6-Month Aortic Valve Implantation of an Off-the-Shelf Tissue-engineered Valve in Sheep

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Abstract

Diseased aortic valves often require replacement, with over 30% of the current aortic valve surgeries performed in patients who will outlive a bioprosthetic valve. While many promising tissue-engineered valves have been created in the lab using the cell-seeded polymeric scaffold paradigm, none have been successfully tested long-term in the aortic position of pre-clinical model. The high pressure gradients and dynamic flow across the aortic valve leaflets require engineering a tissue that has the strength and compliance to withstand high mechanical demand without compromising normal hemodynamics. A long-term preclinical evaluation of an off-the-shelf tissue-engineered aortic valve in sheep model is presented here. The valves were made from a tube of decellularized cell-produced matrix mounted on a frame. The engineered tissue matrix is primarily composed of collagen, with strength and organization comparable to native valve leaflets. *In vitro* testing showed excellent hemodynamic performance with low regurgitation, low systolic pressure, and large orifice area. The implanted valves showed large-scale leaflet motion and maintained effective orifice area throughout the duration of the 6-month implant, with no calcification. After 24 weeks implantation (over 17 million cycles), the valves showed no change in tensile mechanical properties or collagen content. In addition, histology and DNA quantitation showed repopulation of the engineered matrix with interstitial-like cells and endothelialization. New extracellular matrix deposition, including elastin, further demonstrates positive tissue remodeling in addition to recellularization and valve function. Long-term implantation in the sheep model resulted in functionality, matrix remodeling, and recellularization, unprecedented results for a tissue-engineered aortic valve.

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Authors have no conflict of interest.

Introduction

An increasing number of aortic valve replacements (AVRs) are being performed annually in the aging U.S. population (73,327 in 2011 with 30% below the age of 65[1]). Given the current average life expectancy of 79 in the U.S., 30% of AVR patients (22,000 in 2011) would likely receive a mechanical valve rather than a bioprosthetic valve[1]. However, this indefinite durability is at the cost of lifelong anti-coagulation therapy, with associated morbidities. If a tissue-engineered AVR demonstrated both indefinite durability (by virtue of resident cells that maintain the extracellular matrix) and hemocompatibility (via the presence of an endothelium), it could have a major impact on the adult AVR patients younger than 65 year old. The patient population could be much larger (i.e. also include all 51,000 AVR patients aged 65 years and older) if an engineered tissue was available that performed superior to bioprosthetic tissue (i.e. exhibited no calcification).

There have been multiple studies using decellularized native valves for AVR [2–6] including an ongoing clinical trial [7], but for tissue-engineered heart valves grown *in vitro*, despite many studies by multiple investigators spanning 20 years and much progress for pulmonary valve replacements in adult animal models[8–12], including the strategy of decellularization [8, 9], there has been no reported success in demonstrating long-term function as an AVR. In fact, the only report of a preclinical tissue-engineered AVR study based on the cell-seeded polymeric scaffold paradigm of interest here was for a biodegradable synthetic polymer valve pre-seeded with autologous bone marrow mononuclear cells and implanted for up to 2 weeks in the sheep model[13].

We have developed a decellularized (not chemically crosslinked) engineered tissue, either from human or sheep dermal fibroblasts in tubular geometry with diameters ranging from 4–24 mm and length from 2 cm to 15 cm. Decellularization renders it a non-immunogenic extracellular matrix suitable as an allograft. This sheep matrix became extensively repopulated by host tissue cells (including the deposition of collagen and elastin needed for tissue durability) and endothelial cells (needed for hemocompatibility) after 6 months of implantation in the sheep femoral artery[14]. It was also successfully endothelialized with autologous blood outgrowth endothelial cells prior to implantation[15]. These tubular heart valves are created by placing the matrix tubes over a three-pronged frame, which leads to a valvular action as the tube collapses inward between the prongs during the diastolic back-pressure phase of the cardiac cycle. This matrix is formed as a tube with strong circumferential alignment, yielding leaflets also possessing strong circumferential alignment when made into tubular heart valves, consistent with native valve leaflets[16]. In our previously reported *in vitro* characterization, we demonstrated excellent short-term hemodynamic function of this tubular heart valve in a pulse duplicator, for both pulmonary and aortic conditions[16].

Here, we report unprecedented long-term (24 weeks) fatigue resistance and remodeling of these tubular valves in the aortic position in the sheep model. This model has been used extensively to evaluate tissue engineered heart valves in the pulmonary position [8, 9, 11, 12, 17, 18], with the most stringent assessment of scaffold remodeling, recellularization, and performance being based on n=3 to 4 at a maximum time point of 24 weeks[8, 12]. Hence,

we focused our resources on the 24 week time-point with n=3 sheep, while using n=1 sheep at 12 weeks to provide insight into the progression of matrix remodeling and recellularization. Echocardiography was performed at multiple time points and the explanted valves were extensively assessed for matrix durability and remodeling and for recellularization.

Materials and Methods

Engineered tissue tubes

Ovine and human dermal fibroblast (oDF and hDF)-seeded fibrin gel was formed by adding thrombin (Sigma) and calcium chloride in 20 mM HEPES-buffered saline to a suspension of cells (oDF from Coriell, hDF from Lonza) in bovine fibrinogen (Sigma). The final component concentrations of the cell suspension were as follows: 4mg/ml fibrinogen, 0.38U/ml thrombin, 5.0 mM Ca^{++}, and 1 million cells/ml. Cell suspensions were mixed and injected into a tubular glass mold with a 24 mm ID mandrel, 6 mm annulus, and 9 cm length requiring ~24 ml suspension containing 24 million cells and 96 mg fibrinogen. Tubular grafts were cultured in static jars for 2-weeks followed by incubation in custom pulsed-flow-stretch bioreactors for an additional 5-week maturation period as previously described [19].

