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Prototype Anionic Detergent Technique Used to Decellularize Allograft Valve Conduits Evaluated in the Right Ventricular Outflow Tract in Sheep

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Background and aim of the study: **Biodegradable polymeric materials or extracellular matrix scaffolds are used in tissue-engineered heart valve designs, with the expectation of replicating the anatomic, histological and biomechanical characteristics of semilunar valves. The study aim was to evaluate the extent of in-vivo recellularization and the explant pathology findings of a prototype anionic, non-denaturing detergent and endonuclease technique used to decellularize allograft (homograft) valve conduits implanted in the right ventricular outflow tract (RVOT) of sheep, and to identify possible risks associated with tissue-engineered heart valve conduits based on decellularized allograft semilunar valve scaffolds.**

Methods: **Valve conduits were decellularized using a solution of N-lauroylsarcosinate and endonucleases, rinsed in lactated Ringers solution, and stored in an antibiotic solution at 4°C until implanted. Explanted valves and unimplanted controls were examined macroscopically, radiographically (for calcification) and histologically using immunohistochemistry (IHC), routine and special histological stains, transmission electron microscopy (TEM) and polarized light microscopy (evaluation of collagen crimp).**

Results: **Cells and cellular remnants were uniformly absent in the decellularized cusps, but occasional focal sites of arterial wall smooth muscle cells and to a greater extent subvalvular cardiac myocytes were variably retained. The trilaminar histological structure of the cusp was preserved. Valve conduit-related pathology consisted of intracuspal hematoma formation, collagen fraying, thinning of the conduit wall, and inflammatory cells associated with cardiac myocyte remnants. Cuspal calcification was not seen, but elastic fibers in the conduit wall and retained subvalvular cardiac myocyte remnants were liable to calcification. Fibrous sheath formation was present on the luminal surface of the conduit and extended over the cuspal surfaces to a variable extent. Myofibroblast-like cells repopulated the conduit wall and the basal region of the cusp. Re-endothelialization was variably present on the cuspal surfaces.**

Conclusion: **Explant pathology findings showed that in-vivo recellularization occurred, but was focally limited to regions of the arterial wall and cusp base. Safety concerns related to detergent and endonuclease use were identified. Methods to eliminate the potential for structural deterioration and enhance the rate and extent of recellularization of valve conduit tissue are required. Pathology findings showed implantation of valve conduits in the RVOT of juvenile sheep for 20 weeks to be a reliable animal model for the initial in-vivo assessment of decellularized valves. A 20-week period may be insufficient however to evaluate the long-term safety and effectiveness of a tissue-engineered valve conduit, as these depend on effective and phenotypically appropriate recellularization accompanied by sustained cell viability and function.**

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Cryopreserved allograft (homograft) heart valves remain as the standard for ventricular outflow tract reconstruction in infants, children and young adults (1,2). Although the long-term safety and clinical performance of cryopreserved allografts is encouraging (3), their performance characteristics are not ideal in

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The opinions and assertions are the private views of the authors and are not to be construed as conveying either an official endorsement or criticism by the U. S. Department of Health and Human Services or the Food and Drug Administration.

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children due to the reduced availability of small-diameter allografts, the lack of allograft heart valve somatic growth, and reduced long-term durability due to fibrosis and calcification. The application of emerging tissue engineering concepts may provide a solution to this current limitation, through the development of a tissue-engineered (TE) valve conduit that remains viable for the life of the patient as well as undergoing somatic growth as the child grows (4-6).

Two TE heart valve approaches are being explored which are based on the use of either biodegradable polymeric materials (7-13) or decellularized valve conduits (e.g. pulmonary and aortic valve allografts or composite porcine aortic valve xenografts) (14-19) intended to replicate the anatomic, histological and biomechanical characteristics of semilunar heart valves. Current TE heart valve designs rely on the use of a three-dimensional structure (referred to as a scaffold) that consists of either a biodegradable polymer, a tissue-derived biomaterial, or allograft tissues. In addition to the use of polymeric degradable materials, tissue-derived biomaterials and decellularized tissues, fibrin gel, gelatin, folded cell sheets (myofibroblasts; collagen) and elastin-collagen composite scaffolds are also being designed (20-23). All of these TE heart valve designs are based on the expectation that the scaffold material will be recellularized by the patient's autologous cells, either before implantation (i.e. by in-vitro cell seeding) or after implantation. The recellularized scaffold would then be capable of renewing valvular cellular and extracellular matrix (ECM) components, resulting in a viable replacement heart valve.

