Published in final edited form as:

Biomaterials. 2010 February ; 31(6): 1114. doi:10.1016/j.biomaterials.2009.10.019.

THE ROLE OF ORGAN LEVEL CONDITIONING ON THE PROMOTION OF ENGINEERED HEART VALVE TISSUE DEVELOPMENT IN-VITRO USING MESENCHYMAL STEM CELLS

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Abstract

We have previously shown that combined flexure and flow (CFF) augment engineered heart valve tissue formation using bone marrow-derived mesenchymal stem cells (MSC) seeded on polyglycolic acid (PGA)/poly-L-lactic acid (PLLA) blend nonwoven fibrous scaffolds (Engelmayr, et al., *Biomaterials* 2006; vol. 27 pp. 6083–95). In the present study, we sought to determine if these phenomena were reproducible at the organ level in a functional tri-leaflet valve. Tissue engineered valve constructs (TEVC) were fabricated using PGA/PLLA nonwoven fibrous scaffolds then seeded with MSCs. Tissue formation rates using both standard and augmented (using basic fibroblast growth factor [bFGF] and ascorbic acid-2-phosphate [AA2P]) media to enhance the overall production of collagen were evaluated, along with their relation to the local fluid flow fields. The resulting TEVCs were statically cultured for 3 weeks, followed by a 3 week dynamic culture period using our organ level bioreactor (Hildebrand et al., *ABME*, Vol. 32, pp. 1039–49, 2004) under approximated pulmonary artery conditions. Results indicated that supplemented media accelerated collagen formation (~185% increase in collagen mass/MSC compared to standard media), as well as increasing collagen mass production from 3.90 to 4.43 pg/cell/week from 3 to 6 weeks. Using augmented media, dynamic conditioning increased collagen mass production rate from 7.23 to 13.65 pg/cell/week (88.8%) during the dynamic culture period, along with greater preservation of net DNA. Moreover, when compared to our previous CFF study, organ level conditioning increased the collagen production rate from 4.76 to 6.42 pg/cell/week (35%). Newly conducted CFD studies of the CFF specimen flow patterns suggested that oscillatory surface shear stresses were surprisingly similar to a tri-leaflet valve. Overall, we found that the use of simulated pulmonary artery conditions resulted

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substantially large collagen mass production levels and rates found in our earlier CFF study. Moreover, given the fact that the scaffolds underwent modest strains (~7% max) during either CFF or physiological conditioning, the oscillatory surface shear stresses estimated in both studies may play a substantial role in eliciting MSC collagen production in the highly dynamic engineered heart valve fluid mechanical environment.

INTRODUCTION

Surgical replacement of diseased heart valves by mechanical and tissue valve substitutes is now commonplace and enhances the survival and quality of life for many patients. However, repairs of congenital deformities require very small prosthetic heart valve sizes that are not commercially available. Further, in pediatric applications which seek to correct congenital anomalies of the pulmonary valve, accommodation of somatic growth is essential to eliminate multiple reoperations. Tissue engineered pulmonary valves (TEPV) are one such approach that have the potential to develop into a permanent replacement [1]. A fundamental aspect of the development a TEPV capable of physiologic function at the time of implant is knowledge of the biological and mechanical stimulatory requirements to form mechanically robust, biomimetic valvular tissues *in-vitro* [2–3].

It has become axiomatic that *in vitro* mechanical conditioning promotes engineered tissue formation *in-vitro*. However, the underlying mechanisms remain largely unknown, as well as a means to systematically translate these phenomena to the organ level. This lack of knowledge remains a major limiting factor in the rational development engineered heart valve tissues, especially when considering that valve leaflets are subjected to complex, time varying loading environments [4–12]. Thus, many questions remain regarding the exact nature and timeframe of *in-vitro* conditioning necessary for optimal tissue formation. This optimization process must also take into consideration multiple factors, such as leaflet shape and scaffold material properties that will provide the construct with sufficient *in vivo* functionality. At a minimum, sufficient tissue formation, with the appropriate physical qualities, needs to occur prior to implantation so that the TEPV construct can functionally sustain the *in vivo* hemodynamic environment.

Previous work by Mol et al. [10] reported that enhanced tissue formation was observed in conditioned leaflets. Hoerstrup et al. [8] reported on the use of a pulse duplicator system to condition tissue engineered valves at ramping pressure conditions from 30 to 55 mm Hg over a 28 day period. They further reported on substantial increases in the net collagen and DNA content of their conditioned constructs over controls using primary myofibroblasts and endothelial cells [8,10]. However, the specific nature of fluid induced stresses acting on the leaflet surfaces in these studies remains unknown.

Native and prosthetic heart valves are subjected to highly complex, time-varying flexural, tensile, and fluid-induced stresses [13]. Clearly, studies utilizing continuous dynamic stimulation of TEPVs would provide insights into the coupled effects of these stresses during the *in-vitro* conditioning process. The need for such studies has thus far prompted several developments in the form of custom-made devices or bioreactors that can provide biomimetic mechanical conditioning in the heart valve tissue engineering context [4,7,10,14]. However, an optimal conditioning protocol, and the underlying models to develop them for valvular development, remains unknown. Such approaches are particularly important to develop bioreactor-based conditioning regimes using novel (potentially clinically feasible) cell sources [7,15–18] suitable for functional TEPV development.

