Bioreactors for Development of Tissue Engineered Heart Valves

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Abstract—Millions of people worldwide are diagnosed each year with valvular heart disease, resulting in hundreds of thousands of valve replacement operations. Prosthetic valve replacements are designed to correct narrowing or backflow through the valvular orifice. Although commonly used, these therapies have serious disadvantages including morbidity associated with long-term anticoagulation and limited durability necessitating repeat operations. The ideal substitute would be widely available and technically implantable for most cardiac surgeons, have normal hemodynamic performance, low risk for structural degeneration, thrombo-embolism and endocarditis, and growth potential for pediatric patients. Tissue engineered heart valves hold promise as a viable substitute to outperform existing valve replacements. An essential component to the development of tissue engineered heart valves is a bioreactor. It is inside the bioreactor that the scaffold and cells are gradually conditioned to the biochemical and mechanical environment of the valve to be replaced.

Keywords—Heart valves, Bioreactor, Preconditioning, Endothelial cells, Valvular cells.

INTRODUCTION

Millions of people worldwide are diagnosed each year with valvular heart disease, resulting in hundreds of thousands of valve replacement operations. Replacement valves are mechanical or bioprosthetic and intended for the aortic position ($>200{,}000$ annually) or mitral position (~70,000 annually) with pulmonary valve replacements occurring in much smaller numbers.[2,35] From 1998 to 2005, an estimated 330,000 aortic or mitral valve procedures were performed in the United States (repair, $n = 46{,}342$; replacement, $n = 287{,}989$)[5] at a mean cost of \$141,120 and an in-hospital death rate of 4.98%.[1] Most heart valve replacements are mechanical. They can last 20–30 years or longer and generally save and lengthen a patient's life. However, depending on the kind of valve and its position in the heart, complications include blood clot formation on the valve surface, carrying serious risk for stroke or emboli that impede blood flow to vital organs. Mechanical replacements require patients to take anticoagulants for the rest of their lives, which carries dangerous bleeding risks. Bioprosthetic heart valves are made from animal or human cadaver tissue treated with preservatives and sterilized for implantation. They do not carry the same clotting risks but wear out faster (every 10–15 years). Serious complications with mechanical and tissue prosthetic valves at an average annual rate of ~2–4% include thrombo-embolism, size mismatch, dysfunction, endocarditis, and hemolysis.[39] The ideal prosthetic valve would be widely available and technically implantable for most cardiac surgeons, have normal hemodynamic performance, low risk for structural degeneration, thrombo-embolism and endocarditis, and growth potential for pediatric patients.

Tissue engineered heart valves (TEHV) are an exciting prospect for future treatment of valvular disease. Recent efforts to create TEHV have focused on identifying appropriate scaffold materials, valvular cell sources, and seeding techniques. The ideal TEHV scaffold would be perfectly biocompatible, support millions or billions of flow and pressure cycles, allow only one-way flow, impart strain signals to elicit non-pathologic cellular responses, provide suitable porosity

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for interstitial cells, and degrade after valvular cells produce their own extracellular matrix. The valve must also grow and remodel in pediatric patients. Material options may be synthetic or natural. Synthetic scaffolds include polymers of galactin and glycolic acid and have complex construction requirements but have been used to engineer valves with reasonable short-term results.[36] Among natural materials, decellularized xenografts that are not fixed in gluteraldehyde, including porcine aortic roots, are very attractive for such reasons as low cost, ready availability, similarity in size and geometry to human valves, and similarities in pressure and flow patterns. These valves have shown some success in preclinical testing in animal models.[17,19,26] While risk of transmitting zoogenic infection is of theoretical concern, bacteria and viruses have not been detected in this tissue.[29] Vascular endothelial cells can be seeded onto these valves to help prevent thrombosis. Repopulation of the scaffold by host cells can occur passively and actively, when cells are forced onto the matrix. Active seeding presents many technical obstacles, but early results with passively seeded valves, like Synergraft, suggest that active seeding will be required.[3]

The belief that TEHV will outperform existing valve technology is based on the idea that defective valvular tissue will be replaced by living functional tissue and will remodel itself to adapt to the physiologic requirements of the patient. These valves will be required to surpass their mechanical and bioprosthetic counterparts to be a viable clinical and commercial success.[38]

Current TEHV are fabricated in a laboratory from three essential components. First is a structural scaffold of some biologically compatible material, second are the valvular cells that inhabit the scaffold, and third is a bioreactor. It is inside the bioreactor that the scaffold and cells are gradually conditioned to the biochemical and mechanical environment of the valve to be replaced.

