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Effects of hypergravity on adipose-derived stem cell morphology, mechanical property and proliferation

Alireza Tavakolinejad [a], Mohsen Rabbani [b, *], Mohsen Janmaleki [a]

[a] Medical Nanotechnology and Tissue Engineering Research Center, Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Biomedical Engineering, University of Isfahan, Isfahan, Iran

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ABSTRACT

Alteration in specific inertial conditions can lead to changes in morphology, proliferation, mechanical properties and cytoskeleton of cells. In this report, the effects of hypergravity on morphology of Adipose-Derived Stem Cells (ADSCs) are indicated. ADSCs were repeatedly exposed to discontinuous hypergravity conditions of 10 g, 20 g, 40 g and 60 g by utilizing centrifuge (three times of 20 min exposure, with an interval of 40 min at 1 g). Cell morphology in terms of length, width and cell elongation index and cytoskeleton of actin filaments and microtubules were analyzed by image processing. Consistent changes observed in cell elongation index as morphological change. Moreover, cell proliferation was assessed and mechanical properties of cells in case of elastic modulus of cells were evaluated by Atomic Force Microscopy. Increase in proliferation and decrease in elastic modulus of cells are further results of this study. Staining ADSC was done to show changes in cytoskeleton of the cells associated to hypergravity condition specifically in microfilament and microtubule components. After exposing to hypergravity, significant changes were observed in microfilaments and microtubule density as components of cytoskeleton. It was concluded that there could be a relationship between changes in morphology and MFs as the main component of the cells.

1. Introduction

Being exposed to mechanical stimulations affects different cells in terms of cell signaling and leads to the appearance of different behaviors in cells [1]. Cell mechanical stimulations include mechanical stretch, compression, hydrostatic pressure, microgravity and hypergravity. Microgravity is the absence of gravity usually exists in spaceflights to different planets or moons. Hypergravity against microgravity refers to conditions that have gravity force more than gravity of the earth that could be experienced by living cells in some planets and during human high accelerated flights. In some studies, effects of hypergravity on cell behaviors such as proliferation [2], gene expression [3], differentiation [4], cytoskeleton reorganization, adhesion and motility [5], and morphology were evaluated.

F-actins or microfilaments actin (MFs) and microtubule (MTs) are the main subunits of the structural components of the cytoskeleton. Beneath the cell membrane is where MFs are most concentrated [6]. Resisting tension and maintaining cellular shape are what MFs in addition to MTs are responsible for. MTs represent platforms for intracellular transport and are participate in a variety of cellular processes.

There have been, to date, a number of techniques introduced that allow for both quantitative and qualitative measurements of cellular mechanical properties. The techniques comprise of two major approaches, one of which is regarding the probe of only small parts of cells that allows for a quantitative analysis. This approach depends significantly upon the measurement location for obtaining its results. An atomic force microscope (AFM) is the most applicable device within this category. On the other hand, the second method investigates cell as a whole body. Micropipette aspiration (MA) is the most feasible and convenient ones in this category. Both methods have been employed frequently to evaluate mechanical features of cells [7].

In this study, the effects of hypergravity on the elongation and the reorganization of the human adipose-derived stem cell (hADSC) were evaluated. Furthermore, cell proliferation and mechanical properties of these cells were assessed. To the best of our

* Corresponding author.
E-mail address: m.rabbani@eng.ui.ac.ir (M. Rabbani).

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knowledge, this method for morphology assessment of hypergravity on cells is considered as a novel method.

2. Materials and methods

2.1. Cell culture

ADSCs were isolated from adipose tissue according to recommended protocols [8] in which some under skin adipose tissue was removed during orthopedic surgery and washed in sterile phosphate buffer saline (PBS). Next, it was cut in pieces and type-I collagenase was added to facilitate lipid digestion. The indigested tissue was removed after centrifuging. The obtained ADSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM-Gibco, USA) supplemented with 10% Fetal Bovine Serum (Gibco, USA) and 1% Penicillin-Streptomycin (Gibco, USA). Cells were cultured in an incubator of 37 °C in a humidified atmosphere containing 5% CO_2. When cells reached considerable confluence, they were trypsinized and divided for evaluation into two categories of test and control group.