Previous studies have explored the use of bone marrow derived mesenchymal stem cells (MSCs) as a potential autologous cell source for engineered heart valve tissues, since they can

be obtained in a minimally invasive fashion [19]. Further, while yet to be comprehensively demonstrated, MSC pluripotency may allow them to serve as a clinically feasible cell source without raising ethical questions associated with embryonic stem cells. With respect to in-vitro tissue conditioning using MSCs, we have demonstrated that they respond favorably to mechanical stimuli [20–24]. Sutherland et al. [18] also demonstrated that under extended implantation periods (>4 months), TEPVs in an ovine model using MSCs seeded onto polyglycolic acid (PGA)/poly-L-lactic acid (PLLA) nonwoven, biodegradable scaffolds underwent extensive remodeling leading to structures resembling the native valve.

Development of optimal in vitro conditioning regimes are dependent on a wide range of variables, including type/magnitude of stresses applied, leaflet motion and geometry, conditioning timeframes, cell source(s), scaffold materials, and media formulation, to name a few. All of these variables (and potentially many more) will require knowledge of their respective effects on both initial and subsequent post-implant tissue formation. Thus, in order to develop a rational, mechanism-based approach directed towards understanding the processes that occur during in-vitro engineered heart valve tissue development, our laboratory has focused on elucidating the relative contributions of the major modes of heart valve deformation: flow, flexure, and stretch, which are central to valve dynamics [19,25]. In particular, utilizing a specialized bioreactor we have demonstrated that combined cyclic flexure and flow (CFF) conditions result in significantly higher collagen content (~75% increase) in comparison to isolated flex and flow conditions alone [19]. Interestingly, in contrast to vascular smooth muscle cell-seeded scaffolds prepared under static and cyclic flexure-alone environments [5], MSC-seeded scaffolds accumulated less collagen [19]. While these studies have provided initial insight into the complexities of optimization of engineered heart valve tissue properties, the underlying mechanisms of how external fluidic forces and internal deformations modulate tissue formation remain unknown. In particular, the potential to scale-up the phenomenon observed in our CFF and related studies to the complex physical milieu of a functional tri-leaflet valve under physiological hemodynamic conditions remain unproven.

As a next step, we conducted the following study to determine if the extracellular matrix (ECM) growth patterns observed in our previous CFF studies [19] could be duplicated in a functional tri-leaflet valve. Specifically, we utilized ovine MSCs to seed nonwoven PGA/PLLA fabrics and imposed pulmonary artery conditions on intact tri-leaflet valve constructs and compared them to valves grown under static tissue culture conditions. The resulting formed tissues were studied using histological and biochemical measures. In addition, it has been shown that media containing ascorbic acid-2-phosphate (AA2P) and basic fibroblast growth factor (bFGF) augmented tissue formation [18]. Owing to the critical role of collagen in the functional requirements of engineered heart valve tissues [5,26–28], we assessed the coupled effects of ascorbic acid 2-phosphate (AA2P) and basic fibroblast growth factor (bFGF) on collagen and GAG formation. Next we combined these biochemical enhancements with physiological conditioning on tri-leaflet heart valve constructs to evaluate our ability to perform scale-up at the organ level. Finally, in an effort to gain insight into the potential stimulatory mechanisms of MSC seeded TEVC tissue formation, we developed detailed computational fluid dynamic (CFD) models of the CFF bioreactor specimens to specifically delineate the nature in which fluid-induced shear stresses act CFF specimens, and related them to similar patterns reported for the native tri-leaflet valve.

METHODS

Isolation and Characterization of MSCs

Ovine MSC isolation was performed following previously reported protocol by Pittenger et al. [29]. In brief, 10 ml/kg of whole bone marrow was aspirated from the iliac crest of neonatal sheep. Cells were centrifuged on a Ficoll gradient in order to obtain the mononuclear cell

fraction. Red blood cells were lysed with ammonium chloride, and the remaining mononuclear fraction was plated onto bacteriologic plates. Medium was initially changed after 48 hours, and adherent cells were passaged.

MSC characterization was performed using the methodology reported by Perry et al. [12]. Briefly, a sub-population of these cells were grown and plated at 10,000 cells/cm², and after initial culture in standard medium, cells were grown in media known to differentiate MSCs into fat cells (adipogenic medium), bone cells (osteogenic medium) or cartilage (chondrogenic medium). After three weeks, cells were found to differentiate into the three cell types. Subsequently, characterized MSCs were cryopreserved in culture medium supplemented with 5% dimethyl sulfoxide (DMSO) and were shipped to the University of Pittsburgh on dry ice.

MSC culture and AA2P and bFGF supplementation Studies

MSCs were expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic plus HEPES buffer (all purchased from Invitrogen, Carlsbad, CA). Cells were at passage 8 to 10 when sufficient numbers were available for scaffold seeding. A needled nonwoven scaffold consisting of 50:50 blend of PGA and PLLA fibers (Concordia Fibers, Coventry, RI) was utilized as the scaffold for all experiments. The PGA/PLLA scaffolds had an initial fabric thickness of 1.53 mm and density of 59.8 mg/ml. Scaffolds were cut into 5 mm diameter disks with a biopsy punch (Acuderm, Inc., Fort Lauderdale, FL) and cold gas sterilized with ethylene oxide prior to seeding. One scaffold was placed into each of 12 seeding tubes. Each seeding tube consisted of a 50 cc centrifuge tube (Product # 91050; TPP, Trasadingen, Switzerland) fitted with a vent filter cap from a 50 cc tissue culture tube (Product # 91253; TPP), thereby allowing for gas exchange during seeding. Seeding solution (12 ml) was added to each tube to yield a seeding density of $\sim 17 \times 10^6$ cells/cm². The tubes were rotated at 8 rpm (Labquake rotisserie rotator, Barnstead-Thermolyne, Dubuque, IA) inside an incubator operating at 37°C and 5% CO₂ for 30 hours, with the medium was changed every 6 h.

