Allogeneic Heart Valve Storage Above the Glass Transition at −80°C

Kelvin G.M. Brockbank, PhD, Gregory J. Wright, BS, Hai Yao, PhD, Elizabeth D. Greene, LATg, Zhen Z. Chen, MS, and Katja Schenke-Layland, PhD

Cell & Tissue Systems, Inc, North Charleston, South Carolina; Georgia Tech/Emory Center for the Engineering of Living Tissues, The Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia; Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, South Carolina; Clemson University, Clemson-MUSC Bioengineering Program, Charleston, South Carolina; Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB), Department of Cell and Tissue Engineering, Stuttgart, Germany; and Inter-University Centre for Medical Technology (IZST) and Department of Thoracic, Cardiac and Vascular Surgery, Eberhard Karls University, Tübingen, Germany

Background. **Cryopreserved allogeneic heart valves are usually stored and transported below −135°C; however, such methods require expensive equipment for both storage and transportation.**

Methods. **In this study, vitrified porcine aortic valves were stored on either side of the cryoprotectant formulation's glass transition temperature (−119°C) at −80°C and −135°C, using a newly formulated vitrification solution (VS83) consisting of a combination of 4.65M dimethyl sulfoxide, 4.65M formamide, and 3.30M 1,2-propanediol. Three groups of valves were studied: (1) fresh; (2) VS83-preserved, stored at −80°C; and (3) VS83-preserved, stored at −135°C.**

Results. **Using the VS83 cryoprotectant concentration formulation, cracking was not observed during valve storage. No ice-related events were detectable during 5°C rewarming by differential scanning calorimetry. All cryopreserved tissue samples demonstrated significantly less viability than fresh samples ($p < 0.01$). No significant viability differences were observed between the VS83-preserved groups stored at −80°C and −135°C. Material testing did not reveal any significant differences among the three test groups. Multiphoton imaging of VS83-preserved heart valves stored at −80°C and −135°C demonstrated similar collagen and elastin structures.**

Conclusions. **These results indicate that VS83-preserved heart valves can be stored and transported at temperatures in the vicinity of −80°C with retention of extracellular matrix integrity and material properties. The VS83 preservation of heart valves at −80°C without the need for liquid nitrogen should result in both decreased manufacturing costs and reduced employee safety hazards. Moreover, it is anticipated that low cell viability may result in less immunogenicity in vivo.**

(Ann Thorac Surg 2011;91:1829–35)

Transplantation of allograft heart valves was first introduced clinically in 1962 [1]. They have demonstrated exceptionally good initial hemodynamic characteristics, hardly any thromboembolic events without anticoagulation, and better resistance to endocarditis compared with bioprosthetic or mechanical valve substitutes [2, 3]. Allografts have especially benefited children with congenital heart disease. They are also used in young adults, women of child bearing age, and less frequently in older patients with memory problems who may not be relied upon to keep up with the medications required for mechanical valves. Initially, the allograft valves were collected and immediately transplanted [4]. Due to logistic issues, grafts were subsequently stored at 4°C in tissue culture medium with antibiotics for up to 6 weeks prior to implantation [5]. Eventually, cryopreservation with dimethyl sulfoxide (DMSO) and fetal bovine serum was introduced to enable long-term storage and improve infectious disease screening [6]. For the last 20 years freezing has been the worldwide choice for cryopreservation of human heart valves [7].

In pediatric patients allograft function is limited by early structural deterioration, necessitating more frequent reintervention procedures [8–10]. A variety of reasons for pediatric allograft heart valve failure were discussed in the past and most investigators have emphasized immunologic issues [11, 12]. We have previously proposed the hypothesis that the rapid deterioration observed in some allograft heart valve recipients is due to disruptive interstitial ice damage that occurs during cryopreservation and subsequently leads to accelerated valve degeneration and calcification upon implantation [13–15]. This hypothesis led to the development of cardiovascular tissue preservation methods that avoided ice formation by vitrification [13, 16–18] based upon a 55% weight/volume vitrification solution (VS55) originally developed for preservation of kidneys at the Holland Laboratories of the American Red Cross [19–21]. Vitrification, in contrast to traditional cryopreservation employing freezing methods, uses high concentrations of a cryoprotectant solution

Accepted for publication Feb 14, 2011.

Address correspondence to Dr Brockbank, Cell & Tissue Systems, Inc, 2231 Technical Pkwy, Ste A, North Charleston, SC 29406; e-mail: kbrockbank@celltissuesystems.com.

Published by Elsevier Inc

0003-4975/$36.00
doi:10.1016/j.athoracsur.2011.02.043

Ann Thorac Surg
2011;91:1829–35

Abbreviations and Acronyms

DMSO = dimethylsulfoxide
DSC = differential scanning calorimetry
ECM = extracellular matrix
PBS = phosphate buffered saline
QMR = qualitative mean rating
RFU = relative fluorescent units
SHG = second harmonic generation
VS55 = vitrification solution 55%
VS83 = vitrification solution 83%

to promote amorphous solidification rather than crystallization to restrict the amount of ice crystal formation.

