Effects of Uniaxial Cyclic Stretch Loading on Morphology of Adipose Derived Stem Cells

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Adipose derived stem cells (ADSC) are good candidates for the replacement of bone marrow derived mesenchymal stem cells due to their abundance, multipotency property, and easier accessibility. In order to explore the behavior of these cells in response to mechanical stimulation, in this study we have investigated the effects of uniaxial dynamic mechanical loading on ADSC's morphology. Stem cells derived from the fat tissue of human and after an overnight culture were seeded on a silicone rubber strips. Afterwards, cells were subjected to a uniaxial dynamic loading in three different groups. Cell images were evaluated considering different morphological parameters. Fractal dimension decreased significantly after loading while in control groups there were a significant increase ($p < 0.05$), approving that cyclic strain would lead to more aligned and organized cells. Cell orientation also increased significantly ($p < 0.05$). Moreover cells' orientation angle, 24 hour after loading does not change compared to the observations immediately after loading, which attests to the practicality of the cyclic strain in functional tissue engineering. Cell width decreased and cell length increased which led to a significant increase in cell shape index ($p < 0.05$). Results confirmed that uniaxial dynamic loading affects cell morphological parameters comparing their values before and after loading. In addition, the number of cycles are also an important factor since different number of cycles lead to different amounts of certain morphological parameters. Conclusively, cyclic strain can be a practical method in the field of functional tissue engineering.

Tissue Eng Regen Med 2016;13(4):396-402

Key Words: Adipose derived stem cell; Cyclic uniaxial mechanical loading; Cell morphology; Tissue engineering

INTRODUCTION

Tissue engineering is an emerging field of study which holds great promises for the medical issues. It is based on the usage of either stem cells or biological products to treat or replace the defected body parts [1]. Many studies in the field of tissue engineering consider the biochemical cues as the deciding factor of the stem cells fate; however, there are some studies showing the crucial effects of the mechanical loading on the cell proliferation and differentiation path [2]. Developing new treatments using the stem cells requires more insight into their isolation, proliferation, and differentiation and factors affecting them.

Stem cells are classified into several categories that each one of them is isolated from different body parts and their differ-

entiation capacity and characteristics differ significantly. Bone marrow derived mesenchymal stem cells (BM-MSC) has been the most common source of stem cells for studies while there are certain obstacles to the medical usage of these stem cells, namely the number of available cells per volume of bone marrow, pain during the aspiration, and associated morbidity. Adipose-derived stem cells (ADSCs) are easier to isolate, available in bigger amounts (500 times more stem cells in the 1 g of adipose tissue than MSCs in one g of bone marrow) and have been shown to have useful differentiation potentials [3-5]. ADSCs have shown adipogenic, osteogenic, myogenic, and neurogenic potentials [6-8]. Fischer et al. [9] showed that human ADSCs are capable of showing human endothelial cells' characteristics after differentiation under physiological shear stress and vascular endothelial growth factor, thus ADSCs have been the subject of interest as a substitute for MSCs as autologous stem cell for tissue engineering [10].

The cells in human body are always under mechanical loading. Endothelial cells are exposed to the blood flow induced

Received: June 15, 2015

Revised: September 16, 2015

Accepted: October 5, 2015

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shear stress and blood pressure induced tensile strain. Arterial smooth muscle cells are stretched periodically because of the pulsatile blood pressure. This mechanical loading is critical for the development and functionality of the cells. It is well documented that alongside the biochemical factors, mechanical loading is critical for the differentiation, functionality, and phenotypic state of the stem cells *in vivo* [2,11]. This has been also shown in a recent study of ours concerning the functionality of bone under the effects of gravity loading on cells [12]. The results showed that gravity alteration could lead to morphology and proliferation changes in stem cells.

The cells *in vivo* have a specific morphology and are aligned due to the mechanical loading. For example, the endothelial cells under normal shear stress develop fusiform shapes and are aligned due to the flow whereas in the regions with low (<0.4 Pa) and oscillatory shear stress cells become randomly oriented with cobble-stone shape and high turn-over rates [13]. The effects of mechanical loading alongside the biochemical factors on the stem cell (both MSCs and ADSCs) proliferation, gene expression, and other factors that influence the cell's fate have been studied by many researchers [14-22]. Lee et al. [23] studied the effects of mechanical loading and transforming growth factor- β 1 on ADSC morphology using a 7 day long uniaxial cyclic strain.