Following 30 hours seeding, each MSC-seeded scaffold was moved to an individual well of a 6-well dish (Costar Ultra Low Attachment; Corning, Inc, Corning, NY) and maintained in static culture for 3 or 6 weeks prior to analysis (n = 6 specimens/group/time point). For the control group, basal culture medium used for culturing the MSCs was maintained. For the bFGF/AA2P group, the basal medium was supplemented with 2 ng/mL (Peprotech, Inc., Rocky Hill, NJ) of bFGF, consistent with previously utilized amounts [18] and 82 µg/mL of AA2P (Sigma, St.Louis, MO). Note that the concentration (w/v) of AA2P was chosen to maintain the same molar concentration of a typically used 50 µg/mL concentration for L-ascorbic acid (i.e., 0.28 mM), accounting for the higher molecular weight of AA2P versus L-ascorbic acid (289.54 (anhydrous basis) versus 176.12 g/mole).

Construct fabrication and seeding

PGA/PLLA nonwoven scaffolds were cut into a leaflet shape (Fig. 1-a), with the three leaflets (total surface area = 22.3 cm² surface area, total volume = 3.41 cm³) sewn onto a plastic frame (Fig. 1-b) to form the tissue engineered valve construct (TEVC). Each TEVC was placed in a hybridization tube (Fisher Scientific, Pittsburgh, PA) and subjected to Ethylene Oxide gas sterilization for 16 hours. A seeding solution netting 6×10^6 cells/cm² was introduced to each TEVC, with the seeding solution and tissue culturing medium identical to the culturing media utilized in the bFGF/AA2P supplementation studies. Hybridization tubes were subsequently placed on a rotisserie (Fisher Scientific). The tubes were rotated at 8 rpm inside a standard cell culture incubator operating at 37°C and 5% CO₂. During the first week following scaffold seeding, the spent media was centrifuged to salvage as many cells still unattached to the leaflets and the cell pellet was added to fresh media. Media changes were done daily during this first

week, with the size of the cell pellet negligible after this period. Media changes beyond the first week were performed twice weekly.

Dynamic conditioning

Dynamic conditioning was performed through the use of an organ-level heart valve bioreactor (Fig. 1-c) previously developed in our laboratory [14]. In brief, this device consists of a vented atrium which passively fills a pneumatically driven ventricle with a mechanical inflow valve. This device has the ability to regulate pressure and pulsatile flow conditions. After scaffold cell seeding, the TEVC was mounted onto the threaded stent holder (Fig 1-c) in the bioreactor system. Flow rate in the bioreactor was measured using an ultrasonic flow sensor and flowmeter system (Transonic Systems Inc., Ithaca, NY) and was maintained at an average of 1.15 LPM. Peak and mean fluid pressure across the valve were set to 45 mmHg and 20 mmHg to simulate normal pulmonary artery pressure conditions. A pulse rate (analogous to heart rate) of 60 beats/min was prescribed. Total media volume in the bioreactor was maintained at approximately 2.5 LPM with media changes on average being performed once weekly.

The overall study design for dynamic and static groups is summarized in Table 1. All valves cultured underwent static (i.e., no mechanical conditioning) rotisserie culture for a period of 3 weeks and a total of 6 leaflets were terminated at the 3 week timeframe. Another 12 leaflets were allowed to continue developing for a total culture period of 6 weeks, in which 6 were subjected to static culture conditions and 6 to dynamic culture conditions.

Histological assessment

Histology for bFGF/AA2P supplementation studies was performed as described previously [30–31]. In brief, a thin sample of each MSC-seeded scaffold ($\sim 25 \times 1 \times 1$ mm) was fixed in 10% formalin and embedded in paraffin. Serial sections (6 μ m) from 3-week old samples were stained with hematoxylin and eosin (H&E) for morphology and from 6-week old samples with rabbit polyclonal anti-human primary antibodies against collagen I (1:100; AB292, Abcam, Cambridge, MA), and collagen III (1:200; AB778, Abcam). Immunostaining was performed by the Avidin-Biotin-peroxidase-Complex (ABC) method with biotinylated goat anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA) and using 3-amino-9-ethyl carbazole (Dako Corporation, Carpinteria, CA) as a substrate. Sections were counterstained with Gill's hematoxylin solution (Sigma). For specificity controls, an appropriate nonimmune IgG was substituted for the primary antibody. TEPV leaflets were subjected to histological evaluation for collagen as a precursor to detailed biochemical analysis. Leaflets were fixed in 10% formalin and embedded in paraffin. Serial sections (6 μ m) were prepared and subsequently stained with picro sirius red for qualitative assessment of collagen alignment using polarized light microscopy.

DNA and Biochemical assays

Specimens from the basal culture and bFGF/AA2P treated medium groups were removed following 3 (n=6/group) and 6 (n=6/group) weeks and subjected to DNA and collagen biochemical assays. In addition, 5 leaflets in each of the 3 organ-level TEPV categories (3-week static, 6-week static and dynamic) were cut into smaller parts (between 5 to 10 pieces/leaflet) and weighed. DNA, sulfated glycosaminoglycans (S-GAG) and collagen were quantified similar to methods described previously [5]. For DNA content, each specimen was digested with a buffered 0.125 mg/ml papain solution for 10 h in a 60 °C water bath. The extracts were then assayed using the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR) per the manufacturer's instructions and using the blue channel of a TBS-380 Mini-Fluorometer (Turner Biosystems, Sunnyvale, CA). A portion of extract from the papain digest was reserved for the S-GAG assay which was performed according to the guidelines provided

with the Blyscan™ assay kit ((Biocolor Ltd., Newtownabbey, N. Ireland) using a Genesys 20 spectrophotometer (Thermo Spectronic, Rochester, NY) set to an absorbance of 656 nm.

