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

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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

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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.

2.6. Proliferation assessment

To evaluate the proliferation rate of cells, they were cultured in 96-well plate and after exposure to the hypergravity, cells were incubated for 2 h in MTT (a yellow tetrazole). The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan. Afterward, the cells were shaken 20 min in DMSO (Dimethyl Sulfoxide) to get the purple color. The absorbance of this colored solution can be quantified by measuring at 550–600 nm wavelength by a micro-plate reader. In this assessment, the greater the absorption is the more proliferation occurred. This assessment was done 4 times on each groups.

2.7. Statistical analysis

At last, statistical analysis of these data was performed using One Way ANOVA and Post Hoc test (Tukey HSD) in SPSS software and T-Test in Excel software with significance of set at p-value < 0.05.

3. Results

3.1. Cell morphology

An ANOVA test demonstrated that in contrast to cell length and width ($P > 0.5$), CEI is a consistent parameter in all control groups ($p = 0.299$). Comparisons of CEI in test groups to their control groups showed significant increase ($p < 0.05$). In other words, all hypergravity test conditions (10 g, 20 g, 40 g and 60 g) caused cells to be more slender ($(CEI_t)/(CEI_c) > 1$).

Fig. 1(A) illustrates the ratio of CEI_t to CEI_c for all test groups, which are 1.27, 1.30, 1.39 and 1.27 corresponding to hypergravity of 10 g, 20 g, 40 g and 60 g, respectively. Morphology alteration is observable in Fig. 2 containing images taken from tests groups and theirs related control groups.

3.2. Cytoskeleton alterations

It is implied from the calculated value of CTCFs that relative fluorescence of MFs in different level of hypergravity decreased compared with control conditions; although, this trend is not significantly observed in MTs of cells except in 40 g and 60 g tests as shown in Fig. 1(B) and (C).

The ratio of RFAM of test group cells to RFAM of control group cells is indicated in Fig. 1(D). Slight reduction can be seen in all groups except 40 g group.

As it is demonstrated in Fig. 3, the MFs content of cells after hypergravity are less and more slender than control group. It seems that MFs area and width are dependent on the intensity of gravity that cells are exposed to. In other words, the more gravity level the cells exposed to, the more area and concentration decreased.

As shown in this part, there seems to be a correlation between the results of cytoskeleton changes in case of MFs and morphology results.

3.3. Mechanical properties

Cells were exposed to hypergravity and probed by AFM, the differences observed in morphological changes of 60 g group were shown Fig. S1 (images for 10 g, 20 g, 40 g are not shown). Moreover, there is a decrease in elastic modulus of cells after hypergravity loading (Fig. 4A). In addition to data for mechanical properties, the height of cells was measured by AFM. So, significant increase was observed in height of cells in all test groups in comparison to the control group (Fig. 4B).

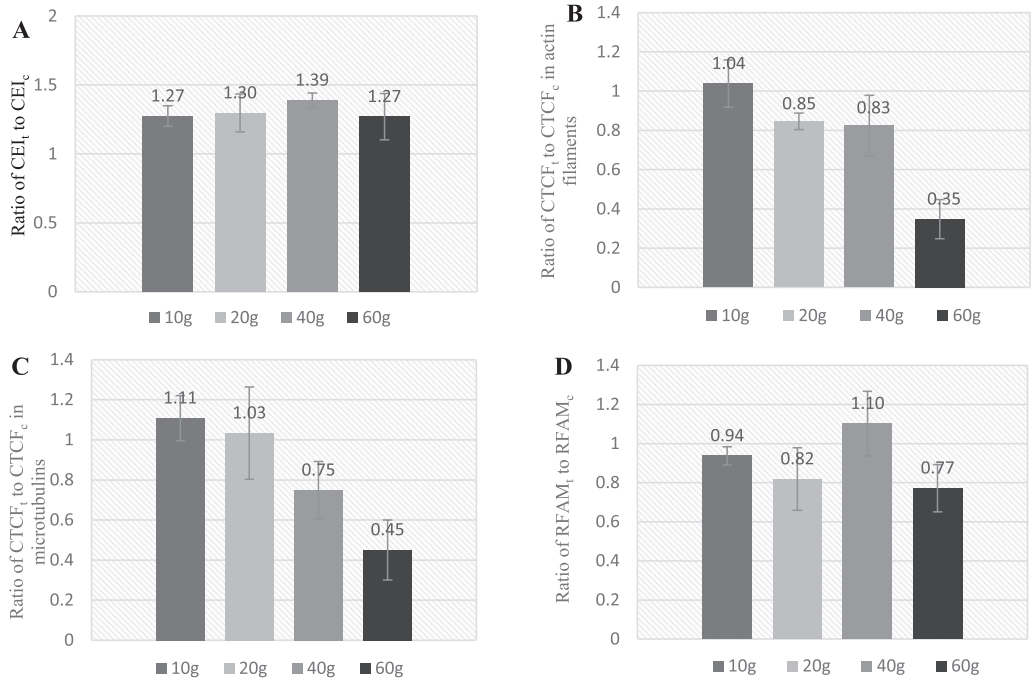

Fig. 1. Ratio of CEI_t to CEI_c (A), Relative decline in CTCF of MFs of test groups to CTCF of their control groups especially observable in 20 g, 40 g, 60 g groups (B), Ratio of CTCF of MTs of test groups to CTCF of their control groups. No significant changes observed in this case except in 40 g and 60 g (C), Reduction of RFAM of test groups to theirs control groups except in 40 g group (D).

