Decellularization reduces calcification while improving both durability and 1-year functional results of pulmonary homograft valves in juvenile sheep

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Objective: The juvenile sheep functional valve chronic implant calcification model was used to compare long-term calcification rates, functional performance, and durability for 3 types of right ventricular outflow tract implants: classically cryopreserved homografts and 2 decellularized pulmonary valved conduits.

Methods: Fifteen juvenile sheep were randomly assigned to one of 3 study arms and underwent pulmonary valve replacement. The arms included the following: (1) cryopreserved ovine pulmonary valves; (2) cryopreserved, decellularized, saline (1°C–10°C)–stored ovine pulmonary valves; and (3) cryopreserved, decellularized, glycerolized (–80°C) stored ovine pulmonary valves. Animal growth, serial echocardiographic results (with valve performance assessment), dimensions, and tissue-specific calcification measurements were compared with preexplant angiographic analysis and right ventricular outflow tract pressure measurements, cardiac magnetic resonance imaging, specimen radiographic analysis, gross explant pathology, and histopathology. Parametric and nonparametric statistical analysis were performed.

Results: All but 2 study animals receiving implants thrived postoperatively, with similar growth rates, explant valve dimensions, ventricular functions, cardiac output, and indices during the study. As determined by means of echocardiographic analysis, 3 animals in arm 1 (and one in arm 2) had leaflet dysfunction. Valve regurgitation was recognized in 1 survivor each from both arms 1 and 2. Although 1 arm 1 animal died with calcified subacute bacterial endocarditis, and the other 4 had leaflet and conduit wall calcification by the time of death, no arm 2 or arm 3 animals demonstrated leaflet calcium, and no arm 3 and only 1 arm 2 animals had calcium in the conduit wall over the entire year, as determined with any measurement method. All cryopreserved conduit walls had calcium by 20 weeks, whereas only 1 of 10 decellularized conduits (arms 2 plus 3) had wall calcium.

Conclusion: Cryopreserved–decellularized–glycerolized valves retained normal valve function, with absent leaflet and minimal wall calcifications 1 year postoperatively, as opposed to classically cryopreserved allografts. These results might be predictive of the prolonged durability and functionality of a cryopreserved–decellularized–glycerolized allograft valve.

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Supplemental material is available online.

Cryopreserved homograft valves retain donor cells with varying degrees of metabolic activity. Cells become apoptotic as a result of harvest, transport, and cryopreservation, rendering the valve essentially acellular 9 to 12 months after implantation.[1] Retained antigenic, apoptotic, necrotic, donor cells/cellular debris lead to calcification, chronic inflammation, or both, promoting valve failure.[2-5] Efficiently decellularized, tissue-engineered homografts might prolong durability by reducing recipient inflammation, immune responses, fibrous scarring, and calcification, ultimately decreasing the number of patients requiring reconstructive cardiac surgery.[6]

The juvenile sheep chronic valve implant model is an excellent predictor of the durability and performance of biologic heart valves as affected by calcification.[7,8] This model was used in this study to compare long-term calcification rates, functional performance, and putative durability between classically cryopreserved allograft valves (arm 1) and cryopreserved–decellularized allograft pulmonary valve conduits preserved by means of 2 different methods. Arm 2 was comprised of decellularized valves stored in saline (4°C), and arm 3 consisted of decellularized valves stored in glycerol (–80°C) for at least 2 weeks before implantation.

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Abbreviations and Acronyms
ANOVA = analysis of variance
EOA = effective orifice area
MRI = magnetic resonance imaging
PRA = panel reactive antibody

MATERIALS AND METHODS

Homograft Harvest and Processing

Donor valves were harvested from sheep of analogous size, age, and weight but from different breeding stock than recipients. All valves were obtained by means of aseptic excision and in a manner similar to tissue harvest procedures.[9] Warm ischemic time was less than 1 hour. Each heart was placed in Lactated Ringers solution containing antibiotics and shipped to LifeNet Health (Virginia Beach, Va) on wet ice at 1°C to 10°C. Cold ischemic time before processing did not exceed 24 hours.

On receipt, pulmonary valves were dissected and disinfected by using standard clinical methods (4°C for 24 ± 2 hours with cefoxitin, lincomycin, polymyxin B, and vancomycin antibiotics). Subsequently, valves were incubated in RPMI 1640 containing 10% dimethyl sulfoxide and 10% fetal calf serum and cryopreserved at –1°C/min by using a computer-controlled freezing profile. Valves were then held in vapor-phase liquid nitrogen for at least 48 hours before decellularization.[10]

Decellularization

Before decellularization, valves were thawed and diluted of cryoprotectant by using a standard clinical protocol. The valve conduits were decellularized by LifeNet Health as in Appendix E1.