Explant pathology findings have been reported by others for the following three distinctly different pulmonary and aortic valve decellularization methods: (i) Sodium dodecyl sulfate, an anionic detergent; (ii) trypsin/EDTA (serine protease/divalent cation chelation); and (iii) deionized water. The first two methods were used to decellularize canine and sheep allograft valves, respectively (14,15), while deionized water has been used to decellularize a gamma-irradiated cryopreserved composite porcine aortic valve xenograft bioprosthesis (16). This unique composite bioprosthesis consisted of three non-coronary cusps, their corresponding arterial wall segments, and anterior mitral valve leaflets. A combination of decellularization (deionized water) and cryopreservation has also been used to process human allograft heart valves (17). All of these decellularized heart valve ECM scaffolds have been implanted as pulmonary artery interposition grafts in canine and sheep models (14-18).

The aim of the present investigation was to evaluate the extent of in-vivo recellularization (conduit wall and cusp), and to describe the explant pathological findings for allograft valve conduits decellularized with a prototype anionic non-denaturing detergent-endonuclease technique implanted in the RVOT of juvenile sheep for 20 weeks as functioning pulmonary valve replacements. Secondarily, these results could be used to identify risks and novel failure modes that were potentially generic to tissue-engineered valves, based on decellularized allograft tissues.

Materials and methods

Animal model

Donor and recipient sheep (*Ovis aries*) were 5 and 10 months old, and weighed 20 and 40 kg, at the time of allograft valve conduit harvest and implantation, respectively. Decellularized valve conduits (see below) were implanted aseptically with cardiopulmonary bypass support (pulmonary, n = 3; aortic, n = 3) in the right ventricular outflow tract (RVOT) as interposition grafts in juvenile sheep after excising the native pulmonary valve cusps and using standard surgical techniques (24,25). The investigational protocol used in this study was approved by the Animal Use and Care Committee of the Brown Medical School, and the animals received humane care in compliance with the principles stated in the *Guide for Care and Use of Laboratory Animals* (NIH Publication No. 85-23).

Valve conduit tissue processing

The harvested ovine valve conduits (aortic, n = 3; pulmonary, n = 3) were disinfected using previously reported standard clinical methods (4°C, for 24 h, with cefoxitin, lincomycin, polymyxin B and vancomycin antibiotics) before the decellularization tissue processing was initiated (26). The cold ischemic times - that is, the time that tissues were stored at 4°C between valve conduit harvesting and antibiotic disinfection - were recorded for three of the six conduits, and ranged from 2 to 27 days (pulmonary valve, 2 and 19 days; aortic valve, 27 days). The cold ischemic times were not recorded for the other three valves; however, this parameter was within the range of the first three valves. In the context of cryopreservation harvest and transport, it has been shown previously that the valves show a marked resilience to prolonged cold storage (27-29). The valve conduits were decellularized using an anionic, non-denaturing detergent (N-lauroylsarcosinate; Sigma, St. Louis, MO, USA) solution containing a recombinant endonuclease (Benzonase; Sigma), which was recirculated at room temperature for 24 h. Following detergent and endonuclease treatment, the valve conduits were rinsed (at room temperature) using recirculated lactated Ringers solution (90 min) and stored in an antibiotic solution (as described above) at 4°C until implantation. The storage interval ranged from 17 to 69 days.

Morphological studies

The valve conduits were explanted at 20 weeks after implantation and fixed with phosphate-buffered 10% formalin. Before being sectioned for histological studies, the explants were examined both macroscopically (dissecting microscope) and radiographically (25 kVp, 5-8 min; Faxitron X-Ray Cabinet, Wheeling, IL, USA). The specimens were then cut longitudinally through the native artery, the proximal and distal anastomoses and the central third of the valve extending from the base to the free edge of the cusp, and embedded in paraffin. Twenty consecutive sections were cut from each block for histological (hematoxylin and eosin, Movat pentachrome staining) and immunohistochemical (IHC) studies. All IHC tissue sections were exposed to an antigen retrieval method (antigen retrieval solution; Vector Laboratories, Burlingame, CA, USA) for 10 min at 90°C. The following primary antibodies (all from DAKO, Carpentia, CA, USA) were used: Factor VIII-related antigen (rabbit polyclonal); alpha smooth muscle actin (mouse monoclonal); and vimentin (mouse monoclonal). The primary antibodies were incubated overnight (4°C), the slides washed, and exposed to either conjugated peroxidase (vimentin-VIP substrate) or alkaline phosphatase (alpha smooth muscle actin or Factor VIII-related antigen-red substrate) secondary antibodies (Vector Laboratories). These antibodies were determined to have cross-reactivity with formalin-fixed ovine vascular tissue (i.e. native aortic wall). In addition, the vascular smooth muscle cells, endothelial cells and fibroblasts present in granulation tissue surrounding the explanted valve conduits served as a positive IHC control in each tissue section. Appropriate negative IHC controls (i.e. omission of primary antibodies; the substitution of normal mouse or rabbit IgG for the primary antibody) were performed and gave negative results. Transmission electron microscopy (TEM) studies (JEOL 100 CX; JEOL USA, Peabody, MA, USA) were conducted on tissues treated with Trump's fixative and processed in a routine manner.