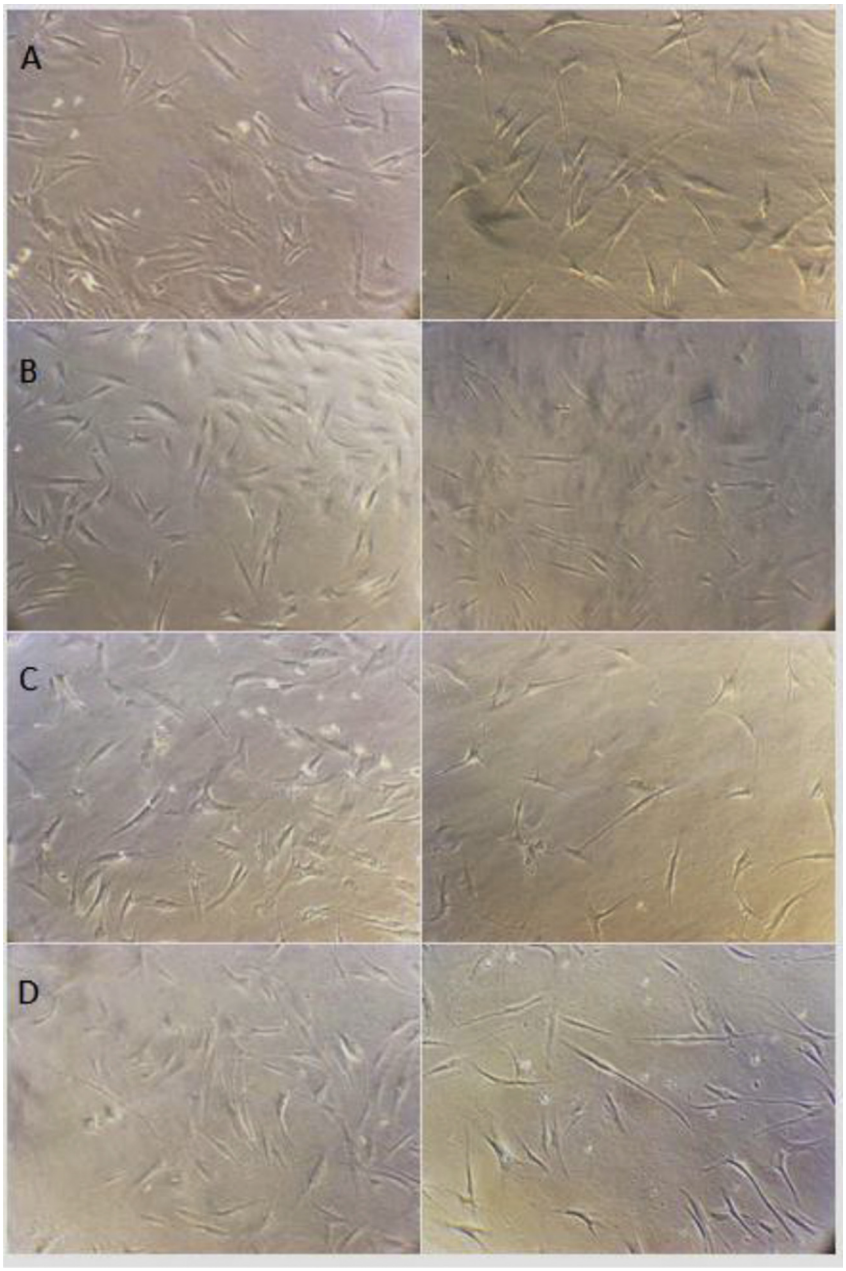

Fig. 2. (Left) cells in control groups and (right) cells in test group (A:10 g, B:20 g, C:40 g, D: 60 g). 24 h after hypergravity exposure, cell have a more slender shape.

3.4. Cell proliferation results

It is shown that hypergravity is a mechanical item that enhances the rate of proliferation in ADSCs (Fig. 4C). In all test groups cells proliferate more than the control group.