Bioreactors for TEHV are designed for two purposes. The first is to elucidate the mechanisms by which the biochemical or biomechanical environment influences the interaction between the valvular cell types or between cells and scaffold material. The second purpose is to prepare an entire cell-seeded TEHV for implantation (preclinically or clinically) into a living animal. This article reviews the bioreactors used for mechanistic studies as well as the bioreactors used to condition TEHV prior to implantation.

BIOREACTORS FOR STRETCH, FLEXURE, AND FLOW STUDIES

Normal heart valve tissue is subjected to cyclic circumferential stretch, oscillating shear stress, and cyclic flexure as the valve leaflets open and close. Informed design of TEHV necessitates an understanding of how these forces influence the development and function of cell-seeded scaffold material. Several investigators have studied the cellular response to these forces by decomposing this complex mechanical environment into components of stretch, flex, or flow. Specialized bioreactors are needed for this purpose.

Stretch

A commercially available and popular bioreactor for studying the effects of stretch in a cell culture environment is known as Flexcell (Flexcell International, Hillsborough, USA). Cells are cultured on a silastic flexible membrane representing the bottom surface or a multi-well plate. A vacuum is applied to the under surface of this membrane stretching the membrane statically or cyclically in uniaxial or multiaxial stretch. Ku et al.[28] cultured valve interstitial cells (VIC) as well mesenchymal stem cells in a Flexcell bioreactor and showed that cyclic stretch upregulated collagen synthesis in valve interstitial cells as well mesenchymal stem cells. Instead of culturing valvular cells on flexible membrane, Merryman et al. constructed a stretch bioreactor for studying cyclically stretched samples of porcine aortic valve leaflets containing endothelial and interstitial cells at 7 and 14 days. Cyclic stretch was applied at a magnitude of 15% and frequency of 1 Hz to circumferentially oriented tissue strips by sealed computer-controlled linear actuator. They reported that cyclic stretch altered cellular stiffness via collagen biosynthesis. They also reported that the presence transforming growth factor-b1 (TGF-b1) in a cyclic stretch environment could potentially result in an altered matrix architecture and compromised valve function, underlining the importance of cyclic stretch in regulating valve structure and function.[32] Balachandran et al. constructed a similar stretch bioreactor to test rectangular tissue strips of fresh porcine aortic cusps by subjecting them to cyclic stretch at 10, 15, and 20% strain at 1 Hz for 48 h along the circumferential direction of the cusps. They showed that elevated levels of cyclic stretch result in a highly activated cell phenotype stimulating altered cell turnover and increased MMP and cathepsin expression potentially setting the stage for valve degeneration.[4] Another stretch bioreactor developed by Gupta et al. used mitral valve interstitial cells or chordal cells seeded within collagen gels. The seeded gels were subjected to 10% strain at 1.167 Hz for 24 h, followed by no cyclic stretch (relaxation) for 24 h. This stretching and relaxation was repeated two more times for a total of 7 days. GAG secretion was significantly upregulated during cyclic stretch and downregulated

during relaxation. The proportional composition of distinct GAG classes remained fairly consistent for chordal cells but tended to change for VICs, which reflects the leaflet VICs' adaptation to high cyclic tensile strains.[18]

Flexure

Dynamic flexure bioreactors allow for the study of isolated segments of TEHV or cell-seeded valve scaffold material in bending. A dynamic flexure bioreactor was designed by Engelmayr *et al.*[13] to compare the effects of flexure on two TEHV scaffold materials—polyglycolic acid (PGA) fibers, and a non-woven mesh of PGA and poly l-lactic acid (PLLA) fibers. Two tissue chambers each with 12 culture wells contained stationary posts to secure material sections. A bifurcating cross-arm attached to the chambers provided three-point bending. Frequency (1 Hz) and strain rate were controlled in this bioreactor. No cells were seeded onto the scaffold strips. Results showed a decrease in stiffness for both materials after 2, 3, and 5 weeks of flexure. Englemayr *et al.*[14] then seeded a scaffold material with ovine smooth muscle cells and placed in cyclic flexure to examine extracellular matrix production and cell development. Cyclic flexure increased stiffness, collagen production, and transmural cell distribution to a much greater extent than in static controls. These results underscore the importance of mechanically conditioning cell-seeded TEHV scaffolding in a bioreactor. The coupled effects of cyclic flexure and laminar shear stress were investigated by Englemayr *et al.*[15] in using sheep bone marrow-derived mesenchymal stem cells. As in the study of Englemayr,[14] the cells were seeded onto strips of synthetic scaffold material. The combined flexure simulating valve leaflet motion and laminar flow yielded collagen production and stiffness greater than static controls.