Histological and IHC sections were studied using a light microscope equipped with a digital camera (Axiphot microscope; Axiocam, Karl Zeiss, Thornwood, NY, USA). The digital images were further processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Results

Unimplanted decellularized valve conduits

Nuclei and cellular remnants were not present in the decellularized cusps (compare Fig. 1A, native valve cusp and Fig. 1B, decellularized cusp); however, arterial wall smooth muscle cells (Fig. 1C) and subvalvular cardiac myocytes were variably retained. The trilaminar histological features of the semilunar cusps (ventricularis, spongiosa, fibrosa) were preserved, though small circular voids were observed in the fibrosa and to a lesser extent in the ventricularis (Fig. 1B). These voids may represent previous sites of interstitial cells removed by the decellularization process.

Polarized light microscopy demonstrated the retention of collagen crimp (waviness) within the fibrosa, and similar to the repeating birefringent pattern seen in native semilunar cusps (Fig. 1D). Small microscopic kinks or folds were observed in the decellularized cuspal tissue using this optical technique. Whether these represent sites previously occupied by interstitial cells

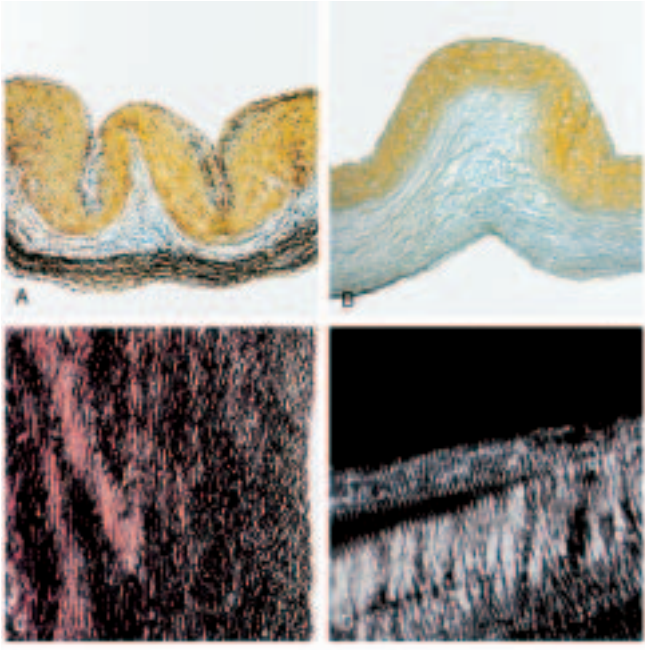

Figure 1: Light micrographs depicting native aortic valve and decellularized valve conduit histological features. A) Trilaminar histological structure of the native aortic valve demonstrating the extracellular matrix features of the ventricularis (elastic fibers), spongiosa (proteoglycans) and fibrosa (collagen). Movat pentachrome stain: collagen shown as yellow; proteogylcans as blue; elastic fibers as black. Original magnification, ×100. B) Decellularized cusp demonstrating the retention of the characteristic trilaminar architecture of the native aortic valve. Note the absence of cellularity and the presence of small voids in the tissue (most apparent in the fibrosa). Movat pentachrome stain. Original magnification, ×200. C) Histological characteristics of the decellularized aortic wall. Smooth muscle cell remnants (pink) are retained in the media. Movat pentachrome stain. Original magnification, ×100. D) Transmitted polarized light micrograph demonstrating the presence of collagen crimp (waviness) in the fibrosa of a decellularized aortic valve cusp. Note the presence of kinks or folds in the tissue. Original magnification, ×100.

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or reflect changes in ECM composition or biomechanical properties of the cuspal tissue remains to be determined.

The tissue processing method used in the present study also resulted in collagen fibril fraying (Fig. 2), as demonstrated by TEM. The ultrastructural features of these frayed collagen fibrils are consistent with type 1 collagen.

Explanted decellularized allograft valve conduits

Inflammatory response

The cuspal and conduit wall components (ECM and cell remnants) did not elicit an inflammatory response; however, chronic mononuclear inflammatory cells were observed surrounding cardiac myocyte remnants present in the subvalvular regions of the decellularized allograft (Fig. 3A). The identification of the specific cell types involved in this inflammatory response was not possible due to the inability to obtain B-cell or T-cell ovine-specific antibodies.

Structural deterioration

Dystrophic calcification: Cuspal calcification was not observed, though variable amounts of calcification occurred in the conduit wall (i.e. elastic fibers), subvalvular cardiac myocyte remnants and thrombus surrounding anastomotic suture materials (Fig. 3B-D).

