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Myocardial Scaffold-based Cardiac Tissue Engineering: Application of Coordinated Mechanical and Electrical Stimulations

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Abstract

Recently, we have developed an optimal decellularization protocol to generate 3D porcine myocardial scaffolds, which preserved natural extracellular matrix structure, mechanical anisotropy, and vasculature templates, and also showed good cell recellularization and differentiation potential. In this study, a multi-stimulation bioreactor was built to provide coordinated mechanical and electrical stimulations for facilitating stem cell differentiation and cardiac construct development. The acellular myocardial scaffolds were seeded with mesenchymal stem cells (10^6 cells/ml) by needle injection and subjected to 5-azacytidine treatment (3 μ mol/L, 24 h) and various bioreactor conditioning protocols. We found that, after 2-day culture with mechanical (20% strain) and electrical stimulation (5 V, 1 Hz), high cell density and good cell viability were observed in the reseeded scaffold. Immunofluorescence staining demonstrated that the differentiated cells showed cardiomyocyte-like phenotype, by expressing sarcomeric α -actinin, myosin heavy chain, cardiac troponin T, connexin-43, and N-cadherin. Biaxial mechanical testing demonstrated that positive tissue remodeling took place after 2-day bioreactor conditioning (20% strain + 5 V, 1 Hz); passive mechanical properties of the 2-day and 4-day tissue constructs were comparable to the tissue constructs produced by stirring reseeded followed by 2-week static culture, implying the effectiveness and efficiency of the coordinated simulations in promoting tissue remodeling. In short, the synergistic stimulations might be beneficial not only for the quality of cardiac construct development, but also for patients by reducing the waiting time in future clinical scenarios.

1. Introduction

Myocardial infarction (MI) and heart failure are the leading causes of mortality globally.¹ When coronary arteries are blocked, the perfusion to the downstream heart muscle is inadequate and can result in cell death.² The pathological progression of MI includes inflammatory responses, cardiomyocyte death, scar formation, expansion of infarcted region, thinning and dilation of left ventricular (LV) wall.^{3, 4} Cardiac function deteriorates

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along with the pathological progression of MI, and at the end stage arrhythmias, mitral regurgitation, and heart failure can occur and are often fatal.^{5, 6}

Heart transplantation is an effective treatment for patients with end-stage heart failure, but is limited by the shortage of donor hearts.⁷ Standard treatments following acute MI involve prompting revascularization with thrombolytic/fibrinolytic therapy, coronary angioplasty, and coronary artery bypass grafting; those treatments are effective in preventing the extension of the infarction, but limited in restoring the cardiac function lost due to heart muscle death.⁸ Recent studies on MI treatment are focused on either avoiding scar formation/triggering tissue remodeling with stem cell injection,^{9, 10} or replacing formed scar tissue with functioning cardiac construct.^{11, 12} The hope of those new approaches is to not only prevent further LV dilation and pathological remodeling, but also regenerate myocardial tissues and consequently restore cardiac function. Among the new approaches, cardiac tissue engineering has attracted significant interest in the past three decades due to a great promise of creating viable myocardial tissues.¹³⁻¹⁵ Interesting progress has been made in cardiac tissue engineering with contributions from research groups of a variety of disciplinary backgrounds, including stem cells, biomaterials, tissue-derived scaffolds, etc.¹⁶⁻¹⁸ However, the complexity of myocardium, both structurally and functionally, still presents many challenges for tissue engineering endeavors.

The myocardium is highly organized in structure and possesses unique electrophysiological and mechanical properties.¹⁹ The myocardial fibers have a complex multilayered helical architecture that is essential for well coordinated heart contraction.²⁰ Among the individual myocardial fibers is a 3-dimensional (3D) network of myocardial extracellular matrices (ECM) that are critical to structural integrity of myocardium, tethering of myocytes, transfer of muscle contractile forces, as well as prevention of excessive stretch of muscle fibers.²¹⁻²⁴ To engineer contractile and functional equivalent of the native myocardium, the challenges not only fall into the generation of high density functional cardiomyocytes, but also the integration of the cells within a 3D ECM/scaffold.²⁵⁻²⁷ The mixture of functional cardiomyocytes with ECM/scaffolds needs to reach a delicate balance in order to generate an optimal myocardial construct.^{12, 28-30} It can be understood logistically that the approaches starting from a high density of cells experience good cell-to-cell connection and capability to propagate electrical propagation, but see hurdles in structural strength and mechanical properties.^{15, 31-33} On the other hand, the approaches utilizing polymeric scaffolds and tissue-derived scaffolds often have good mechanical behavior in the construct, but encounter challenges in reaching high reseeding density and cell-to-cell connections, and hence lack contractility and electrical transduction.^{14, 34}

Recently, the potential of acellular myocardial scaffolds have been revealed by Ott *et al.* in their work of a revitalized beating rat heart made of a decellularized intact rat heart perfused with cardiac and endothelial cells.³⁵ Early stage scale-up research on whole pig heart was also reported and discussed by Badylak *et al.* and Taylor *et al.*^{36, 37} Our group, however, has undertaken an effort to harness the potential of decellularized porcine myocardium as a scaffold material (e.g., for making cardiac patch).^{38, 39} Up to now, studies have emerged not only on acellular porcine and rat myocardial scaffolds,³⁸⁻⁴⁰ but also on acellular human myocardial scaffolds.⁴¹ As shown in our previous publications,^{38, 39} we have developed an optimal decellularization protocol to generate 3D porcine acellular myocardial scaffolds, in which 3D cardiomyocyte lacunae and ECM networks were well preserved along with mechanical anisotropy and vasculature templates;^{38, 39} the acellular myocardial scaffolds also showed good stem cell recellularization and differentiation potential and experienced positive tissue remodeling manifested by a recovering trend in tissue mechanical properties.³⁸

