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Design and Testing of a Pulsatile Conditioning System for Dynamic Endothelialization of Polyphenol-Stabilized Tissue Engineered Heart Valves

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Abstract

Heart valve tissue engineering requires biocompatible and hemocompatible scaffolds that undergo remodeling and repopulation, but that also withstand harsh mechanical forces immediately following implantation. We hypothesized that reversibly stabilized acellular porcine valves, seeded with endothelial cells and conditioned in pulsatile bioreactors would pave the way for next generations of tissue engineered heart valves (TEHVs). A novel valve conditioning system was first designed, manufactured and tested to adequately assess TEHVs. The bioreactor created proper closing and opening of valves and allowed for multiple mounting methods in sterile conditions. Porcine aortic heart valve roots were decellularized by chemical extractions and treated with penta-galloyl glucose (PGG) for stabilization. Properties of the novel scaffolds were evaluated by testing resistance to collagenase and elastase, biaxial mechanical analysis, and thermal denaturation profiles. Porcine aortic endothelial cells were seeded onto the leaflets and whole aortic roots were mounted within the dynamic pulsatile heart valve bioreactor system under physiologic pulmonary valve pressures and analyzed after 17 days for cell viability, morphology, and metabolic activity. Our tissue preparation methods effectively removed cells, including the potent α -Gal antigen, while leaving a well preserved extra-cellular matrix scaffold with adequate mechanical properties. PGG enhanced stabilization of extracellular matrix components but also showed the ability to be reversible. Engineered valve scaffolds encouraged attachment and survival of endothelial cells for extended periods and showed signs of widespread cell coverage after conditioning. Our novel approach shows promise toward development of sturdy and durable TEHVs capable of remodeling and cellular repopulation.

Keywords

Tissue engineered heart valves; Stabilization; Endothelial cells; Biaxial mechanical testing; Scanning electron microscopy

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ELECTRONIC SUPPLEMENTARY MATERIAL

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INTRODUCTION

Valvular heart disease results in approximately 275,000 valve replacement procedures performed annually worldwide.²² Structural deterioration, calcification, tissue overgrowth, and thromboembolism frequently lead to complications that contribute to the failure of native heart valves. Existing heart valve replacements provide major improvements in cardiac function and life expectancy, but have significant limitations and eventually require surgical replacement within 15–20 years.²² These risks are particularly prominent in pediatric patients, who naturally outgrow their mechanical implants and where tissue derived non-living valves degenerate and calcify rapidly.³¹ Limitations of current heart valve replacements call for implants capable of self-repair and growth. Tissue engineered heart valves (TEHVs) developed with autologous cells would potentially meet the needs.

One major obstacle to the creation of such tissue engineered heart valves seen in many experimental approaches of biodegradable polymer scaffolding is inadequate mechanical properties to withstand *in vivo* forces immediately following implantation. Conversely, many stabilized tissues have more than adequate mechanical properties, but are unable to degrade appropriately to facilitate the formation of a natural valve or have chemical properties that do not facilitate cellular in-growth. As a result, research is being focused on decellularized valves that will allow the recipient patient's cells to infiltrate the extra-cellular matrix, repopulate the valve, and eventually replace the slowly degrading³² donor scaffold with newly fabricated extra-cellular matrix.^{21,22,31}

Bioreactor systems have been developed that provide physiological pressure and flow profiles to developing valvular constructs, as well as the biological factors needed for cell proliferation and differentiation, to allow the tissue engineered valves to become “preconditioned” *in vitro* before *in vivo* implantation.^{2,11,16,22–25,29,30,32} Biologic scaffolds have been used either without stabilization (fully biodegradable) or with extensive chemical cross-linking (non-biodegradable biomaterials). To slow down the degradation process while still allowing cellular infiltration, alternative stabilization agents such as penta-galloyl glucose (PGG) can be used. By virtue of its polyphenolic residues, PGG has a high affinity for Proline-rich proteins such as elastin and collagen and in doing so it protects proteins from the action of hydrolytic enzymes. PGG is a reversible matrix stabilizing agent^{4,13,34} capable of limiting degradation in the absence of permanent crosslinking and thus can be considered promising agent for heart valve tissue engineering. The objective of this work was to prepare acellular reversibly stabilized porcine aortic valve scaffolds, design a bioreactor that would facilitate testing of endothelial cell-seeded valves and analyze cellular interactions with the treated valves after dynamic conditioning under physiologic conditions.

