

Research Paper: Investigating Morphologic Changes and Viability of Rats' Bone Marrow Mesenchymal Stem Cells in Microgravity



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ABSTRACT

Introduction: Mesenchymal Stem Cells (MSCs) are multipotent cells capable of duplication and auto-recovery and distinction from various cells including chondrocytes, adipocytes, chondroblasts, fibroblasts, and osteoblasts. Human stem cells are always subject to local and external mechanical loads. External loads are caused by physical activity in external environment loading to infliction of static and dynamic loads on the body and internal loads are typically caused due to body physiological function. Mechanical factors can affect different parameters such as morphology, proliferation, migration, metabolism and death as well as chemical changes in cells and lead to chemical changes in extracellular matrix and intracellular environment, besides distinction of cells.

Methods: MSCs were isolated from rat's bone marrow, then cultured in microgravity conditions. Morphologic changes of cells were analyzed by taking pictures at different times.

Results: Results indicated a reduction in cell area and an increase in cell aspect ratio, in microgravity conditions. No significant difference was observed in cell angle of rotation at different time measurements. Also, in measuring viability of these cells using MTT test it was found that microgravity reduces viability of stem cells, considerably.

Conclusion: Microgravity conditions have a considerable impact on morphology of MSCs. Furthermore, viability of MSCs decreased significantly after 48 h, under microgravity conditions.

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1. Introduction

More than a century had passed since man's journey to the moon and it is known that microgravity affects almost all human physiological systems. Physiological changes like osteoporosis, and anemia and immune system changes are only some effects of the long-term space journeys on human body [1]. These physiological changes prevent man from inter-planetary journeys. Osteoporosis is among most important physiological changes occurring during space journeys. Space journeys can lead to about 2% bone density loss per month in any astronaut [2]. Some astronauts have even lost up to 20% of their bone density during their mission [3].

Generally, there are 4 types of cells in bones, classified based on morphology, and performance, as follows: MSCs, osteoblasts, osteocytes, and osteoclasts. MSCs are able to differentiate into adipocytes, osteoblasts, and chondroblasts [4]. When mechanical load is increased in bones, osteoblasts create bone and when the mechanical load decreases, osteoclasts decrease bone density [5].

MSCs are multipotent cells that can differentiate into various cell types [6, 7]. Microgravity exposure results in extensive physiological changes in MSCs [8]. Lack of gravity in space can considerably reduce mechanical pressure. Thus, this condition dramatically decrease osteogenesis and increase adipocyte cells growth rate by MSCs [9]. Also, studies indicate that the scale and function of mesenchymal cells do not return to former state upon being in normal gravity. Reestablished gravity conditions fail to entirely restore bone texture. Therefore, it is necessary to conduct extensive investigations in this regard to prevent loss in ossification capacity of bone marrow [10, 11].

Designing laboratories to emulate space-based illumination conditions is very challenging, due to the unique space environment. Environmental conditions change quickly in such state. Therefore, certain equipment are required to simulate these environmental conditions, well [12]. Conducting biologic tests in microgravity conditions on earth is very difficult due to the lack of microgravity conditions on earth. Special equipment are needed to emulate such condition on earth. The present study used Random Positioning Machine (RPM) to emulate microgravity conditions [13].

Cells have different behaviors, in respond to various chemical and physical conditions. Morphology and orientation of cells, proliferation gene expression, and dif-

ferentiation into other cell types could be affected by environmental conditions. Environmental temperature, stiffness of culture membrane and amount and duration of stress imposed on the cell are among physical environment properties. Culture media type, chemical growth factors and pH of the environment are important chemical conditions of cell culture. Texture engineering has addressed the effect of physiological factors on cell metabolism and biology. Also, problems with texture maintenance and organ transplant has encouraged researchers for stem-cell engineering. Elucidating the mechanobiology and empirical observations in this field can significantly impact the future. This study aimed to quantitatively explore the microgravity effect as an independent mechanical parameter on morphology, orientation and viability of rats' bone marrow MCS.

2. Materials and Methods

Animals

Male Sprague Dawley rats (250 to 300 gr) were obtained from the Razi Institute (Karaj, Iran) and housed under standard laboratory conditions. They were kept at constant room temperature ($21\pm 2^\circ\text{C}$) under 12:12 h light-dark cycle with free access to food and water.

