Cryopreservation of Rat Aortic Valves Results in Increased Structural Failure

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**Background**—The cause of valve allograft failure is most likely multifactorial and may include mechanical, immunological, and other factors. Cryopreservation of these valves is often used to extend storage times. However, there has been considerable confusion as to the effects of cryopreservation on valve durability. Our objective was to determine the effects of cryopreservation on histopathological changes in rat aortic valve grafts.

**Methods and Results**—Syngeneic rat aortic valve grafts (Lewis to Lewis; n=24) and allogeneic rat aortic valve grafts (Brown Norway to Lewis; n=24) were implanted infrarenally, either fresh or after cryopreservation. At 7, 14, and 28 days, the valves were explanted, and histological and immunohistochemical examinations were performed in a blinded fashion. Fresh syngeneic graft leaflets retained their normal structure for the 28-day period of observation. Cryopreserved syngeneic grafts showed retrovalvar thrombus formation, with leaflet destruction at 7, 14, and 28 days. Fresh allogeneic graft leaflets showed significant leaflet thickening and progressive destruction at 14 and 28 days. Cryopreserved allogeneic grafts had evidence of retrovalvar thrombus formation with leaflet destruction at 7, 14, and 28 days. Cryopreserved syngeneic grafts resulted in significant infiltration of mononuclear (ED1⁺) cells not seen with fresh syngeneic grafts but similar to fresh allogeneic grafts. All allogeneic grafts resulted in significant infiltration of T-lymphocytes (CD3⁺, CD8⁺, CD43⁺).

**Conclusions**—Cryopreservation appears to predispose syngeneic and allogeneic rat aortic valve leaflets to accelerated injury and destruction. This mode of failure resembles that of fresh allogeneic valve grafts. *(Circulation. 2000;102[suppl III]:III-75-III-78.)*

**Key Words:** valves ■ rejection ■ leukocytes ■ lymphocytes

A llograft heart valves (homograft) have been widely used and shown to have superior hemodynamics, low thromboembolic rates, and resistance to infection compared with mechanical valves and xenografts.¹ Furthermore, allograft valves have become the valved conduit of choice used in pediatrics to reconstruct the right ventricular outflow tract.² However, despite these advantages, allograft valves ultimately fail. This is particularly true in children, in whom evidence of structural deterioration may be as high as 56% at 4 years.³

Preservation methods were first developed to standardize tissue specimen availability and to preserve the structural integrity of the allograft valves before implantation.⁴,⁵ Preservation methods included chemical preservation, irradiation, and freeze drying but resulted in an unacceptable incidence of structural deterioration.⁶ Improved results were then obtained with antibiotic sterilization⁷ and cryopreservation. Many centers now use cryopreserved heart valves based on work by O’Brien et al.⁸

However, significant controversy remains regarding valve preservation, because the effects of the various preservation techniques on allografts have not been clearly defined. Preservation may affect the antigenicity of the graft, the immunogenicity of the graft, and the viability of the cellular elements.⁹,¹⁰ All of these factors could have an impact on the durability of the allograft after implantation. Some centers continue to advocate the use of fresh valves maintained at 4°C and implanted within hours to a few days. These grafts have been referred to as homovitals.¹¹

It remains unclear whether the process of cryopreservation itself is detrimental to the cellular elements of the valve. It has been shown that cryopreserved heart valves have some demonstrable cellular viability and, most importantly, are structurally preserved at the time of implantation.¹² There also is evidence that like fresh valve allografts, cryopreserved valve allografts are immunogenic and result in donor-specific immune responses in the host.¹³,¹⁴ However, very few studies have isolated cryopreservation and evaluated its impact on long-term valve allograft survival.⁹

In the present study, we evaluated the histopathological changes seen in fresh and cryopreserved rat valve grafts implanted into syngeneic recipients. We compared the damage to these grafts during the next 4 weeks with the immune-mediated damage to allografted valves.
Methods

Experimental Animals

Inbred male Brown Norway (RT1.A^+^) and Lewis (RT1.A^+^) rats (weight 250 to 350 g) were purchased from Harlan-Sprague Dawley and housed in the Medical Sciences animal care facility with food and water ad libitum for 1 week before experimentation, in accordance with the guidelines of the Canadian Council of Animal Care.

Aortic Valve Allograft Implantation

The technique of aortic valve allograft transplantation was performed as first described by Yankah et al. An intraperitoneal injection of 65 mg/kg sodium pentobarbital was used to anesthetize the rats.

Donor Operation

A midline upper abdominal incision was made and extended as a median sternotomy. The aortic valve with some myocardium and ≈8 mm of ascending aorta was dissected free, and the coronary ostia were ligated with 9-0 nylon (Sharpoint) sutures. The graft was then rinsed with cold isotonic saline. Both the donor and recipient operations were performed with a Weck OM-1206 mounted operating microscope.