For collagen, samples were digested with a solution of 0.5M acetic acid (Sigma) and pepsin (1 mg/ml Pepsin A (P- 7000); Sigma). Digestions were carried out overnight (~16 h) on a rocker (Orbitron Rotator™; Boekel Scientific, Feasterville, PA) at 4°C. Collagen extracts were then assayed according to the guidelines provided with the Sircol™ assay kit (Biocolor Ltd.) using the same spectrophotometer used in the S-GAG assay (Thermo Spectronic) set to an absorbance of 540 nm.

Mechanical evaluation

While our optical system was capable of imaging the TEVC leaflets directly, the use of the phenol red in the media (necessary for verifying any pH changes) limited our ability to visualize the leaflet surfaces directly. While use of non-dye media was considered, the novelty of the present study made it necessary to use the dye in all experiments to verify cell viability and if bacterial contamination occurred. However, we have shown [26] that a polyacrylamide gel can simulate effective tissue properties (under the assumption of tissue mechanical isotropy) in PGA/PLLA scaffold-gel composite scaffolds.

Hence, in the present study an equivalent scaffold-only TEVC control was prepared for imaging, and an agarose gel (AG) applied onto the leaflet scaffolds to simulate the presence of tissue for mechanical characterization and subsequent visualization. Specifically, the AG was prepared by dissolving agarose powder (Type VII, low gelling temperature, Sigma) in phosphate buffered saline (Invitrogen) at the desired concentration to produce a 6% weight per unit volume mixture. The mixture was then heated to 85°C to allow complete dissolution and poured onto a tri-leaflet TEPV construct. The construct was then allowed to cool at 4°C for 15 min. The 6% AG used provided an equilibrium aggregate modulus of 138 kPa [32], and the construct has an effective stiffness (E) of ~1000 kPa at this gel modulus. Gel and scaffold-only TEPVs were mounted into the organ-level bioreactor and subjected to a peak static pressure of 20 mmHg to simulate full diastolic loading.

To ensure that our TEVC leaflet analogs mechanically behaved in a manner similar to engineered heart valve tissue [5], stiffness properties of the former were directly measured. AG-leaflets (with 6% agarose gels) and scaffold only specimens were mechanically tested in the preferred direction of fibers within the scaffold. Similar to previous work [27], a device capable of imposing three-point bending on tissue was employed to characterize the flexural stiffness of the scaffold-only and scaffold-gel composites. Both negative and positive curvature directions (n = 6 measurements, 3 in each direction) was imposed on samples during testing.

Leaflet surface reconstruction

A design goal of the TEVC used in this study was to approximate a semi-cylindrical geometry to maintain geometric similarities to the cylindrical shape of fully bent, rectangular CFF bioreactor specimens. While not intended to be an exact match, we sought to maintain an overall geometric similarity. To verify this leaflet geometry, 3D reconstructions of the leaflet surface were carried using previous methods [33] to determine the shape of the AG-leaflets. In brief, a 14 mW diode laser module with an interchangeable optical pattern generator head (Lasiris Inc., Canada) was used to project a matrix of 19×19 dots on to the leaflet surface. A custom-built index-matching chamber housing the TEPV stent was filled with 60:40 water:glycerin solution for refractive index matching purposes. The loop of the organ-level bioreactor was also filled (~2.5 L) with the same water-glycerin solution.

Image acquisition was performed through the use of two cameras (Basler, A504k, Sydney, Australia), with image capture and subsequent acquisition for each camera occurred simultaneously for the two views and was initiated and controlled using commercially available image capture software (Advanced Digital Vision Inc, Natick, MA). Captured images were analyzed using SigmaScan image processing software (Systat Software Inc., Chicago, IL). The images were first processed to enhance contrast; then the location of each laser dot in each image was recorded as the centroid of the laser dot. Corresponding laser dot locations in each view were identified, and a calibrated standard discrete linear transform (DLT) [33] was used to convert these pixel locations into three-dimensional (3-D) spatial coordinates. Leaflet, 3-D surface meshes (using triangular elements) were created from the 3-D points using Gambit (Fluent, Lebanon, NH) meshing software.

Computational Fluid Dynamic (CFD) analysis

To gain a more detailed information of the surface flow and shear stress patterns acting on the CFF specimens under increasing flexure states than that performed in [25], we conducted the following quasi-static analysis (Fig 2-a). Details on the discretization of the sample geometry at each new position have been presented previously [34]. In brief, we assumed that the flexure of rectangular scaffold strips in the CFF bioreactor followed a parabolic profile. The bent configuration of the samples was determined numerically based on the rate of specimen displacement. Additionally we assumed that the sample along its length (x-direction) conformed to the equation of an arc length with the constraint of a fixed length (L) at any time point of 25 mm, given by:

$$\int_a^b \sqrt{1+(2Cx)^2} dx = L \quad (1)$$

where a is the position of the stationary post attachment to a CFF bioreactor specimen, b is the new position of the moving post attachment to the specimen, as it moves towards the stationary post (determined from the prescribed actuator motion) and C is the constant that changes according the new position b . The new configuration of the sample due to bending was hence computed (Mathcad v.14 PTC, Needham, MA).