Cell culture

Rats were initially anesthetized using diethyl ether and their femur and tibia were detached and then bone surface textures were cleaned completely. Next, bone ends were cut and bone marrow cells were transferred to the falcon pipe by flushing technique. The falcon pipe was centrifuged for 5 minutes at 1200 rpm and pellet was poured into 1 ml of the DMEM after supernatant removal. The surface media was transferred to a flask and supplemented with 10% FBS. Then, it was incubated at 37°C and CO_2 concentration of 5%. After 24 hours, the surface media was removed and cells attached to the bottom of culture flask were washed via PBS and the new culture media was added. Then, for a period of 14 days, all the 4 culture media were exchanged and when the flask bottom reached confluence, the cells were passaged using 0.25 Trypsin/EDTA

Cell culture under microgravity conditions: After seventh passage, cells were divided into 2 groups of control and test. The control group was placed under static conditions (9.8 g) in incubator and the test group was placed in an incubator with RPM machine installed under the gravity of 0.01 with 30 rpm. The cells of both groups were removed from incu-

bators at 12, 24, and 48 h. Then, the microscopic photos for morphological changes were obtained.

Investigation of cells morphology

Photos taken of cells were quantitatively investigated for morphological changes considering the parameters of cells area, length-to-width ratio, and cell angle of rotation using ImageJ software. Data were compared, accordingly.

Viability measurement

Metabolic activity of the cells was measured using MTT test. MTT is a yellow water-soluble tetrazolium resuscitated by mitochondrial dehydrogenase enzymes and precipitated in live cells as insoluble formazan crystals. The amount of these produced insoluble purple crystals is proportionate to cell activities. Metabolically active cells perform MTT resuscitation and are considered as live cells. To perform MTT, cells were washed twice with PBS after 21 days in osteogenic media culture and FBS-free culture media was added to them. Then, per 100 microliter of the culture media, 10 μ L of MTT solution (5 mg/mL) was added to every well of the plate and the plates were incubated at 37°C for 4 hours. After this period, the supernatant was removed slowly and the Dimethyl Sulfoxide Solution (DMSO) was added to resulting formazan crystals and light absorption of the resulting solution was read using spectrophotometer at the wavelength of 505 nm.

Statistical analysis

Statistical analysis was performed using ANOVA by SPSS. $P \leq 0.05$ was considered as the level of significance.

3. Results

Cell morphology was measured using quantitative parameters and ImageJ software. The obtained data showed that culture of MSCs has a profound impact on

these cells morphology in microgravity conditions (Figure 1). Cell area, cell aspect ratio, and cell angle of rotation were measured. These parameters were evaluated at 12, 24, and 48 h (Table 1).

One of the morphological parameters on which the effect of microgravity conditions was evaluated was cell areas. Figure 2 shows the extent and quality of cell area changes at 48 h after starting the process. Results show that cell areas have decreased in microgravity. The area occupied by cells under the controlled conditions has increased slightly by 12% over 48 h. However, area of cells under loading had reductions in the first 12 h but the difference was insignificant. The cell area decreased significantly to 59%, after 48 h. It seems that microgravity conditions lead to reduced area of MSCs.

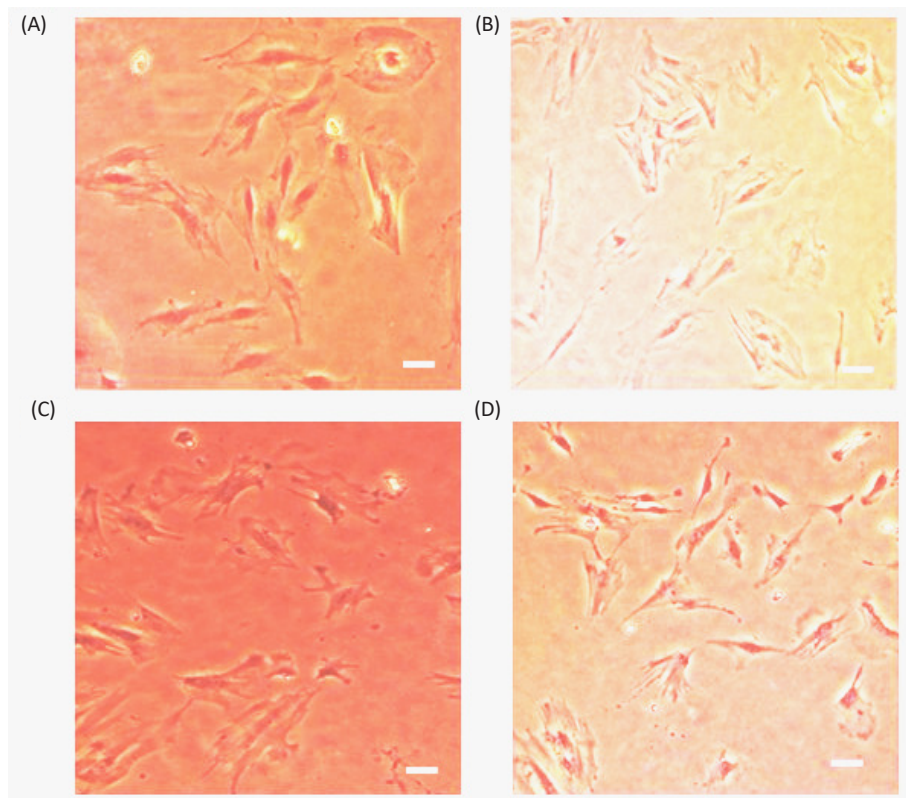
The next morphological parameter evaluated over time was cell aspect ratio. This ratio had 9% increase in the control cells at different times. However, in cells under loading, no significant difference was observed over the first 12 hr from the control group, aspect ratio increased significantly at 24 and 48 h, with the increase after 48 h reaching about 38%. This ratio indicates that cells show a more elongated form under microgravity conditions (Figure 3). To evaluate the effect of microgravity on cell orientation, cell rotation angle was calculated in both groups of test and control using ImageJ software. As shown in Figure 4, lack of significant difference between the 2 groups demonstrates that being exposed to microgravity conditions does not have a considerable impact on orientation and alignment of cells.