Recipient Operation

A midline laparotomy was performed, the bowel was eviscerated to the right, and the abdominal aorta was exposed. The aorta was mobilized from the level of the renal artery to the aortic bifurcation and divided between clamps. The aortic valve graft was then anastomosed between the stumps of the recipient aorta with interrupted 9-0 nylon sutures flushed intermittently with heparinized saline. Once hemostasis was secured, the abdominal contents were returned to the peritoneal cavity, and the wound was closed in layers. Strain combinations for experimentation consisted of syngeneic (Lewis to Lewis) and allogeneic (Brown Norway to Lewis).

Donor Valve Grafts

Two types of aortic valve grafts were used: (1) cryopreserved and (2) fresh. A 44.7 ± 6.1-minute period of warm ischemia was common to both types of valves, allowing the donor operation (10.6 ± 2.7 minutes) and recipient operation (34.3 ± 4.9 minutes) to be performed. (1) Cryopreservation was performed at the Queen Elizabeth II Regional Tissue Bank in the same manner as for human valve allografts. The explanted aortic valves were incubated in Hanks’ balanced salt solution (GIBCO) containing gentamicin and ceftazolin at 4°C for 24 hours and then placed in Hanks’ balanced salt solution with 10% dimethyl sulfoxide (Ficher) as a cryoprotectant. The valves were cooled in a controlled-rate freezer (Planer KRYO10) at −1°C/min to a temperature of −80°C and stored in vapor phase liquid nitrogen below −135°C. Before implantation, valves were thawed in a warm water bath (room temperature) and then rinsed with saline solution (0.9% normal saline). (2) Fresh tissue valves were harvested from donor animals, rinsed with saline solution, and implanted without delay into recipient animals.

Tissue Analysis

Tissue was harvested for histology at 7, 14, and 28 days and immersion-fixed (10% formaldehyde) at 4°C for 12 hours. Grafts were then paraffin embedded, and 5-μm sections were cut for histological and immunocytochemical examinations. Each tissue block was serially sectioned to ensure valve leaflets would be visualized. Tissue sections were stained for histology with hematoxylin-eosin (Harris hematoxylin) or Verhoeff elastin stain (5% hematoxylin, 10% ferric chloride, and Lugol’s iodine). Tissue slides from each animal were then examined with light microscopy (Nikon; Optophot Canada Inc.).

Immunohistochemistry

Sections were deparaffinized, endogenous peroxidase was quenched (0.06% HOOH/MeOH), and nonspecific staining was blocked with normal horse serum; sections were subsequently incubated with primary monoclonal antibodies for 1 hour at 37°C. The antibodies that we used were anti-CD43 (W3/13; Cedarlane), which is specific for a cell surface molecule present on most rat leukocytes; anti-CD3 (R73; Cedarlane), which is specific for αβ T-cell receptor positive bearing cells; anti-CD8 (MRC OX-8; Cedarlane), which is specific for a cell surface molecule present on CD8+ T cells; and anti-ED1 (ED1; Cedarlane), which is specific for a cytoplasmic molecule found in monocytes and macrophages. Sections were then washed in PBS containing 1% BSA (Sigma Chemical Co) 3 times before incubation with biotinylated secondary antibody and labeling with peroxidase avidin/biotin complex, with 3,3’-diaminobenzidine used as the chromogen (Vector Laboratories).

Results

Before implantation, both fresh and cryopreserved valve grafts were structurally normal, with thin, cellular leaflets (Figure 1). There was no cellular infiltration and no histological evidence of cell loss.

Fresh aortic valve grafts implanted into syngeneic animals (Lewis-Lewis) resulted in preservation of leaflets at all time points: 7, 14, and 28 days after implantation (Figures 2a to 2c). Leaflets were thin and cellular and had minimal inflammatory infiltration. In contrast, cryopreserved aortic valve grafts implanted into syngeneic animals (Lewis-Lewis) resulted in significant tissue infiltration (leaflet and adventitia) at 7 days with loss of leaflet structural integrity by...
14 and 28 days (Figures 2d to 2f). Only identifiable leaflet remnants were seen by 14 days, associated with significant intimal proliferation and some retrovalvar thrombus formation.

The appearance of the structural failure of cryopreserved syngeneic implants (Figures 2d to 2f) was compared with the failure of fresh allogeneic implants, as we previously reported.17 Fresh allogeneic valve grafts have been shown to have significant tissue infiltration at 7 days (Figure 2g), loss of leaflet cellularity by 14 days (Figure 2h), and loss of structural integrity by 28 days (Figure 2i). Morphologically, the mode of failure appears to be similar for fresh allogeneic and cryopreserved syngeneic grafts; in both cases, the end result was early structural failure by 28 days with complete destruction of the implanted valve (Figures 2f and 2i). These findings were consistent in all animals (4 per group). Cellular infiltration of the leaflets and adventitia early after implantation was most prominent with cryopreserved donor tissue. Characterization of these infiltrates was performed with immunocytochemistry. The Table illustrates that at 7 days after implantation, fresh syngeneic grafts resulted in little or no leaflet infiltration by ED1⁺ cells (macrophages), CD3⁺ T cells, CD8⁺ (cytotoxic T-lymphocytes [CTLs]), and CD43⁺ cells (primarily T-lymphocytes), with only moderate adventitial infiltration by ED1⁺ cells. However, at 7 days after implantation, cryopreserved syngeneic valve grafts resulted in significant infiltration of ED1⁺ cells (leaflet and adventitia) with few CD3⁺ T cells, CD8⁺ CTLs, and CD43⁺ cells. In contrast, at 7 days after implantation, cryopreserved allogeneic valve grafts resulted in significant infiltration of ED1⁺ cells, CD3⁺ T cells, CD8⁺ CTLs, and CD43⁺ cells (leaflet and adventitia). Representative photomicrographs of syngeneic and allogeneic cryopreserved valve grafts stained with anti-ED1 and anti-CD8 antibodies are shown in Figure 3.