4. Discussion

The cells of the human body are continuously in contact with multiple stresses and thus respond appropriately to the mechanical stimulus. The results of the previous researches and the present study indicate a change in morphology and cell structural properties as a result of hypergravity. However, no single study has aimed to do tackle this issue; therefore, the present study dedicated its focus on the effects of hypergravity in different magnitudes on ADSC.

It seems though according to the significant results of the morphological changes, cells became slender. Also the significant results show an increase in the height of the cells in all the experimental groups. It could be concluded that cells became more slender and swollen as a result of hypergravity. However, the increase in height of the cells was in contrast with the results of Van Loon et al. (2009) which had focused on the change in cell height under hypergravity of 2–3 g. This could be due to the difference in cell type, the method of hypergravity implication and the hypergravity magnitude. For instance, gravity varies in the present study from 10 g to 60 g; although in Van Loon study there was an exposure of 2 g–3 g hypergravity [11].

Not much research has been done on the effect of hypergravity on cell morphology, but in a study, the size of Paramecium cells decreased in 20 g hypergravity. Meaning that there was a decrease in both length and width of the cell compared with the control

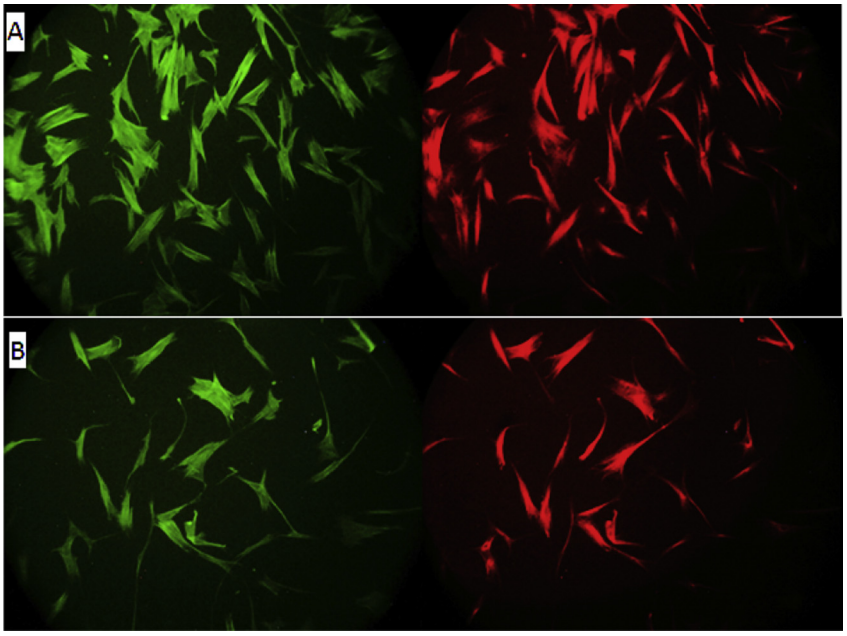

Fig. 3. MFs and MTs of cells under hypergravity test (10X), (A) control group, (B) test group, Left: stained MFs, Right: stained MTs.