Flow

Jockenhoevel *et al.*[24] used a pump to apply steady fluid shear stress to myofibroblasts seeded on polymeric non-woven PGA in the main stream and a second pump for nutrient exchange on the opposite side. No shear stress values were reported. However, hydroxyproline production increased as a result of shear suggesting an improvement in tissue strength. In two reports by Butcher *et al.*[7,8] the effects of steady fluid shear on porcine valvular and vascular endothelial cells cultured on glass slides, differences in morphology and transcriptional profiles were shown. Vascular ECs aligned parallel to the flow direction whereas valvular ECs aligned perpendicular to the flow direction. The pattern of focal adhesion was different in the different cells.[7] The transcriptional profiles showed that under a steady shear of 20 dynes/cm^2 valvular ECs were less intrinsically inflammatory than vascular ECs but both cell types expressed similar antioxidant and anti-inflammatory genes in response to shear stress. Shear stress also appeared to have a protective effect against calcification. Butcher *et al.* also reported two steady shear models, one with valvular interstitial cells cultured in a collagen gel and the second being a co-culture of valvular ECs and valvular interstitial cells.[6] The first model (only interstitial cells) showed a mitogenic response with proliferation under 20 dynes/cm^2. The co-culture model showed inhibition of glycosaminoglycan loss along with increased protein synthesis. Valvular endothelial cells appear to regulate interstitial cell phenotype and shear flow enhances regulation.

Flexure, Stretch, and Flow

Engelmayr *et al.*[16] described the development of a bioreactor capable of applying flexure, stretch, and shear to engineered heart valve tissues independently or in combination. The flex–stretch–flow (FSF) bioreactor consisted of two identically equipped chambers, each having the capacity to hold up to 12 rectangular tissue specimens with a "spiral-bound" technique. Specimens could be subjected to changes-in-curvature up to 50 mm^{-1} and uniaxial tensile strains up to 75%. Steady laminar flow could also be applied by a magnetically coupled paddlewheel system. This article detailed the bioreactor design without biologic results.

These kinds of mechanistic studies are tremendously informative in understanding the biologic consequence of well-defined force components in isolation or in combination. This parametric approach to TEHV design is limited in simulating the true three-dimensional biomechanical environment. Specifically, the complexity of leaflet stretching and deformation will be difficult simulate accurately particularly with non-native materials. Non-laminar flow structures as found in the coronary sinus region of the aortic valve are difficult to simulate in isolation from other mechanical effects like pressure-induced vessel stretch. The overall fluid and solid mechanical effects of leaflet coaptation may be difficult to simulate in a mechanistic bioreactor setting. In spite of these challenges, the design of flex, stretch, and flow bioreactors will likely be driven toward increasingly realistic simulations of the *in vivo* heart valve environment resulting in the ability to better assess ideal conditioning regimes for TEHV.

BIOREACTORS FOR CONDITIONING TEHV

Bioreactor studies that describe the independent or combined effects of flexure, stretch, and shear provide

a wealth of information to be used in the conditioning of entire TEHV prior to implantation. The choice of scaffold material, magnitude and frequency of loading, duration of conditioning can all be evaluated using aforementioned bioreactors. Unfortunately, when cell-seeded whole TEHV scaffolds are placed into bioreactors, it is difficult to accurately quantify these mechanical forces. However, estimates of the desired magnitudes of flex, stretch, and shear imparted to whole TEHV in a bioreactor can be made from existing knowledge of valve fluid and solid mechanics.