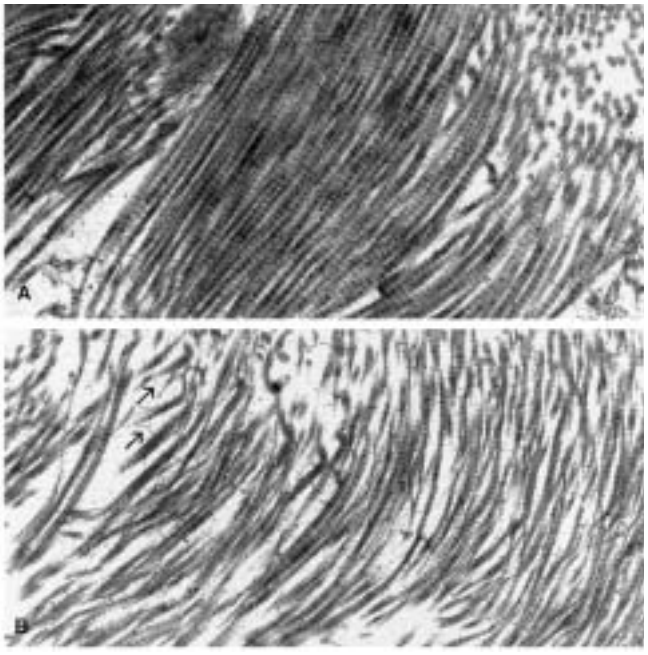

Figure 2: Ultrastructural morphology of collagen fibrils in the fibrosa of the native aortic valve (A) and the decellularized cuspal tissue (B). Collagen fibril fraying (arrows) in the decellularized tissue is present in regions of crimped collagen. Lead citrate and uranyl acetate staining. Original magnification, ×28,000.

Abrasive wear: Evidence suggestive of cuspal tissue abrasive wear was in the form of intracuspal hematoma formation, and collagen bundle fraying was also observed in 20% of the cusps. These findings were consistently observed in the region of the nodulus Arantii (aortic valve) and nodulus Morgagni (pulmonary valve), which are located in the middle portion of the cuspal free edge. Repetitive contact between the adjacent noduli of the semilunar cusps is believed to result in abrasive tissue damage in the form of collagen fraying, tissue dissection and infiltration of red blood cells (intracuspal hematoma) (Fig. 4A).

Arterial wall thinning: Marked thinning of the conduit wall following implantation in the RVOT was noted in this series of explants (Fig. 4B). Histological sections in which the smooth muscle cells were essentially absent from the arterial wall resulted in compressive thinning of the conduit after implantation (Fig. 4C). In regions where removal of smooth muscle cells was incomplete, thinning of the conduit wall was less apparent.

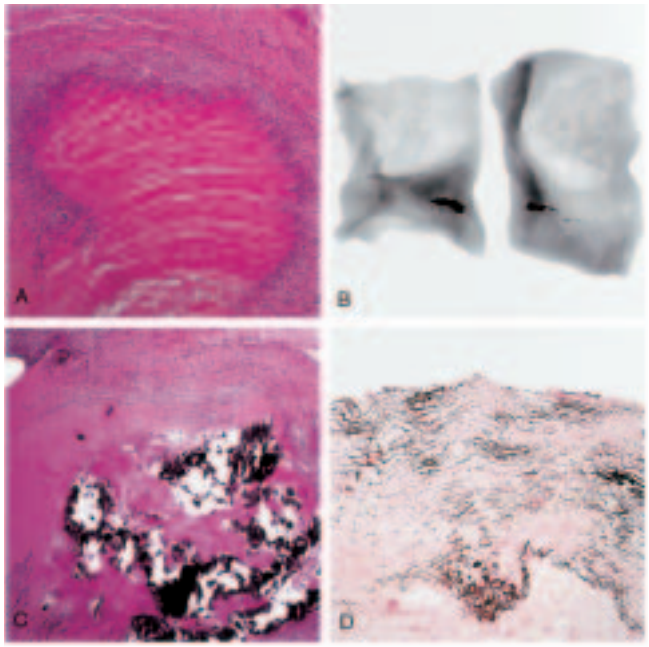

Figure 3: Morphological features of the conduit wall following implantation for 20 weeks. A) Chronic inflammatory cells associated with subvalvular remnants of cardiac myocytes. H & E stain. Original magnification, ×100. B) Radiograph demonstrating the presence of calcification in the subvalvular region of the valve conduit and focally along the proximal anastomosis. Note the absence of cuspal and conduit wall calcification. Original magnification, ×200 C) Histological section demonstrating calcification associated with cardiac myocytes remnants. H & E stain. Original magnification, ×100. D) Microscopic calcification (black) of elastic fibers present in the conduit wall. Von Kossa stain. Original magnification, ×100.