For decellularization, the tubes were incubated on an orbital shaker at room temperature for 6 hr with 1% sodium dodecyl sulfate (SDS, Sigma) followed by 1% Triton X-100 (Sigma) for 30 min, extensively washed with PBS for 168 hours, and then incubated in 2U/ml deoxyribonuclease (Worthington Biochemical, DR1) in DMEM supplemented with 10% FBS overnight.

Valve design

The decellularized 24 mm ID engineered tissue tube was mounted onto a Mitroflow® 21mm frame (Sorin Group). The engineered tissue tube was cut to ~16 mm length, slightly longer than the frame height, and secured to the frame using interrupted 4-0 prolene sutures. The larger diameter tissue tube compared to the frame diameter (24 mm *vs.* 21 mm) was utilized to allow for full coaptation of the valve during diastole.

Pulse duplicator testing

A customized pulse duplicator system was designed based on a commercial wave generator and pump (ViVitro Systems). It was described in detail previously [16] (schematic and image in Supplementary Figure 1). Each valve (human and ovine tissue derived AVR) was tested with pressure conditions to mimic aortic conditions of an adult human (120 over 80mmHg with diastolic transvalvular pressure of 100 mmHg) and flow rate of ~5 liter per minute at 70 beats per minute.

During valve testing, end-on camera (Canon) images were obtained at 60fps for video capture. Images extracted from the video were imported into ImageJ® software to measure the open area of the valve during systole to report the geometric orifice area (GOA). The theoretical effective orifice area (EOA) was calculated based on following two equations from ISO standard 5840 [20].

$$q_{v,RMS} = \sqrt{\frac{\int_{t1}^{t2} q_v(t)^2 dt}{t_2 - t_1}}$$

$$EOA = \frac{q_{v,RMS}}{51.6 \sqrt{\frac{\Delta P}{\rho}}}$$

Valve Implant in Ovine Model

Tissue-engineered ovine AVR were implanted as aortic valve replacements in n=4 Dorset sheep (weight exceeding 65kg to accommodate 21mm valve, age at implant: 1.9 yr old). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Approved protocol number 1306-30668A) and conform to NIH guidelines on Care and Use of Laboratory Animals. The surgeries were performed by the University of Minnesota's Experimental Surgical Services. For all animals, anesthesia was induced by administering 10mg/kg Ketamine (IM) and 2–6 mg/kg propofol (IV). Animals were then intubated and maintained on isoflurane at 2–4% for the duration of surgery and monitored for heart rate, mean blood pressure, fixed pupil location, corneal reflex absence, and oxygen saturation to ensure proper anesthesia. The heart was exposed by a left lateral thoracotomy with dissection through the intercostal space. The valve was implanted using interrupted 3-0 braided polyester, inverted mattress stitches. An epicardial echocardiogram was performed post-implant (within 1 hr) after animals were off bypass. Post-surgery, animals received subcutaneous 1500IU heparin BID for duration of the study. For pain, animal received ketoprofen 1–2mg/kg (IM) every 12–24 hours as directed by post-operative Veterinarian, additionally two doses of buphenorphine 0.01–0.03 mg/kg (IM) was given at implant and 6 hrs post-op. Animals for the study were numbered as TEV1, TEV2, TEV3 and TEV4. At 12 and 24 weeks post-implant, transthoracic echocardiogram was performed under manual restraint. One valve (TEV 4) was explanted at 12 weeks to assess intermediate remodeling and three at 24 weeks (TEV 1, TEV 2, and TEV 3). Animals were euthanized with beuthanasia given intravenously at 87–90 mg/kg. Explanted valves were photographed, and then dissected into strips for histology, biochemical and mechanical quantitation.

Mechanical testing

Tissue strips cut from the engineered tissue tube and ovine aortic valve leaflets of dimension ~2 mm × 10 mm were tested for tensile properties in both the circumferential and radial directions with respect to the valve "leaflets" (for the tubular valve, the circumferential and radial directions of the "leaflets" are the circumferential and axial directions of the tube, respectively). The tangent modulus (E) was determined as the slope of the linear region of the stress-strain curve prior to failure. The peak stress was defined as ultimate tensile strength (UTS). Mechanical anisotropy was defined as the ratio of the modulus of tissue samples cut in the circumferential direction to the modulus of samples cut from the tissue in the axial direction.

Tissue composition and DNA analysis

The collagen mass content was quantified using a hydroxyproline assay previously described [21] assuming 7.46 mg of collagen per 1 mg of hydroxyproline. The total protein content

was measured using the ninhydrin assay [22]. The tissue sample volume was calculated using the measured length, width, and thickness of the strips. Collagen and protein concentrations were calculated as mass per unit volume. The DNA content was quantified with a modified Hoechst assay for DNA [23].

Histology and Immunostaining

Each explanted valve was evaluated for histological and immunological staining with multiple strips cut to cover all regions of interest from each valve. Circumferential tissue strips of fresh and explanted valve were fixed in 4% paraformaldehyde, embedded in OCT (Tissue-Tek), and frozen in liquid N_2. Cross sections of 9-µm thickness were stained with Lillie's trichrome, Alcian blue, and picrosirius red stains. Images were taken at 10x magnification. Histological sections were stained for αSMA (Sigma, A5228), vimentin (Abcam, ab80667), vWF (Abcam ab6994), CD45 (US Biological C2399-07B), elastin (Abcam ab21599), collagen IV (Abcam, ab6586) and laminin (Abcam, ab11575). All samples were blocked with 5% normal donkey serum, incubated in primary antibody at 5ug/ml and stained with a Cy5-conjugated, species-matched secondary antibody (Jackson Immunoresearch). Nuclei were counterstained with Hoechst 33342 (Invitrogen H3570).