To further improve the effectiveness and efficiency of cell differentiation and tissue remodeling of the stem cell-reseeded acellular myocardial scaffolds, in this study we have explored the effect of physical stimulations on the outcomes of the tissue construct fabricated with the acellular myocardial scaffolds and mesenchymal stem cells. As pointed out by Vogel et al., physical stimulations can affect the cell proliferation, differentiation, and migration, and consequently the biological, mechanical, and other properties of the cardiac construct.^{28, 42} For cardiac tissue engineering, two types of physical simulations are important to assist in the development of tissue construct: one is mechanical stretching and the other is electrical current stimulation. In an *in vitro* study, Zhuang et al.⁴³ reported that 10% cyclic uniaxial stretch produced upregulation of connexin-43 and N-cadherin in the intercellular junctions and increased the propagation velocity. Yamada et al. also reported that the cyclic stretch (10%) upregulated expression of both the electrical junction protein (connexin-43) and the mechanical junction proteins (plakoglobin, desmoplakin, and N-cadherin) via integrin-dependent activation of focal adhesion kinase (FAK).^{44, 45} Moreover, Zimmerman et al.⁴⁶ found that the encapsulated neonatal rat cardiomyocytes in a gel matrix formed an intensively interconnected, longitudinally oriented cardiac muscle bundles under uniaxial stretching, and the tissue construct showed contractile properties similar to the native myocardium. For electrical stimulation, Radisic and Vunjak-Novakovic observed that spontaneous construct beating took place in a construct (collagen sponge) reseeded with rat cardiomyocytes under ~5 V and 1 Hz electrical stimuli.^{47, 48} In Ott's study,³⁵ electrical stimulation of 5-20 V (10 ms) pulse was also applied to the recellularized rat heart using epicardial leads.

The synergistic effects of the mechanical and electrical stimulations on cardiac patch tissue engineering have not yet been fully investigated. We thus designed and fabricated a bioreactor to provide multi-stimulations to the tissue construct made of the acellular myocardial scaffolds and mesenchymal stem cells (MSCs). We hypothesize that the combined mechanical and electrical stimulations will more efficiently promote the cell repopulation, cardiomyocyte differentiation, and remodeling of the engineered cardiac construct. In order to sort out the effects of the applied mechanical and/or electrical stimulations during *in vitro* culture, acellular myocardial scaffolds were reseeded with rat MSCs and subjected to various bioreactor conditioning. The *in vitro* conditioning parameters examined include mechanical stretching, electrical stimulation, and tissue culture times. The hope is to shed light on how physical stimulations play a role in tissue remodeling of the engineered cardiac construct derived of acellular myocardial scaffolds.

2 Materials and Methods

2.1 Design of a Multi-stimulation Bioreactor

The working principle of the multi-stimulation bioreactor, which was capable of delivering both mechanical and electrical stimulations to the cardiac construct, was shown in Figure 1-a,b. Linear movement was applied by the movable arm driven by an Xslide assembly and a stepper motor (Velmex, New York City) (Figure 1-c). Electrodes were made from Teflon-coated silver wire of 75 μ m diameter (A-M Systems, Carlsborg, WA). Teflon insulation was stripped from the end of the wire and the naked wires were inserted into the two opposite edges of the tissue construct. To simulate what myocardial tissue experiences, electrical pulses were applied when each unloading cycle started (Figure 1-b). The frequency and amplitude of the cyclic stretches and electrical pulses were controlled by a custom written LabView program. The program was capable of delivering multiple protocols of mechanical stretching and various waveforms of electrical stimulation (Figure 1-d).

The bioreactor chamber and parts were designed using Solidworks 3D CAD software (Solidworks Corp., Concord, MA). The structural elements of the device were machined

from either acrylic or polysulfone that provided abrasion-resistance and excellent thermal/chemical stability. The bioreactor consisted of one tissue culture chamber, in which two to four pieces of tissue constructs (20 mm × 20 mm × ~3 mm) could be mounted between a fixed clamp and a movable clamp (Figure 1-c). Ti-corn blue sutures (#0) were used for connecting the sample with two clamps. The cover of the tissue culture chamber was fabricated with 1/4" thick clear polycarbonate (Small Parts, Inc. Logansport, IN). A hole 2 cm in diameter was made on the cover and then sealed with the pentafluoroisopropenyl fluoromethyl ether (PIFE) membrane with 0.2 µm pore size (Millipore, Billerica, MA) to enable air exchange (Figure 1-c).

2.2 Preparation of Acellular Myocardial Scaffolds, MSC Reseeding, and Cardiomyocyte Differentiation

Preparation of acellular myocardial scaffolds—The decellularization method for porcine myocardium was the same as our previous study.³⁹ Forty fresh pig hearts were harvested from ~6-month old pigs and transported from the local slaughter house to our laboratory in phosphate Buffered Saline at 4 °C. From the middle region of the anterior left ventricular wall, we were able to trim square-shaped myocardium samples (20 × 20 × ~3 mm), of which one edge aligned along the muscle fiber preferred direction (PD) and the other edge aligned along the cross-fiber preferred direction (XD); note that the PD direction was determined based on overall muscle fiber texture and heart anatomy.³⁹ Two edges of the square sample were then perforated with 27G × 31/2 BD Quincke Spinal Needles that were later affixed by two rectangular plastic frames.³⁹ The purpose of this frame-pin supporting system was to prevent contraction of tissue macrogeometry and collapse of internal cardiomyocytes lacunae.³⁹ The mounted myocardium samples were then decellularized in a rotating bioreactor using 0.1 % sodium dodecyl sulfate (SDS) (Sigma Aldrich Inc., St. Louis, MO) with 0.01 % trypsin (VWR), 1 mM phenylmethylsulfonylfluoride (PMSF, protease inhibitor) (Sigma Aldrich Inc., St. Louis, MO), 20 µg/ml RNase A (Sigma), 0.2 mg/ml DNase (Sigma Aldrich Inc., St. Louis, MO), and 100 U/ml penicillin and 100 µg/ml streptomycin at room temperature for 2.5 weeks. Ten-minute ultrasonic treatment (50 Hz, Branson) was applied each day; the solution was changed every two days to avoid contamination and tissue deterioration.

