Mechanical Stretch Regimen Enhances the Formation of Bioengineered Autologous Cardiac Muscle Grafts

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Background—Surgical repair of congenital and acquired cardiac defects may be enhanced by the use of autologous bioengineered muscle grafts. These tissue-engineered constructs are not optimal in their formation and function. We hypothesized that a mechanical stretch regimen applied to human heart cells that were seeded on a three-dimensional gelatin scaffold (Gelfoam) would improve tissue formation and enhance graft strength.

Methods and Results—Heart cells from children undergoing repair of Tetralogy of Fallot were isolated and cultured. Heart cells were seeded on gelatin-matrix scaffolds (Gelfoam) and subjected to cyclical mechanical stress (n=7) using the Bio-Stretch Apparatus (80 cycles/minute for 14 days). Control scaffolds (n=7) were maintained under identical conditions but without cyclical stretch. Cell counting, histology, and computerized image analysis determined cell proliferation and their spatial distribution within the tissue-engineered grafts. Collagen matrix formation and organization was determined with polarized light and laser confocal microscopy. Uniaxial tensile testing assessed tissue-engineered graft function. Human heart cells proliferated within the gelatin scaffold. Remarkably, grafts that were subjected to cyclical stretch demonstrated increased cell proliferation and a marked improvement of cell distribution. Collagen matrix formation and organization was enhanced by mechanical stretch. Both maximal tensile strength and resistance to stretch were improved by mechanical mechanical stretch.

Conclusion—The cyclical mechanical stretch regimen enhanced the formation of a three-dimensional tissue-engineered cardiac graft by improving the proliferation and distribution of seeded human heart cells and by stimulating organized matrix formation resulting in an order of magnitude increase in the mechanical strength of the graft. (Circulation. 2002; 106[suppl 1]:I-137-I-142.)

Key Words: congenital cardiac defect ■ cardiac tissue engineering ■ mechanical stimulation

The feasibility of seeding autologous cells on a three-dimensional (3D) biodegradable scaffold to form a viable and contractile TEG for surgery has been established.2–9 Fetal rat ventricular cells seeded on a 3D biodegradable collagen matrix formed viable cardiac tissue that contracted spontaneously both in tissue culture and after subcutaneous implantation.7,8 The right ventricular outflow tract has been successfully replaced in rats with an autologous cell-seeded TEG.9 The graft cells survived in the right ventricular outflow tract, the matrix scaffold dissolved, and the inner surface endothelialized. Although encouraging, replacement within the left ventricle will require a stronger and thicker tissue construct. Even with state-of-the-art techniques, the mechanical properties of current TEGs are considerably weaker than the native tissues they replace.10

A clinically applicable TEG will require conditions that promote the extensive proliferation and integration of cells within all three dimensions of the scaffold, the replacement of the biodegradable scaffold with an organized extracellular matrix and, lastly, mechanical properties that mimic the replaced muscle. Mechanical stress is a potent stimulus for cell proliferation and hypertrophy, as well as extracellular matrix formation and organization.11–13

We hypothesized that the application of a mechanical stretch regimen to human heart cells seeded on a 3D gelatin scaffold (Gelfoam) would improve tissue formation and enhance the mechanical properties of the bioengineered graft.

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DOI: 10.1161/01.cir.0000032893.55215.fc
Methods