Statistics

Statistical significance for differences between two groups was determined using Student's t-test when comparing two groups and ANOVA with tukey post-hoc analysis for more than two groups. Paired symbols are used in figures to represent statistical difference. Any reference to a difference in the Results and Discussion sections implies statistical significance at the level $p < 0.05$.

Results

Tissue-engineered aortic heart valve properties

Tissue-engineered matrix tubes fabricated from ovine and human dermal fibroblasts (ovine and human tubes, respectively) were evaluated for tensile mechanical and biochemical properties (Table 1). In general, human tubes were 33% thinner than ovine tubes (1.2±0.1 mm). As shown in Fig. 1f, human tubes lacked residual fibrin (stains red) while ovine tubes (Fig. 1e) had a band of fibrin visible on the lumenal surface. Mechanically, both human and ovine tissue had tensile strength and stiffness similar to ovine native aortic valve leaflets. Most importantly, the tissue tubes had mechanical anisotropy, with stiffness being 3-fold higher for ovine tubes and 2-fold higher for human tubes in the circumferential direction. The total collagen concentration was similar for human and ovine tubes. The DNA content after decellularization was less than 99% when compared to native valve leaflets.

Both human and ovine tubes were mounted on the 21mm Sorin Mitroflow® valve frame (Fig. 1a–c) and tested in a pulse duplicator at physiological aortic conditions. For both human and ovine valves, the systolic pressure drop was less than 5 mmHg, effective orifice area (EOA) of 1.6 cm² and diastolic regurgitation was less than 5% (Table 2, SF1, SV1, and SV2). ISO standard guideline for a 21 mm valve requires maximum regurgitation not to exceed 10% and a minimum EOA of 0.85 cm².[20]

Aortic valve implant evaluation with ultrasound over 24 weeks

All four animals had uneventful recovery and no symptoms throughout the duration of the study. At implantation (Fig. 1d), once animals were off bypass, a transapical echocardiogram was performed to observe leaflet motion and valve function (leaflet here refers to the three regions of the collapsing tube between the three frame posts, as a tubular valve does not possess anatomically distinct leaflets as in native valves). All four valves had three functional leaflets, with complete valve opening and closing and laminar flow through the valve. Trivial to mild regurgitation was determined for 2 of 4 valves at implantation. Representative end-on (aortic view) and side-view images are shown in Fig. 2a–d (supplementary video SV3). At 12 weeks post-op, all valves showed functional leaflets with no thickening, stenosis, or evidence of calcification (brighter ultrasound speckle) with transthoracic echocardiography (Fig. 2e–g, supplementary video SV4). As shown in Fig. 3a, the aortic insufficiency (AI) grading increased for 3 of 4 valves at 12 weeks, but remained unchanged between 12 and 24 weeks. One valve (TEV4) was harvested at 12 weeks for an intermediate assessment. At 24 weeks, the remaining three valves were evaluated. No stenosis was observed, confirmed with measurement of EOA showing no change between 12 and 24 weeks (Fig. 3b). At 24 weeks, all 3 valves had uniform leaflet thickness and motion (Fig. 2h–j, supplementary video SV5). The EOA values were also comparable to the pre-implant theoretical EOA value. The mean systolic pressure drop was 48 ± 16 mmHg (n=4) at 12 weeks and 45 ± 16 mmHg at 24 weeks (n=3). Measured aorta size at both 12 and 24 weeks was 27 ± 1 mm.

Explanted valve gross pathology

The top view of the explanted valves is shown in Fig. 3c–f, with TEV1, TEV3, and TEV4 exhibiting completely coapting leaflets. In the case of TEV3, there was no change in its AI value of 1 over the 24 weeks, which is consistent with gross pathology of the valve. The development of mild to moderate regurgitation in TEV1 and TEV4 was likely due to a small tear at one of the posts leading to leaflet prolapse. TEV2 also developed mild to moderate regurgitation but there was no tear developed at any location. However, the frame posts are more exposed in TEV2, which was due to the entire tube sagging downwards on the frame during the course of implant, leading to lack of coaptation (Fig. 3d). This was supported by observation of the three leaflets belly regions sagging below the bottom of frame. Trichrome-stained sections of leaflets of the explanted valves in Fig. 3g–j revealed similar thickness and organization of the leaflets as well a dominant collagen content and absence of the residual fibrin present at implantation (c.f. Fig. 1f). Alcian blue staining revealed proteoglylcans co-localized in regions with invading cells (Fig. 3 l,n), but not in areas still devoid of invading cells (Fig. 3 k,m) or in the matrix prior to implantation (Fig. 3o).

Explanted valve mechanical and biochemical properties

The explanted valves at 12 and 24 weeks had reduced matrix thickness by 35% and 24%, respectively (Fig. 4a). The UTS, modulus, and stiffness anisotropy showed no change over the 24 week implantation (Fig. 4b,c). The total collagen concentration at 24 weeks was 48 ± 8 mg/ml and the total protein concentration was 76 ± 14 mg/ml (Fig. 4d,e), with collagen concentration being higher than the pre-implant value. The total DNA content increased

during implantation, reaching 141 ± 121 μg/ml by 24 weeks (Fig. 4f). In comparison, the native aortic valve has DNA content of 621 ± 55 μg/ml (Table 1).

