

## DYNAMIC FLEXURE INDEPENDENTLY STIMULATES ENGINEERED HEART VALVE TISSUE DEVELOPMENT

George C. Engelmayr, Jr. (1), Elena Rabkin (2), Fraser W. H. Sutherland (3)  
Frederick J. Schoen (2), John E. Mayer, Jr. (3), and Michael S. Sacks (1)

(1) Engineered Tissue Mechanics Laboratory  
McGowan Institute for Regenerative Medicine  
University of Pittsburgh  
Pittsburgh, PA

(2) Department of Pathology  
Brigham and Women's Hospital  
Harvard Medical School  
Boston, MA

(3) Department of Cardiovascular Surgery  
Children's Hospital  
Harvard Medical School  
Boston, MA

**Abstract**—The independent effect of dynamic flexure on the development of tissue engineered heart valves (TEHV) was investigated. Engineered heart valve tissues were constructed by seeding ovine smooth muscle cells onto rectangular scaffold strips and incubating the resultant constructs in a dynamic flexure bioreactor or under static conditions for three weeks. Tests indicated a trend of higher effective stiffness, increased collagen accumulation ( $p < 0.05$ ), improved mid-thickness cellularity, and increased expression of vimentin in flexed vs. static samples, with comparable levels of glycosaminoglycan accumulation and alpha-actin expression. These results suggest that dynamic flexural stimulation can independently reproduce certain aspects of TEHV development observed in pulse duplicators.

### INTRODUCTION

Pulse duplicator bioreactors have been successfully implemented to stimulate the development of tissue engineered heart valves (TEHV) [2]. Due to their coupled mechanical stimuli, however, they are not directly amenable to biomechanical studies which aim to isolate the independent effect of simple modes of mechanical stimulation. Dynamic flexure represents a simple mode of deformation experienced by TEHV during *in vitro* development in pulse duplicators, as well as *in vivo* following implantation. In order to investigate the isolated effect of dynamic flexure on the development of TEHV, we designed a bioreactor with the capacity to provide cyclic three-point bending to 12 rectangular samples of TEHV biomaterial [1]. Our previous study demonstrated that dynamic flexure leads to both quantitative and qualitative changes in the flexural mechanical properties of non-woven mesh scaffolds coated with poly-4-hydroxybutyrate (Tepha, Inc., Cambridge, MA). In the present study, we aimed to isolate the effect of dynamic flexure on the mechanical property changes due to tissue deposition from changes due to scaffold degradation. To this end, we used a scaffold that does not demonstrate a significant change in flexural mechanical properties over a three-week period.

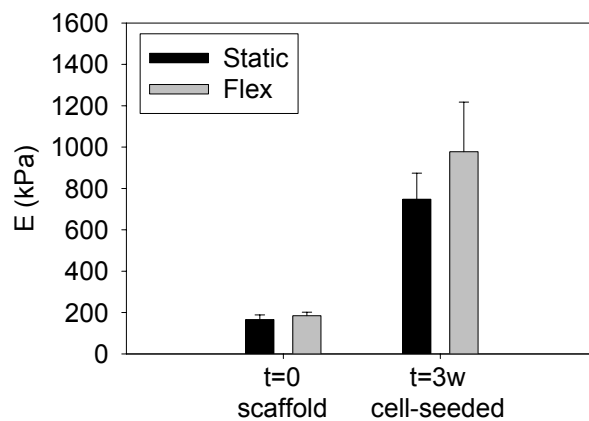


Figure 1 - Dynamically flexed samples demonstrate a trend of increased effective stiffness compared to static controls.

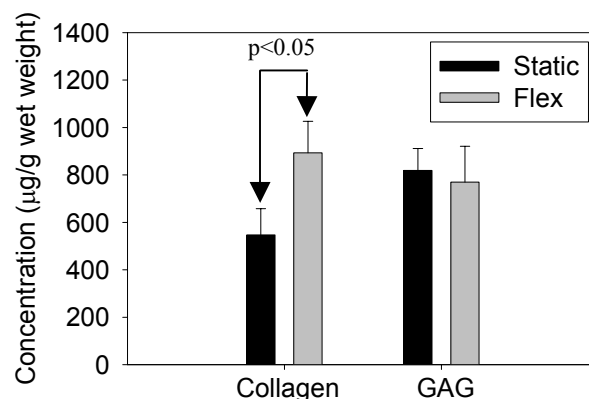


Figure 2 - Dynamically flexed samples accumulated significantly more collagen than static controls.

## MATERIALS AND METHODS

### Bioreactor

The bioreactor used in this study has been described previously [1]. In brief, the bioreactor relies on a linear actuator to provide cyclic three-point bending to 12 rectangular samples situated in individual, sterile culture wells. The entire device operates inside a humidified incubator operating at 37 °C and 5% CO<sub>2</sub>.

### Scaffolds

Scaffolds were a non-woven, 50:50 blend, mesh of polyglycolic acid (PGA) and poly-L-lactic acid fibers (PLLA) (Albany International Research, Mansfield, MA). Twenty-four 2.5 x 0.75 x 0.1 cm scaffolds were subjected to initial bending testing and cold gas sterilized in ethylene oxide prior to use.