Cell Isolation and Culture

Human heart cells were isolated and cultured as previously described. Briefly, during surgical repair of Tetralogy of Fallot in children, ventricular tissue was obtained from the right ventricular outflow tract (25 to 100 mg wet weight). Permission to obtain myocardial biopsy from patients was approved by The Hospital for Sick Children and The Toronto General Hospital Human Experimentation Committee. The resected tissue was promptly immersed in culture medium (Iscove’s modified Dulbecco’s medium (GIBCO Laboratory, Life Technologies, Grand Island, NY), with 10% fetal bovine serum, 0.1 mmol/L of β-mercaptoethanol, 100 U/mL of penicillin, and 100 μg/mL of streptomycin). Visible fibrous tissue was removed and the remaining myocardial sample was minced with fine scissors. The fragments were incubated in phosphate-buffered saline (PBS) solution containing trypsin (0.25%), collagenase type II (0.1%), and glucose (0.02%) with agitation at 37°C. After 10 minutes of digestion, the cell suspension was transferred into 30 mL of culture medium. The digestion process was repeated 2 times. The combined cell suspension was centrifuged at 600g for 5 minutes and the cell pellet was cultured at 37°C in 5% carbon dioxide and 95% air. Passage 3 human pediatric heart cells were detached from the culture dishes with 0.05% trypsin in PBS. After cell counting with an automatic cell counter (Coulter Co., Miami, FL) the cells were pelleted by centrifugation, washed, and resuspended for seeding on the scaffold.

Graft Seeding

Cells were seeded on a sheet of gelatin sponge (Gelfoam: Pharmacia & Upjohn Co., Kalamazoo, MI) as we previously described. The gelfoam scaffold (30×20×3 mm for mechanical testing and 20×20×3 mm for the other studies) was incubated in culture media for 5 days before cell seeding. The gelatin scaffolds were then attached to Bio-Stretch cell culture dishes, which are rectangular and allow for fixation of the gelatin sponge by clamping it to the inner aspect of the dish margins. The opposite free margin of each patch is clamped to a cylindrical coated steel bar. Cells in suspension were seeded onto each Gelfoam scaffold (3×10^6 cells/patch for cell distribution and cell proliferation analyzes, 15×10^6 cell/patch for the extracellular matrix formation analyzes and 30×10^6 cell/patch for the tensile strength measurements). The stepwise addition of culture medium to the chamber until TEG submersion prevented disruption of the seeded cells from the gelatin scaffold.

Application of Mechanical Stretch

Cyclical mechanical stress was applied to the seeded TEGs using the Bio-Stretch apparatus. This technique has been described in detail previously. The Bio-Stretch culture dish is placed in front of a magnet, and the movement of the steel bar is controlled by dynamically changing the magnetic field on the controller. Stretch patterns of variable frequency, duty cycle, and magnitude can be designed for each stretch regimen. In this study, the Bio-Stretch apparatus was programmed to deliver a 20% stretch length at a frequency of 80 cycles/min for a 14 consecutive days’ duration. Culture medium was replaced each day in both groups.

Analysis of Cell Proliferation and Distribution

At the end of the culture period, the cells within the TEG were counted to compare the cell proliferation under stretched (n=7) versus static culture (n=7) conditions. After 3 brief rinses in PBS, each TEG was incubated in 10 mL of PBS solution containing collagenase type II (0.08%) and glucose (0.02%) at 37°C with agitation until completely dissolved (10 to 12 minutes). Cell number was determined with an automatic cell counter (Coulter Co., Miami, FL) and compared between groups.

For the analysis of cell distribution, the TEGs (stretch: n=9, control: n=8) were rinsed 3 times in PBS and fixed in 10% phosphate-buffered formalin solution for 5 days. The TEGs were cut in cross-section fixed in 5% glacial acetic acid and methanol, embedded in paraffin, and sectioned at a 10-μm thickness. The sections were stained with hematoxylin-eosin. Three random images of each TEG in cross-section were taken at 25× magnification using a light microscope and digital imaging system (Axiolab microscope, Carl Zeiss, Germany; Power HAD 3 CCD video camera, Sony, Japan). On each digital image, a grid was superimposed (Corel), dividing the cross section into 3 equally sized, horizontal areas (seeded surface=top area, center area, and unseeded surface=bottom area). Each area was graded for its cellular density by 3 observers, who were blinded to the study methods. Grading was performed with a rating system from 1 to 5, with 1 resembling an area with no or hardly any cells and 5 resembling an area with a high density of cells. An inverted duplicate version of each image was used as an internal control to determine intra- and interobserver reliability. Spearman’s analysis of 3 independent observers gives a correlation coefficient of 0.90 as a measure of intraobserver reliability. The interobserver reliability correlation coefficient was calculated between 0.87 and 0.90. The average cell density grade for each area was compared between control and stretch groups.