MSC Preparation, Reseeding, and Cardiomyocyte Differentiation—Well characterized Lewis rat mesenchymal stem cells (MSCs, fourth passage) were obtained from the Stem/Progenitor Cell Standardization Core (SPCS) at the Texas A&M Health Science Center (NIH/NCRR grant). These cells were re-suspended in mesenchymal stem cell medium (L-DMEM, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) and seeded in 175-mm flasks at a density of 3×10^3 cells/cm². The medium was changed twice a week. After confluency was reached, aliquots of the MSCs were prepared for reseeded and differentiation.

Before cell reseeded, the square samples of acellular scaffolds were mounted between the fixed clamp and the movable clamp using the surgical Ti-corn blue sutures (#0). The acellular scaffolds were then sterilized in 70% ethanol in the tissue culture chamber for 2 hours and rinsed with sterilized Phosphate-Buffered Saline (PBS) four times. The entire tissue culture chamber with the mounted scaffolds was further sterilized with UV light for 20 minutes. After completion of the sterilization protocol, each scaffold sample was injected with 1 ml MSC solution at a concentration of 10^6 cells/ml using a syringe (1 ml; 26G Permanent needle, BD Inc.). 1 ml MSC solution was injected evenly at nine points (~0.1 ml/point; located as a 3 × 3 array) in the middle region of the square sample (Figure 1-a).

After cell injection, the tissue constructs were cultured in 5-azacytidine differentiation medium (L-DMEM, 10% FBS, 3 $\mu\text{mol/L}$ 5-azacytidine (MP Biomedicals Inc.), cardiac myocyte growth supplement (ScienCell Research Laboratories), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin) for the first 24 hours. After the 5-azacytidine treatment, the medium was changed to the complete medium (L-DMEM, 10% FBS, cardiac myocyte growth supplement (ScienCell Research Laboratories), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin) for the remaining tissue culture protocols.

2.3 Protocols for *in vitro* Bioreactor Conditioning

To compare the influence of mechanical and electrical stimulations on the tissue engineered cardiac construct, we randomly divided all the acellular scaffolds into four groups, which were designed as follows: (i) the control group in static culture; (ii) 20% strain stimulation; (iii) 5 V electrical stimulation; and (iv) 20% strain + 5 V electrical stimulations. For the control group (group (i)), samples were injected with the same amount of MSCs and were placed in the same culture chamber without mechanical or electrical stimulations. For tissue constructs subjected to only stretch conditioning (groups (ii)), samples were mounted between the fixed clamp and the movable clamp and immersed in cell culture medium. For the group given only electrical stimulation (group (iii)), the positive and negative electrodes were mounted on the two opposite sides of the sample without application of the clamp stretch. For group (iv), the stretch and electrical stimulations were simultaneously applied. Note that, for the applied stimulations, both the triangular strain waveform and square wave electrical pulse were set at a frequency of 1 Hz, which simulates the physiological frequency experienced by the heart muscles. In each group, 4 tissue constructs were fabricated and used for characterizations such as cell viability, histology, and immunofluorescence staining. For groups subjected to tissue mechanical assessment, 4 additional tissue constructs were produced and used for biaxial testing.

2.4 Cell Viability, Histology, and Immunohistological Characterizations

Cell viability—Cell viability in the tissue construct was examined by a Live/Dead assay (L3224, Life Technologies Inc., Grand Island, NY) according to the manufacturer's instructions. Briefly, calcein-AM (2 mM) and ethidium bromide homodimer (4 mM) were mixed in PBS. The tissue constructs were then incubated in the above solution for 20 minutes at room temperature, and rinsed in PBS three times for laser scanning confocal microscopy (LSCM) (Zeiss LSM 510). Ten regions were selected randomly in each sample under high magnification (40 $\times$) for analyzing cell viability. The ratio of living cells was estimated by living cells divided by the total amount of cells (green color indicates living cells and red color indicates dead cells).

Histology—Samples prepared for histology were fixed in 2% paraformaldehyde solution for 2 hours, embedded in paraffin, subjected to sectioning, stained with Hematoxylin and eosin (H&E) or Masson's trichrome, and imaged with bright field light microscopy (Nikon EC600). Histological images were taken randomly in each section at 40 $\times$. The number of reseeded cells was counted in each image, and the cell density was estimated by normalizing cell number to the area of the selected regions.

Immunofluorescence staining—For immunofluorescence staining, after rehydration and antigen retrieval with 0.05% Trypsin for 10 min at 37°C, tissue sections were blocked with 1% bovine serum albumin (BSA) for 2 hours. Tissue sections were then incubated at 4°C overnight with primary antibodies targeting myosin heavy chain (GenWay), sarcomeric α -actinin (Sigma), cardiac troponin T (abcam), Connexin-43 (Sigma), or N-Cadherin (Sigma). After thorough rinsing, secondary antibodies, Cy3 AffiniPure goat anti-mouse IgG, Cy5 Affini-Pure donkey anti-mouse IgM, DyLight 649 goat anti-mouse IgG, and DyLight

549 AffiniPure Donkey Anti-Mouse IgG (JacksonImmuno Research) were applied at room temperature for 1 hour. Lastly, all tissue sections were stained with Hoechst (Invitrogen) for cell nuclei. Immunofluorescence slides were observed with an inverted LSCM (Zeiss LSM 510). Positive controls were prepared by following the same protocols with sections of native porcine heart. Negative controls were prepared by following the same protocols with the omission of all the primary antibodies.

2.5 Biaxial Mechanical Characterization of Tissue Constructs

Biaxial mechanical testing has high sensitivity to capture mechanical behavior changes resulting from subtle structural/compositional alterations in tissues.^{38, 49, 50} Details for biaxial testing such as system setup and testing protocols can be found in the previous publications.^{49, 50} In this study, biaxial loading was applied along muscle/scaffold fiber-preferred direction (PD) and cross-preferred direction (XD). After 10-cycle preconditioning, the tissue construct was subjected to an equibiaxial tension protocol ($T_{PD}:T_{XD} = 60:60$ N/m), where T_{PD} and T_{XD} were the applied tensions along PD and XD, respectively. Extensibility of the tissue construct was characterized by maximum stretch along PD (ϵ_{PD}) and maximum stretch along XD (ϵ_{XD}) at equibiaxial tension of 60 N/m. Tissue constructs were tested in a PBS bath at 37°C.