Fibrous sheathing: Fibrous sheath formation was present on the luminal surface of the conduit, and extended over the cuspal surfaces (inflow side > outflow side) to a variable distance (approximately 20-30% of the length of the inflow cusp; 0-5% or less on the outflow cuspal surface) (Fig. 4D). The thickness of the fibrous sheath decreased as it progressed from the base of the cusp toward the free edge. The fibrous sheath was predominantly covered by a continuous layer of endothelial cells (Factor VIII-related antigen immunoreactive). A focal endothelial cell distribution was also present, primarily on the outflow surface, in the distal regions of the cusp not covered by fibrous sheath.

Recellularization

Recellularization was variably present in the conduit wall, and limited to the basal region of the cusp. Microscopically, autologous cells migrated through the anastomotic sites and adventitial side of the conduit (Fig. 5A and B).

The cellular repopulation of the basal region of the cusp appeared histologically to be an extension of the tissue ingrowth occurring through the proximal anastomosis. Cuspal recellularization occurred as the result of cell migration into the spongiosa (middle histological region of the cusp), which is composed of loose connective tissue rich in proteoglycans. Autologous

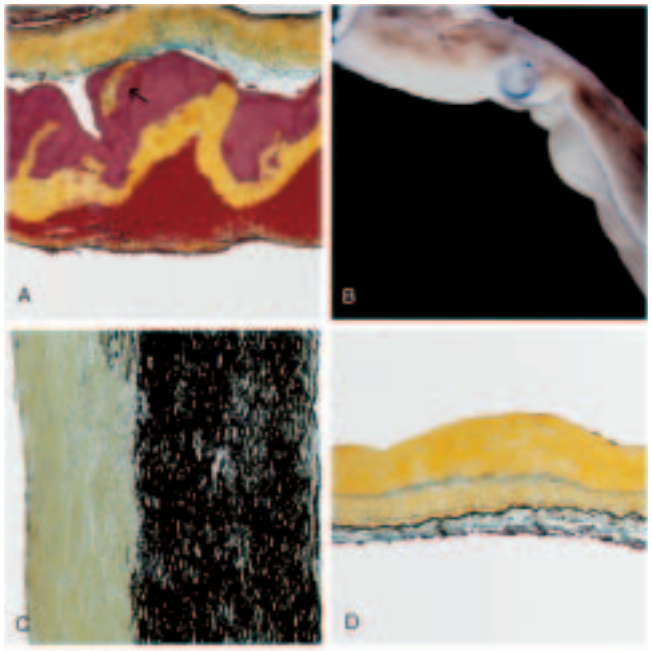

Figure 4: Series of micrographs demonstrating the structural changes occurring in cuspal and conduit wall tissues of a decellularized valve conduit implanted for 20 weeks. A) Structural deterioration of a decellularized cusp: 1) collagen bundle fraying (arrow); 2) intracuspal hematoma formation (spongiosa- bright red region); and 3) thrombus on the outflow surface of the cusp associated with collagen bundle fraying. Movat pentachrome stain. Original magnification, ×100. B) Thinning of the conduit wall. Prolene suture (blue) marks the site of the distal anastomosis. Compare the conduit wall thickness of the native pulmonary artery to that of the conduit wall. Original magnification, ×100. C) Histological section taken through the thin region of the conduit wall, illustrating compression of the decellularized media and a lack of recellularization of this region of the conduit wall. Original magnification, ×100.D) Micrograph showing loss of the spongiosa in the mid-region of a decellularized cusp. Fibrous sheath is present on the inflow surface of the cusp. Movat pentachrome stain. Original magnification, ×100.