Explanted valve histological analysis

No evidence of calcification was detected in any of the valves, as determined by Von Kossa stain performed on 7 regions from each valve. Figure 5 shows three representative regions from TEV3, the commissure and regions of high and low cellularity. Recellularization occurred predominately near the base of the leaflet at 12 week (Fig. 6a1–a4). The invaded cells were positive for interstitial phenotype (αSMA and vimentin positive) and CD45. In addition there was partial endothelialization. As seen in Fig. 4f and 6b1–b4 and 6c1–c4, by 24 weeks implantation, there was increased cellularity in the leaflets compared to 12 weeks in 2 of 3 valves (TEV2,3), with extensive recellularization at the base and lower belly region of the leaflet and partial recellularization towards the free edge of the leaflets. Cellular marker expression was heterogeneous based on region/degree of recellularization. In the fully recellularized region, most cells were negative for αSMA and CD45, while positive for vimentin, and the surface was extensively endothelialized. In comparison, towards the free edge of the leaflet, where there was partial recellularization, cells were primarily αSMA/vimentin or CD45 positive, with partial endothelialization of the surface (similar to the base of the leaflets after 12 weeks implantation).

Elastin was detected, including some organized fiber structures, in the recellularized region of TEV2 and TEV3 at 24 weeks (Fig. 7a,d). Moreover, laminin and collagen IV deposition were pronounced at the endothelialized surface (Fig. 7b,c) and only present in the recellularized regions (Fig. 7e–f). None of these proteins were detectable under exposure-matched settings in the matrix prior to implantation (Fig. 7g–i), indicating their deposition originated from invading host cells.

Discussion

With an aging world population and increased life expectancy, more patients are outliving implanted bioprosthetic heart valves. The bioprosthetic valve typically fails after 10–20 years primarily due to degradation and calcification of the fixed extracellular matrix. Therefore, young adults in need of aortic valve replacement currently have no optimal bioprosthetic valve option[24, 25]. A longer-lasting aortic valve solution could benefit the 30,000 patients in the United States, who receive a mechanical valve with lifelong anticoagulation therapy annually. As a possible remedy for these shortcomings of current prosthetic valves, here we present a novel tissue-engineered tubular heart valve based on a completely-biological matrix tube.

While the tissue-engineered heart valve field has progressed well in the last decade for the pulmonary valve replacement[8, 9, 12], very little success has been reported in developing an aortic valve replacement. This has been primarily due to high durability requirements for the material under the mechanical loading associated with pressure gradients in the aortic position. While cell-seeded tissue assures cellularity upon implantation, it limits the commercial potential of tissue-engineered heart valve because it requires autologous cells. In order to address both the requirement of high mechanical strength and off-the-shelf

availability, we have developed allogeneic decellularized engineered tissue tubes[16]. Based on our decellularized engineered tissue tube technology, previously demonstrated as ovine femoral artery grafts[14], we developed a tubular heart valve design from larger diameter tubes[16].

In order to develop an allogeneic valve for an eventual clinical trial as well as preclinical studies, we developed human and ovine valves in parallel. Decellularized tissue tubes fabricated from human and ovine dermal fibroblasts showed comparable collagen concentration and tensile mechanical properties, and, following mounting of the tissue tubes on a Sorin Mitroflow® frame, comparable valve performance in a pulse duplicator under aortic conditions. Both ovine and human tubes have greater stiffness in the circumferential direction, similar to anisotropy found in native aortic valve leaflets. Theoretically, this anisotropy would lead to improved hemodynamic performance compared to an isotropic matrix. In pulse duplicator testing, both the ovine and human valves showed excellent leaflet motion with complete valve opening during systole and small pressure drops. We used ISO standards sets for bioprosthetic valve to evaluate this engineered valve. Overall, the measured metrics for the valve, such as regurgitation, systolic pressure drop, and effective orifice area were all well within the standards.

For in vivo evaluation, we used the longest reported time point for tissue engineered heart valve implantation, 24 weeks. In a previous study by Gottlieb *et al*, n=3 valves were explanted at a longest time point of 20 weeks [12], while in study by Mol *et al*, n=4 valves were explanted at a longest time point of 24 weeks [8]. Based on these two studies, we thus focused resources on the 24 week time-point with n=3 while performing longitudinal echocardiography on each animal. Echocardiography, immediately after valve implantation, showed excellent leaflet motion, large orifice area and laminar flow in systole, and normal heart function. However, 2 of 4 valves exhibited trivial to mild aortic insufficiency at implantation. In one case, echocardiography had clear resolution to show that the leak was primarily due to one of the leaflets prolapsing below the other two in the closed valve position. This was determined due to a design flaw in mounting the tissue tube onto the frame where one leaflet had larger area than the other two. Additional echocardiography at 12 and 24 weeks showed no further change in any measured parameters over time, indicating stable valve performance and no major matrix deterioration or valve stenosis between 12 and 24 weeks. The systolic pressure drop was recorded high in all animals primarily due to mismatch between 21 mm valve and ~27 mm aortic lumen and accelerated heart rate with increased cardiac output during manual restraint for ultrasound, but didn't change between two ultrasounds performed at 12 and 24 weeks, confirming no stenosis development.

Explanted valves revealed the original matrix in macroscopically pristine condition, with no change in tensile mechanical properties or total protein concentration, and no evidence of calcification, as confirmed with Von Kossa stain performed on multiple strips of tissue from each valve. The leaflet thinning that occurred by 12 weeks is consistent with the 12.5% reduction in leaflet thickness reported for just 2 weeks of simulated aortic function for these valves in a pulse duplicator [16]. This 12-week thinning can thus be attributed to matrix consolidation/water expulsion, although it did not result in an increase in total protein or

collagen concentrations at 12 weeks, indicating some degree of matrix degradation, as would be expected. A small increase in collagen concentration evident at 24 weeks presumably reflects the increase in cellularity, consistent with the deposition of elastin discussed further below. There was increased cellularity in 2 of 3 explants at 24 weeks in comparison to 12 weeks. The lack of cellularity in one of the valves (TEV1) could be due to a barrier presented by the frame's polyester sewing cuff to cell migration from the aortic root since the tube was placed on the outside of the frame. The cells were present mostly on the aortic side, with a graded cellularity from the base to the free edge of the leaflets. This clearly indicates that migration from the aortic root was the dominant mechanism of recellularization; hence, it would be important in a future iteration of the valve design to eliminate or wrap the polyester cuff and allow direct matrix to aortic root contact for rapid cellular migration. Also, based on sparse cellularity on the ventricular side at 12 and 24 weeks, and the gradient of cells towards the free edge being dominant on the aortic side at 24 weeks, it can be concluded that very few if any cells were from the circulating blood.