MATERIALS AND METHODS

Bioreactor Design

Development of the bioreactor was approached as a design project and thus formal methods of design⁵ were used to determine the desired qualities of the system. In designing our system, we decided to pursue 10 important conditions. These are (1) To allow mounting of various sizes and shapes of free-standing (“stentless”) and stented valve designs into the bioreactor, (2) To ensure that the valve opens and closes cyclically due to measurable changes in trans-valvular pressure, (3) To maintain the desired concentration of gases and nutrients and waste removal in/from the culture medium, (4) To expose cell-seeded tissue engineered scaffolds to physical stimuli similar to *in vivo*, including trans-valvular pressures, pulsatile forces, flow rate, frequency, stroke rate, stroke volume and sheer stresses on the valve surfaces, (5) To allow full control over parameters and to allow for implementation of progressive adaptation protocols, (6) To ensure maximum visibility and the ability to

continuously and remotely monitor and record valve function, (7) To use materials which are non-toxic, non-degradable, and easy to sterilize, (8) To ensure reasonable durability, easy setup, and compact size to fit in standard cell culture incubators, (9) To maintain the system at 37 °C, 5% CO₂, and 95% humidity in sterile conditions and (10) To yield repeatable results while fulfilling all of the above conditions.

The valve bioreactor we have developed is based on a functional principle proposed by Hoerstrup *et al.*⁹ with numerous modifications. The pneumatic-driven conditioning system (Fig. 1c) consists of a three-chambered heart valve bioreactor (1), an optional pressurized compliance tank (2), a reservoir tank (3) with sterile filter (4) for gas exchange, one-way valves (5), resistance valves (6), pressure transducers, a web-cam (7), and a ventilator pump routinely used in clinics for intensive care and anesthesia (Siemens 900E, Soma Technology, Bloomfield, CT). The entire system (except ventilator) can be sterilized using conventional methods (ethylene oxide gas for the acrylic and high-density polyethylene components and autoclave for the silicone, PVDF, and stainless steel components) and accommodates all clinically relevant sizes of stented or stentless biological, mechanical, or tissue engineered valve substitutes. The valve bioreactor is made of acrylic, composed of three compartments and measures 6 in. in diameter and 8.5 in. in height and is completely transparent (Fig. 1a). The three parts of the bioreactor are held together by stainless steel screws.

The air chamber is connected to the external pump and is the only chamber not filled with culture medium. It is separated from the ventricular chamber by a clear silicone rubber membrane. During the inspiration phase of the ventilator (ventilator causing the patient to breath air in), air is pushed from the ventilator into the air chamber, the membrane bulges into the ventricular chamber, and the residing media is pushed through the heart valve into the aortic chamber, opening the valve. Once completed, the exhalation phase of the ventilator begins (ventilator relaxes pressure to allow the natural patient exhalation) and the ventilator releases pressure in the air chamber, allowing the hydrostatic pressure of the remaining fluid in the ventricular chamber to push down on the silicone membrane, creating vacuum pressure inside the ventricular chamber. The resultant pressure gradient between the ventricular chamber and the reservoir causes culture medium to flow from the reservoir tank, through the one way valves, and into the ventricular chamber in preparation for the next cycle (Fig. 1b).

During the pumping phase, the curvature of the ventricular chamber and angle of media inlets contributes to the circulation of culture medium throughout the chamber. Once through the valve, the medium enters the aortic chamber then flows through a compliance chamber and into the reservoir. Both the aortic and ventricular chambers have multiple ports for easy access of pressure transducers, as inlets and outlets for media change, or for other probes.

The special removable valve holder in the design is able to adapt to valves of all sizes and types (Fig. 1). The valves reported in these experiments were stentless, decellularized valves that were held in place by 6–8 sutures to a silicone ‘aortic root’ that was clamped inside the bioreactor. Our mounting method involves a clamping mechanism to hold the valve base between two rings. Some other examples of materials that can be clamped between the rings are the Dacron sheath (“sewing skirt”) of bioprosthetic heart valves and mechanical valves, the excess myocardial tissue and native mitral valve cusp of fresh aortic valves, the structural components of a polymer-based valve, and other materials from the scaffolding of tissue-engineered materials. Once the valve base was clamped between the two rings, the valve was inserted onto the holder and the holder mounted to the inferior side of the aortic

chamber with stainless steel screws. On the opposite side of the valve, the holder is adapted with two external o-rings which fit snugly inside the ventricular chamber to create a seal.

Figure 1d shows two bioreactors set up in one standard size incubator. The clear, flat top of the aortic chamber facilitates the viewing of the functioning of the heart valve via the webcam. A permanent fluorescent lamp mounted inside the incubator provides lighting. Using websites such as livestream.com and others, continuous broadcast of the bioreactor and valve performance is possible over the internet, allowing the investigators remote viewing of valve performance from any computer and sharing of information with collaborators. For the current study we have built two identical systems and used them in tandem.

Bioreactor Testing

To test the capabilities of the heart valve bioreactor, pressure and flow were measured in a mock system using double-distilled water and a porcine glutaraldehyde fixed stented aortic bioprosthetic heart valve (25 mm, Biocor Mitral, St. Jude Medical, Inc.). The aortic chamber fluid outlets were connected by silicone tubing to an air-tight pressurizing reservoir (compliance chamber), which in turn was connected to a second reservoir exposed to atmospheric air via one top outlet fitted with a 0.22 μm air filter. Finally, the second reservoir was connected to the ventricular chamber via one-way valves. The compliance chamber described here is used for obtaining higher pressures and is not utilized in the experiments reported in this study. It is included to describe the higher pressures the conditioning system is able to reach.