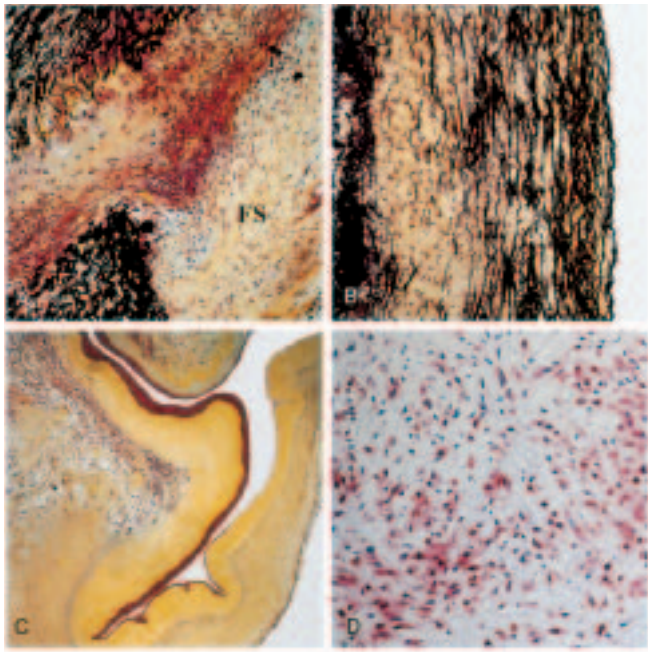

Figure 5: Representative micrographs depicting valve conduit recellularization following 20 weeks of implantation. A) Host cells migrating from the fibrous sheath (FS) present on the luminal surface of the conduit wall through the proximal anastomosis. Note the cellularity of the collagen-rich extracellular matrix and the presence of cells migrating between the elastic fibers (stained black) in the native artery and conduit wall. Movat pentachrome stain. Original magnification, ×200. B) Histological section of the conduit wall. Host cells are present between the elastic fibers, and appear to migrate into the conduit wall from the adventitial side of the graft. Movat pentachrome stain. Original magnification, ×200. C) Histological section demonstrating the limited migration of host cells into the basal region of a decellularized cusp. The luminal surface of the conduit wall and the outflow side of the cusp are covered with thrombus. Movat pentachrome stain. Original magnification, ×100. D) Micrograph demonstrating the presence of alpha smooth muscle actin-immunoreactive cells in the basal region of the cusp at 20 weeks after implantation. Alkaline phosphatase stain, red substrate. Original magnification, ×200.

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cells were observed to migrate into the wide spongiosa present in the base of the cusp, but were not seen in the thin spongiosal layer which continues a short distance toward the free edge of the cusp (Fig. 5C). This observation was in contrast to the findings noted in the distal regions of the cusp, where the ventricularis and spongiosal layers were markedly compressed to the point where elastic fibers predominantly comprised the ventricularis, but the spongiosa was completely absent (Fig. 4D). Recellularization of the cusp did not progress from the spongiosa into either the fibrosa or ventricularis, with the exception of the presence of focal regions of cell migration in the basal region of the cusp. The ventricularis and the fibrosa may in fact be anatomic barriers preventing complete recellularization of the cusp (e.g. fibrosa - dense, circumferentially oriented collagen bundles; ventricularis - dense, radially oriented elastic fibers).

The non-inflammatory cells repopulating the decellularized conduit wall and the basal regions of the cusp had a fibroblast-like histological appearance. The majority of the cells were alpha smooth muscle actin- (Fig. 5D) and vimentin-immunoreactive, suggesting a myofibroblast phenotype (30,31). The ultrastructural features of the recellularized regions of the valve conduit were consistent with both fibroblasts (no basement membrane, abundant endoplasmic reticulum) and myofibroblasts (incomplete basement membrane, peripherally located intermediate filaments) (32); these findings indicated that a mixed cell population consisting of fibroblasts and myofibroblasts was repopulating the decellularized tissue.

Discussion

Decellularized (also referred to as acellular or devitalized) aortic and pulmonary valve tissues are emerging as investigational scaffolds for the creation of TE heart valves. Various techniques have been reported for the removal of cellular components from semilunar valve tissue while retaining the majority of the ECM components (primarily collagens, elastin and the less water-soluble proteoglycans). The following decellularization methods effectively remove endothelial cells and cuspal interstitial cells: (i) detergents; (ii) trypsin/EDTA; and (iii) deionized water (14-19). These agents are frequently used in combination with protease inhibitors. In addition to removing the cellular components, residual nucleic acids are also removed from the tissue by endonuclease digestion. The tissues are then further washed to facilitate removal of cellular remnants and tissue-processing reagents.

Although endothelial cells and cuspal interstitial cells are effectively removed by the above-mentioned decellularization methods, few reports have been made describing the effectiveness of these methods to remove cardiac myocytes and arterial wall smooth muscle cells that are also present in valve conduit tissues. The present findings, based on the use of N-lauroylsarcosinate - an anionic, non-denaturing detergent - showed that the subvalvular remnants of cardiac myocytes were consistently retained, while the arterial wall smooth muscle cells were markedly reduced, but variably present. Cuspal cells were consistently removed by the N-lauroylsarcosinate detergent tissue processing.

These histologic findings indicated that modifications of decellularization techniques are required to facilitate complete removal of the cellular components present in the valve conduit, and consequently the following approaches are under investigation:

- determination of the optimal duration of the ischemic cold time (i.e. minimizing the potential for uncontrolled tissue autolysis while enhancing the positive effect of soaking tissue in an electrolyte solution, thus facilitating tissue penetration by decellularization agents);
- use of non-ionic versus anionic detergents;
- optimal tissue exposure times to decellularization agents;
- methods for increasing tissue permeability; and
- the use of standard methods for the evaluation of cytotoxicity (ISO 10993-5:Cytoxicity).

The decellularization method employed in the present study altered the ultrastructural morphology of type I collagen, as shown by the presence of fibril 'fraying' in the unimplanted valve conduit tissue. In addition to the observed ultrastructural alterations in collagen morphology, evidence of non-calcific structural deterioration was also seen, ranging from abrasive wear (e.g. intracuspal hematoma formation, collagen bundle fraying) to conduit wall thinning. These findings serve as a warning that any technique which accomplishes aggressive decellularization may compromise valve conduit safety (e.g. aneurysm formation, cuspal abrasion and tearing) and performance (e.g. cuspal wear, saccular dilation of the arterial wall, resulting in the loss of cuspal coaptation).