2.6 Statistical Analysis

Mean $\pm$ standard deviation was used for presenting experimental data. One-Way Analysis of Variances (ANOVA) was applied in statistical analyses, with Holm-Sidak test for post hoc pair-wise comparisons or comparisons versus the control group (SigmaStat 3.0, SPSS Inc., Chicago, IL). The differences were considered statistically significant when $p < 0.05$.

3 Results

3.1 Acellular Myocardial Scaffold

Acellular myocardial scaffold was obtained after 2.5-week decellularization treatment. The morphological difference between the acellular myocardial scaffold and the native myocardium was shown in Figure 2-a, in which the acellular myocardial scaffold exhibits bright white color of typical collagenous materials. Complete removal of cellular contents and good preservation of subtle ECM structure have been verified in our previous publications.^{38, 39} The acellular myocardial scaffolds were completely removed of cells, cell debris, DNA fragments, and α -Gal porcine antigens, while preserving the myocardial ECM and microstructures such as cardiomyocyte lacunae, blood vessel templates, cardiac elastin, etc.^{38, 39} Figure 2-b and c showed the histological comparison of the native myocardium and the acellular myocardial scaffold by Mason's trichrome staining.

3.2 Effect of the *in vitro* Bioreactor Conditioning on Construct Recellularization

Cell density, distribution, and viability after 2-day tissue culture were compared among various bioreactor conditioning protocols using H&E and Live/Dead cell staining (Figure 3). H&E staining showed that the cell densities in 20% strain stimulation group (group (ii)) (Figure 3 - c), 5 V electrical stimulation group (group (iii)) (Figure 3 - e), and 20% strain + 5 V combined stimulations (group (iv)) (Figure 3-g) are higher than the static control group (group (i)) (Figure 3 -a). Moreover, the Live/Dead staining results further verified this observation, and the ratios of living cells in the bioreactor conditioning groups (group (ii, iii, iv)) (Figure 3 - d, f, h) were also higher than the static control group (group (i)) (Figure 3 - a).

Compared with the static control group (group (i)) (Figure 3 - a, b), higher cell density and certain cell alignment were observed in the 20% strain stimulation group (group (ii)) (Figure

3 - c, d); high cell density was also found in the 5 V electrical stimulation group (group (iii)) (Figure 3 - e, f), although there was no obvious cell alignments in this group. In the multi-stimulation group ((20% strain + 5 V, group (iv)), higher cell density was observed, and the reseeded cells exhibited better morphology and alignment (Figure 3 - g, h). Statistical analysis showed that there was significant difference when comparing group (iv) with group (iii), group (ii), or group (i) ($p < 0.05$, pair-wise comparison using Holm-Sidak method) (Table 1).

It could be seen from the above observation that the multi-stimulation (20% strain + 5 V, group (iv)) resulted in better construct recellularization. We further assessed the cell morphologies of the multi-stimulation group (20% strain + 5 V, group (iv)) after 1-day, 2-day, and 4-days tissue cultures. Mason's trichrome staining showed that, after 2-day of the combined stimulations, the cells showed good morphology of cell aggregation (Figure 4 - b); this trend was even more obvious in the 4-day combined stimulation group (Figure 4 - c).

3.3 Cell Phenotype Characterizations

As shown in section 3.2, we found that the combination of 20% strain and 5 V electrical stimulation (group (iv)) generated the best result when compared with other conditioning protocols. The tissue constructs subjected to 20% + 5 V combined stimulations were thus processed for cell phenotype characterization using immunofluorescence staining. Figure 5 showed that the differentiated cells demonstrated cardiomyocyte-like phenotype, by expressing myosin heavy chain, sarcomeric α -actinin, and cardiac troponin T (Figure 5 - a, b, c). The existence of electrical gap junction protein and adherens junction protein were also verified by positive staining of the connexin-43 and N-cadherin, respectively (Figure 5 - d, e). There was no positive staining in all the negative controls, which indicates the immunohistological images (Figure 5) showed specific staining.

3.4 Biaxial Mechanical Properties

The biaxial tissue behavior of the native myocardium, decellularized myocardial scaffolds, 2-day and 4-day static control groups (group (i)), and 2-day and 4-day combined stimulation groups (20% strain + 5 volt, group (iv)) were evaluated, and the averaged stress-strain curves were plotted in Figure 6. The tissue extensibilities were compared by the maximum stretches in PD direction and XD direction (Table 2). Both PD and XD directions of the decellularized myocardium scaffold showed stiffer stress-strain responses ($p < 0.01$) (Figure 6-b compared with Figure 6-a). By examining the stress-strain curves in Figure 6, we found that panel (f), i.e., 4-day *in vitro* condition with 20% strain and 5 Volt electrical simulations, showed the nonlinear anisotropic mechanical behavior close to the biaxial behavior of the native myocardium (Figure 6-f compared with Figure 6-a). This recovery trend of biomechanical behavior was more evidenced when comparing the native myocardium (Figure 6-a), acellular myocardial scaffolds (Figure 6-b), 2-day multi-stimulation conditioning (Figure 6-e), and 4-day multi-stimulation conditioning (Figure 6-f) together. The 2-day and 4-day static control groups (Figure 6-c,d) showed a small trend of softening when compared with the acellular myocardial scaffolds (Figure 6-b), which can be explained by the remodeling of the injected cells. However, we noticed that the tissue constructs of the static control group (Figure 6-c,d) exhibited less anisotropy when compared with the multi-stimulation group (Figure 6-e,f).

4. Discussion

In our previous studies, the rotating bioreactor³⁸ allowed better oxygenation of the recellularized scaffolds than the static tissue culture; however, the efficiency of recellularization and cell differentiation were still relatively low. In this project, the multi-

stimulation bioreactor has been designed and built to provide mechanical stretch to the tissue construct periodically, as well as apply electrical pulses to stimulate cell differentiation and construct development. During the study, the bioreactor was able to ensure good cell viability under sterile conditions for an extended period of time, and experiments showed excellent reproducibility. Other advantages of the designed multi-stimulation bioreactor are easy sample mounting, visibility of the sample chamber, and flexibility in implementing complicated protocols.