Since a clinical ventilator was used as the driving force for the valve bioreactor, ventilator settings were translated into cardiac settings as follows. The ventilator was set to have an inspiration time of 50% (translating into equal open and close portions of the valve cycle), pause time of 0% (no pause between cycles), and working pressure of 108 cmH_2O (amount of pressure applied during pressurization of air chamber), while functioning at 60 bpm (beats per minute, bpm). Pressure transducers (0–50 psi, Cole-Parmer, Vernon Hills, IL) were mounted onto the aortic and ventricular chambers of the bioreactor and a data acquisition system (OMEGA, Stamford, CT) set at 180 Hz. The aortic pressure transducer was approximately 7.5 cm above the valve and the ventricular pressure transducer was approximately 8.0 cm below the valve. The inspiration tidal volume of the ventilator was 138 ± 2 mL. The total volume of fluid in the system was estimated to be about 1600 mL. This fluid was distributed between the bioreactor and two reservoirs: one to be pressurized and one to serve for gas exchange. Pressure values were recorded under no pressurization, after an initial pressurization of the top reservoir using compressed air, and after a second pressurization of the top reservoir.

To measure fluid flow, we used a flow meter (300–3000 mL/min, Cole-Parmer, Vernon Hills, IL) on each of the two outlet lines from the aortic chamber. The top and bottom fluid levels in the reservoir, with respect to the bottom of the bioreactor were 13 and 9.5 cm, respectively. Following 1 min of cyclic flow, the values were recorded to determine the flow per minute and stroke volumes attainable at these conditions. The actual inspiration tidal volume was adjusted while keeping all other variables constant to determine flow capabilities. The experiment was repeated two times and the readings averaged.

Tissue Collection and Decellularization Procedure

Fresh porcine aortic roots with intact ascending aorta were collected from adult pigs at a local abattoir and stored in sterile saline with 2% antibiotics/antimycotics/ antifungals (Penicillin, Streptomycin, Amphotericin B for cell culture) on ice during transportation to

the laboratory. The valves were cleaned over ice, the aortic root was trimmed to a length of about one inch, and valves were decellularized on an orbital shaker. Decellularization steps consisted of hypotonic shock (ddH₂O, 24 h, 4 °C), extraction of cell fragments (0.05 M NaOH, 2 h, 22 °C), decellularization solution (0.05% SDS, 0.5% Triton X-100, 0.5% Na-Deoxycholate, and 0.2% EDTA in 10 mM TRIS, pH 7.5, 24 h, 22 °C), and removal of nucleic acid remnants (deoxyribonuclease & ribonuclease treatment, 24 h, 22 °C). To reduce bio-burden, each extraction step was preceded by a 70% ethanol treatment for 20 min. After valve decellularization, the coronary arteries were ligated with Vicryl 4–0 sutures to facilitate cell seeding. The roots were incubated in 70% ethanol for 24 h at 22 °C and stored in sterile 0.02% sodium azide (NaN₃) solution at 4 °C.

To test for decellularization completeness, cusp tissue samples were weighed wet, DNA extracted and purified with a specific kit (Qiagen, Valencia, CA), and DNA quantified by reading absorbance at 260 nm. Quantities of DNA were normalized to weight and expressed as ng/mg wet tissue. For histology studies on decellularized cusps and samples obtained from bio-reactor studies, cusp samples were fixed in 10% for-malin, embedded in paraffin, sectioned in the radial direction at 5 µm, and stained with Hematoxylin & Eosin (H&E), and Movat's Pentachrome. To detect Galα1–3Gal (α-Gal), the main porcine antigen responsible for acute rejection of xenotransplants, we used biotinylated *Griffonia simplicifolia* (GS) lectin immunohistochemistry.^{1,33}

PGG Treatment

High purity 1,2,3,4,6-Penta-*O*-galloyl-beta-D-glucose (penta-galloyl glucose, PGG) was a generous gift from N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium (www.omnichem.be). Acellular valve scaffolds were rinsed in sterile PBS and then treated 24 h at 22 °C with sterile 0.075% PGG in 50 mM dibasic sodium phosphate buffer in saline containing 20% iso-propanol, pH 5.5. At the onset of fixation, cusps were lightly stuffed with sterile cotton balls pre-soaked in PGG solution to preserve the valve conformation in “closed” position. After rinsing in sterile PBS three times, scaffolds were treated for 20 min in 70% ethanol and stored in sterile 0.02% NaN₃ at 22 °C until seeding with endothelial cells.