Dystrophic calcification was essentially limited to regions of the tissue containing cardiac myocyte remnants, which extend into the semilunar valve annulus to the base of the cusp, although focal microscopic calcification was present in conduit wall elastic fibers. No evidence of cuspal calcification was observed, which suggested that the detergent treatment was effective in mitigating dystrophic calcification of cuspal tissue, as reported previously with detergent (surfactant) treatments of bioprosthetic heart valves (34). The limited

recellularization of valve cuspal tissue was an unanticipated finding of the present study. Cytotoxicity associated with residual chemicals used in the decellularization process, or their inconsistent removal during tissue washing, must be considered as a possible factor contributing to the limited in-vivo recellularization of valve conduit tissue. As part of the initial development of this prototype technique, decellularized valve conduit tissues (wall and cusp) were indirectly screened for the presence of residual cytotoxic agents by seeding processed tissues with cuspal interstitial cells using routine in-vitro cell culture methods (31,33). Cytotoxicity was not observed, suggesting that residual agents - if present - were not responsible for the limited in-vivo recellularization of the cuspal tissue observed in this series of explants.

The present histologic findings showed that cuspal recellularization was essentially limited to the base of the cusp, although fibrous sheath formation occurred on the cuspal surface (inflow > outflow). However, recellularization of the arterial wall was consistently observed on the adventitial side of the conduit wall as well as occurring through the anastomoses, indicating that recellularization was primarily the result of recipient tissue ingrowth, as is typical of the healing response. However, focal endothelialization was observed on the cuspal outflow surface in regions not covered by fibrous sheath, suggesting that circulating endothelial progenitor cells may have repopulated the cuspal surface. Although cuspal recellularization has been reported in the literature, few comments have been made concerning the extent and distribution of cuspal recellularization; however, fibrous sheath formation has been misinterpreted as cuspal recellularization.

In the context of tissue engineering, recellularization should be clearly defined as the process in which phenotypically appropriate cells capable of reconstituting native tissue structure and function migrate into a scaffold material. This definition is clearly different from less rigorous criteria based merely on the presence of cells. The findings of the present study reflect true recellularization only focally, but generally a salubrious and functional fibrous sheath formation (a response to fluid shear stress on the luminal surface of the conduit wall and the cuspal surfaces) and a healing response characterized by fibrous tissue ingrowth into the conduit wall. The healing response observed in this series of explants was similar to that typically observed following the implantation of cryopreserved heart valve allografts, prosthetic heart valves, vascular grafts, and decellularized blood vessels in various chronic animal models (35). In addition to cytotoxicity, other possible mechanisms for the limited cuspal recellularization observed in the present study include: the presence of anatomic barriers (e.g. elastic fibers in the ventricularis; compression and loss of the spongiosa after implantation); the removal of proteins and glycosaminoglycans that may facilitate cell migration; mechanical factors such as bending stresses present in the basal region of the cusp; and/or perhaps the limited duration of implantation (20 weeks) (36).

Among published findings describing decellularized allograft valve recellularization, another method which involves the treatment of valvular tissue with trypsin/EDTA and static seeding of the upper surface of the valve with autologous myofibroblasts (six days) followed by endothelial cells (two days) before implantation in sheep (pulmonary artery interposition graft), has also demonstrated some encouraging results. This method reportedly resulted in complete cuspal recellularization and the formation of a confluent endothelial cell layer on the surface of the cusp in vivo, while partial degeneration was observed in the unseeded decellularized valves, without evidence of cuspal interstitial cell repopulation (15); indeed, these findings were not too dissimilar to those of the present investigation. An in-vivo model was selected initially to qualify this prototype decellularization method, as in-vivo correlates of in-vitro surrogate markers of structural deterioration based on tissue mechanics (e.g. Young's modulus, maximal tensile strength, stress relaxation constants) have not been validated, and neither have tissue mechanics 'pass/fail' criteria been established (FDA Replacement Heart Valve Guidance Document - Draft, 1994; ISO 5840 Cardiovascular implants - Cardiac valve prostheses, 1996) for tissue-derived biomaterials used in the fabrication of bioprosthetic heart valves.

It is acknowledged that tissue mechanical properties are a critical component in the characterization of tissue-derived biomaterial properties, as well as biochemical and morphological studies. The documentation of the tissue mechanical properties will be incorporated into ongoing comparative studies of investigational decellularization methods being evaluated in the authors' laboratory. The identification of tissue mechanics endpoints would provide a means of monitoring and comparing the effects of tissue processing and the alteration of tissue mechanical properties as a result of decellularization.