Despite differences in cellularity, the measured mechanical properties, leaflet thickness, and collagen concentration were comparable among the four valves, proving durability of the original matrix to withstand extensive (17M) cyclic loads without deterioration. Of the four implanted valves, TEV3 showed no change in AI from a value of 1 (trace) over the course of 24 weeks, while TEV1, TEV2, and TEV4 showed an increase in AI from implantation to 12 weeks post-op. However, the AI did not progress between 12 weeks and 24 weeks, indicating some initial loss of function but not a progression. The initial change was likely due to problems near the top of the frame struts, whether it was the entire tissue tube sagging downwards on the frame (TEV2) or a small tear at one of the struts (TEV1 and TEV4). This presumably can be eliminated by improving the suture pattern near the top of the struts. In the case of TEV4, the tear occurred at the exact location of the suture needle hole. However, it is noteworthy to mention that despite tears in the matrix at one strut, the failure did not propagate any further into the leaflet or grossly compromise valve function.

The area of recellularization in TEV2 and TEV3 at 24 weeks showed deposition of new proteoglycans and extracellular matrix proteins including elastin, a critical component of native heart valves. The cells in the lower belly region in these two 24 week did not express CD45 positive and αSMA, but were vimentin positive. Additionally, there was an endothelium, with substantial basement membrane components in these regions. This indicates that by 24 weeks, an extensively recellularized region that mimics native leaflet cell types resulted, including interstitial cells that lack αSMA and express vimentin[26]. The rare presence of CD45 positive cells is consistent with the hypothesis that favorable remodeling coincides with the resolution of inflammation and an absence of immunogenicity of this tissue-engineered matrix. It also agrees with the results of our 24 week femoral artery grafts studies, which also used similar tissue-engineered matrix in the sheep model[14].

There are few other technologies being explored for creating aortic heart valves. Most recently, a synthetic polymer valve seeded with autologous stem cells was evaluated for up to 2 weeks in an ovine model[18]. Its success requires matrix production by the cells *in vivo* matching polymer degradation. Extensive efforts have been made with decellularized native

valves (xenogeneic and allogeneic), mostly tested as pulmonary implants. Xenogeneic decellularized valves showed immune reaction and failure in a recent clinical trial[27]. Hence, the focus is now primarily on allogeneic technology, which showed promising early to midterm results in one clinical trial for pulmonary valve replacement[28],[29]. However, due to severe shortage of donors, allogeneic native valves have limitations as a practical solution.

Valves fabricated using engineered matrices offer numerous advantages over decellularized native tissue, assuming lab grown matrix meets the mechanical durability and compliance requirements, which are strongly demonstrated in this study. In addition to the benefits in sourcing the matrix and creating a size-matched valve for each patient, both *in vitro* and *in vivo* studies have shown better recellularization/regeneration potential of the engineered matrix. In our previous *in vitro* study, using mesenchymal stem cells, it was demonstrated that decellularized engineered tissue could be completely recellularized within 3 weeks[30, 31]. However, very limited cellularization occurred in a decellularized native leaflet over 5 weeks[30]. Similar *in vivo* results were reported by Weber *et al*, where decellularized tissue-engineered valves and a decellularized native valve were implanted in a primate pulmonary valve model and showed 20-fold higher cellularization in the engineered leaflets compared to the native leaflets[9].