A laminar flow, quasi-static CFD simulation was performed using commercially available software (Fluent, Ansys Inc, Lebanon, NH). 11 different time points in a cycle of 0.5 Hz were simulated, wherein CFF bioreactor specimens initiated from a fully straight configuration to increasing levels of flexure. A structured grid consisting of 1.43 million hexahedral elements (1.54 million nodes) was constructed (Ansys). The density and viscosity material properties were set to 1010 kg/m³ and 0.00076 kg/m-s consistent with previously utilized values [25] for cell culture media. Convergence criteria for continuity and 3D equations of motion were set to residual values of $\leq 10^{-9}$. A plug flow inlet velocity boundary condition of 5.5 cm/s (equivalent to an operational speed of 2000 RPM for the flow source component of the device) was prescribed and allowed to develop through the addition of an extended entrance length connected to the main configuration of the CFF device (Fig 2a). Post-processing (Tecplot 360, Tecplot Inc, Bellevue, WA) was performed in order to visualize the shear stresses contours and friction streamlines (shear stresses tangent to the shear stress vector field) on the sample walls.

Data Analysis and Statistics

The CFF bioreactor provided a platform in which the effects of different combination of TEPV variables such as coupled/decoupled CFF stress states, scaffold material(s) and cell sources can be optimized in a straightforward manner. However, a precursor to using the device for this purpose was to first establish if tissue formation at the organ-level was reproducible in

rectangular, CFF bioreactor specimens. Thus, linearized rates of collagen production were utilized to compare the effects of bFGF/AA2P inclusion, and organ versus CFF level bioreactor conditioning. In addition, the amount of collagen produced at 6 weeks by combined flow and cyclic flexure in the CFF bioreactor was predicted based on its linear rate over a 3 week period. DNA content in CFF studies was assumed to be the same as the amount determined after 3 weeks of static culture in bFGF/AA2P supplemented media. Justification for this was based on the findings of DNA levels found (see results section) in dynamic and static groups in the organ level studies. Assay results are presented as mean $\pm$ SEM. Group comparisons were performed using the Student t-test, with statistical significance taken as $p < 0.05$.

RESULTS

Leaflet shape and scaffold mechanical behavior

The surface reconstruction of a representative leaflet at end diastole (maximum closure phase) demonstrated that the leaflet conformed to a circular cross-section hence, which is the nature of bending that occurs during flexure of straight, rectangular-shaped, CFF-bioreactor specimens (Fig. 2-b). We were thus able to approximately approximate our CFF specimen geometry [19] in the TEVC. In the preferred direction of fibers, a flexural modulus of 161 ± 8 kPa (mean $\pm$ SEM) was determined for the scaffold-only, consistent with previous findings [5]. A much higher flexural modulus of 940 ± 56 kPa was found for the AG-gel scaffold. This amount were comparable to previously predicted effective stiffness values (e.g., $E \cong 1000$ kPa) [26] for TEPV analogs using a polyacrylamide gel suggesting functional equivalence between the two materials. In addition, as a measure of suitability to serve as TEPV analogs, the AG-gel leaflets had a comparable flexural modulus to previously reported values ($E = 978 \pm 228$ kPa) [5] for engineered heart valve tissue derived from ovine smooth muscle cells subjected to cyclic flexure conditioning for 3 weeks.

Leaflet histology

After 3 weeks of culture, H&E staining demonstrated enhanced tissue formation in the bFGF/AA2P group versus the basal media cultured tissue (Fig. 2a) which served as a control group. Specifically, tissue formation appeared to be sparse in the basal media group (Fig. 3a), with cells and extracellular matrix (ECM) localized primarily near the scaffold perimeter. In contrast, abundant cells and ECM were observed throughout the scaffolds, including the central region, in the bFGF/AA2P group (Fig. 3b). In addition, immunostaining for collagen I appeared to be enhanced in the bFGF/AA2P group (Fig. 3c) over the controls (Fig. 3d). Collagen III staining demonstrated a similar pattern of expression (data not shown). Collagen alignment (Fig. 3e) was observed through histological staining of TEPV sections taken from the 6-week static group. The abundant presence of collagen in the microstructure found after 6 weeks of incubation was consistent with previous studies performed in our laboratory that showed evidence of sparse collagen aggregates after being cultured for half (3 weeks) the culture time [5].

Biochemical and DNA assays

Initial DNA content in the basal media and bFGF/AA2P supplemented groups was determined to be ~ 500 μg DNA/g wet weight. Subsequently, dramatic DNA loss was observed (at 3 weeks: 375 ± 29 μg DNA/g wet weight for basal culture media group and 369 ± 28 μg DNA/g wet weight bFGF/AA2P group; at 6 weeks: 287 ± 17 μg DNA/g wet weight for basal culture media group and 274 ± 47 μg DNA/g wet weight for bFGF/AA2P group). A similar degree of cellularity between the groups for both culture durations ($P > 0.05$, for both 3 and 6 weeks) was found. The progressive loss of cellularity following MSC seeding is similar to previous observations [19,35] that suggested a possible inefficiency in attachment of the MSCs to serum proteins adsorbed to the scaffold PGA and PLLA fibers. Consistent with H&E staining,

biochemical assays demonstrated AA2P/bFGF stimulated significantly higher collagen concentrations versus basal culture medium ($p < 0.05$, +180% at 3 weeks and $p < 0.05$, +77% at 6 weeks). As shown in Fig. 4a, the net collagen production/DNA was also found to be significant between the 2 groups ($p < 0.05$, +185% at 3 weeks and $p < 0.05$, +85% at 6 weeks).

Biochemical assays of the TEVC leaflets revealed nearly a 4-fold increase in collagen production (Fig. 4b) when subjected to additional 3-weeks of dynamic conditioning under physiologically-relevant pulmonary artery pressure conditions (20/45 mmHg mean/peak pressure). Comparisons of the dynamic conditioning group with collagen levels found in both the 3 and 6 week static groups (Fig. 4b) were found to be statistically significant ($P < 0.05$). No significance ($p > 0.05$) in net collagen production was observed between the 3 and 6 week static groups.