A chronic animal study evaluating the safety and performance of cardiac valve prostheses is traditionally considered to be 'preclinical' (or 'non-clinical') in nature (ISO 10993-2), and is intended to identify potential valve design-related failure modes and valve-related pathology. However, there is a different and very important role for in-vivo testing in less mature technologies such as tissue-engineered constructs. In the initial phase of development of novel technologies,

potential and unique risks must be identified and subsequent modifications made to minimize the risks associated with an investigational technology or device. It is the early phase (or initial qualifying study) to which the experiments reported herein belong. The present study was performed to assess the in-vivo recellularization by autologous cells of a prototype decellularized ECM scaffold, and to define potential generic safety concerns related to the use of detergent-based decellularization methods.

While all animals survived, and there were no catastrophic failures of the functioning valve conduits - that is, all cusps were present and pliable at explant, and there were no right-heart failures or conduit wall ruptures - the explant pathology findings described herein have identified several potential safety concerns that might contribute to the long-term failure of detergent-based decellularized valve conduits, including the use of non-denaturing detergents. The increase in tissue processing time (e.g. 24 h of tissue rinsing at room temperature), storage of the valve conduit in a physiological solution at 4°C, and the use of an antibiotic-disinfected decellularized ECM, as compared to a sterile bioprosthetic heart valve, raises concerns of a possible increase in the incidence of endocarditis associated with the use of decellularized valve conduits. Modest dimensional changes (i.e. arterial wall thinning) were observed in valve conduits implanted in the RVOT; however, based on the observed morphology, the potential for significant dimensional changes (e.g. saccular aneurysm formation or even rupture) would be possible in valve conduits used in left ventricular outflow tract (high-pressure) reconstructions. In addition, evidence of decreased cuspal resistance to abrasive wear (intracuspal hematoma formation, collagen bundle fraying) was observed, and this might affect the long-term durability of the detergent-decellularized valve conduits. The presence of cellular remnants, including subvalvular cardiac myocytes and arterial wall smooth muscle cells, would be expected to elicit an immune response, which may contribute to fibrous sheath formation and calcification.

Although other detergent-based methods have not been perfected, previous reports have suggested that, as a general methodological approach, this technique shows good promise. Thus, the purpose of the present investigation was initially to evaluate a previously unreported anionic detergent, N-lauroylsarcosinate, as a valve conduit decellularization agent based on an in-vivo feasibility and safety study. The following primary endpoints were assessed: (i) The extent of preimplantation decellularization; (ii) feasibility, as defined by the rate and extent of recellularization; and (iii) safety, based on the identification of valve conduit-related pathology as the consequence of implantation in the RVOT. In this respect, the present study was extremely valuable.

The results of the present study clearly demonstrated the effectiveness of an anionic, non-denaturing detergent (N-lauroylsarcosinate) to remove endothelial cells and cuspal interstitial cells, while retaining the ECM histologic features of the semilunar cusp. The observed retention of cardiac myocytes (base of cusp) and focal sites of smooth muscle cell remnants present in the conduit wall highlight the need to balance efforts for complete decellularization of tissue against the potential safety concerns associated with the loss of structural integrity. For example, the chronic inflammatory response observed at the base of the cusp due to retained donor cardiac myocytes may not be clinically significant, as this is where the sutures - which themselves elicit an inflammatory response - are placed. A most basic qualification is that the cusps must be completely devoid of donor cells and with minimal residual debris or loss of integrity. Any method which results in the incomplete decellularization of arterial wall (due to the potential for cell remnants to elicit immune and inflammatory responses) may contribute to the failure of decellularized valve conduits. While the tested technique proved to be encouraging, the study results identified potential failure modes, and these must be specifically addressed when bioengineered heart valve tissue processing techniques are further developed. The explant pathology findings presented herein have identified safety concerns associated with valve conduits decellularization strategies. Lastly, the explant pathology findings reported showed that implantation of valve conduits in the RVOT of juvenile sheep for 20 weeks is a useful chronic animal model for the initial feasibility and safety evaluation of decellularized valve conduit tissue-processing techniques. However, the traditional 20-week period of implantation - which in the past has been a reliable model for assessing calcification as a major risk failure mode in manufactured bioprostheses - may be insufficient to evaluate adequately the long-term safety and effectiveness of tissue-engineered valves, the advantages of which depend on the ultimate extent of recellularization, cell viability, and cell function. On balance, detergent-endonuclease-based decellularization methods appear promising as a means of providing a functioning, decellularized allograft valve conduit scaffold on which to base a recellularized tissue-engineered valve. The potential risks and consequences of such treatments tend to be different from the well-known mechanisms of valve structural deterioration associated with glutaraldehyde-crosslinked bioprostheses. Although modifications and pretreatments to avoid new and novel failure modes require further investigation, the emerging

decellularization methods for allograft valve conduits may prove to be useful as the foundation on which to build a tissue-engineered heart valve.

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