Excellent preservation was also reported using VS55 to maintain chondrocyte viability in rabbit articular cartilage [22]. However, when thicker porcine cartilage was employed it was necessary to utilize a more concentrated formulation, an 83% weight/volume vitrification solution (VS83), to maintain chondrocyte viability [23]. In this report, we present further studies using the VS83 formulation in which we investigated storage of heart valves below and above the formulation's glass transition temperature (−119°C) at −80°C and −135°C, respectively.

Material and Methods

Sample Preparation

Porcine hearts were obtained from a local slaughterhouse as bona fide excess tissues. The pigs were approximately 90 kg in weight, equally distributed male to female, and ranged in age from 4 to 6 months. These pigs were immature, since maturity is achieved at weights greater than 200 kg and 2 years of age. The postmortem warm ischemia time was approximately 30 minutes. All hearts were then rinsed with 4°C phosphate buffered saline (PBS; MediaTech, Herndon, VA), placed in PBS in sterile bags on ice, and immediately transferred to the laboratory for dissection. Aortic heart valves were excised under sterile conditions and gently rinsed free of any residual blood in sterile PBS. The valves were further dissected of adherent fat and most of the myocardium, leaving a thin ridge of subvalvular cardiac muscle tissue and a length of ascending thoracic aorta. All valves were incubated in an antibiotic solution consisting of Dulbecco's modified Eagle medium, containing 4.5 g/L glucose (MediaTech), and 1% penicillin-streptomycin (Sigma, St. Louis, MO) for approximately 24 hours at 4°C prior to use.

Cryopreservation Protocol

All valves were gradually infiltrated with precooled vitrification formulations of DMSO, formamide, and 1,2-propanediol in EuroCollins solution at 4°C in six steps of at least 15 minutes duration, consisting of 0, 12.5, 25, 50, 75, and 100% of each formulation to achieve a final cryoprotectant concentration of 55% or 83% weight per volume. The final concentration of each cryoprotectant was either 3.10M DMSO, 3.10M formamide, and 2.21M 1,2-propanediol (VS55), or 4.65M DMSO, 4.65M formamide, and 3.30M 1,2-propanediol (VS83). All tissue specimens were placed in polyethylene bags containing approximately 80 to 90 mL of precooled vitrification solution. A thermocouple was inserted into a separate dummy sample of the same vitrification solution and its output monitored by a digital thermometer throughout the cooling process. Samples were cooled to −100°C by placing the samples in a precooled bath containing isopentane in a −135°C mechanical storage freezer. Upon achieving −100°C, the specimens were removed from the bath and placed at −135°C in the mechanical storage freezer. When the heart valves reached −135°C they were transferred to their final storage temperatures of −80 or −135°C for 1 to 30 days. The tissues were rewarmed in two stages; first, slow warming to −100°C by placing them at the top of a mechanical storage freezer and then warming to −30°C in a 30% DMSO in water bath at room temperature. Tissues stored at −80°C were warmed using the second step only. After rewarming, the vitrification solutions were removed in seven sequential 15-minute steps at 4°C into Dulbecco's modified Eagle medium culture medium as previously described [16, 22, 24].

Biomechanical Testing

Fresh control samples were obtained from the noncoronary leaflet of aortic valves; the remaining tissue including the coronary leaflets were then bisected, cryopreserved in 50 mL of solution as described above, and randomized to each experimental storage group (n = 5 to 6). A radial strip of tissue was removed from each fresh control leaflet and experimental leaflet sample after the rewarming and cryoprotectant removal procedure described above. Each strip was cut using uniformly spaced blades to obtain a width of 4.5 mm. The thickness of each sample was measured using a custom-designed current sensing micrometer. After samples were dissected they were immediately placed in cold PBS until mechanical testing.

The biomechanical test methods were based upon published testing and calculation methods [25–27]. Tests were conducted on a soft tissue mechanical testing system (Bose ELF 3200; Bose Corporation, Eden Prairie, MN), exposing the tissue uniaxially to tension until failure. Samples were tested at room temperature and kept hydrated during testing using PBS. An initial tare load of 0.02N was applied to the tissue for preconditioning and the sample was loaded at a rate of 10 mm/minute until failure. Failure was considered to be a 20% reduction in force. Data were recorded as force versus displacement. The initial gauge length of the specimen was measured after preconditioning and used for calculating strain. The initial gauge length was approximately 9.0 mm for the samples tested. The initial cross-sectional area measured during dissection was used for stress calculations. Young's modulus (E) was calculated as the slope of the linear elastic portion of the stress-strain