Analysis of Matrix Content and Organization

Formalin-fixed sections of stretched (n=7) and control (n=7) TEGs were stained with 0.1% picrosirius red (PSR; Sigma Chemical Company, St. Louis, MO) to observe fibrillar collagen matrix (type I and III isoforms). The seeded cells were identified by nuclear counter-staining with celestine blue. Identification of the newly formed fibrillar collagen matrix secreted by the human heart cells was obtained with polarized light microscopy (Axiolab microscope). Although PSR stained both the newly formed matrix and the gelatin scaffolding background, only the newly formed collagen matrix secreted by the cells had birefringence that could be observed with polarized light. Images were obtained with a computer-linked digital optical system (Sony digital camera) and compared qualitatively by 3 observers who were blinded to the study.

For the determination of the matrix organization, formalin-fixed sections of stretched (n=7) and control (n=7) TEGs were pretreated with 0.2% phosphomolybdic acid for 30 minutes before PSR staining. Sections were examined with a BioRad MRC 1024ES scanning confocal laser imaging system (BioRad, Toronto, ON), fitted with an argon/krypton mixed-gas laser with excitation wavelengths of 488, 567, and 647 nm, filters for fluorescein isothiocyanate (FITC) and rhodamine excitation, and 2 filter blocks (K and K2) for double labeling with simultaneous excitation of FITC and rhodamine using a double dichroic beam splitter. Images were obtained by serial optical sectioning at 0.5-μm intervals. PSR staining was detected with the FITC setting (508 nm laser wavelength) and autofluorescent seeded cells were detected with the FITC setting (488 nm laser wavelength). Digitized images were exported to Adobe Photoshop (version 5.0; Adobe Systems, San Jose, CA) and compared qualitatively by 3 observers who were blinded to the study.

Analysis of Tensile Strength

Uniaxial tension testing was performed with the Instron 8501 Dynamic Biomechanical Testing System. This system required a minimum patch area of 6 cm². Rectangular gelfoam segments with a larger surface area were required than the previous studies (30×20×3 mm) and more cells were required for seeding (30×10⁶ cells/patch) to maintain the proportion of surface area to seeded cells between experiments. In brief, TEGs were attached on opposite ends to the test apparatus. One arm of the test apparatus progressively stretched the TEG at 0.5 mm/minute until failure (complete tear). A computer continuously recorded the passive tensile strength of each TEG during the displacement. These data were plotted for each TEG as a displacement versus force graph and compared between groups. Maximal graft stress (index of maximal tensile strength) was determined by the peak of the curve whereas tissue resistance (inverse of elastance) to stretch was determined by the slope of the curve.

Measurement of Surface Area

After fixation for 5 days in 10% phosphate-buffered formalin solution, all TEGs were photographed (FX-35 camera: Nikon, Japan) and the patch surface area was determined by computed planimetry.
Statistical Analysis
All values are shown as mean±SD. Continuous variables were compared between groups by one-way ANOVA and categorical variables were compared by Chi-square analysis using SAS (SAS Institute, Cary, NC).

Results

Cell Analysis

Cell Proliferation
Cyclical stretching resulted in a marked increase in cell proliferation as revealed by the higher total cell number in grafts subjected to mechanical stretch after 14 days in culture (5.5±0.44x10^6 cells versus 4±3.1x10^6 cells; P<0.01), despite an equal number of cells at the time of seeding (3.0±10^6 seeded cells in both groups at Day 0) (see Fig. 1).

Cell Distribution
Cellular infiltration was observed throughout the three dimensions of the TEG in stretched and control groups. In both groups, cell density was highest at the seeded surface with a decreasing gradient toward the nonseeded surface of the TEG. In the control group, seeded cells formed a thick multicellular layer on the seeded surface with a significant decreasing gradient of cellular density toward the nonseeded surface of the patch (Fig. 2A). In the bottom (unseeded) third of the patches, only few, mostly isolated cells were observed with no visible organization. This observation indicated a limited capacity for cell migration throughout the TEG in the unstretched group.