Decreased GAG production (Fig. 4c) was observed over time ($p > 0.05$, dynamic versus 6-week static; $P < 0.05$, 3-week static versus 6-week static or dynamic groups). This decrease in the formation of GAGs was consistent with those observed when flexure and flow conditioning was coupled on CFF bioreactor samples [19]. While a temporal decrease in the net DNA of the TEVC leaflets occurred ($p < 0.05$, 3-week static versus 6-week static groups), dynamically conditioned constructs were able to maintain their level of DNA (Fig. 4d) to their initial levels determined at the end of first three weeks of static culture ($p > 0.05$, 3-weeks static versus dynamic groups).

Collagen production on TEVC leaflets versus CFF-bioreactor specimens

The inclusion of bFGF/AA2P was found to have a ~3-fold increase in collagen production/ MSC over basal media alone at 3 weeks of culture (Fig. 5). From the 3 to 6 week period, dynamic mechanical conditioning effects coupled with bFGF/AA2P supplementation on TEVCs resulted in a 33% increase in collagen production/ MSC. Our previous studies with the CFF-bioreactor [19] did not include the use of bFGF/AA2P. At 3 weeks, the effect of dynamic conditioning alone in that study resulted in 23% less collagen produced/ MSC versus culturing with bFGF/AA2P media supplementation alone.

To allow comparisons of tissue formation at 6 weeks with the CFF-specimens, we first noted that in our bFGF/AA2P studies that no significant differences ($p > 0.05$) of DNA levels were found at 6 weeks between basal media and bFGF/AA2P supplemented groups. In addition, the DNA level was maintained beyond the initial 3 week period when dynamic organ-level conditioning was used suggestive of DNA preservation by conditioning. Hence for comparison with CFF bioreactor conditioned specimens, we assumed no change in DNA content beyond the 3 week time point and linearly extrapolated the amount of MSC collagen production to 6-weeks.

We determined an approximate 12% increase in MSC collagen production was found at 6 weeks in the CFF-specimens over use of bFGF/AA2P alone, while dynamic conditioning at the organ-level produced an ~18% increase over dynamically conditioned CFF specimens (Fig. 5). bFGF and AA2P supplemented media accelerated collagen formation during at 3 weeks (~185% increase in collagen mass/ MSC compared to non-supplemented media), as well as increasing collagen mass production from 3.90 to 4.43 pg/cell/week from 3 to 6 weeks (Fig. 6). Using supplemented media, TEVC physiologic conditioning increased collagen mass production rate from 7.23 to 13.65 pg/cell/week (88.8%) during the dynamic culture period, along with greater preservation of net DNA (Fig. 6). Moreover, when compared to our previous CFF study under combined cyclic flexure and sub-physiological flow, physiologic conditioning increased collagen production rate from 4.76 to 6.42 pg/cell/week (35%).

CFD analysis

CFD analysis was able to converge to solutions at the specified criterion (residual values $\leq 10^{-9}$). The CFD analysis were performed on fully bent CFF bioreactor specimens indicated that there was a strong oscillatory component associated with the flow field, particularly on the inner wall of the sample (Fig. 7). The magnitude of shear stresses was higher on the outer wall of the specimens as compared to the inner wall. However, owing to the low inlet velocity prescribed for the CFD model (prescribed based on the experimental conditions of flow source velocity = 2000 RPM reported previously [19]), the shear stresses were in the sub-physiologic range [36], consistent with the findings of a previous CFD study on the CFF bioreactor [25].

As a means to quantify the magnitude of oscillatory shear that occurs, the following oscillatory shear index, as proposed by He and Ku [37], was used

$$OSI = \frac{1}{2} \left(1 - \frac{|\int_0^T \tau_w dt|}{|\int_0^T |\tau_w| dt|} \right) \quad (2)$$

where τ_w is the wall shear stress, T is the period, and t is time. The OSI has a maximum value of 0.5 that represents pure oscillatory flow. As described in Fig. 8, spatial variations in OSI were quantified between two landmark vortices labeled A and B along the inner wall, over 11 instantaneous time points in a 0.5 Hz cycle from fully straight to increasingly bent CFF bioreactor specimen configurations. As shown, a large portion of the specimen inner wall experiences a high level of oscillatory shear.

Previous work by Sotiropoulos and Ge [13] has described the complex surface flow patterns that native tri-leaflet valves. As observed in the CFF specimens, flow reversal also occurs in the native leaflet (Fig. 9), with oscillatory fluid-induced stress effects residing on the aortic side. Other dominant features of the flow field are the relative lower shear stresses on the aortic side of the valve compared to the ventricular side. The overall flow patterns and magnitude of fluid-induced surface shear stresses were similar between the inner wall of the CFF-bioreactor specimens and the aortic side of the leaflet. However, the commissure and basal attachment regions of the ventricular side of a native aortic valve experiences ~62 to 82% higher shear stresses over the peak fluid-induced stresses that were experienced by the CFF specimens conditioned at sub-physiological flow conditions.

DISCUSSION

There is general agreement that mechanical conditioning provides the necessary stimuli to promote proper tissue formation in engineered tissues. We have shown that mechanical stimulation augmented both collagen intrinsic stiffness and mass [3]. This result demonstrated that basic approaches to promote tissue formation by physical stimulation have effects beyond just the up-regulation of tissue component mass production. The mechanical quality, regulated by how the collagen is laid down (form and content), is also modified. Moreover, these results cannot be obtained by 2D tissue systems; full 3D approaches like that used herein are clearly required to determine how cellular stimulation translates into specific ECM formation.