Cell orientation angle and cell shape index for cells under mechanical loading show the impact of the mechanical loading on cytoskeleton. The effects of the mechanical loading without any biochemical factors on the cell morphology have not been explored thoroughly in the literature. Haghighipour et al. [24] studied the effects of different cyclic strain waveforms on the endothelial cells' morphology. Goli-Malekabadi et al. [25] showed the different impacts of the static and dynamic loading on MSCs' morphology. Based on the previous findings, cells under mechanical strain align in a direction that would lead to minimal deformation on the cytoskeleton [24,26,27] and the orientation angle is highly dependent on the amplitude, strain rate, and number of loading cycles [24].

To the best of our knowledge no one has studied the effects of different number of strain cycles and the following no-loading period on cell's morphology. In this study we evaluated the effects of uniaxial cyclic strain loading by various number of cycles with a following no-loading period on the morphology of ADSCs. The cell morphology under mechanical loading has been evaluated using parameters such as fractal dimension (FD), cell surface coverage (CSC), cell orientation angle, and cell shape index. Studying cells with utilizing these parameters provides us with a new perspective on the cell morphology and alignment due to mechanical loading which is very important considering the *in vivo* conditions of cells that is applicable for functional tissue engineering.

MATERIALS AND METHODS

Cell extraction and cell culture on the elastic strips

Adipose tissue is excised from patients during orthopedic knee surgery with informed consent considering ethical issues. ADSCs were extracted from adipose tissue according to recommended protocols by density gradient centrifugation [28]. The extracted tissue was rinsed in the digesting solution containing 2 mg/mL of type-I collagenase in phosphate buffer saline. Then, it was centrifuged at 1400 rpm for 5 minutes (Eppendorf, Hamburg, Germany). The supernatant solution containing adipose cells was incubated 30 minutes at 37°C for further digestion. Subsequently, the solution was centrifuged at 2000 rpm for 5 minutes and the remaining pellet was re-suspended and transferred to Glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 15% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin. After 24 hours, homogeneous ADSCs were attached while adipose cells were suspended and discarded.

Cells were characterized using flow cytometry by their stem cell-surface antigens. Cells were positive for CD105, CD166, CD90, and CD44 as stem cell markers and negative for CD34, and CD45 as hematopoietic markers. ADSCs express surface antigens similar to bone marrow-driven mesenchymal stem cells when they are maintained for extended periods as undifferentiated cells [29].

Cells were cultured in low-glucose DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, inside a CO₂ incubator at 37°C with 5% CO₂ and finally third passage of cells were used for mechanical stretch tests.

Strips with 0.25 mm thickness was made from medical grade silicone rubber (Wacker Chemie AG, Munich, Germany), utilized as the substrate of cell culture. Back of strip was marked with 16 small dots that gave us 9 squares with the same size. After sterilization, the elastic strip was coated with 5 μ g/cm² collagen type I [24]. ADSCs were cultured on the strip. After 24 hours overnight incubation, strip were assembled into the tensile device for exposure to cyclic stretch.

Mechanical stimulation

A custom made test apparatus (Fig. 1) was designed and manufactured for application of cyclic tension capable of operating inside an incubator with strain up to 25%, frequency range of 1-3 Hz and modifiable cycle numbers. The tensile device consists of electrical and mechanical units.

The electrical unit includes a power supply, stepper motor driver, the motor interface and programmable logic control. Data acquisition is performed using a computerized program.

Figure 1. The custom made stretch apparatus. (A) The schematic of the loading apparatus. (B) The cyclic stretch device.

The mechanical unit contains a step motor, ball screw, connector rod, an encoder, and mobile and fixed grippers for stretch of elastic membrane according to a programmed pulsatile algorithm. In this study, 10% strain with 1 Hz frequency has been applied on the strips.

Experimental procedures

Cells have been subjected to dynamic mechanical loading with three different numbers of cycles, 5000, 10000, and 15000 cycles. Each set with a specific cycle number consists of 3 pairs of test-control groups. Both test and control groups are the same in all conditions except mechanical loading that is applied just to the test group. In each pair, test group has been subjected to the mechanical loading whereas the control group has not been applied with mechanical loading. The cyclic loading is applied by utilizing above mentioned custom-made device to impose uniform tensile strain of 10% on cultured cells with the specific cycle numbers. A new digital image analysis method is designed to evaluate morphological changes of ADSCs.