In contrast, TEGs subjected to 14 days of cyclical mechanical stretch showed improved spatial cell distribution throughout the gelatin scaffold of the TEG (Fig. 2B). Using a 5-point grading system for cell density (1 for lowest cell density, 5 for highest), 3 independent observers blinded to the groups evaluated the cell distribution within the TEG on multiple representative histological images (Fig. 3). A cell density score of 3 or greater was considered the minimal cell density required for adequate tissue formation within the TEG. In the seeded area, cellular density was not significantly different between the 2 groups (3 or greater cell density: stretched 99.48% versus unstretched 95.9%; P=NS).

Similarly, the middle areas of the TEGs in both groups were not significantly different between the 2 groups (3 or greater cell density: stretched 99.48% versus unstretched 95.9%; P=NS).

Figure 1. Heart cell proliferation within cyclically stretched matrix scaffolds (biostretch) and static scaffolds (control) after 14 days in culture. The biostretch (n=7) and control (n=7) scaffolds were initially seeded with 3x10^6 cultured heart cells. Cyclic stretching increased cell proliferation within the scaffold compared with the control scaffolds. Results are expressed as mean±1 SD.

Figure 2. Hematoxylin-eosin staining of representative TEG cross-sections. Both the static control (A) and cyclically stretched (B) grafts were seeded with 3x10^6 cultured heart cells. In both groups, the cell density was highest at the seeded surface. The cyclically stretched graft had an improved spatial heart cell distribution throughout the scaffold compared with static control grafts with a cell distribution predominantly in the cell-seeded surface and middle layers. Black arrows indicate the cells, open arrows indicate the gelatin scaffold (magnification×25).

Figure 3. Analysis of cell density at the top (seeded area), center (middle area), and bottom (unseeded area) of the stretched and control cell-seeded grafts. On randomized images of TEG cross-sections cell density was graded on a 5-point scale by blinded observers. Cell density graded 3 or greater is presented. The cell density in the scaffolds was greater (P<.001) in the unseeded area of the TEGs subjected to mechanical stretch compared with the static controls. The results are expressed as mean±1 SD, n=7 for each group.
significantly different (3 or greater cell density: stretched 87.7% versus unstretched 84.7%; \( P = 0.05 \) NS). However, the most striking effect of the stretch regimen was determined in the bottom third of the patches, which represent the nonseeded surface, the area requiring the greatest degree of cell migration. Under stretched conditions, cell density in the unseeded areas of the TEGs was considerably increased (3 or greater cell density: stretched 25.3% versus non-stretched 2.1%; \( P = 0.001 \)). The TEGs subjected to stretch also showed a thin layer of cells covering the nonseeded surface. This was not seen in the control TEGs.

Matrix Analysis

**Formation of De Novo Matrix**

With polarized light microscopy, the new fibrillar collagen matrix formed by the seeded human heart cells in the TEGs was observed distinct from the gelatin scaffold. Newly formed fibrillar collagen matrix was observed in both groups. However, a marked increase in total amount of newly synthesized collagen matrix was observed in all the stretched TEGs compared with seeded control TEGs. The observed extend of this increase was not quantified, but the difference between the stretched and control cell-seeded TEG was evident to the 3 blinded observers. A representative image of each group is presented in Figure 4. Newly formed matrix was colocalized to areas of the highest cell density in both groups.

**Matrix Organization**

Confocal microscopy revealed the structural organization of the newly formed fibrillar collagen matrix secreted by the human heart cells within the TEGs (Fig. 5). An extensive network of fibrillar collagen filled the interstitial space and bridged the gap between the cells and the porous gelatin scaffold. Fibrillar collagen fibrils surrounded and embedded individual human heart cells. Importantly, the new matrix organized into a fibrillar collagen network that mechanically linked adjacent heart cells to each other within the grafts (Fig. 6). Only in the stretched cell-seeded group was the collagen fibers aligned parallel to the axis of stress (figure not shown).
Figure 6. A confocal laser scanning micrograph of the organization of the fibrillar collagen network in a cyclically stretched human heart cell-seeded gelatin scaffold. The newly synthesized collagen fibrils linked adjacent heart cells to each other. Collagen appears in red (picrosirius red stained) and heart cells in green (autofluorescence).