Stabilization Studies

To assess stabilization of scaffolds by PGG, treated and untreated controls were subjected to collagenase and elastase digestion as follows. Cusp samples were lyophilized and dry weight recorded. About 5–10 mg dry scaffold ($n = 12$) were incubated in 1 mL collagenase solution (5 Units of collagenase/mL in 50 mM Tris, 1 mM CaCl₂, 0.02% NaN₃, pH 7.8). A similar group of scaffolds ($n = 12$) underwent treatment with elastase (6.25 Units/mL in 100 mM Tris, 1 mM CaCl₂, 0.02% NaN₃, pH 7.8). After 3 and 7 days, samples ($n = 6$ per time point) were rinsed three times in ddH₂O by centrifugation at 12,000 rpm for 5 min and finally lyophilized and weighed. Percent mass loss was calculated from the following: $100 \times (\text{scaffold weight before enzyme} - \text{scaffold weight after enzyme}) / \text{scaffold weight before enzyme}$.

Biaxial Mechanical Testing

Samples of the native, decellularized, and decellularized & PGG-treated cusps were subjected to biaxial mechanical testing. A 12 mm × 12 mm square was cut from a central region of the cusp, with one edge aligned along the circumferential direction and another edge aligned along the radial direction. The biaxial testing method has been reported in great detail previously.^{7,18,27} Briefly, four markers were placed in the center of the specimen to track tissue deformation. A total of 8 loops of 000 polyester suture of equal length were attached to the sample via stainless steel hooks, with two loops on each side of the square

specimen. Specimens were first preconditioned for 10 contiguous cycles, then loaded up to 60:60 N/m equibiaxial tension. 60 N/m tension was found to correspond with the physiological loading experienced by an aortic valve cusp at peak diastolic load.³ Tissue extensibility was characterized by λ_{circ} and λ_{rad} , the maximum stretch ratio along circumferential and radial direction, respectively. The tests were implemented with the samples completely immersed in PBS (pH 7.4) at physiological temperature (37 °C).

Differential Scanning Calorimetry

Three specimens from each group of the native, decellularized, and decellularized & PGG-treated cusps were subjected to differential scanning calorimetry (DSC, model 131 Setaram Instrumentation, Caluire, France) to determine the thermal denaturation temperature (T_d). Specimens were tested at a heating rate of 10 °C/min from 20 to 110 °C in a N₂ gas environment. T_d is defined as the temperature at the endo-thermic peak and is a well-known indicator of degree of collagen crosslinking.³⁴

Endothelial Cell Seeding and Bioreactor Conditioning

The PGG-treated acellular valve scaffolds were rinsed with sterile PBS, then mounted onto silicon mounting rings using Vicryl 4–0 sutures and incubated overnight at room temperature in sterile Dulbecco's Modified Eagle Medium with 50% fetal bovine serum (Atlanta Biological, Atlanta, GA) and 2% antibiotics/ antimycotics/antifungals to neutralize unbound PGG and enrich the scaffolds with growth factors and adhesion molecules. After aspirating the neutralization solution, the valves were stood upright in individual sterile specimen cups and seeded with 300,000 porcine aortic endothelial cells (Cell Applications, Inc, San Diego, CA, grown on fibronectin-coated plates, $p < 5$) in 500 μL of cell culture medium per cusp. Only the arterial aspects of the valve scaffolds (*fibrosa*) were seeded for this study. Any cell culture media that leaked out of the valve during seeding was repeatedly reapplied to the cusps for 1 h. Valves were then fully immersed in cell culture medium and kept under static conditions in the cell culture incubator for 2 h. The heart valve bioreactor components were sterilized separately using ethylene oxide gas (acrylic and high-density polyethylene parts) and autoclaving (silicone, PVDF, and stainless steel parts) and the entire system assembled in a sterile biohood. Cell-seeded valves were then mounted in the bioreactor in sterile Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 2% antibiotics/antimycotics/antifungals and subjected to cyclic functioning at 60 bpm, 40/20 mmHg (diastolic/systolic), 10 mL stroke volume (600 mL/ min) for 17 days. Before reaching these working parameters, the cell-seeded valves were subjected to a progressive adaptation protocol starting with 24 h of functioning at 20 bpm then moving to 24 h of 40 bpm. Media was changed every 7–8 days. A total of four cell seeded valves were tested under identical conditions ($n = 4$). For analysis, valves were removed from the bioreactor, cusps carefully dissected from their aortic wall insertions, and subjected to scanning electron microscopy (SEM), Live/DEAD[®] staining, and metabolic assays as described below. Static controls consisted of cell-seeded valves ($n = 3$) which were not subjected to dynamic conditions and analyzed 1 day after seeding ("time zero" samples).