Critical to the application to any TEHV approach is an understanding of the time evolution of the ECM stiffness and how it is modulated by physical conditioning. Although such approaches have been established for a range of tissues (e.g. [38–44]), particular challenges are encountered for heart valves, which exist in a highly dynamic, fluidic environment that induces very large tissue stresses. Moreover, these functional demands must be met at the time of implantation

– no stress shielding or other protective interventions are possible. For engineered heart valve tissues, the modeling of such approaches is still in its infancy.

To achieve this task from a bio-mimetic standpoint, appropriate organ-level studies are required wherein the valves are compelled to cyclically open and close, and in doing so are subjected to the complex coupled flow, flexure and stretch stress states that native valves experience. Related studies [18–19] concerning the potential of MSCs in heart valve tissue engineering, in this study, we seeded MSCs on tri-leaflet valve-shaped PGA/PLLA scaffolds. Subsequently, we investigated the effects of dynamic organ-level valve conditioning at physiological, pulmonary artery pressures on bulk engineered tissue formation, namely the collagen and GAG production. In addition, we conducted these studies by simultaneously attempting to improve tissue culture media conditions by supplementing it with bFGF/AA2P.

Collagen and GAGs have unique function in heart valves [2], and inclusion of these two components will be essential for the development of any engineered heart valve tissue, even if the specific distribution and architecture differs from native valves [3]. We were able to demonstrate, prior to any mechanical conditioning, that media supplementation with bFGF/AA2P augments collagen production substantially at the initial (first 3 weeks) culture times. Further, it provides at a minimum a threshold amount of collagen that may likely contribute towards adequate stiffness characteristics of the construct.

The present TEVC studies demonstrated more robust collagen formation compared to our previous CFF studies (~35% increased rate of collagen production/cell [25]). The DNA content of the TEPV constructs were maintained beyond the initial 3-week static culture period when dynamic conditioning was performed for a subsequent 3 weeks. These assay results demonstrate that either, a larger number of viable MSCs are retained and/or the ability of the cells to secrete more collagen increases with physiological pressure-based conditioning.

Consistent with previous studies performed in our laboratories [19], there was a reduction in GAG concentration (i.e. in proportion with the total tissue mass) with an increase in culture time. Flanagan et al. [45] have shown that incorporation of a GAG component to the scaffold environment results in enhanced extracellular matrix production. Another study [46] showed that GAG production approached near native valve levels in tissue engineered heart valves under hypoxic and insulin-rich environments. Further studies will be required to better understand the role of GAGs in TEPV development.

The role of oscillatory flow patterns on MSC ECM production

Fully unidirectional forward flow when CFF specimens were straight reduced with increasing specimen flexure (Fig. 7). At greater flexure states large regions of flow reversal were found and, in particular, complete flow reversal on the inner walls of the samples was observed. The large extent to which the flow is reversed during CFF specimen flexure points towards a high oscillatory component that is associated with the fluid-induced shear stresses that act on the surface of the specimens. We were able to confirm this by quantifying the purely oscillatory nature of the surface fluid flow ($OSI = 0.5$) in regions of the inner wall (Fig. 8).

In order for scaffold deformations to induce significant tissue formation, sufficient cellular strains must occur for cellular transduction events to occur. However, that the nonwoven PGA-PLLA scaffolds used in this study undergo relatively modest strains under tension (unpublished observations) and thus experience small strains when TEVC is in the fully closed, loaded position. Further, in flexure they undergo an estimated at most $\pm 7\%$ tensile/compressive strain [47].

Taken as a whole, it is unlikely that scaffold straining alone can be sole underlying contributor to augmented tissue formation found to occur under combined flexure and flow conditions. This observation is further supported by the modest levels of tissue formation detected in the flexure-only experiments, which were also comparable in magnitude to the steady flow alone group [19]. More importantly, in both the previous and current studies, only with time-varying movement (flex-flow or organ level conditioning) were the rates of collagen formation substantially increased over the flex and flow (wherein the flow fields were relatively static) conditions alone. Our current CFD results suggest that the only remaining extrinsic physical factor was the presence of time-varying surface shear stresses that occur with time-varying movement (flex-flow or organ level conditioning). We thus hypothesize that *oscillatory surface shear stresses* induced by the time-varying flow patterns induced by the deforming scaffolds or leaflets play a major role in stimulating engineered tissue formation in MSC-seeded scaffolds.

Support for this hypothesis exists in the bone tissue engineering literature, where oscillatory fluid flow preserves healthy osteogenic tissue, upregulates transcription factors involved in multiple differentiation pathways of MSCs, and has been found to increase MSC proliferation [48–50]. While the specific molecular mechanisms involved in each of these pathways are still largely unknown, it has been recently shown that non-canonical Wnt and N-Cadherin related β -Catenin signaling are involved in the osteogenic pathway of MSCs [48]. In addition, Arnsdorf *et al* [49] demonstrated that oscillatory flow regulates multiple genes (*runx2*, *sox9* and *PPAR γ*) in MSCs that can commit the cells to osteogenic, chondrogenic and adipogenic lineages. MSC signaling was induced by the direct effect of oscillatory fluid flow exerting a greater tensile load on the actin cytoskeleton. On the other hand, pure chemical inducement of actin tension alone was not sufficient to upregulate gene expression.

These findings suggest that unique, external mechanical conditioning regimes that have fluid-induced oscillatory shear stresses may play a critical role in engineered tissue development that utilize MSCs. Practically, such simulation regimes could be exploited to assist in engineered heart valve tissue formation using optimized bioreactor protocols. The extent to which these regimes impact MSC trans-differentiation and phenotype, as well as the underlying cell-cell signaling occurring within the engineered tissue, remains to be elucidated.