Images and image processing

Cell images were obtained from test and control groups utilizing a digital camera (Sony, Tokyo, Japan) installed on a phase-contrast inverted optical microscope (Nikon, Tokyo, Japan) with 100 $\times$ magnification. Images were captured from marked area on the substrate before loading, immediately after loading and 24 hours after loading from both control and test groups. Four zones of each substrate were chosen for images to obtain a reliable image of the whole. The FD of surface covered by cells of ADSCs before, after and 24 hours after loading was examined using an image processing algorithm which was developed utilizing ImageJ program (v. 1.44a) [30]. An 8 bit format of the images of cells was prepared, the background was subtracted and specific filter was applied. Finally the binary mode was applied for morphological evaluations.

Defining morphological parameters

FD was calculated by using "box counting" method as it is adopted by most studies on FD analysis of cell morphology [31] and CSC was calculated using "measure" feature in the ImageJ [30].

Other parameters including cell alignment, length and width of cells were measured by a program that has been developed in MATLAB (The MathWorks Inc., Natick, MA, USA). Shape index is defined by the ratio of length to the width.

Statistical method

Statistical analysis was carried out to investigate differences of the parameters in test and control groups while data were presented in mean $\pm$ SD.

At last, the gathered data were statistically analyzed using one way ANOVA, Post Hoc test (Tukey HSD) and t-test. The significance level was set to 0.05; thus differences were considered statistically significant for *p*-values smaller than 0.05.

RESULTS

Effects of mechanical loading on ADSCs morphological parameters including FD, orientation, length, width, and shape index were studied by evaluation of cell images modes. All results and data in three cycle modes were categorized into 3 main subsets: data that has been gathered before loading (subset A), data gathered immediately after loading (subset B), and the data gathered 24 hours after loading (with a no-loading period after cyclic strain) (subset C). Subset A was chosen as the reference point, while subsets B and C are mostly evaluated relative to subset A. Figure 2 demonstrates cell morphology of control group in comparison to test group while exposed to 15000 loading cycles in all three subsets (before, after, and 24 hours after loading).

Fractal dimension

Applying cyclic stretch resulted in a decrease in FD of test

Figure 2. Cell culture images taken from control groups of 15000 cycle loading in left column (A, C, and E) and test groups in the right column (B, D, and F). (A) Before loading control group. (B) Before loading test group. (C) After loading control group. (D) After loading test group. (E) 24 hours after loading control group. (F) 24 hours after loading test group.

group images that has been taken immediately after loading (subset A) ($p < 0.05$). This decreased FD testifies to a more aligned and organized cells [24,32]. For test groups belonging to subset C, FD has been increased compared to the subset B, yet such rise in FD for all cycle numbers was statistically insignificant ($p > 0.05$) (Fig. 3A). This shows the significance of the mechanical loading for cell alignment and achieving a particular structure for desired cell morphology. In addition, after comparing the results from subsets A and C, there are still significant differences which means that the effects of cyclic mechanical loading will not wear off after 24 hours without any stimulus.

Cell surface coverage

The results demonstrate that CSC in all three cycle numbers has been decreased significantly by cyclic stretch ($p < 0.05$) which can be explained by the remodeling that happened to the cell shape due to the mechanical loading. Results for control group from subset B compared to subset A show no significant change in CSC which attests to the role of mechanical loading and related cell shape remodeling as CSC decreased in test groups.

CSC increased significantly 24 hours after loading (subset C) ($p < 0.05$) compared to subset B (Fig. 3B). Also, for control groups, there were statistically significant changes comparing sub-

Figure 3. Data from subset B (immediately after loading) and subset C (24 hours after loading) relative to subset A (before loading). (A) A significant decrease in the FD immediately after loading has been observed, yet no significant change can be observed comparing the subsets C and B. (B) CSC has been decreased in subset B while there is a significant increase in subset C. (C) A significant increase in the angle is observed immediately after loading which has been preserved (non-significant change) after 24 hours of non-loading period. (D) There is a significant increase in the cell shape index after loading but no changes comparing to subsets C (after 24 hours of non-loading). Note that test groups are indicated with small letters and control groups are indicated by capital letters. The figures for each parameter summarizes the comparison between group A, B, and C within each group of cycles and the control group. FD: fractal dimension, CSC: cell surface coverage.

sets B and C. This significant increase of CSC in both control and test groups from subset B and subset C may be the consequence of the cell proliferation regardless of loading period [32].

