allograft heart valve recipients may be secondary to disruptive interstitial ice damage that occurs during cryopreservation by freezing. The results of the present calcification studies have provided weak support for this proposal, because vitrified specimens exhibit more calcification than fresh valves, but in marked contrast the explanted rat heart valve graft morphological studies presented herein do not support this hypothesis at all. Large animal studies comparing allogeneic aortic heart valves cryopreserved by freezing and by ice-free vitrification have been initiated in a sheep model. It is anticipated that valve performance and explant analyses from these large animal studies may determine whether or not ice formation during cryopreservation results in a predisposition to structural deterioration.

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