Valve Fluid and Solid Mechanics

The normal fluid and solid mechanics of the recipient hosting the TEHV should provide benchmark values of flow rate, flow rate waveform, pressure variations, cyclic stretch, and cyclic flexure. The pulmonary and tricuspid valves control flow on the right side of the heart and are subject to pressures of about 30 mmHg. By contrast the mitral and aortic valves must support pressures that exceed 120 mmHg. Tissue engineered heart valves should be designed to operate under this cyclic mechanical stress combined with the oscillating fluid mechanical shear stress of blood flow potentially over as many as 3×10^9 cycles. The fluid and solid mechanical forces on the valve leaflets are thought to contribute to the development and maintenance TEHV leaflets.[21] Understanding these forces is essential to reproducing them in a TEHV bioreactor. As the left ventricle contracts at the beginning of systole, blood is ejected through the aortic valve during the first third of the cardiac cycle. Blood is accelerated to approximately 1.4 m/s and then decelerates.[42] Vortices form downstream of the valve during this deceleration phase. Diastolic pressure reaches approximately 80 mmHg and the valve closes with very little backflow into the ventricle in healthy individuals. Weston et al.[41] used laser Doppler measurements from inside and downstream of a polyurethane valve to quantify maximum wall shear stress. Inside the valve was found to be 79 dyne/cm^2 and downstream of the valve was 10–17 dyne/cm^2 at a flow rate of 7.5 L/min. At a flow rate of 22.5 L/min the shear stress was 52–104 dyne/cm^2. Shear stress on valve leaflets is dependent upon the moment in the cardiac cycle and the angle of the valve leaflet with respect to the flow axis. This work defines an upper limit for shear stress that is important to consider in bioreactor development and testing protocols for tissue engineered valves. The pulmonic valve flow behaves similarly to that of the aortic valve flow but the magnitude of the velocity is not as great. Typical peak velocities for healthy adults are 0.75 ± 0.15 m/s and slightly higher for children at 0.9 ± 0.2 m/s.[41]

In addition to the fluid mechanical forces imparted to valvular endothelial cells, solid mechanical factors contribute to TEHV cell adaptation and scaffold material fatigue. In particular, flexural stresses developed during valve opening and closing are thought to play a role in calcification and material damage of bioprosthetic valves. Lyengar et al.[31] used a laser light projection technique to quantify valve leaflet deformation during opening and closing. A bovine pericardial bioprosthetic valve was placed in a pulsatile flow loop and the valve leaflet motion was imaged using points of light and a boroscope. Their findings showed complex bending motion in the central region of the leaflets both in the opening and closing phases. Strains were not computed.

Current Bioreactors

Although many types of bioreactors have been used for conditioning of TEHV, there is a paucity of studies demonstrating that this technique is needed, and what the effects of bioreactor use are. Bioreactors for whole TEHV provide an environment to condition cells or cell-seeded scaffolds in an in vitro environment. Within a bioreactor, flow rate, pressure, temperature, and oxygen diffusion can all be controlled to provide a physiologic environment for the purpose of enhancing extracellular matrix formation, cellular proliferation, and cellular alignment.

The fundamental components of any bioreactor for conditioning whole TEHV are (1) a driving force or pump for fluid movement, (2) a reservoir usually containing cell culture media, (3) a test section containing the heart valve or component of heart valve (4), a fluid capacitance to store and release energy at every pump cycle, (5) a resistance element, and (6) the possibility for gas exchange. Pumps can take the form of pneumatically controlled diaphragms, reciprocating pistons, peristaltic pumps, or meshing gears teeth in order to displace fluid. It is desirable for each pump to provide complete control over fluid movement, and these pumps are typically associated with a computer control system. Peristaltic pumps typically create sinusoidal flow rate waveforms (and low flow) while pneumatic, piston, and gear pumps can create higher flow rates with more control over the shape of the waveform. Test sections containing the heart valve tissue or valve segment should be designed to minimize flow disturbances especially at the entrance. This enhances the ability to create laminar flow through the valve. A fluid capacitance helps with energy release following peak system pressure for forward or reverse fluid flow, and a resistance element is useful for controlling the overall system pressure. Gas exchange is essential to create the cell culture media oxygenated.

This can occur by active bubbling of media or simple diffusion at the reservoir surface. Finally, a bioreactor system that minimizes the total volume of culture media will be less expensive to operate.

While all pulsatile bioreactors function in generally the same manner, small differences can produce widely varying hemodynamic environments thus varying results for preconditioned cell-seeded heart valves. Schematic diagrams of five heart valve bioreactors are shown in Fig. 1.