Analysis of Tensile Strength

Maximal Stress and Resistance to Stretch

 Compared with the unseeded stretched and static scaffolds, only the stretched TEG showed increased ($P<.05$) strength and resistance to stretch (Table 1). There were no statistical differences in strength and resistance to stretch among the static control TEG and the unseeded stretched and static grafts. TEGs subjected to stretch were stronger and were more resistant to stretch than TEGs without stretch, but the differences did not reach statistical significance ($P=.08, P=.07$).

Patch Surface Area

After 16 days of culture, TEG surface area in the seeded, unstretched controls was substantially decreased as a result of shrinkage. Cyclical mechanical stretch of the seeded TEGs attenuated the shrinking significantly. Stretched TEGs maintained a surface area of $134\pm17.5$ mm$^2$ versus $90.2\pm12.8$ mm$^2$ of control TEG ($P<.001$).

Discussion

Advances in tissue engineering offer surgeons an exciting opportunity to develop and apply autologous muscle-tissue constructs for children with congenital cardiac malformations. Autologous TEGs are created by isolating and expanding heart cells in vitro and incorporating them into a biodegradable 3D matrix scaffold. The matrix scaffold provides a transient but important structural support for the cells until they can secrete and maintain their own extracellular matrix. The development of a functional tissue construct is dependent on the capacity of the seeded cells to re-establish an architecture that resembles native myocardium. Our observations and those of others indicate that ex vivo heart cells possess an innate capacity to re-establish complex 3D myocardial organization if provided with appropriate environmental cues.$^{2,4-8,14}$ A large focus of the current research in tissue engineering is aimed at stimulating cultured muscle cells to organize into a functional 3D construct that allows for surgical application.

Mechanical stress plays an important role in the regulation of myocardial structure and function, a process well recognized by cardiac surgeons. Increased mechanical stress can stimulate cells to hypertrophy, orient, and increase their contractile state.$^{11,12,20}$ Increased mechanical load can also stimulate the secretion and reorganization of extracellular matrix components. Increased mechanical stress can also stimulate the secretion and reorganization of extracellular matrix components. The extra-cellular matrix is the substrate for cell adhesion, growth, and differentiation, and it provides the mechanical support necessary for effective cardiac contraction.$^{21,22}$ Given the critical role of mechanical stimuli in maintaining effective myocardial structure and function, in this study we examined the ability of a mechanical stretch stimulus to stimulate human heart cells to form functional autologous myocardial tissue constructs.

Using identical culture methods, we previously demonstrated that human pediatric heart cells dedifferentiate in culture, allowing them to divide.$^{14}$ The cells derived from this process are similar to cardiomyocytes but have distinct differences. Heart cells retain the morphology, metabolism, and structural contractile elements of cardiomyocytes, but their myofibrils are disorganized and the cells do not contract.$^{14}$ In contrast, fetal and neonatal heart cells retain their ability to contract, both in culture and after subcutaneous implantation.$^{7,23}$ The isolated pediatric heart cells in this study were not contractile. In this study, we produced an autologous bioengineered muscle graft, which could have been used to repair the right ventricular outflow tract of children undergoing repair of Tetralogy of Fallot. Using fetal or neonatal heart cells may produce a contracting bioengineered muscle graft, which would permit pulsatile pulmonary perfusion. A bioengineered autologous patch has advantages over synthetic materials because the patch will grow and remodel as the child develops.$^{24}$ This study demonstrated that mechanical stretching increased cell proliferation and induced matrix remodeling in vitro. The resulting autologous TEG may grow and remodel in children with Tetralogy of Fallot. In addition, the heart cells may differentiate and begin contracting after implantation.

The application of cyclical mechanical stress to human heart cells within a gelatin scaffolding enhanced tissue formation, resulting in improved tensile strength. The mechanical stretch increased the proliferation and distribution of the heart cells in the seeded TEGs.