Limitations

The PGA-PLLA scaffolds utilized here were chosen on the basis of their well characterized mechanical behavior [26] and extensive use in ours and other laboratories [3]. However, PGA/PLLA scaffolds are not elastomeric, do not permit large deformations, and are not able to produce a TEVC that duplicated native valve motion. However, it is unlikely that these limitations qualitatively affected our results, but rather our findings represented a lower bound tissue formation likely to be found under fully simulated valve dynamics.

CONCLUSIONS

Overall, we found that when MSC-seeded TEVC are subjected to dynamic simulated organ level conditions they exhibited increased collagen mass production levels and rates compared to our earlier CFF study. Moreover, given the fact that the seeded scaffolds underwent at most modest strains during either CFF or physiological conditioning, the oscillatory surface shear stresses estimated in both studies suggest that they may play a significant role in eliciting MSC collagen production in the highly dynamic engineered heart valve fluid mechanical environment. This speculation is supported by quasi-static CFD simulations that indicated specific flow features, such as flow reversal previously reported in native valves, are replicated in a fully bent CFF bioreactor specimen. With respect to of native leaflets, flow vortices on the aortic side increase speed and efficiency of valve closure by impinging on the leaflet in the

direction that favors coaptation, thereby assisting valve dynamics. From a tissue engineering viewpoint, oscillatory shear stresses may be exploited to enhance collagen production within the complex heart valve fluidic environment during the in-vitro tissue formation phase.

Acknowledgments

Funding for this work was provided by NIH R01s HL-68816 and HL-089750, and American Heart Association Scientist Development Grants 0830061N (Ramaswamy) and 0635620T (Sales). The authors would like to thank Mr. Steven M. Boronyak for his assistance with the custom-built index-matching chamber and performing the digitization of leaflet images. Finally, the authors also thank Mr. Chad Eckert for conducting the three-point bending tests.

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Figure 1. Details of the TEVC showing (a) leaflet dimensions, (b) final TEVC. Also shown in (c) is the organ-level bioreactor [14] used for dynamic conditioning.

Figure 2.

(a) CFF bioreactor geometry taken from [25] showing specimen locations. Note that an entrance length was added to the inlet of the CFD model and 4 specimens were simulated. (b) Representative TEVC leaflet shape under diastolic loading showing the cylindrical shape.

Figure 3.

Representative H&E sections of MSC-seeded scaffolds incubated for 3 weeks in: (a) basal culture medium and (b) basal culture medium supplemented with bFGF/AA2P (Scale bar = 500 μ m). Representative collagen I immunostained sections of MSC-seeded scaffold incubated for 6 weeks in: (c) basal culture medium and (d) basal culture medium supplemented with AA2P and bFGF (Scale bar = 100 μ m). e) Evidence of collagen alignment (by polarized light microscopy) after 6 weeks of static tissue culture.

Figure 4.

(a) Effects of bFGF/AA2P supplements to the media on net soluble collagen/DNA ($\mu\text{g collagen}/\mu\text{g DNA}$) after 3 and 6 weeks of static tissue culture. Also shown are the normalized concentrations of (b) Collagen, (c) GAG and (d) DNA content in TEVC leaflets grown for 3 and 6 weeks in static culture only, along with results for the 3 week static followed by 3 week dynamic tissue culture environment group (dynamic).

Figure 5.

A compilation of the temporal evolution of collagen mass production for all groups investigated at the tissue and organ levels. Note that we projected collagen mass formation for the dynamically conditioned (combined modes of cyclic flexure and sub-physiologic shear stress levels (average ~ 1.1 to 1.8 dynes/cm² [25]) specimens [19] for comparison.

Figure 6.

Linearized rates of collagen mass formation in TEVC at physiologically representative pulmonary artery pressure scales *and* in comparison to specimens conditioned (combined modes of cyclic flexure and sub-physiologic shear stress levels (average ~ 1.1 to 1.8 dynes/cm² [25]) in a CFF bioreactor [19]. Data computed from the information shown in Fig. 5.

Figure 7.

CFD analysis performed on CFF bioreactor geometry previously developed by Engelmayer et al, [25] showing the number, location and bent configuration of specimens that were utilized for the CFD model in this study. As illustrated by the friction streamlines on the color maps, flow reversal occurs on bent rectangular specimens. Note the increasing presence of this phenomenon on the inner wall of the CFF specimens as the level of bending in the specimens is increased.

Figure 8. Oscillatory shear index (OSI) plot (as defined by He and Ku [37]) from flow vortex “A” and “B” as shown. Note a large portion of the line connecting the 2 vertices undergoes pure oscillatory flow, i.e., OSI = 0.5.

Figure 9. Wall shear stress magnitudes and friction streamlines previously observed in a native aortic valve [13]. Note the flow vortices on the aortic side of the valve and the ability to reproduce this phenomenon at the CFF bioreactor scale.

Table 1

Summary of endpoints in organ-level TEPV experiments.

GROUP #	Endpoint	Total leaflets (N) (One-side surface area of a leaflet ~ 7.4 cm ²)	BMSC Seeding density	Leaflets utilized for collagen/GAG/dna assays	Leaflets utilized for Histology
GROUP 1	3 week static, rotisserie culture	6 leaflets	6 × 10 ⁶ million/cm ²	5	1
GROUP 2	6-week static, rotisserie culture	6 leaflet	6 × 10 ⁶ million/cm ²	5	1
GROUP 3	3-week static followed by 3-week dynamic pulmonary artery pressure conditioning (Peak pressure = 45 mmHg Mean pressure = 20 mmHg)	6 leaflets	6 × 10 ⁶ million/cm ²	5	1