To increase the efficiency of cell reseeding, we employed a needle injection method for cell implanting with a total cell amount of 10^6 cells/scaffold in this study. The scaffold was injected at nine points (~ 0.1 ml/point) that separated evenly within the central square region (~ 1 cm $\times$ 1 cm). During the injection, effort was taken to ensure that the implanted cells distributed in an even manner. However, due to the porous structure of the acellular myocardial scaffold, a small amount of leakage did happen during the process of the cell injection. For this kind of situation, we injected the leaked medium back into the same region of the acellular patch.

To further explore the effects of mechanical stimulation alone, electrical stimulation alone, and the combined mechanical and electrical stimulations on cell repopulation and alignment, we compared the cell density, morphology, and viability after the 2-day *in vitro* culture among the static control (group (i)), 20% strain stimulation (group (ii)), 5 V electrical stimulation (group (iii)), and 20% strain + 5 V combined stimulations (group (iv)). Cell density of the control group after 2-day culture was 0.91×10^2 cells/mm², and no obvious cell alignment was found (Figure 3-a). In the bioreactor conditioning groups, cell densities were 2.21×10^2 cells/mm² (group (ii)), 2.37×10^2 cells/mm² (group (iii)), and 3.28×10^2 cells/mm² (group (iv)). Using Holm-Sidak method for pair-wise multiple comparison, we found that there was significant difference when comparing group (iv) with group (iii) or group (ii) ($p < 0.05$), which demonstrated that the combined mechanical and electrical stimulations (group (iv)) promoted the cell repopulation in the myocardial scaffolds (Figure 3-c,e,g, Table 1). While there was no obvious cell alignment in group (iii) (Figure 3-e, f), cell alignment was observed in both group (ii) and group (iv) (Figure 3-c, d, g, h), implicating that the mechanical stimulation likely assisted in cell alignment during *in vitro* conditioning.

Previous studies have shown that cyclic mechanical stimulation can assist in the cell alignment, stimulate ECM formation,^{51, 52} and improve the cardiomyocytes development and function.^{46, 53} Our findings are consistent with the observations of the above-mentioned studies focusing on mechanical stimulation.^{46, 51-53} Since the cell density of the combined stimulations (20% strain + 5 V, group (iv)) (Figure 3-g) was higher than the 20% strain stimulation (group (ii)) (Figure 3-c), it might indicate that electrical stimulation, along with mechanical stimulation, seems to have a synergistic effect. Electrical stimulation is believed to be able to induce transient calcium levels which will in turn further improve the amount and organization of sarcomeres in the cardiac tissue.⁵⁴ Furthermore, electrical stimulation has been found to facilitate cell proliferation and promote the formation and localization of electric gap junctions.^{55, 56} Our results show that synergistic treatment with both mechanical and electrical simulations did promote the repopulation and alignment of the reseeded cells.

Biaxial testing results further show that the 20% strain + 5 V stimulations (group (iv)) generated good tissue remodeling trend (Figure 6-e, f) and desirable nonlinear anisotropic tissue construct behavior (Figure 6-f), i.e., the 4-day *in vitro* conditioning of group (iv) resulted in a biaxial stress-strain behavior (Figure 6-f, Table 2) similar to that of the native myocardium (Figure 6-a, Table 2). Our observation in recellularization and tissue

mechanical behavior all demonstrate positive tissue remodeling taking place in group (iv) (20% strain + 5 V) (Figure 6, Table 2).

To further understand the synergistic effects of multi-stimulation on stem cell differentiation, we performed immunofluorescence staining on the tissue constructs produced by 2-day multi-simulations (20% strain + 5 V, group (iv)). Our results indicated that the differentiated cells clearly demonstrated cardiomyocyte-like phenotypes, by abundantly expressing myosin heavy chain, sarcomeric α -actinin, and cardiac troponin T (Figure 5-a, b, c). Connexin-43 and N-cadherin were also observed in tissue constructs (Figure 5-d, e). As we know, connexin-43 is the major electrical gap junction protein, and N-cadherin is the fascia adherens junction protein in heart muscles. Those junction proteins play important roles to synchronize and coordinate the myocardium contraction.^{57, 58} The myocardium contracts synchronously since ion currents conducted through the tissue by intercellular gap junctions, which directly couple the cytoplasmic compartments of adjacent cells.^{57, 58} Yamada et al. found that mechanical stretch could upregulate the expression of both electrical and mechanical junction proteins.⁴⁴ Zhuang et al. also proved that *in vitro* pulsatile linear stretch could upregulate the expression of both electrical and mechanical junction proteins.⁴³ Our study also demonstrated that the combined mechanical and electrical stimulations facilitated the formation of electrical gap junctions and mechanical junctions.

5. Conclusions

In this study, we have successfully built a bioreactor that is able to apply both mechanical and electrical stimulations for facilitating tissue construct development. We found that the recellularization, cardiomyocyte differentiation, and tissue remodeling were more effectively and efficiently promoted by the combined mechanical and electrical stimulations (20% strain + 5 V, group (iv)). The benefit of this combination of stimulations (20% strain + 5 V) was evidenced by good cell viability, repopulation, differentiation, and positive tissue remodeling within a short period of time (2 - 4 days). In future clinical settings, patients will have to wait for cell expansion, tissue culture, and cell reconstruction until a regenerated functional tissue can be applied in surgical treatment. Our results show that the synergistic stimulations might be beneficial not only for the quality of cardiac construct development, but also for patients by reducing the waiting time in future clinical scenarios.

6. Limitations and Future Studies

Due to the structural complexity of the bioreactor, we adopted 70% ethanol sterilization for the acellular myocardial scaffolds and the culture chamber; for the other parts of the bioreactor that could not be immersed in ethanol, we used UV light for sterilization. Yet, the sterilization protocol can be further optimized to reduce sterilizing treatments to a minimal level. As for mechanical stretch, the applied tissue construct strain was estimated by normalizing the displacement of the movable arm to the original distance of two sample mounting arms. The use of the clamp-to-clamp displacement in strain estimation was not an ideal method to accurately measure the tissue construct strain. Our future improvement will be real time tracking of markers on the tissue construct using a camera.