Cells orientation

Cell orientation has been significantly ($p < 0.05$) increased in all three test groups (5000, 10000, 15000 cycles). A semi-linear rise in orientation angle can be seen by increasing the number of cycles. Increase of loading cycles from 5000 to 10000 and 15000 cycles led to the orientation angles of 61.58°, 65.25°, and 68.71°, respectively, which means more alignment and larger orientation angle between cells and the uniaxial stretching direction (topological remodeling of cultured endothelial cells by characterized cyclic strain). In addition, cell orientation for test groups from subset C did not show significant changes compared to subset B (Fig. 3C), confirming that cell orientation is stable for this period of time without any mechanical stimulus. This indicates that cells which are oriented neither lose their orientation nor migrate except along the cell longitudinal axes. It seems that cells in test groups are less capable of

migration unless it is in the direction of cell longitudinal axis.

In contrast to test groups which were exposed to cyclic stretch, cell orientation shows unorganized cells with insignificant changes within three corresponding groups from subset A, B, and C. As seen in Figure 2, cells after their stretch are conditioned with unidirectional shape in contrast to cells in control group which spread in three or more directions with their lamellipodia.

This morphological changes are due to cytoskeleton remodeling in response to substrate cyclic deformation. Cytoskeleton remodeling is based on minimizing cytoskeleton deformation in response to the straining of underlying substrate [25,32]. This phenomenon could describe cell orientation along optimum angle that could be in correlation with Poisson's ratio of silicone substrate (0.35) [33,34]. Cell orientation changes during a remodeling process. Cells orient to make approximately a 60 degree angle with the uniaxial strain direction [33]. It seems that the remodeling process for a duration of time, changes the orientation of cells but finally we should expect a stable angle while cells are fully remodeled and compatible with environ-

mental loading [25].

Shape Index

Cells after cyclic stretch (subset B) were longer and thinner statistically compared to the cells before stretch (subset A) and cells 24 hours after stretch remained long and thin. This can also be seen roughly and qualitatively in Figure 2B and D for the cells loaded with 15000 cycles. Cell shape index significantly increased by mechanical stretch. Observations on cell shape index of ADSCs immediately after loading (subset B) has shown a significant ($p < 0.05$) change for all cyclic modes (5000, 1000, 15000 cycles), and alterations in the shape index of cells 24 hours after loading (subset C) was not significant ($p > 0.05$) compared to subset B.

Although cell width and length increased significantly after 24 hours without mechanical loading, cell shape index in subset B and subset C were similar with no significant changes ($p > 0.05$) (Fig. 3D). This evidence confirms that cell shape index could be stable for at least 24 hours after stretch. However, in control groups, there were no significant changes in cell width, length and shape index during or after the test period.

DISCUSSION

Cyclic stretch affects cell morphological parameters. This influence on cells are sometimes dependent on number of cycles since more cycles could lead to larger orientation angle of cell with respect to stretching direction. Also cells can preserve changes of cell orientation and shape index until 24 hours after loading. In other words, cyclic stretch is a proper method to align cells while the changes are preserved for a period of time. This evidence proves cyclic strain as a practical method in tissue engineering with the focus on oriented cells and development of functional tissue considering that the cyclic stretch could be interrupted for a period of time such as 24 hours without losing the cell orientation.

Cells grew during 24 hours without stretch and consequently their length and width changed but shape index had non-significant changes explaining almost a constant shape for cells independent of cells' size. In other words, cell growth and consequent increase in projection dimensions do not change shape indexes. This event shows that cyclic strain as a mechanical stimulus is useful for cell engineers who are interested in cells with specific shape and morphology. So cell growth after cyclic stretch is modulated and guided in a way that makes them in consistent proliferation and spreading.

Conclusively, cyclic stretch is an efficient method which prepares cells with specific shapes, in specific direction while these two characteristics could be maintained even after unloading

and this is convenient and interesting for *in-vitro* engineering of different functional tissues.

Although the cell culture substrate was loaded with a uniaxial stretching, the cultured cells were loaded biaxially considering the 2D deformation of the substrate. This is a good approximation of tissues such as muscle and skin.

Our study is based on the two dimensional cell culture yet the body tissue is three dimensional. The study of the morphological parameters of cells that are cultured on a 2D substrate can be a good start to study effects of cyclic stretch and the numbers of cycles on cells' morphological parameters. Future studies could consist of revalidating the results for the 3D tissues.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

Adipose tissue is excised from patients during orthopedic knee surgery with informed consent considering ethical issues.

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