There were no significant differences between the control and test groups. To evaluate changes in cell morphology, cell viability was evaluated under microgravity conditions using MTT test. As illustrated in Figure 5, viability of mesenchymal stem cells decreased significantly over time under microgravity conditions, expressing

Table 1. Results of measuring different parameters related to cell morphology

Time		Area		Shape Index		Angle		Optical Density	
		Test	Control	Test	Control	Test	Control	Test	Control
12 h	Mean	3568.64	3632.37	2.51	2.61	73.37	73.63	0.71	0.81
	SD	308.89	183.82	0.19	0.14	4.51	2.08	0.04	0.05
24 h	Mean	2994.13	3844.31	3.24	2.81	66.64	65.11	0.64	0.92
	SD	134.72	81.44	0.18	0.07	4.51	4.01	0.04	0.06
48 h	Mean	1838.63	4137.63	3.86	2.97	70.32	70.12	0.57	0.92
	SD	284.84	148.13	0.34	0.15	7.51	3.04	0.06	0.03

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Figure 1. Morphological changes in MSCs cultured under microgravity conditions

A. Cells cultured under static conditions; B. Cells cultured under microgravity conditions after 12 h; C. After 24 h; and D. After 48 h; Bar represents 20 μm ($\times 40$)

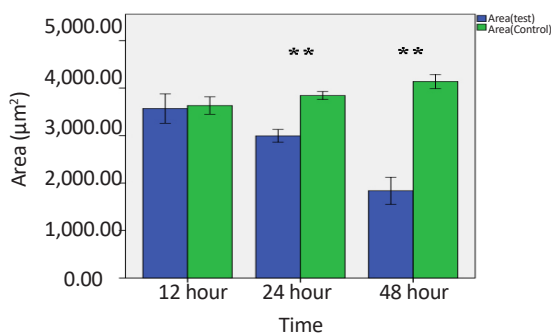
that microgravity conditions lead to reduced viability of MSCs. Viability of cells exposed to microgravity conditions after the start of loading, reduced in a descending fashion, in which after 48 hours, this criterion was about 39% less, compared to cells in static-condition.

4. Discussion

MSCs are capable of long-term proliferation and differentiation into various types of stromal cells. Various

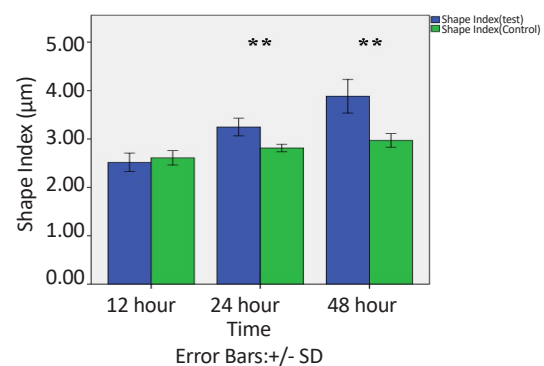
factors such as microgravity, cellular microenvironment and soluble mixtures affect MSCs. When people get hospitalized for a long time or spend a long time under microgravity conditions and different mechanical motives do not affect the cells of this area of body, these cells osteogenesis is weakened [14, 15].