### Discussion

In the present study, we used genetically identical inbred animals in the transplantation of aortic valve grafts, thus isolating cryopreservation of donor tissue as the only variable between fresh and cryopreserved valve grafts. Therefore, with the assumption that all other variables are controlled, we provide strong evidence to suggest that cryopreservation itself may be harmful and predispose aortic valve grafts to injury and early failure in this rat model. We show that in syngeneic cryopreserved valve grafts, there was early tissue infiltration and loss of structural integrity by 14 days in all animals. In contrast, fresh syngeneic valve grafts had normal preservation of leaflets for the duration of experimentation (28 days).

Cryopreservation of heart valve tissue in humans was established as a method of storage and believed by some to maintain the viability of the fibroblasts within the leaflets with the aim of subsequently enhancing valve durability,⁴,¹² Despite a body of literature that documents the preservation of viable fibroblasts up to 9 years after implantation,⁸ it appears that cryopreservation can damage cells and affect cell viability. The process involved for cryopreservation is relatively complex and has many variables: harvesting (warm ischemia), sterilization (antibiotic media for 24 hours), freezing (fluid shifts and ice crystal formation), storage, and thawing. At each step, there is a potential for cellular injury.⁴ Indeed, this technique is well known to result in reduced endothelial cell viability.¹⁸ The protocol for cryopreservation that we used in the present study is the standard protocol used for human valve tissue at our institution. During cryopreservation, ideally there should be a uniform rate of freezing, minimal fluid shift, and little crystal formation. Based on this, the relatively small pieces of rat tissue should exhibit more uniform cryopreservation than the much larger human valves. This should in theory result in less tissue injury.

Our experiments also suggest that morphologically, the appearance of structural failure with syngeneic cryopreserved valve grafts shows some similarities to that of fresh allogeneic valve grafts. In all cases, there was early infiltration at 7 days and leaflet destruction by 28 days despite differences in the characteristics of immune infiltrating cells. Structural failure of leaflets in an allogeneic environment with fresh valve grafts has been described in previous work performed by us and others.¹⁷,¹⁹ Based on this observation, it appears that injury to leaflet stromal cells, whether immune mediated

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<th>Valve Grafts 7 Days After Implantation</th>
<th>ED1⁺</th>
<th>CD3⁺</th>
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<td>Fresh syngeneic</td>
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- - indicate no, minimal, mild, moderate, and severe cellular infiltration, respectively.
(allogeneic/T cell driven) or mechanical (syngeneic cryopreserved tissue/monocyte macrophage driven), will result in tissue infiltration and the loss of structural integrity. In the present study, cryopreservation resulted in early infiltration of a significant number of mononuclear cells (ED1⁺), which are known to be important early effector cells in response to injury and to initiate a response to injury process. In the present study, they may be mostly responsible for the loss of leaflet integrity. In support of this observation, fresh syngeneic grafts had no leaflet infiltration throughout the study (28 days).

Cryopreservation does not appear to protect against immune cell activation, because allogeneic valve graft implantation resulted in T-cell (CD3⁺, CD8⁺, CD43⁺) immune activation and infiltration not observed with syngeneic grafts. The majority of infiltrating T-lymphocytes (CD3⁺) were also CD8⁺ (CTLs). These findings are in agreement with previous reports of donor-specific immune responses generated after allograft implantation in animal models and in humans.¹⁻³,¹⁴,¹⁻²¹

Taken together, there appears to be 2 important processes that may predispose cryopreserved valve grafts to structural failure. First, there is an early infiltration with myeloid cells regardless of allostimulation. Second, there is concomitant immune activation with infiltration of alloreactive T cells, more particularly, CD8⁺ CTLs. Both of these processes are followed by structural failure of cryopreserved allograft valves, suggesting that valve leaflet infiltration and associated tissue injury may cause structural failure of the implanted valve.

Clinically, these findings have important implications in terms of designing and timing therapeutic approaches to prolong allograft survival and durability. There appear to be 2 processes that could be targeted: early mononuclear cell infiltration in response to injury and lymphocytic infiltration associated with allostimulation. Standard clinical practice does not attempt to minimize or control either of these responses. Immunosuppression or anti-inflammatory agents have been sporadically tried, but no rigorous, prospective study has been performed.²,²²

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References