The field of heart valve tissue engineering has shown promising growth in the last few years, especially with several clinical trials for pulmonary valve replacement using decellularized native valves[28]. This study demonstrated that tubular valves made from decellularized engineered tissue can be created with durability sufficient to withstand aortic conditions in a sheep model for over 17 million cycles. There was substantial recellularization/endothelialization, elastin deposition, and no evidence of calcification or valve stenosis. These are promising results for an off-the-shelf tissue-engineered aortic valve in a preclinical model and pave the way for future clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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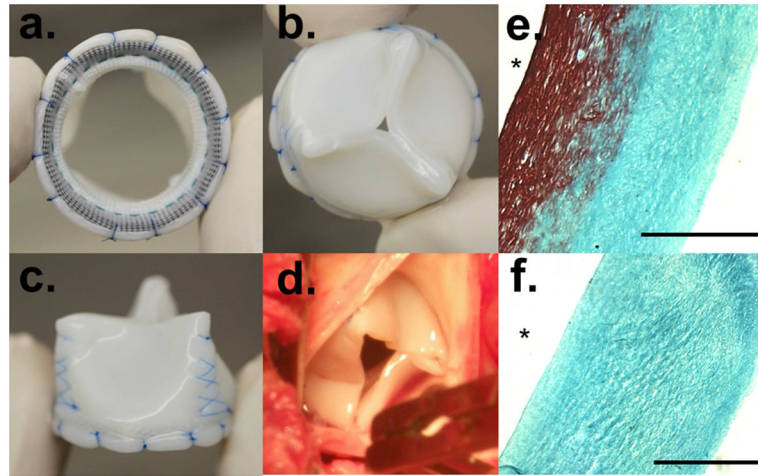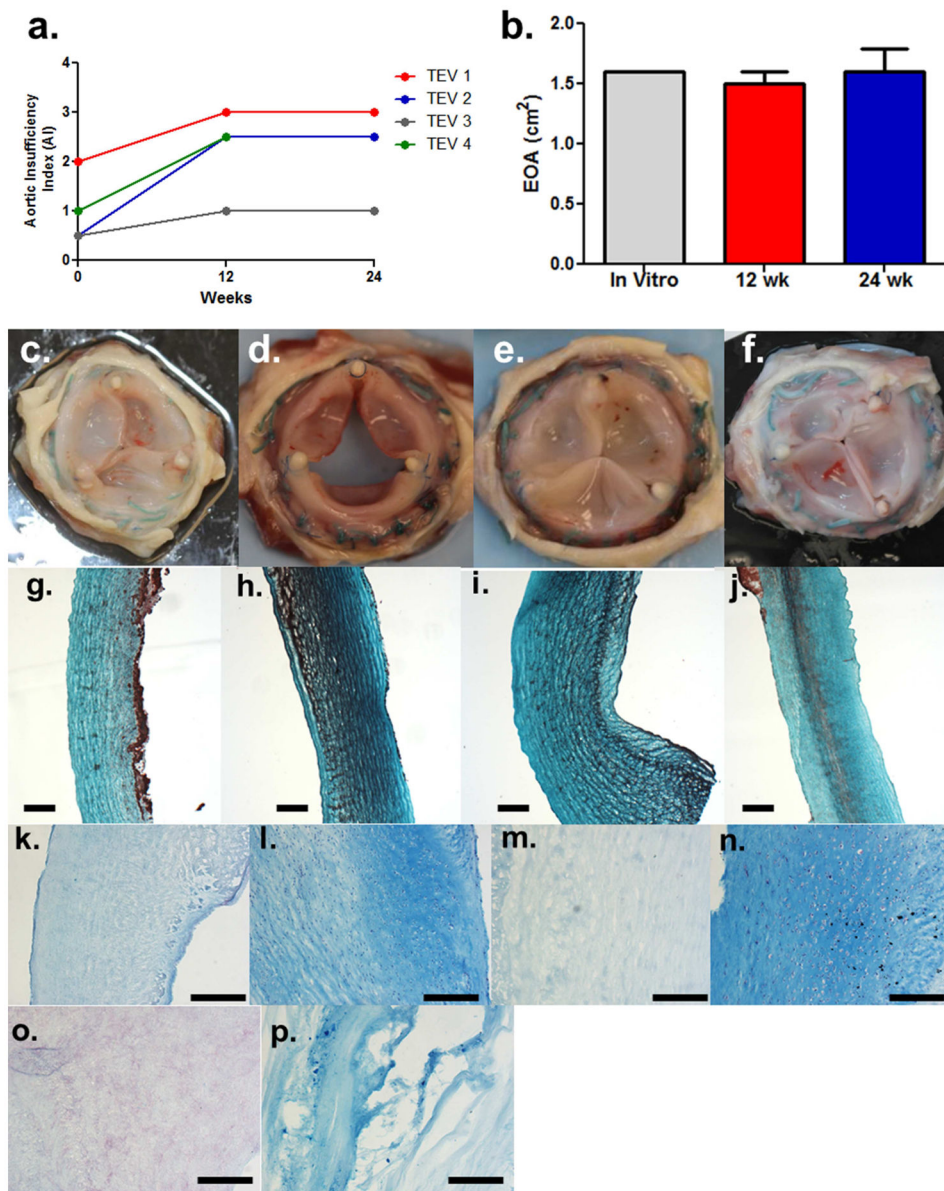
Figure 1.
Tissue-engineered aortic valve mounted on Sorin's Mitroflow® Frame with **a.** bottom, **b.** top and **c.** side view shown, **d.** Top view of implanted valve in the ovine aortic positon, **e.** A trichrome cross section of the ovine fibroblast produced decellularized tissue and **f.** A trichrome cross-section of the human fibroblast produced decellularized tissue, with * representing the lumenal side, which becomes the ventricular side of the implanted valve. A 500 μm scale bar is shown in black on the trichrome sections.
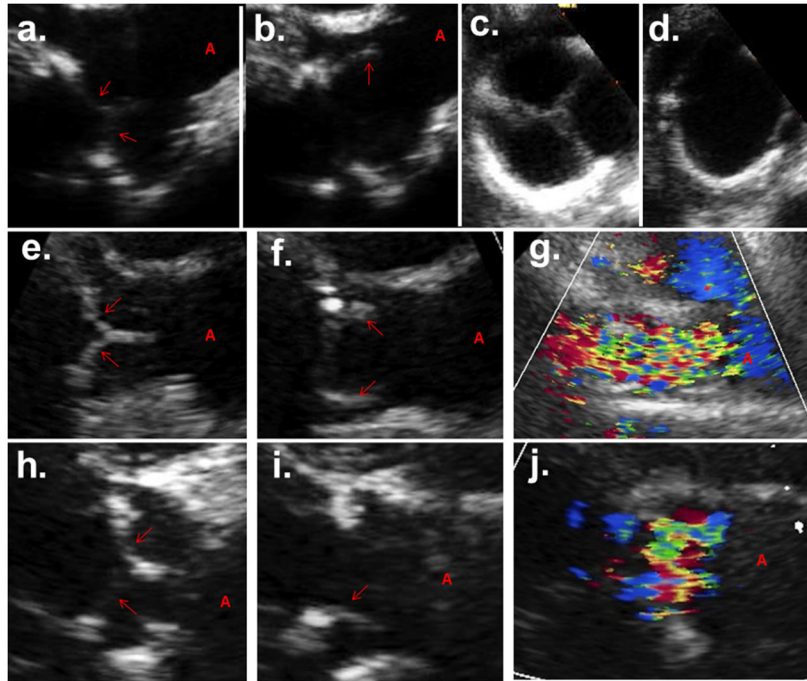
Figure 2.
Ultrasound imaging of the implanted aortic valve at time of implant with **a.** side view in closed position, **b.** side view in open position, **c.** end-on view in closed position, and **d.** end-on view in open position. At 12 weeks, side view in **e.** closed and **f.** open position, **g.** color Doppler side view showing unhindered flow through completely open leaflets. At 24 weeks, side view in **h.** closed and **i.** open position, **j.** color Doppler side view at 24 weeks showing unhindered flow through completely open leaflets.