Multiple investigators have created pulsatile bioreactors for conditioning intact TEHV. Hoerstrup et al.[22] created one of the first compact heart valve bioreactors. It consisted of an air chamber, media chamber, and perfusion chamber that held the heart valve. A reciprocating pneumatic diaphragm located between the air and media chambers provided pulsatile flow. Flow rates ranging from 50 to 2,000 mL/min were achieved as well as pressure between 10 and 240 mm Hg. In a later study by Hoerstrup et al.[23] PGA TEHV constructs were seeded with carotid ECs and carotid myofibroblasts. All valves were successful in sheep after 20 weeks. Increased collagen production, total DNA, and similar mechanical strength to native valves over control valve (*in vitro*) were shown. Another bioreactor designed by Zeltinger et al.[43] included a medium reservoir, pneumatic pressure chamber with elastomeric bladder, timer mechanism and pressure source to control varying pressures, and a chamber to hold the heart valve. A bladder was placed below the heart valve, which produced a back flow on the valve due to negative pressure allowing it to close. Pressure was controlled by an air pressure source with a timer-controlled solenoid switch. Weston et al. created a tubular pulsatile flow bioreactor that tested sectioned leaflets, which were sutured to a tube.[40] Pumps were used to produce steady and pulsatile flow through the closed loop system. A heat exchanger and gas infusion filter were used to allow for the system to physiologically function outside of an incubator. Schenke-Layland et al.[34] created a system similar to Zeltinger et al.[43] which used a pressure vacuum to control a bladder which in turn controlled fluid pressure and flow. Unlike Zeltinger the heart valve was
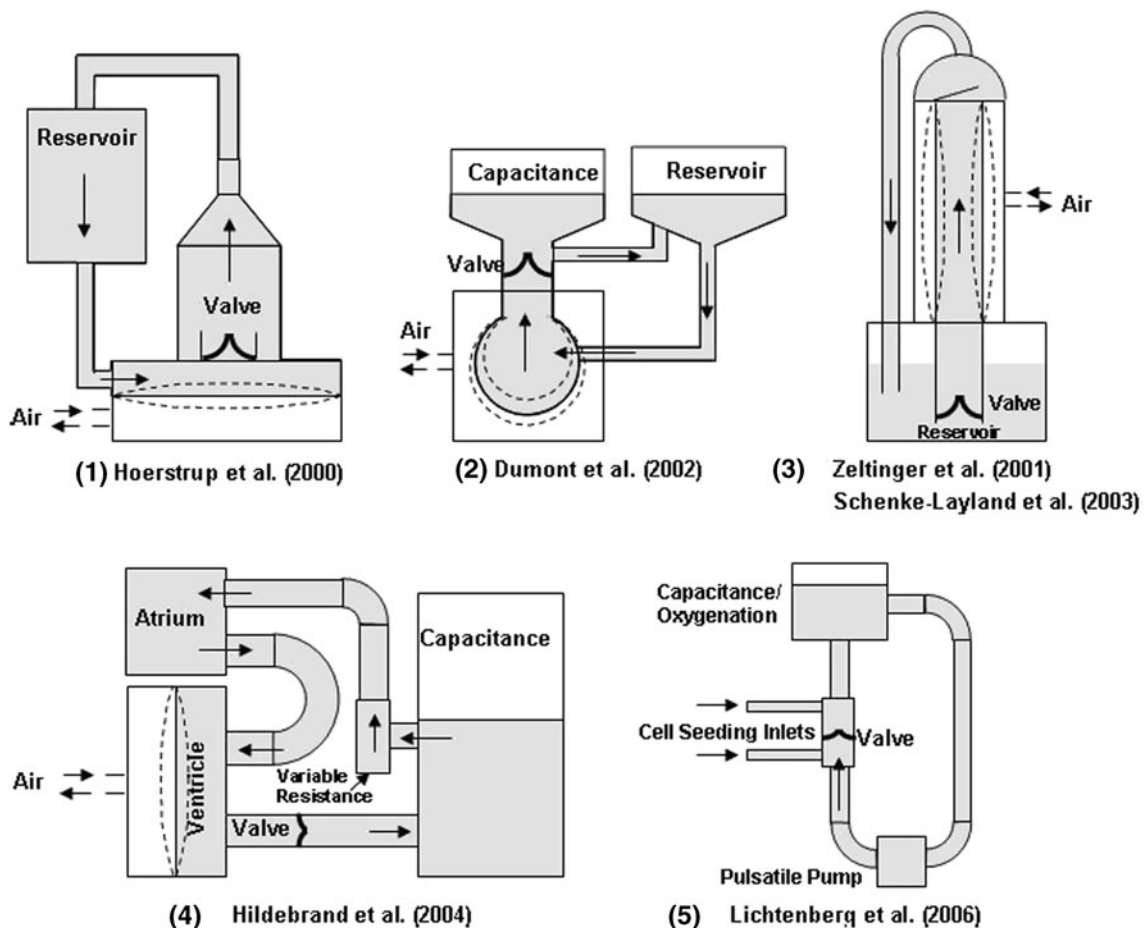