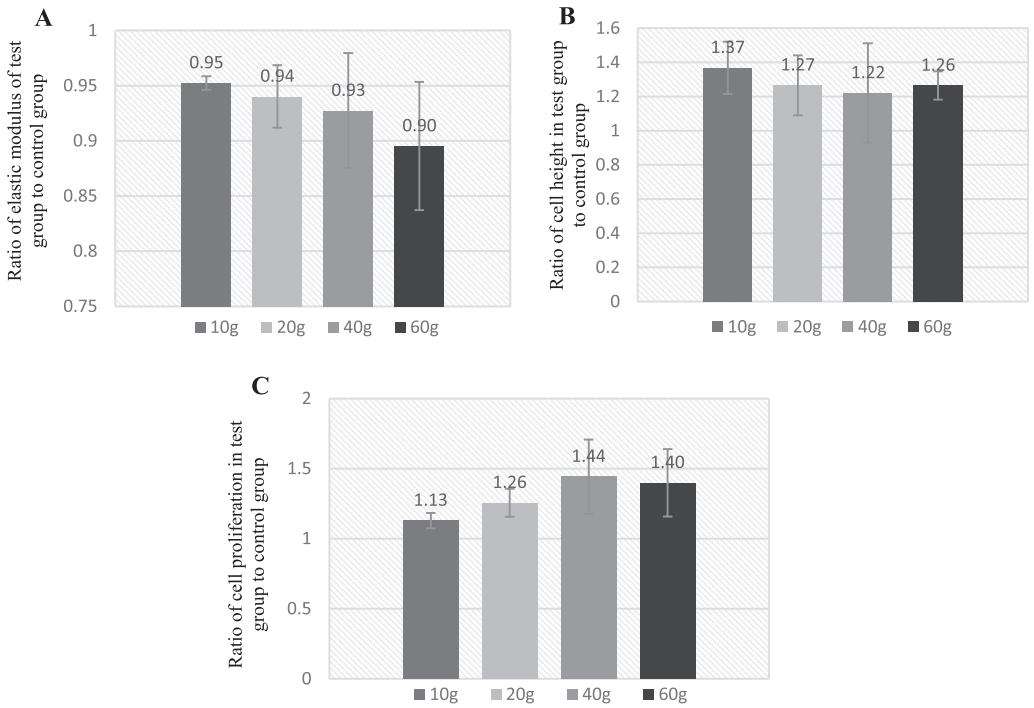

Fig. 4. Decrease in elastic modulus of test cells groups in comparison to control cell group (A), Significant increase in test cell height group in comparison to control cell height group (B), Increase in proliferation of test cell groups in comparison to control cell group (C).

group, leading to a decrease in cell volume [12]. In a further study, there were no significant differences in bovine aortic endothelia cell (BAEC) morphology after exposing to 10 g hypergravity [13]. Gebken et al. (1999) has revealed that no morphological changes occurred as a result of hypergravity of 13 g on human cells [14].

In the case of cytoskeleton, the results showed that hypergravity affects the dynamic of MFs, apparently. It causes significant modification in major cytoskeleton constituents [13]. The outcome of the studies done by Kacena et al. (2004) [15] and Morrbidelliet al. (2009) [13] confirm that these changes are towards increase of width and number of MFs, which is in accordance with the result of rearrangement of the cell structure in previous studies.

In the present study, MFs and MTs were both measured after fluorescence staining; however, there was significant difference in MFs and MTs which resulted in more slender cells with lower density of MFs and MTs content compared to those of the control group. It seems safe to claim that these changes in the MFs are in correlation with the morphology changes. These results are in

accordance with Breuls et al. (2008) study which indicates that morphological changes in cells are due to cytoskeletal changes of cells [16].

A further study indicated an increase in width of MFs of cardiocyt cells exposed to 2 g hypergravity for 2–48 h. However, no significant difference was reported in MTs [17]. The present findings are in contrary to the results obtained from other studies, since Rotsch et al. (1997) stated a 49% increase in the width of MFs in skeletal cells exposed to 4 g hypergravity for 2 days [18]. Moreover, in a study carried out on rat myoblast cells (C2C12) in 5 g–10 g–20 g hypergravity, there was a significant increase in thickness of MFs [19]. In another research, the MFs in skeletal cells were more sensitive to hypergravity and as a result became more observable. This 2 g hypergravity, however, had no significant effect on MTs [20]. Results of this study showed that cell exposure to hypergravity conditions causes remodeling or impairment of cytoskeletal organization in a way that leads to slender and longer hADSCs.

The elasticity of different types of cell is supposed to be changed under conditions that affect actin network and cytoskeleton of the cell. In this study, it was observed that by exerting hypergravity on cells, the concentration and distribution of MFs and MTs decreased and MFs fibers become slender. The results of different studies that evaluate actin network in different ways including applying shear stress [21] or evaluating actin profile [22] confirm the correlation between elastic response of cell with actin network. There seems to be a correlation between the decrease of MFs of test cells and the decrease of elastic modulus. This assertion is based on the claim that the increase of MFs density of cells also results in an increase in elastic modulus [18]. In other words, MFs seemed to have a more prominent role in mechanical properties of cells in comparison to MTs [17]. Evaluating cytoskeleton of cells by using medicine that affects the cell's cytoskeleton has been done in many studies [18,23]. The results prove the role of cytoskeletal elements in formation of the mechanical properties of cells.

When it came to the cell proliferation, the results indicated that hypergravity exposure led to an increase in proliferation compared to the control group. This result is also in line with a number of previous research, some of which are as follows: An increase in the proliferation of MC3T3-E1 cells in 2–5 g hypergravity [23], human umbilical vein endothelia cells (HUVEC) in 20 g hypergravity [24], A6 cells extracted from epithelial cells from the tissues obtained from frog's kidney in 5 g hypergravity [25], MC3T3-E1 cells in both 20 g and 40 g hypergravity, HeLa and JTC-12 cells in 40 g hypergravity [26], and myoblast cells C2C12 of rat (18). In contrary, Gebken et al. (1999) found a slight reduction in the number of osteoblast like cell after hypergravity of 13 g [14]. Also, no significant changes observed in proliferation of cells underwent spaceflight test in other studies [27,28].

The differences in results obtained by different researchers can be justified due to the differences in cell type, method of gravity exposure, level of gravity from 2 g to 2000 g, and hypergravity exposure time from some minutes to days, hypergravity exposure methods, and even the interval between the exposure periods.

In conclusion, we found that ADSCs cells exposed to discontinuous hypergravity stimulation illustrate changes in morphology, cytoskeleton re-organization, changes in cell proliferation and elastic modulus.

Ethical statement

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.160.

Transparency document

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