Figure 3.

a. Aortic insufficiency grade over 24 week with 1/2/3/4 reporting trivial/mild/moderate/ severe insufficiency, **b.** Measured EOA with average of all 4 valves at 12 week and 3 valves at 24 weeks. The 12 week values are based on replicate measurements of all valves (TEV1– 4) and the 24 week values indicate the means and associated standard deviations based on 3 valves (TEV1,2,3). Top view of 24 week explanted valves **c.** TEV1, **d.** TEV2, **e.** TEV3, and **f.** 12 week explanted valve (TEV4). Trichrome-stained sections of the explanted valve leaflets for valves **g.** TEV1, **h.** TEV2, **i.** TEV3, and **j.** TEV4 showing organized collagen. Alcian blue staining showing **k.** proteoglycans in recellularized regions in leaflet of TEV2, but **l.** absent in regions still acellular, with similar staining pattern for **m.** recellularized and **n.** acellular regions in leaflet of TEV3, as well as **o.** the decellularized tissue pre-

implantation. **p.** Positive control intervertebral disc for Alcian blue staining. Scalebars in all histology slides = 200 μm.
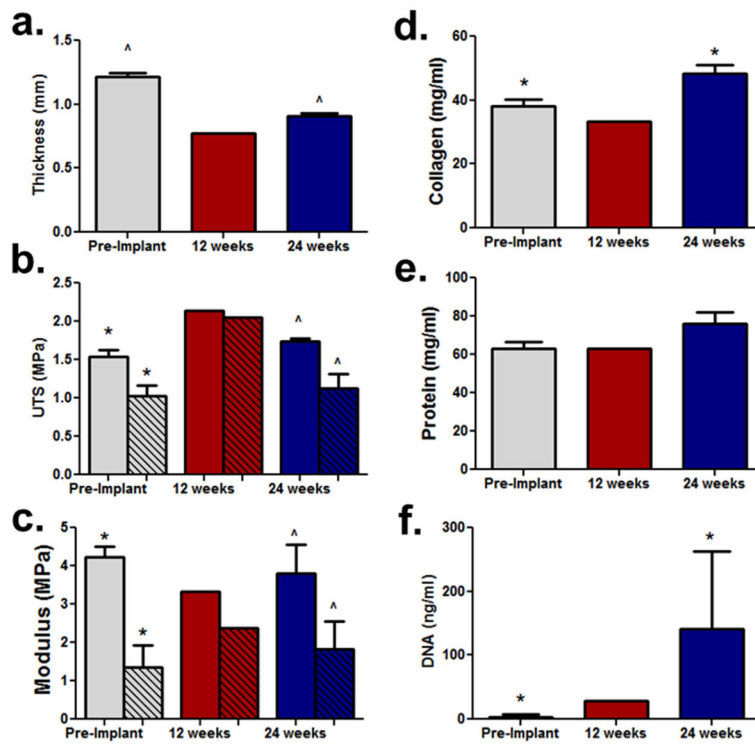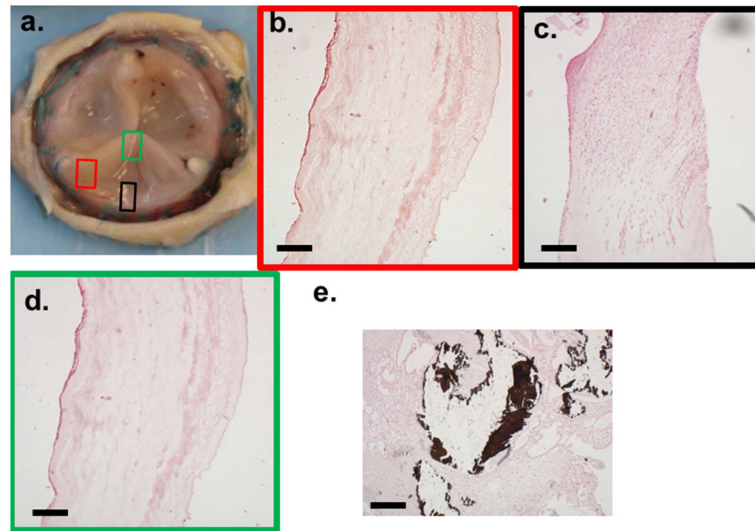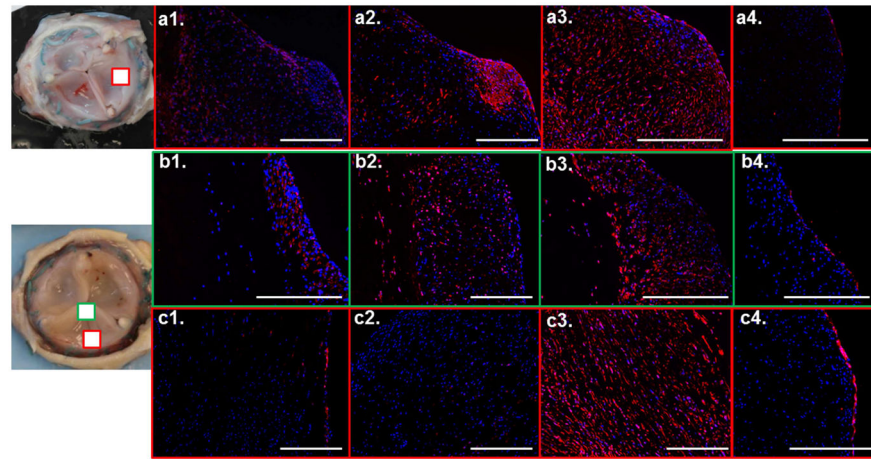
Figure 4.
Mechanical and biochemical measurement of pre-implant and explanted valve tissue with **a.** thickness, **b.** UTS, **c.** Modulus, **d.** Collagen, **e.** Total protein and **f.** DNA concentration. The 12 week values are based on replicate measurements of 1 valve (TEV4) and the 24 week values indicate the means and associated standard deviations based on 3 valves (TEV1,2,3).
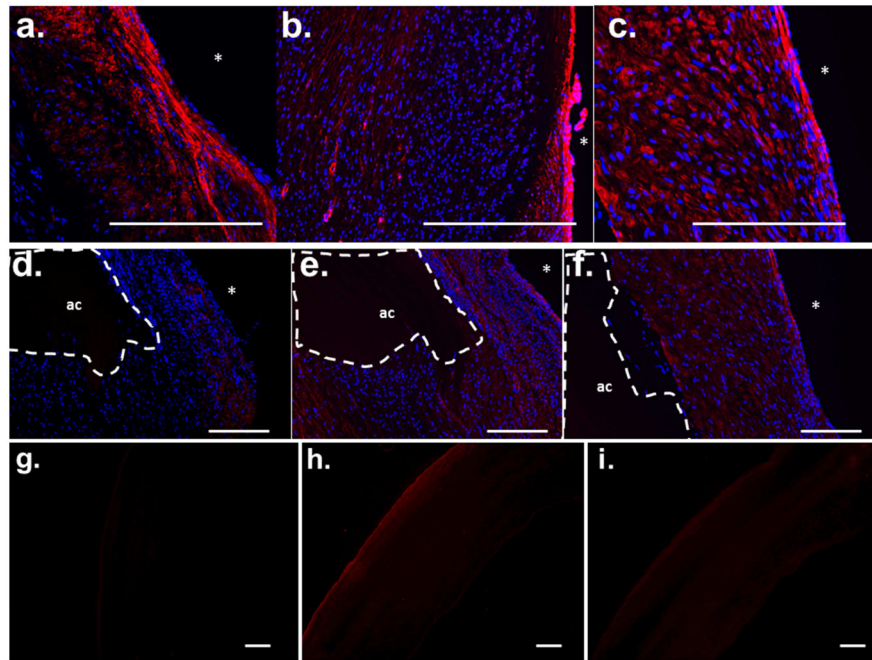
Figure 5.
Von Kossa staining on TEV 3 explanted valve with **a.** image of TEV3 showing region of histological analysis, **b. c. & d.** sections with Von Kossa stain, the color border represent the corresponding region of the valve, and **e.** positive control (calcium control slide, Cat#csc015P, American MasterTech). Scalebars in all histology slides = 200 μm