### Cell Culture and Seeding

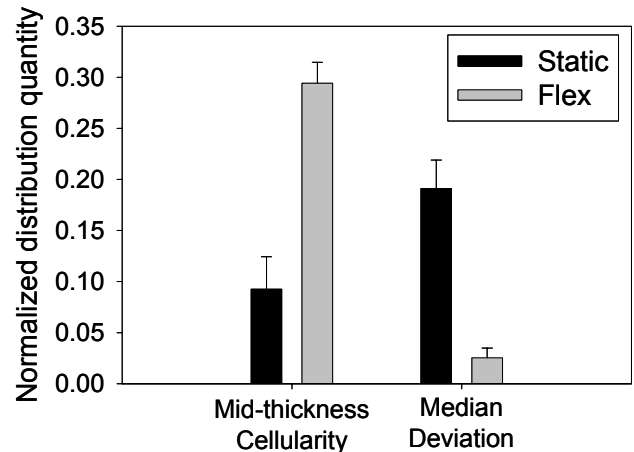
Ovine smooth muscle cells were isolated as previously described [2], expanded in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic (Gibco), and 2 ng/ml basic fibroblast growth factor (bFGF; BD Biosciences, Bedford, MA). A total of 54 confluent 185 cm<sup>2</sup> flasks (passage 13) (Nuclon™Δ SoLo flask, Nalge-Nunc, Rochester, NY) were trypsinized (Gibco), and the cells were resuspended to yield a seeding solution of 8x10<sup>6</sup> cells/ml. Three scaffolds were placed into each of four 50 cc centrifuge tubes fitted with a vent filter cap (TPP, Trasadingen, Switzerland). 12 ml of seeding solution was added to each tube to yield a seeding density of approximately 17x10<sup>6</sup> cells/cm<sup>2</sup>. The tubes were rotated at 8 rpm (Labquake rotisserie rotator, Barnstead-Thermolyne, Dubuque, IA) inside an incubator operating at 37°C and 5% CO<sub>2</sub> for 30 hours, during which time the culture medium was changed every 6 hours. Following seeding, each scaffold was cultured under static conditions for 4 days in 6 ml of medium in 6-well plates (Costar Ultra Low Attachment, Corning, Corning, NY). Following this period, six constructs (and un-seeded controls) were moved into the bioreactor and subjected to unidirectional flexure at 1 Hz and a central displacement of 0.25 inches for 3 weeks. Medium was changed once daily.

### Mechanical Testing, Tissue Composition, and Histology

The methods used in our lab to measure effective stiffness (E) have been described previously [1, 3]. E was measured in both the “with-flexure” (WF) and opposing “against-flexure” (AF) directions. Collagen, elastin, and glycosaminoglycan (GAG) isolation was adapted from Brown et al [4], and quantified using commercially available kits according to the manufacturer's guidelines (Biocolor Ltd., Newtownabbey, N. Ireland). Histology was carried out as previously described [5].

### Transmural Cell Distribution Analysis

Panoramic images of transmural cross-sections were generated by concatenating 12-17 400x digitized photomicrographs of Movat-stained histological sections using Image Expert 2000 (Jasc Software, Inc., Eden Prairie, MN). RGB values corresponding with cell nuclei were determined using SigmaScan Pro (SPSS, Inc., Chicago, IL), and the images were analyzed using a custom program written in Matlab (The MathWorks, Natick, MA) to yield the coordinates of the cell nuclei normalized to the transmural thickness. The program yielded the median of the cell distribution, as well as the fraction of cells located in the middle-thickness of the cross-section (0.35-0.65 of the normalized thickness), hereafter referred to as the “mid-thickness cellularity”. One transmural cross-section from the center of one 6 μm histological section was analyzed for each of the 12 samples.



**Figure 3 – Mid-thickness cellularity and deviation of the cell distribution median from 0.5 were significantly improved ( $p < 0.01$ ) in the flexed group, suggested that dynamic flexure promotes a more homogeneous cell distribution.**

## RESULTS AND DISCUSSION

Effective stiffness (E) results are summarized in Figure 1. Three weeks incubation of the cell-seeded constructs resulted in a significant increase (~ 4 fold) in E over the baseline E of the scaffolds. Moreover, dynamically flexed samples exhibited a trend of increased E compared to static controls at this early time point. In contrast, un-seeded scaffolds exhibited no significant changes in E under dynamic flexure or static incubation conditions (data not shown), indicating that any changes in mechanical properties of the cell seeded constructs is due exclusively to tissue accumulation. Collagen and glycosaminoglycan (GAG) assay results are summarized in Figure 2. Dynamically flexed samples demonstrated significantly more collagen accumulation ( $p < 0.05$ ), and a profound increase in vimentin expression (data not shown), compared to static controls, with comparable levels of GAG and alpha-actin expression. Photomicrographs of Movat-stained histological sections suggested that dynamic flexure induces increased mid-thickness cellularity compared to static controls. This finding prompted us to quantify the transmural cell distribution. The results of the analysis are summarized in Figure 3. Quantitative analysis of the histological sections suggested that dynamic flexure can induce a more homogenous transmural cell distribution than static incubation, with a significantly higher proportion of cells located in the middle thickness of the construct. It is unclear if this effect is directly related to mechanical stimulation, or an indirect effect associated with improved mass transport. Overall, the morphology, composition, and cell phenotype of the dynamically flexed samples was comparable to 14 day pulse duplicator TEHV [5], suggesting that dynamic flexure can independently yield certain aspects of development observed in the complex mechanical environment of pulse duplicators.

## ACKNOWLEDGEMENTS

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