In the bioreactor conditioning protocols, note that only simple combinations were examined in this study due to an excessive workload. More complicated combination of stimulations will be investigated in the future since change in parameters in mechanical and electrical simulation (e.g., various stretch level, voltage magnitude, stimulation frequency) will likely affect structural alignment, cell-scaffold interaction, and construct remodeling accordingly. Moreover, we have not observed the electrophysiological response in the engineered cardiac tissue constructs using a microelectrode array technique developed for *in vitro*

measurement.⁵⁹ The lack of electrophysiological response is likely caused by the still low cell density (compared with native tissue) and lack of cell-to-cell connections both locally and globally in our current tissue constructs. In short, future investigation is warranted to identify the optimal reseeded and bioreactor conditioning protocols in order to produce functional cardiac tissue. Lastly, a future study will assess the potential of the engineered construct in cardiac repair/regeneration using a well-established rat model.⁶⁰

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References

1. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation*. 2012; 125(1):e2–e220. [PubMed: 22179539]
2. Raab W. The Nonvascular Metabolic Myocardial Vulnerability Factor in "Coronary Heart Disease". *Fundamentals of Pathogenesis, Treatment, and Prevention*. *Am Heart J*. 1963; 66:685–706. [PubMed: 14083792]
3. Baroldi G. Coronary heart disease: significance of the morphologic lesions. *Am Heart J*. 1973; 85(1):1–5. [PubMed: 4682003]
4. Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW, Khaw BA. Apoptosis in myocytes in end-stage heart failure. *N Engl J Med*. 1996; 335(16):1182–9. [PubMed: 8815940]
5. Cleutjens JP, Blankesteijn WM, Daemen MJ, Smits JF. The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. *Cardiovasc Res*. 1999; 44(2):232–41. [PubMed: 10690298]
6. De Celle T, Cleutjens JP, Blankesteijn WM, Debets JJ, Smits JF, Janssen BJ. Long-term structural and functional consequences of cardiac ischaemia-reperfusion injury in vivo in mice. *Exp Physiol*. 2004; 89(5):605–15. [PubMed: 15258119]
7. Cooper DK. Donor heart resuscitation and storage. *Surg Gynecol Obstet*. 1975; 140(4):621–31. [PubMed: 1093270]
8. Topol EJ. Current status and future prospects for acute myocardial infarction therapy. *Circulation*. 2003; 108(16 Suppl 1):III6–13. [PubMed: 14605014]
9. Laflamme MA, Zbinden S, Epstein SE, Murry CE. Cell-based therapy for myocardial ischemia and infarction: pathophysiological mechanisms. *Annu Rev Pathol*. 2007; 2:307–39. [PubMed: 18039102]
10. Sharma R, Raghur R. Stem cell therapy: a hope for dying hearts. *Stem Cells Dev*. 2007; 16(4): 517–36. [PubMed: 17784827]
11. Fujimoto KL, Tobita K, Merryman WD, Guan J, Momoi N, Stolz DB, Sacks MS, Keller BB, Wagner WR. An elastic, biodegradable cardiac patch induces contractile smooth muscle and improves cardiac remodeling and function in subacute myocardial infarction. *J Am Coll Cardiol*. 2007; 49(23):2292–300. [PubMed: 17560295]
12. Leor J, Aboulafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y, Cohen S. Bioengineered cardiac grafts: A new approach to repair the infarcted myocardium? *Circulation*. 2000; 102(19 Suppl 3):III56–61. [PubMed: 11082363]
13. Langer R, Vacanti JP. *Tissue Engineering*. *Science*. 1993; 260:920–926. [PubMed: 8493529]

14. Zimmermann WH, Melnychenko I, Eschenhagen T. Engineered heart tissue for regeneration of diseased hearts. *Biomaterials*. 2004; 25(9):1639–47. [PubMed: 14697865]
15. Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G, Freed LE. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. *Am J Physiol*. 1999; 277(2 Pt 2):H433–44. [PubMed: 10444466]
16. Orlic D, Hill JM, Arai AE. Stem cells for myocardial regeneration. *Circ Res*. 2002; 91(12):1092–102. [PubMed: 12480809]
17. Giraud MN, Armbruster C, Carrel T, Tevæearai HT. Current state of the art in myocardial tissue engineering. *Tissue Eng*. 2007; 13(8):1825–36. [PubMed: 17518754]
18. Akhyari P, Kamiya H, Haverich A, Karck M, Lichtenberg A. Myocardial tissue engineering: the extracellular matrix. *Eur J Cardiothorac Surg*. 2008; 34(2):229–41. [PubMed: 18502661]
19. Zimmermann WH, Didie M, Doker S, Melnychenko I, Naito H, Rogge C, Tiburcy M, Eschenhagen T. Heart muscle engineering: an update on cardiac muscle replacement therapy. *Cardiovasc Res*. 2006; 71(3):419–29. [PubMed: 16697358]
20. Zhang S, Crow JA, Yang X, Chen J, Borazjani A, Mullins KB, Chen W, Cooper RC, McLaughlin RM, Liao J. The correlation of 3D DT-MRI fiber disruption with structural and mechanical degeneration in porcine myocardium. *Ann Biomed Eng*. 2010; 38(10):3084–95. [PubMed: 20499182]
21. Walker CA, Spinale FG. The structure and function of the cardiac myocyte: a review of fundamental concepts. *J Thorac Cardiovasc Surg*. 1999; 118(2):375–82. [PubMed: 10425017]
22. Weber KT. Cardiac interstitium in health and disease: the fibrillar collagen network. *J Am Coll Cardiol*. 1989; 13(7):1637–52. [PubMed: 2656824]
23. Holmes JW, Borg TK, Covell JW. Structure and mechanics of healing myocardial infarcts. *Annu Rev Biomed Eng*. 2005; 7:223–53. [PubMed: 16004571]
24. Humphery, JD. *Cardiovascular Solid Mechanics*. Springer Verlag; 2002.
25. Wall ST, Walker JC, Healy KE, Ratcliffe MB, Guccione JM. Theoretical impact of the injection of material into the myocardium: a finite element model simulation. *Circulation*. 2006; 114(24):2627–35. [PubMed: 17130342]
26. Chen QZ, Bismarck A, Hansen U, Junaid S, Tran MQ, Harding SE, Ali NN, Boccaccini AR. Characterisation of a soft elastomer poly(glycerol sebacate) designed to match the mechanical properties of myocardial tissue. *Biomaterials*. 2008; 29(1):47–57. [PubMed: 17915309]
27. Simpson D, Liu H, Fan TH, Nerem R, Dudley SC Jr. A tissue engineering approach to progenitor cell delivery results in significant cell engraftment and improved myocardial remodeling. *Stem Cells*. 2007; 25(9):2350–7. [PubMed: 17525236]
28. Radisic M, Park H, Gerecht S, Cannizzaro C, Langer R, Vunjak-Novakovic G. Biomimetic approach to cardiac tissue engineering. *Philos Trans R Soc Lond B Biol Sci*. 2007; 362(1484):1357–68. [PubMed: 17594967]
29. Kellar RS, Shepherd BR, Larson DF, Naughton GK, Williams SK. Cardiac patch constructed from human fibroblasts attenuates reduction in cardiac function after acute infarct. *Tissue Eng*. 2005; 11(11-12):1678–87. [PubMed: 16411813]
30. Schachinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C, Abolmaali ND, Vogl TJ, Hofmann WK, Martin H, Dimmeler S, Zeiher AM. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. *J Am Coll Cardiol*. 2004; 44(8):1690–9. [PubMed: 15489105]
31. McSpadden LC, Kirkton RD, Bursac N. Electrotonic loading of anisotropic cardiac monolayers by unexcitable cells depends on connexin type and expression level. *Am J Physiol Cell Physiol*. 2009; 297(2):C339–51. [PubMed: 19494239]
32. Bian W, Liao B, Badie N, Bursac N. Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues. *Nat Protoc*. 2009; 4(10):1522–34. [PubMed: 19798085]
33. Bursac N, Loo Y, Leong K, Tung L. Novel anisotropic engineered cardiac tissues: studies of electrical propagation. *Biochem Biophys Res Commun*. 2007; 361(4):847–53. [PubMed: 17689494]
34. Zimmermann WH, Eschenhagen T. Cardiac tissue engineering for replacement therapy. *Heart Fail Rev*. 2003; 8(3):259–69. [PubMed: 12878835]

35. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, Taylor DA. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med.* 2008; 14(2):213–21. [PubMed: 18193059]
36. Badylak SF, Taylor D, Uygun K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Annu Rev Biomed Eng.* 2011; 13:27–53. [PubMed: 21417722]
37. Wainwright JM, Czajka CA, Patel UB, Freytes DO, Tobita K, Gilbert TW, Badylak SF. Preparation of cardiac extracellular matrix from an intact porcine heart. *Tissue Eng Part C Methods.* 16(3):525–32. [PubMed: 19702513]
38. Wang B, Borazjani A, Tahai M, Curry AL, Simionescu DT, Guan J, To F, Elder SH, Liao J. Fabrication of cardiac patch with decellularized porcine myocardial scaffold and bone marrow mononuclear cells. *J Biomed Mater Res A.* 2010; 94(4):1100–10. [PubMed: 20694977]
39. Wang B, Tedder ME, Perez CE, Wang G, de Jongh Curry AL, To F, Elder SH, Williams LN, Simionescu DT, Liao J. Structural and biomechanical characterizations of porcine myocardial extracellular matrix. *J Mater Sci Mater Med.* 2012; 23(8):1835–47. [PubMed: 22584822]
40. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, Glower DD, Kraus WE. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med.* 1998; 4(8):929–33. [PubMed: 9701245]
41. Godier-Furnemont AF, Martens TP, Koeckert MS, Wan L, Parks J, Arai K, Zhang G, Hudson B, Homma S, Vunjak-Novakovic G. Composite scaffold provides a cell delivery platform for cardiovascular repair. *Proc Natl Acad Sci U S A.* 108(19):7974–9. [PubMed: 21508321]
42. Vogel V, Sheetz M. Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol.* 2006; 7(4):265–75. [PubMed: 16607289]
43. Zhuang J, Yamada KA, Saffitz JE, Kleber AG. Pulsatile stretch remodels cell-to-cell communication in cultured myocytes. *Circ Res.* 2000; 87(4):316–22. [PubMed: 10948066]
44. Yamada K, Green KG, Samarel AM, Saffitz JE. Distinct pathways regulate expression of cardiac electrical and mechanical junction proteins in response to stretch. *Circ Res.* 2005; 97(4):346–53. [PubMed: 16037569]
45. Shanker AJ, Yamada K, Green KG, Yamada KA, Saffitz JE. Matrix-protein-specific regulation of Cx43 expression in cardiac myocytes subjected to mechanical load. *Circ Res.* 2005; 96(5):558–66. [PubMed: 15705967]
46. Zimmermann WH, Melnychenko I, Wasmeier G, Didie M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat Med.* 2006; 12(4):452–8. [PubMed: 16582915]
47. Radisic M, Euloth M, Yang L, Langer R, Freed LE, Vunjak-Novakovic G. High-density seeding of myocyte cells for cardiac tissue engineering. *Biotechnol Bioeng.* 2003; 82(4):403–14. [PubMed: 12632397]
48. Radisic M, Yang L, Boublik J, Cohen RJ, Langer R, Freed LE, Vunjak-Novakovic G. Medium perfusion enables engineering of compact and contractile cardiac tissue. *Am J Physiol Heart Circ Physiol.* 2004; 286(2):H507–16. [PubMed: 14551059]
49. Grashow JS, Yoganathan AP, Sacks MS. Biaxial stress-stretch behavior of the mitral valve anterior leaflet at physiologic strain rates. *Ann Biomed Eng.* 2006; 34(2):315–25. [PubMed: 16450193]
50. Liao J, Joyce EM, Sacks MS. Effects of decellularization on the mechanical and structural properties of the porcine aortic valve leaflet. *Biomaterials.* 2008; 29(8):1065–74. [PubMed: 18096223]
51. Rubbens MP, Driessen-Mol A, Boerboom RA, Koppert MM, van Assen HC, TerHaar Romeny BM, Baaijens FP, Bouten CV. Quantification of the temporal evolution of collagen orientation in mechanically conditioned engineered cardiovascular tissues. *Ann Biomed Eng.* 2009; 37(7):1263–72. [PubMed: 19415496]
52. Akhyari P, Fedak PW, Weisel RD, Lee TY, Verma S, Mickle DA, Li RK. Mechanical stretch regimen enhances the formation of bioengineered autologous cardiac muscle grafts. *Circulation.* 2002; 106(12 Suppl 1):I137–42. [PubMed: 12354723]