Cell Analysis After Bioreactor Conditioning

Scanning Electron Microscopy (SEM)—For SEM analysis, cusps were fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.4) for at least 24 h. Tissues were then dehydrated in increasing ethanol concentrations, stored in 100% ethanol for up to 12 h, immersed in hexamethyldisilazane (Polysciences, Inc., Warrington, PA) for 15 min, and air dried before being coated with platinum (Hummer 6.2, Anatech LTD, Union City, CA) for 2 min. The cusps were imaged using a Hitachi

S4800 scanning electron microscope (Clemson University Electron Microscope Facility, Anderson, SC).

Live/DEAD—Presence and viability of cells on the surface of cusp scaffolds was analyzed by LIVE/DEAD[®] stain (Molecular Probes, Carlsbad, CA) according to the manufacturer's instructions.

Metabolic Activity Test (MTS Assay)—To evaluate the number of viable cells, cusp segments selected randomly from each valve were analyzed by the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin) which employs reduction of a tetrazolium salt (MTS) by dehydrogenases in metabolically active cells and quantification of the formazan product by measuring absorbance at 490 nm. An MTS standard curve built with porcine aortic endothelial cells was used to calculate cell numbers according to manufacturer's instructions.

Statistical Analysis

Results are represented as means $\pm$ standard deviation. Statistical analysis was performed with one way analysis of variances (ANOVA) and results were considered significantly different at $p < 0.05$. The Holm–Sidak test, which can be used for pair-wise comparisons and comparisons vs. a control group, was used for the *post hoc* comparison.

RESULTS AND DISCUSSION

Bioreactor Design and Testing

The bioreactor and conditioning system have been manufactured by the Clemson University Machining and Technical Services based on CAD designs provided by the authors (Fig. 1). The entire assembly has been tested in more than 30 experiments with various valve designs and four of these experiments are presented in this paper. The system fulfills most of the original conditions, including mounting of various types of valves and excellent valve opening and closing characteristics (Figs. 2 and 6). The system ensured good cell viability under sterile conditions for extended periods of time (see analysis of endothelial cells below) and excellent reproducibility. It also allowed for implementation of progressive adaptation protocols, ensured perfect visibility and the ability to continuously and remotely monitor and record valve function. Notably, we have presented live video of a functioning valve in the heart valve bioreactor running in Clemson, South Carolina during the TERMIS-NA 2008 poster session in San Diego, California. Video images of a functioning bioprosthetic heart valve such as the one shown in Fig. 2a are presented in Video 1 (see supplemental materials).

Flow—Inspiration tidal volumes of the ventilator were adjusted between 7.5 and 130 mL, resulting in incremental stroke volumes from 4.7 to 22.3 mL (Fig. 2b). For the lowest inspiration tidal volume air settings of 7.5 and 10 mL, the valve was only slightly opening. To ensure full opening and closing of the experimental valves, inspiration tidal volume air settings were set to 24 mL, corresponding to about 10 mL of media per stroke.

Pressure—Pressure levels were recorded at 180 Hz, which created “low resolution” pressure profiles of 3 data points/ s (Fig. 2c). Under no added pressurization, systolic and diastolic pressures had an average value of 40/ 15 mmHg, respectively (Fig. 2d). After pressurization, these values increased to a stable 80/70 mmHg (Fig. 2e). As the fluid pushes down on the silicone membrane, a negative ventricular pressure is produced which in turn draws fluid from the reservoir. The drift in peak pressures between 60–120 and 120–180 s is attributed to redistribution of pressures within the conditioning system (i.e. from the

bioreactor to the reservoir). Spikes observed in the traces are due to the low data acquisition rate of the DAQ (3 data points/s). Higher pressures could be attained, but this halted circulatory flow and the pressures were not stable. The pressure limitations were the result of an inability of the system to circulate fluid effectively. A very high level of pressure could be reached if the pumping mechanism could match and overcome that pressure. By using an alternative pumping mechanism that would preserve energy by reducing air compression, modifications could be made to greatly increase the flow to match and surpass even high physiological values.

Comparison with Other Bioreactors—The values reached for stroke volume (flow) and pressure are roughly mid range compared to other bioreactor designs, with some reaching much higher^{8,10} and some reaching much lower levels⁶ than aortic physiological levels. Our stroke volume and pressure do not yet meet aortic physiological levels, but are appropriate for testing valves in pulmonary pressure conditions. Endothelial cell-seeded tissue engineered heart valves must go through steps of progressive adaptation with flow and pressure beginning at values well within levels attainable by the Clemson bioreactor. As the tissue engineering technology improves and valves get closer to implantation stages, we will need to be able to reach the higher values to represent aortic physiological conditions, but the current maximum values of our system are adequate for the initial stages of tissue engineered heart valve development.