### Mechanical Properties of Stretched TEGs Versus Controls and Unseeded Scaffolds

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<th>TEG</th>
<th>Unseeded scaffolds</th>
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<tr>
<td></td>
<td>Stretched (n=3)</td>
<td>Control (n=3)</td>
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<tr>
<td>Graft strength</td>
<td>0.172±.057*</td>
<td>0.086±.031†</td>
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<tr>
<td>[Newton]</td>
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<tr>
<td>Graft resistance</td>
<td>0.54±.017*</td>
<td>0.022±.014†</td>
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*TEG indicates autologous tissue-engineered muscle grafts. Values are mean±SD.
*$P<.5$ versus unseeded scaffolds; †$P=NS$ versus unseeded scaffolds and stretched TEG.
throughout the three dimensions of the graft, allowing for improved tissue formation. Rhythmic stretching of the scaffold would have facilitated nutrient and waste exchange throughout the scaffold by opening and compressing the scaffold pores. An improved metabolic environment within the scaffold might have facilitated cell division and migration throughout the scaffold. In addition the rhythmic changes in matrix pore configuration could have contributed to the migration of the cells or, by mechanically shaking the cells, from the upper seeded surface into the unseeded middle and bottom scaffold layers. Although improved by the stretch mechanism, the distribution of cells was not uniform and areas in the center of the patch and in the unseeded (bottom) portion were poorly populated. Augmentation of the number of seeded cells or the stretch regimen may further improve the cell distribution.

Importantly, the greater cell number and cell distribution in the TEGs subjected to mechanical stretch provided for extensive extracellular matrix formation, a critical component of graft construction. The improved matrix formation may also have had a reciprocal effect on stimulating the cells themselves to proliferate and organize within the graft.23 The organization of the newly formed cardiac matrix was similar to normal human myocardium. There was a distinct pattern of endomysial (surrounds individual cells) and perimysial (surrounds groups of cells) collagen fibers within the graft (Fig. 5). There was evidence of a mechanical linkage between heart cells by the new matrix, which would provide an important substrate for graft contraction and if implanted, it would allow integration with the host matrix and cardiomyocytes. The material properties of myocardium are largely the result of the content and organization of the fibrillar collagen matrix.24 Specifi- cally, orientation of collagen fibers parallel to the axis of stress can significantly increase tensile tissue strength.19 This parallel orientation was observed in TEGs subjected to mechanical stretch. As such, the extensive and highly organized formation of the fibrillar collagen network in the TEGs subjected to mechanical stretch may account for the improved stress-strain responses obtained by mechanical testing.

The TEGs subjected to mechanical stress had a 2- to 3-fold higher tensile strength and were more resistant to stretch than the scaffolding alone or the control TEGs. Although the results were not statistically significant when compared with control TEGs, there was a strong trend given the small number of TEGs subjected to mechanical testing (n=3/group). Additionally, the total graft surface area was increased in the stretched group. A common concern with using cell-seeded gelatin scaffolds has been the significant shrinkage of the graft over time; however, shrinkage was prevented by cyclical stretching. The increased strength and preservation of size are desirable properties for the graft to be secured by sutures and to withstand systemic pressures.

The stimulation of developing TEGs with a unidirectional mechanical stretch regimen can enhance tissue formation resulting in greater tensile strength. The bioengineered tissue construct may grow and remodel after implantation in response to local environmental cues. These autologous grafts can be employed for the repair of the right ventricular outflow tract in children with Tetralogy of Fallot and may improve the long-term results of surgery.

Acknowledgments

Funding was provided by the Heart & Stroke Foundation of Ontario (HSFO; NAA4603) and Canadian Institute for Health Research (CIHR; MOP14795) to RKL. P.A. is a Research Scholar of the German Academic Exchange Service (DAAD). P.W.M.F. and S.V. are Research Fellows of the CIHR and the HSFC. R.K.L. is a Career Investigator of the HSFC.

References

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Circulation. 2002;106:I-137-I-142
doi: 10.1161/01.cir.000032893.55215.fc
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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