Numerous studies evaluated the microgravity effect on bone marrow MSC. Researchers also explored the effect of microgravity conditions on MSCs as well as the



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Figure 2. Cell area changes in the test and control groups
** $P < 0.01$



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Figure 3. Aspect ratio of cells in the control and test groups
** $P < 0.01$

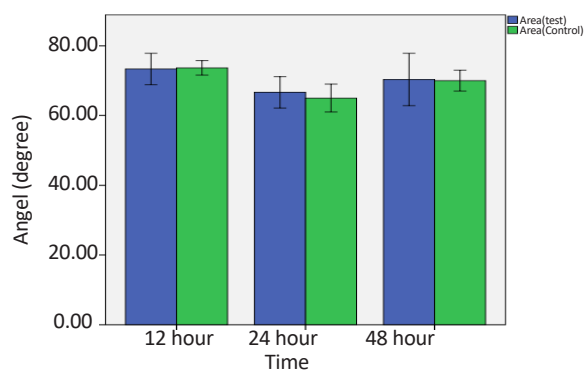


Figure 4. Cell rotation angle changes in the control and test groups

mechanism of this effect. In 2004, researchers evaluated the effect of long-term microgravity conditions on morphology, meiosis rate and expression of certain cellular markers in cultured human mesenchymal cells. They observed the effect of these conditions on cells at different times (1 hour to 10 days). Their obtained results revealed that meiosis rate of these cells reduced considerably under microgravity conditions, compared to normal conditions. In this experiment, cells cultured under microgravity conditions were more plain, which confirms these mesenchymal cells can sense gravity changes and react to these changes with altered function [16, 13].

Alkaline activity known as an osteoblast differentiation marker decreased by 40% in mesenchymal cells of rats' bone marrow that had been placed under microgravity conditions, compared to the control group [17, 18]. Having cultured cells for 20 days in zero gravity simulator, meiosis activity of mesenchymal cells decreased; however, number of large flat cells inside the culture media increased [19-21]. Rucci N, et al. reported that decreased gravity leads to suppressed osteogenesis and increased gravity results in further osteogenesis [22].

Findings suggest that the motive of microgravity suppresses cellular population growth of bone marrow, resulting in the lack of differentiation progress to osteoblasts, which in turn causes physiologic deformities in space explorations [21, 23]. Sheyn et al. clarified that the expression of 882 genes in MSC decrease, compared to the control group and expression of 505 genes increase in microgravity. In this study gene expression related to osteogenesis differentiation was decreased dramatically. However, gene expression was increased in those evolving adipocyte differentiation [24, 15].

According to the literature, microgravity leads to widespread physiological changes in MSCs. The present study,

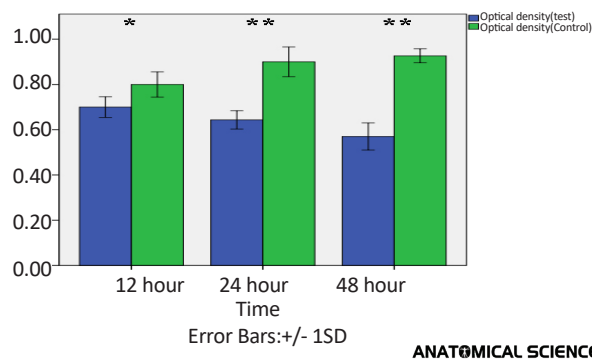


Figure 5. Cell viability changes in the control and test groups measured by MTT test

* $P \leq 0.05$; ** $P \leq 0.01$

quantitatively evaluated the effect of microgravity on morphology and viability of MSCs. Three morphologic parameters including cells area, cell angle of rotation, and cell aspect ratio were evaluated. According to prior studies, microgravity conditions lead to reduced cellular area. Thus, significant changes in the aspect ratio was observed in which it grew significantly and cells become more elongated under microgravity conditions. Furthermore, angle size of MSCs were compared in microgravity conditions with the control group. Statistical non-significant difference between the 2 groups suggests that cells fail to assume a certain orientation in microgravity conditions. Microgravity conditions have a considerable impact on morphology of MSCs. Furthermore, viability of MSCs decreased significantly after 48 h, under microgravity conditions.

Ethical Considerations

Compliance with ethical guidelines

The research which involved animals (rats) was performed in compliance with the principles of the Declaration of Helsinki.

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Authors contributions

The authors contribution is as follows: Samira Monfaredi: Concept study and design; Reza Ahadi: Acquisition of data; Shahram Darabi: Analysis and interpretation of data; Farzad Rajaei, Reza Ahadi: Drafting the manuscript; Samira Monfaredi: Critical revision of the manuscript for important intellectual content; Shahram Darabi:

Statistical analysis; and Farzad Rajaei: Administrative, technical, and material support, and study supervision.

Conflict of interest

The authors certify that they have no affiliation with or involvement in any organization or entity with any financial interest, or non- financial interest in the subject matter or materials dismissed in this manuscript.

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