Several groups have published development of heart valve bioreactors, but very few have described the actual design process, to facilitate development by other investigators. The bioreactors range in design, complexity, and function, performing at various levels and accuracy. Valve mounting methods and modes of assembly also vary, often with little options or ability to accommodate different or abnormal valve shapes. Most heart valve bioreactors in the literature are designed for use with only the valve type investigated in that laboratory. Many facilitate testing of decellularized valves^{26,36} or laboratory-manufactured polymer valves.⁶ However, the Clemson heart valve bioreactor is able to test all clinically relevant sizes and variations of stented or stentless biological, mechanical, or tissue engineered valve substitutes with multiple mounting methods allowing for variations in tissue thickness, engineered material, valve diameter, amount of excess tissue, and presence or absence of aortic sinuses. Many of the mounting mechanisms used in other bioreactors are not explained in depth in the literature. The mechanisms that are described are restrictive on the type of valve that can be accommodated: Hildebrand's bioreactor⁸ demands a totally sutured-in valve, Ruel's bioreactor²⁶ requires tissue inferior of the valve to be clamped, and Zeltinger's bioreactor³⁶ has the valve stapled to a rigid stent. These are all different methods, but cannot be easily adjusted for an alternative type of valve. Our bioreactor's mounting design easily facilitates many different valves in a variety of non-restrictive options.

Valve Decellularization

The sequence of hypotonic shock, alkaline treatment, detergent extractions, and nuclease digestions proved to be a very efficient decellularization procedure. Morphologically, the cusps exhibited a "bleached out" aspect and snow-white color (Figs. 3a–3c). Quantitative DNA analysis showed a 98.5% reduction in DNA content (from 80.6 ± 6.5 to 1.2 ± 0.2 ng DNA/mg wet tissue) which was considered satisfactory for tissue engineering applications and comparable to other decellularization studies.² These results were confirmed by agarose gel/ethidium bromide gel electrophoresis as well as DNA Lab-on-a-Chip analysis on an Agilent 2100 Bioanalyzer (data not shown).

Histological analysis using Movat's Pentachrome (Figs. 3d and 3e) confirmed complete cell removal by our procedure and good preservation of cusp extra-cellular matrix components collagen and elastin, with partial loss of glycosaminoglycans. These results are considered favorable as some studies resulted in incomplete cell removal¹⁵ or major loss of structural components^{15,18} when using trypsin, a common agent in other decellularization protocols. α -Gal staining of fresh cusp sections using GS lectin immunohisto-chemistry (Figs. 3f and 3g) revealed numerous valvular interstitial cells that stained positive while decellularized cusps revealed undetectable α -Gal xenoantigen. These results are consistent with the literature for Triton X-100 based decellularization.^{12,14} We did not conduct analysis of basement membrane components, but studies using SDS and sodium Deoxycholate² analyzed with collagen IV staining showed an intact basement membrane. There are many detergent-based treatments and ours lies among them with complete cell and α -Gal removal with good preservation of collagen, elastin, and glycosaminoglycans.

PGG Stabilization

Enzymatic Resistance—Treatment of decellularized cusps with PGG increased their resistance to protease digestion. After 3 days of incubation, control untreated cusps were almost fully digested by collagenase while PGG-treated cusps lost less than 20% of their mass due to collagenase ($p < 0.05$). At 7 days, PGG cusps lost about 40% of their mass ($p < 0.05$), indicating that PGG binding significantly reduces collagen degradation (Fig. 4a). The increase in collagenase sensitivity from 3 to 7 days was statistically significant, suggesting that PGG-treated collagen is slowly biodegradable. A similar trend was observed for susceptibility of PGG-treated decellularized cusps to elastase digestion (Fig. 4b).

Previous studies³⁴ by our group comparing non-fixed, PGG-fixed, and glutaraldehyde-fixed porcine pericardium had similar results with the percent mass loss of collagen, with PGG-fixed tissue lying in between glutaraldehyde and non-fixed tissue. Alternative cross-linking agents, procyanidins & quercetin, have also been shown^{37,38} to be successful in stabilizing collagen with efficacy similar to that of glutaraldehyde. However, these methods introduce permanent cross links and do not allow for the slow biodegradation of the matrix.

The biaxial tissue responses of the three groups (native, decellularized, and decellularized & PGG-treated cusps) were evaluated by the averaged stress-strain curves (Fig. 5). Tissue extensibilities were compared by the maximum stretches in the circumferential and radial directions at physiological tension (60 N/m) (Table 1). The decellularized cusp showed only slightly higher extensibility along the radial direction (λ_{rad}) when compared with the native group (1.5020 ± 0.0656 vs. 1.3645 ± 0.0513 , $p = 0.046$), while the extensibility along the circumferential direction (λ_{circ}) of the decellularized cusp was only slightly lower than the native group (no statistical significance, $p = 0.12$). These findings are consistent with previous observations of decellularized valves.¹⁸ It was showed that the further increased radial extensibility in the decellularized cusp was likely due to the increased mobility for the circumferentially oriented collagen fibers to rotate toward the radial direction in the decellularized tissue matrix, which was partially disrupted by cell removal agents.¹⁸ It is notable that the biaxial behavior of the decellularized cusp was deviated from that of the native cusp tested at the same condition (Figs. 5a and 5b). PGG treatment of the decellularized cusp reversed the trend of an overly extensible radial direction. Interestingly, the PGG-treated group showed a biaxial behavior very similar to the native cusp group (Fig. 5c). Although in the PGG-treated group, there was a stiffening trend along the radial direction, the difference did not reach a statistical significance ($p = 0.112$). However, the circumferential extensibility of PGG-treated was found higher than the native group (PGG-treated 1.0710 ± 0.0046 vs. Native 1.0510 ± 0.0098 , $p = 0.034$).