53. Fink C, Ergun S, Kralisch D, Remmers U, Weil J, Eschenhagen T. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. *FASEB J*. 2000; 14(5):669–79. [PubMed: 10744624]
54. Huang G, Pashmforoush M, Chung B, Saxon LA. The role of cardiac electrophysiology in myocardial regenerative stem cell therapy. *J Cardiovasc Transl Res*. 4(1):61–5. [PubMed: 21128127]
55. Wikswo JP Jr, Lin SF, Abbas RA. Virtual electrodes in cardiac tissue: a common mechanism for anodal and cathodal stimulation. *Biophys J*. 1995; 69(6):2195–210. [PubMed: 8599628]
56. McDonough PM, Glembotski CC. Induction of atrial natriuretic factor and myosin light chain-2 gene expression in cultured ventricular myocytes by electrical stimulation of contraction. *J Biol Chem*. 1992; 267(17):11665–8. [PubMed: 1376309]
57. Akar FG, Spragg DD, Tunin RS, Kass DA, Tomaselli GF. Mechanisms underlying conduction slowing and arrhythmogenesis in nonischemic dilated cardiomyopathy. *Circ Res*. 2004; 95(7): 717–25. [PubMed: 15345654]
58. Kostin S, Dammer S, Hein S, Klovekorn WP, Bauer EP, Schaper J. Connexin 43 expression and distribution in compensated and decompensated cardiac hypertrophy in patients with aortic stenosis. *Cardiovasc Res*. 2004; 62(2):426–36. [PubMed: 15094362]
59. Villa E, White AS, Liao J, de Jongh Curry AL. An inexpensive alternative bath system for electrophysiological characterization of isolated cardiac tissue. *Conf Proc IEEE Eng Med Biol Soc*. 2011; 2011:8424–7. [PubMed: 22256302]
60. Patten RD, Hall-Porter MR. Small animal models of heart failure: development of novel therapies, past and present. *Circ Heart Fail*. 2009; 2(2):138–44. [PubMed: 19808329]

Figure 1.

(a) Schematic illustration of the engineered scaffold subjected to cell injection, mechanical, and electrical stimulations; (b) Wave forms of the applied mechanical stretch and electrical pulses; (c) The bioreactor placed in the incubator; (d) User interface of the custom written LabView program.

Figure 2.

(a) Native (left) and decellularized (right) porcine myocardial scaffold; (b) Mason's trichrome staining of the native porcine myocardium; (c) Mason's trichrome staining of the decellularized myocardial scaffold showed well preserved cardiomyocyte lacunae. Note - red: cardiomyocytes, blue: collagen.

Figure 3.

(a) H&E and (b) Live/Dead staining of the static control group (group (i)); (c) H&E and (d) Live/Dead staining of the 20% strain stimulation group (group (ii)); (e) H&E and (f) Live/Dead staining of the 5 V electrical stimulation group (group (iii)); (g) H&E and (h) Live/Dead staining of the 20% strain + 5 V stimulation group (group (iv)). Images were taken from the constructs after 2-day's tissue culture.

Figure 4. Mason's trichrome staining of tissue constructs of group (iv) after 1-day bioreactor conditioning (a); after 2-day bioreactor conditioning (b); and after 4-day bioreactor conditioning (c). Group (iv) delivers 20% strain + 5 Volt stimulations.

Figure 5. Immunofluorescence staining of tissue constructs after 2-day bioreactor conditioning (group (iv): 20% strain + 5 V). The reseeded cells exhibited cardiomyocyte-like phenotype by expressing (a) myosin heavy chain (MHC), (b) sarcomeric α -actinin, and (c) troponin T; electrical gap junction protein and adherens junction protein were verified by (d) connexin-43 and (e) N-cadherin staining.

Figure 6.

Biaxial mechanical behavior: (a) native myocardium, (b) acellular myocardial scaffolds, (c) 2-day static control (group (i)), (d) 4-day static control (group (i)), (e) 2-day *in vitro* condition (group (iv): 20% strain + 5 V), and (f) 4-day *in vitro* condition (group (iv): 20% strain + 5 V).

Table 1

Cell density and ratio of living cells of the static control group (group (i)), 20% strain stimulation group (group (ii)), 5 V electrical stimulation group (group (iii)), and 20% strain + 5 V stimulation group (group (iv)) after 2-day *in vitro* culture.

	Groups (i)	Groups (ii)	Groups (iii)	Groups (iv)
Reseeded cell density (cells/mm ²)	0.91×10^2 #	2.21×10^2	2.37×10^2	3.28×10^2 *
Ratio of living cells	51.43% #	80.35%	80.98%	79.38%

* denotes significant difference when comparing group (iv) with group (iii), group (ii), or group (i) ($p < 0.05$, pair-wise comparison using Holm-Sidak method);

# denotes significant difference when comparing group (i) with group (ii), group (iii), or group (iv) ($p < 0.05$, pair-wise comparison using Holm-Sidak method).

Table 2

Biaxial mechanical properties of the native myocardium, acellular myocardial scaffolds, and tissue constructs of group (i) and (iv) after 2-day and 4-day culture.

	Native	Acellular	Group (i)		Group (iv)	
			2-Day	4-Day	2-Day	4-Day
Maximum Stretch (PD)	1.062±0.004	1.024±0.008 *	1.080± 0.008	1.131± 0.015 *	1.115± 0.007	1.065± 0.006
Maximum Stretch (XD)	1.293±0.012	1.102±0.025 *	1.154± 0.006 *	1.186± 0.030 *	1.203± 0.012 *	1.254± 0.021

* denotes significant difference when compared with the native myocardium (p < 0.05).