Figure 6.
Immunostaining of the explanted leaflets with top panel showing **a1.** CD45, **a2.** αSMA, **a3.** vimentin and **a4.** Von Willebrand factor staining for the 12 week explanted tissue (TEV4). The image of the valve on left shows the location from which the images are taken. The other two panels show images from the two regions of a 24 week explanted valve (TEV3) with image of the valve on left showing the locations, **b1.** and **c1.** CD45, **b2.** and **c2.** αSMA, **b3.** and **c3.** vimentin, **b4.** and **c4.** Von Willebrand factor. The white scalebar shown in all images = 200μm.

Figure 7.
Extracellular matrix staining in leaflets after 24 week implantation with **a.&d.** elastin, **b.&e.** collagen IV and **c.&f.** laminin immunostaining at low and high magnification. The acellular region in the low magnification images (d–f) is bordered with a white hash line. Exposure matched immunostaining for the same three proteins in the matrix pre-implantation (**g–i**). The white scalebar shown in all images = 200μm.

Table 1

Tensile mechanical and biochemical properties of engineered tissue tube developed from ovine and human dermal fibroblast (oDF & hDF)

Property	Ovine Aortic Valve Leaflet	Ovine Tubular TEHV	Human Tubular TEHV
Thickness	0.37±0.06 mm	1.2±0.1 mm	0.8±0.1 mm
UTS (circumferential)	1.9±0.6 MPa	1.5±0.8 MPa	2.2±0.6 MPa
Modulus (Circumferential)	3.4±1.1 MPa	4.2±0.3 MPa	4.2±0.7 MPa
Anisotropy	2.8±0.9	3.1±0.2	2.0±0.3
Collagen Concentration	54±7 mg/cm^3	38±4 mg/cm^3	38±9 mg/cm^3
Protein Concentration	70±11 mg/cm^3	63±7 mg/cm^3	56±6 mg/cm^3
DNA content	621±55 μg/cm^3	4±4 μg/cm^3	7±3 μg/cm^3

Table 2

Hydrodynamic properties of oDF and hDF derived engineered tissue valve at aortic conditions.

	Ovine TEHV	Human TEHV
Systolic P	2.9 mmHg	2.8 mmHg
Diastolic P	94 mmHg	83 mmHg
Average Flow Rate	6.6 LPM	5.5 LPM
Peak Flow Rate	14.2 LPM	10.3 LPM
Geometric Area[1] (%)	97 %	95 %
Regurgitant Fraction	1.1 %	3.2 %