Differential Scanning Calorimetry (DSC)—DSC analysis showed T_d values of 75.70 ± 1.55 °C for the native valve cusp, 75.69 ± 2.06 °C for the decellularized cusp and 85.91 ± 3.72 °C for the PGG-treated decellularized cusp, which indicates matrix stabilization. The T_d value of the PGG-treated decellularized cusp was significantly higher than the native group (ANOVA, $p = 0.003$). There was no statistical difference in T_d between the decellularized cusp and the native group.

Valve Functioning in the Bioreactor

Webcams were used to monitor valve function in the bioreactor on a daily basis, and short movies and still pictures were taken periodically to document valve function. Video images of a cell seeded, decellularized PGG-treated valve functioning in our bioreactor are presented in Video 2. There were no significant changes in valve function over the time period tested (data not shown). Among the three cusps, the non-coronary and the left coronary cusps functioned very well, providing a perfect closure with good apposition and coaptation and excellent opening characteristics (Fig. 6). However, the right coronary cusp, lying on the thick muscular shelf, did not move well in systole, while it provided good closure in diastole. The thick muscular shelf is known to be an anatomical particularity in swine and it is not present in human valves.³⁵

Analysis of Endothelial Cells After Bioreactor Conditioning

Scanning Electron Microscopy (SEM)—SEM analysis of decellularized aortic cusps showed very smooth surfaces, completely devoid of endothelial cells and other cell remnants (not shown). A basement membrane was not specifically stained for, but this smooth surface gives some evidence for the presence of a basement membrane.^{2,20} Immediately after seeding, cells did not completely cover all areas of the valve. This is consistent with the literature,^{17,19,20} where moderate flow has been required to achieve cellular confluence.

After seeding with endothelial cells and functioning for 17 days in the heart valve bioreactor, many areas were completely covered with endothelial cells, while other areas were not fully covered (Fig. 7). This aspect may be related to incomplete surface coverage during initial cell seeding and comparatively low initial cell numbers. We used 300,000 cells/cusp in our experiments as compared to as many as 3 million/cusp in other papers.^{17,20,28} While improved coverage would be important for animal implantation studies, in this study we were primarily interested in basic cell adhesion and retention properties. SEM analysis at higher magnifications revealed that most cells attached well on the scaffold surface, spread, and acquired “cobblestone” morphology similar to native valvular endothelial cells (Figs. 7c and 7d).

Viability Tests—Live/DEAD analysis showed that >90% of cells were alive with very few cells staining red (dead), suggesting that the valvular scaffolds were not cyto-toxic. Endothelial cells appeared to have distributed alongside the thick collagen fibers and most cells aligned parallel to the circumferential direction (Figs. 7e and 7j). Based on MTS data normalized to values obtained from time zero samples (cell seeded and analyzed after 24 h), about 55% of seeded cells were retained and were viable on the cusps after 17 days in the bioreactor.

Histology—H&E staining essentially confirmed SEM observations. Areas where cells were found tended to be near the surface of collagen bundles and in some areas, a multi-layered distribution was noted compared to the monolayer desired and reported by others.²⁰