FIGURE 1. Schematic diagrams of five contemporary heart valve bioreactor designs. These designs have been reported to deliver physiologic pulsatile flow and pressure while maintaining the proper temperature, gas exchange, and sterility.

placed below the bladder. This bioreactor was run at a rate of 3 L/min at 60/40 mmHg. Mol et al.[33] created a diastolic pulse duplicator (DPD) which consisted of a valve holding container and a medium container. A magnetic valve controlled the release of air into a cylinder which compressed or decompressed tubing resulting in a pressure differential. This pressure differential produced a pulsatile flow. A syringe filled partly with media and partly with air acted as a compliant section. This system was very compact and it has been shown that six of these bioreactors could be placed on a standard incubator shelf. Lichtenberg et al. engineered a bioreactor which included a pulsatile pump, heart valve reservoir, medium reservoir, and oxygenation/compliance chamber.[30] This closed loop system allowed for direct control of the flow rate by a pulsatile pump. Flow, pressure, and temperature transducers monitored conditions within the bioreactor. Cell seeding inlets were located above and below the heart valve. A roller pump provided a continual flow of gas through the oxygenation chamber. This system was used to precondition heart valves for clinical use. Recently, Karim et al.[25] designed a very compact closed loop system. It consisted of an outer glass cylinder and an inner device for mounting the heart valve. The inlet and outlets of the glass cylinder were connected to a pump and medium reservoir. This reactor could be continuously rotated due to its cylindrical shape. Dumont et al.[12] designed a mock loop based on the left part of the heart. It included a left ventricle chamber, compliance section, area for a TEHV, reservoir, mechanical valve, and aerator. This system allowed for control of stroke volume, compliance, resistance, and beat frequency. Hildebrand et al.[20] reported a bioreactor that included an atrial chamber, ventricle chamber, compliance chamber, variable resistor, stepper motor, pressure sensors, and flow sensors. This system allowed for automatic control of mean pressure, mean flow rate, beat frequency, stroke volume, and the shape of the driving pressure waveform.

Syedain and Tranquillo reported that they developed a controlled cyclic stretch bioreactor for fibrin-based TEHV that leads to improved tensile and compositional properties.[37] The TEHV is mounted inside a latex tube, which was then cyclically pressurized with culture medium. The root and leaflets stretched with the latex. Nutrient delivery was provided with slow perfusion of culture media. The TEHVs were seeded with human dermal fibroblasts and subjected to 3 weeks of cyclic stretching with incrementally increasing strain amplitude. The TEHV possessed the tensile stiffness and stiffness anisotropy of leaflets from sheep pulmonary valves and also showed circumferential fiber alignment. Kortsmit et al. developed a proportional integral-derivative (PID) feedback controller to regulate deformation in their TEHV bioreactor system. Results indicated a good correspondence between the measured and the prescribed deformation values.[27]

Preclinical and Clinical Testing of Bioreactor-Conditioned TEHV

Preclinical evaluation of testing tissue engineered heart valves (TEHV) in animals describes testing the valve in the pulmonary position.[10,11] The rationale for testing TEHV in the pulmonary position is based on avoiding the harsh hemodynamic conditions present in the left heart compared to the right heart. In addition, the consequences of aortic valve failure are much more severe than pulmonary valve failure and it is surgically easier to replace the pulmonary valve than the aortic valve. The ultimate indicator of success of preconditioning TEHV with bioreactors has been the preclinical or clinical outcomes for the recipients of these valves. Cebotari et al. [9] recently reported a clinical result for pulmonary valve replacement using tissue engineered heart valves in two pediatric patients. Autologous endothelial progenitor cells were seeded onto human cadaveric pulmonary valves and preconditioned in a bioreactor for up to 21 days. Follow-up at 3.5 years showed normal patient growth and normal valve growth with no complications.

CONCLUSION

While it is clear that tissue engineered heart valves will need to function under physiologic levels of cyclic stretch, flexure, and shear stress upon implantation, the optimal magnitudes of *in vitro* conditioning have yet to be completely delineated. There is no agreed-upon conditioning protocol for TEHV bioreactors given the paucity of clinical data of TEHV. However, bioreactors should gradually introduce physiologic flex, stretch, and flow in order to condition valvular cells to their final mechanical environment. Future studies should identify those forces and conditions within TEHV bioreactors that will lead to clinical success.

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