Sheep Chronic Implant Model

The investigational protocol used in this study was approved by the animal care and use committee. The animals received humane care in compliance with the principles stated in the ''Guide for the care and use of laboratory animals'' (National Institutes of Health publication no. 85-23). Fifteen juvenile, pre-estrus female domestic sheep *(Ovis aries)* of a Rambouillet, North Country Cheviot, Suffolk breed mix (163 ± 27.9 days of age; range, 126–202 days of age) with body weights 30 to 40 kg were selected as recipients. Age distribution for each arm was as follows: arm 1, 151.2 ± 28.9 days (range, 126–196 days); arm 2, 149.0 ± 20.5 days (range, 128–173 days); and arm 3, 190.4 ± 10.9 days (range, 176–202 days). After achievement of general anesthesia through a left thoracotomy and with cardiopulmonary bypass support, the pulmonary valve was excised, and the test valve was sutured into place as an interposition valved conduit. Animals were matured as a herd for 1 year.

Serial Studies

The following assessments were performed before implantation and 10, 20, 35, and 52 weeks after valve implantation: animal size, echocardiographic analysis, and panel reactive antibody (PRA) analysis.[11] Other assessments were performed 1 week before death.

Animal growth. Sheep growth was assessed by means of weight and length measurements and then converted to body surface area by using the Haycock formula.[12]

Echocardiographic analysis. Valve performance was evaluated with transthoracic 2-dimensional echocardiographic and Doppler assessments. Images were obtained in unsedated sheep held in the right lateral decubitus position with a 2.5-MHz transducer and a SONOS 1000 platform (Hewlett–Packard, Palo Alto, Calif). Echocardiographic protocols are fully described in Appendix E2.

PRA assay. Blood specimens were collected from the jugular veins of the sheep. The full serum PRA protocol is included in Appendix E3.[11]

Pre-explantation Studies

One week before death (week 52), each surviving animal underwent cardiac catheterization with right ventricular and pulmonary arterial angiographic analysis. These techniques are included in Appendices E4 and E5, respectively. All animals in arm 3 (and 1 in arm 1) also underwent cardiac magnetic resonance imaging (MRI), as detailed in Appendix E6.

Valve Explant Studies

Gross examination and measurements. After necropsy, explants were examined macroscopically (with a dissecting microscope) and radiographically (with an OEC 9600; General Electric, Milwaukee, Wis) for calcification and gross abnormalities. Calcium was mapped on a standardized valve diagram, and leaflet morphology, mobility, and configuration were recorded. Conduit dimensions were measured with calipers. The valves were then fixed for histopathology.

Histopathology. Movat's pentachrome, hematoxylin and eosin (Vector Laboratories, Burlingame, Calif), and either Alizarin red S or Von Kossa staining was performed by using standard histologic methods. Immunohistochemistry, confocal microscopy, electron microscopy, and quantitative morphometrics were also performed and will be reported separately.

Statistics

For all variables, descriptive statistics (means and standard deviations for continuous variables and proportions for categorical variables) were computed. Echocardiographic variables were compared by using single-factor analysis of variance (ANOVA). A general linear regression model was used for repeated measures (eg, multiple time points). Multivariate analyses included Pillai's Trace, Wilks' Lambda, Hotelling's Trace, and Roy's Largest Root. The SPSS version 15.0 for Windows Statistical Package (SPSS, Inc, Chicago, Ill) was used.

RESULTS

All but 2 study animals receiving implants thrived postoperatively. One arm 1 animal had bacterial endocarditis at 257 days. One arm 2 animal accidently hanged herself on a feed trough on day 299. All arm 3 animals survived the full 52 weeks. The rate of animal growth was similar for all 3 arms (see Figure E1), with significant increases in body surface area over time (ie, growth, $P = .006$). The sheep demonstrated no significant differences in growth curve shapes over the study ($P = .145$).

Echocardiographic Analysis

Peak and mean pressure gradients, effective orifice area (EOA) and EOA index were not different among arms over the study (see Table E1). Three animals in arm 1 (and 1 in arm 2) had leaflet dysfunction, whereas function remained normal for all arm 3 animals. Any occurrence of leaflet dysfunction in arm 1 valves was associated with leaflet calcification. The arm 2 animal identified with leaflet dysfunction demonstrated wall calcium by means of echocardiographic analysis, angiographic analysis, and explant macro-observations. Overall, 4 of 5 arm 1, 1 of 5 arm 2, and 0 of 5 arm 3 animals had leaflet calcium, wall calcium, or both, as determined by means of echocardiographic