2.2. Hypergravity exposure

Both test and control samples were cultured in 12-well plates for morphology test and cultured in 4 cm wells in diameter for cell staining assay and elastic modulus analysis at a density of 5000 cell/cm^2. In case of cell proliferation, cells were cultured in 96-wellplates to measure optical absorbance in microplate reader (DiaMedEuroGen). Half an hour after cell seeding, test group cells were exposed three times to hypergravity condition with duration of 20 min. and an interval of 40 min at controlled conditions after the first and second exposure. A centrifuge (Eppendorf, Germany) was utilized to apply hypergravity of 10 g, 20 g, 40 g, and 60 g on cultured cells. After the last exposure, both test and control groups were incubated overnight, and were evaluated 24 h after seeding.

2.3. Morphology assessment

In order to assess morphology alterations of cells, digital image processing algorithms were applied. Images were captured by a Sony digital camera (Coolpix, Japan) TE200U assembled on an inverted microscope (Nikon, Japan) and the images were processed for measurement of length, width and cell elongation index (CEI) utilizing a custom made program in Image Processing Toolbox of MATLAB (MathWorks Software, USA). CEI is defined as the ratio of the cell length to its width. Five images from each test group were analyzed. From each image approximately 7 cells which were not near to other cells were chosen in order to ignore the accidental effects of other cells. By this software, 4 points of each selected cell were chosen which the distance between first two points indicate the length of cell and the distance between second two points measure the width of cells in pixel.

This experiment was done 3 times and about 100 cells of these tests were selected for morphology assessment. Finally the length, width and CEI are defined as morphological parameters.

2.4. Cytoskeleton assessment

To observe cytoskeletal changes due to hypergravity experiences, MFs and MTs of cell were stained. Cells were washed with PBS and fixed with 4% para-formaldehyde for 15 min followed by washing with ice cold PBS. Afterward, cells became permeable with 0.25% Triton X-100 for 10 min. After rewashing with PBS, non-specific binding was blocked with 1% bovine serum albumin for 30 min. Cells were incubated in turn with 10% Phalloidin (Sigma P5282) for MFs staining; and for 40 min afterward with monoclonal anti-β-tubulin-Cy3 (Sigma C4585) with the aim of MTs staining. Finally, cells were washed with PBS three times, each one 5 min, and images of MFs and MTs were captured with an inverted fluorescent microscope (Olympus, BX51 with DP72 camera), and were processed with Image J software.

Corrected Total Cell Fluorescence (CTCF) is a criterion of fluorescence level measured with the following formula [7,9]:

$$\begin{aligned}\mathrm{CTCF} = {} & \textit{integrated density of pixel for one cell} \\ & - (\textit{area of the selected cell} \\ & \times \textit{mean fluorescence of background}).\end{aligned}$$

CTCFs were calculated for actin filaments and β-tubulins distinctively of 21 cells of both test and control group. Alteration of cytoskeleton elements in hypergravity exposure is indicated as relative fluorescent of actin filaments to microtubules (RFAM) per cell according to the following equation:

$$\mathrm{RFAM} = \frac{\mathrm{CTCF\ for\ actin\ filaments}}{\mathrm{CTCF\ \ for\ \beta - tubulins}}$$

2.5. Cell mechanical properties

Here, AFM indentation was measured to characterize the elasticity of ADSCs after mechanical loading of hypergravity. After hypergravity exposure, cells were washed with PBS and fixed in glutaraldehyde 0.5% for 1 min and then washed 3 times in PBS, each time 5 min and eventually washed with deionized water.

Afterward, the cells in each group were scanned by AFM system (DME, Denmark) and cell morphology and elastic modulus of cells were evaluated by force–distance curve obtained by DDM software provided by DME Company. For estimating elastic modulus of cells, 3 or 4 cells from each group were selected; then, 4 or 5 points near the core of each cell were chosen to evaluate Young's modulus. For this evaluation, the test was done 4 times. All in all 16 cells from each group were chosen to measure Elastic modulus. Young's modulus of cells was calculated by syntax written in MATLAB according to Hertz mechanical model as up to now lots of research which has been done on Young's modulus of cells was based on Hertz model.

The force on cantilever $F(h)$ is according to the following formula if the tip of AFM's cantilever is somehow spherical in shape with the radius of R:

$$F(h) = \frac{4\sqrt{R}}{3}E^{*}h^{3/2}$$

In which h is the amount of the indentation, E^{*} the effective modulus of a system tip -sample and calculated from the equation below:

$$\frac{1}{E^{*}} = \frac{1-\nu_{tip}^{2}}{E_{tip}} + \frac{1-\nu_{sample}^{2}}{E_{sample}}$$

where E_{tip}, ν_{tip} and E_{sample}, ν_{sample} are Young's modulus and the Poisson's ratio for the materials of cantilever's tip and the sample, respectively [10].

Moreover, cells height distribution was analyzed in both test and control groups using DDM software.

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