CONCLUSIONS

A bioreactor was designed that can test all types of heart valves, including tissue engineered heart valves. It created pressure profiles similar to physiologic pulmonary conditions, causing the valves to open and close appropriately. The decellularization method described effectively removed cells while preserving valve matrix components and eliminating the α -Gal epitope. PGG stabilization provided a scaffold with enhanced mechanical properties over decellularized valves while still allowing slow scaffold degradation. Not as permanent as other fixations, this method of stabilization is a promising solution for the current limitations of heart valve replacements and tissue engineered heart valves. Endothelial cells attached and survived on PGG-fixed valvular scaffolds after 17 days of dynamic conditioning in the bioreactor. Ongoing work in our group is focused on improving coverage with endothelial cells, long term bioreactor studies of endothelialization and assessment of endothelial cell functionality after bioreactor conditioning and further repopulating the inner layers with valvular interstitial cells or alternative cell sources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Heart valve bioreactor and conditioning system. (a) CAD designs (left) and manufactured heart valve bioreactor (right) showing the four main components. A transparent silicone membrane diaphragm is mounted between the air chamber and the ventricular chamber. (b) Air and media flow through the system during systole (left) and diastole (right). Color coding aids identification of the components and black arrows indicate direction of air and media flow. (c) Schematic overview of the entire conditioning system: a three-chambered heart valve bioreactor (1), an optional pressurized compliance chamber (2), a reservoir tank (3) with sterile filter (4) for gas exchange, one-way valve (5), pressure-retaining valve (6), a webcam (7), and a ventilator pump (air pump). The entire setup fits within a standard cell culture incubator. (d) Two identical bioreactor systems (Br 1 and Br 2) with endothelial cell-seeded, functioning valves inside an incubator; the bioreactors are in the front row while their corresponding reservoirs are in the back row. The webcams normally mounted onto the top viewing windows of the aortic chamber have been removed to reveal bioreactor details. Details of mounting procedures for stented valves, mechanical valves and un-stented valves are shown next. (e) The basic valve holder, (f) holder with one o-ring placed in the machined groove (arrow), (g) bottom mounting ring placed on top of the o-ring (arrow). (h) A stented valve was placed on top of the bottom mounting ring (arrow), (i) a second mounting ring placed and o-ring over it and (j) pushed slowly. (k) Shows a mechanical valve (arrow) after mounting between the two rings. (l) Porcine aortic valve root ready for mounting in bioreactor; (m–o) valve mounted into a silicone root using 6–10 bottom sutures and 3–6 upper sutures (arrows). (p) The valve was placed on the holder prepared as shown in (g); (q) a second ring slid onto the root (arrow), (r) a second o-ring added on top and (s) the entire mount inserted into the aortic chamber (arrow).

FIGURE 2.

Bioreactor performances. (a) A porcine bioprosthesis mounted in the bioreactor for testing. Note closure of the cusps during diastole (top) and opening in systole (bottom). (b) Ventilator air output compared to actual stroke volume of media through the valve. (c) Pressurization test of the bioreactor system showing steps of (1) no pressure, (2) added pressure, (3) additional pressurization, and (4) release of all pressure. (d) Aortic and ventricular pressure measurements under no additional pressurization. (e) Aortic and ventricular pressure measurements under initial, stable stage of pressurization.

FIGURE 3.

Heart valve decellularization. Whole aortic root after decellularization showing arterial view (a) ventricular view (b) and inside view after cutting the root open to reveal the three cusps (c). Representative Movat's Pentachrome stain of fresh cusps (d) and decellularized cusps (e) showing elastin (dark red), collagen (yellow), cells (dark blue) and proteoglycans (light blue). GS lectin histochemical staining for α -Gal in fresh cusps (f) and in decellularized cusps (g) showing representative positive stain in brown and cell nuclei counterstained in blue.

FIGURE 4.

Stabilization of valvular collagen and elastin by PGG. Resistance to collagenase (a) and elastase (b) of PGG-treated decellularized cusps as compared to fresh decellularized cusps. Values are expressed as percent mass loss, measured at two time points. For both time points, data obtained for PGG-treated cusps were statistically different from untreated cusps ($p < 0.05$).

FIGURE 5.

Mechanical properties of valvular scaffolds. Biaxial stress–strain curves of the native aortic valve cusps (a), the decellularized valve cusps (b), and the decellularized, PGG-treated cusps (c). 60 N/m equibiaxial tension was applied to simulate the physiological loading that valve cusp experiences. Error bars represent standard deviation of the strain.

FIGURE 6.

Endothelial cell-seeded valvular scaffold functioning in the bioreactor. All four cell seeded valvular scaffolds tested (a–d) are shown during actual functioning in the valve conditioning system. Note the opening during systole (left) and closing during diastole (right). The right coronary cusp is shown on the upper right corner of the valve sinus.

FIGURE 7.

Cell analysis of endothelial cell-seeded valvular scaffolds after functioning in the bioreactor. Representative SEM images showing native cusp surfaces (a, b), at time zero ($T = 0$ d), i.e. immediately after seeding (c, d) and after 17 days ($T = 17$ d) in the conditioning system (e–h). Typical Live/Dead staining showing cells at time zero (i) and 17 days after endothelial cells seeding and bioreactor conditioning (j). Representative H&E staining of sections through time zero endothelial cell-seeded valves (k, l). White arrows depict orientation of thick collagen fibers (cusp circumferential direction).

TABLE 1

Maximum stretch ratios under 60 N/m equibiaxial tension for native valve cusp, decellularized valve cusp, and decellularized PGG-treated cusp.

	Native valves	Decellularized valves	PGG-treated scaffolds
λ_{circ}	1.051 ± 0.009	1.038 ± 0.004	$1.071 \pm 0.004^*$
λ_{rad}	1.364 ± 0.051	$1.502 \pm 0.065^{**}$	1.287 ± 0.040

λ_{circ} and λ_{rad} are the maximum stretch ratios along circumferential and radial directions, respectively. Values are given as mean $\pm$ SD.

Statistically significant difference when compared with the native group

*
 $p = 0.034$,

**
 $p = 0.046$).