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Weightlessness Simulated with Random Positioning Machine Influences the Cytoskeleton and Migration of MC3T3-E1 Cells

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Abstract

Weightlessness conditions result in bone loss, which may be partly attributed to the dysfunction of osteoblast. Random positioning machine (RPM) was often used to simulate the weightlessness effects on animal cells. The aim of this study was to explore the response of MC3T3-E1 osteoblast-like cells to the specific mechanical environment in RPM. The results showed that RPM exposure for 12 and 24 h inhibited the cell proliferation, and changed the cell shape for 12h, but had no effect for 48 h. The stress fibers of MC3T3-E1 cells cultured in RPM for 12 and 24 h disrupted detected by rhodamine-labeled phalloidin, but partially recovered for 36 and 48h. The cytosolic free calcium concentration ($[Ca^{2+}]_i$) of MC3T3-E1 cells cultured in RPM for 12 and 24h increased. The cell migration of MC3T3-E1 cells cultured in RPM inhibited for 12, 24, 36 and 48 h. Based on these findings, it appears reasonable to suggest that RPM affects the osteoblast-like cell shape, F-actin distribution, proliferation for 12 and 24 h, but the cells recovered for 36 and 48 h except cell migration. From these results, we conclude that MC3T3-E1 cells cultured in RPM at early stage could response to the new mechanical stress and display the weightlessness effects.

1. Introduction

Weightlessness conditions in space pose significant health risks for astronauts during long term spaceflights[1], which could induce decreased bone mass[2], skeletal muscle and cardiovascular problems[3]. Bone loss is one of the most serious problems, which appears to block long term space flight. It has been determined that 1-2% of bone mass is lost every month during spaceflight and this loss is site specific occurring mostly in the weight-bearing bones[4].

Bone is a kind of living tissue which is continually adapting to its environment via continuous formation and resorption, in which separately involves osteoblast and osteoclast. Rapid bone loss has been attributed to a decrease of osteoblast function[5].

Because the difficulty to carry out experiments in space to investigate the actual mechanism for bone loss induced by space flight, random positioning machine (RPM) has become critical ground-based simulator to continue space biology studying. RPM is originally designed to study the role of gravity on plants, and then developed to simulate the effects of weightlessness on animal cells. The principle of generating simulated effects of weightlessness by RPM is that the time of gravity changes is faster than the response time of the objects in direction[6]. Uva found that microfilaments of glial cells (C6 line) were highly disorganised by RPM which was similar with the results of real weightlessness effects[7].

Only a few experiments on MC3T3-E1 cells using RPM have been reported. The main purpose of this study was to investigate the changes of the cell shape, microfilament, proliferation, cytosolic free calcium concentration ($[Ca^{2+}]_i$) and migration in MC3T3-E1 osteoblast-like cells induced by RPM.

2. Materials and Methods

2.1 Random-positioning machine

The RPM, manufactured by the center for space science and applied research of Beijing[8], is composed of an inner and an outer frame which can rotate independently of each other in random directions. The samples are fixed as close as possible to the center of the inner rotating frame. The rotation of each frame is randomly and autonomously controlled by the computer system[9].

2.2 Cell Culture

The mouse MC3T3-E1 cells were purchased from the China Center for Type Culture Collection, which obtained the cells originally from American type culture collection (Manassas, VA, USA). The cells were grown in complete MEM culture medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 10% fetal calf serum (FCS) at 37℃, 5% CO_2 in a humidified atmosphere.

To start a RPM experiment, MC3T3 cells (2×10^4 cells/cm^2) were inoculated into the culture flasks. After 24h growth, the culture flasks were filled with medium, tightly capped, taking care to avoid air bubbles, and were fixed onto the RPM, which

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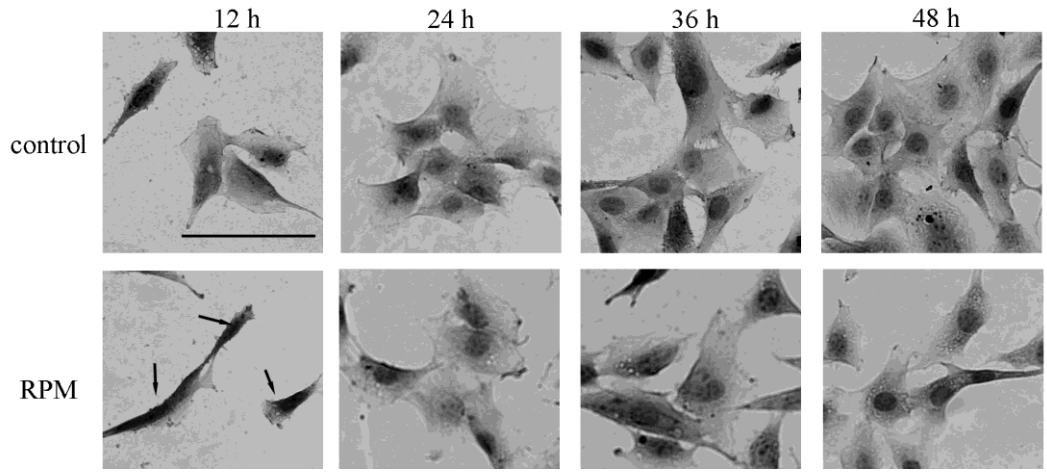

Fig. 1 The morphology changes of MC 3T3-E1 cells cultured in RPM (Bar=100 μm)

was rotated randomly at the range of 0~10 rpm for 12 h, 24 h, 36 h and 48 h. The RPM was located in an incubator providing 37℃. Control groups were also completely filled up with medium and cultured in the incubator at 37℃.

2.3 Hematoxylin and Eosin (HE) Staining

MC3T3-E1 cells were washed twice with phosphate-buffered saline solution (PBS), pH 7.4 and fixed in 4%(w/v) paraformaldehyde solution in PBS for 20 min. The coverslips were placed in 0.5% haematoxylin for 10 min and 0.5% eosin for 5 min respectively. Then the coverslips were dehydrated through an ascending gradient of ethanol, made transparent with dimethylbenzene.

The images were analyzed and quantified by Image-Pro plus 6.0 software.

2.4 F-actin Localization Measurement

Samples of MC3T3-E1 cells grown on cover glass were transferred to 500 μl 4% (w/v) paraformaldehyde in PBS for 10 min. Fixed cells were permeabilised by incubation for 15 min with 0.3 % (v/v) Triton X-100 detergent dissolved in PBS. Cells were then incubated for 60 min with fluorescein rhodamine-labeled phalloidin in PBS solution supplemented with 1 % (w/v) bovine serum albumin (BSA). Staining of actin filaments was measured using a Laser Confocal Scanning Microscope (LCSM, Leica SP5, Germany) with excitation wavelength of 496 nm and emission at 516 nm.

2.3 Cell Proliferation Assays

The cell proliferation was assessed by MTT assay. After clinorotation, the MC3T3-E1 cells were harvested and plated in 96-well tissue culture plates, and 20μL of 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) was added. After incubating for 4 h at 37℃ in 5% CO_2, dimethyl sulfoxide (200 μL) was added to each well. The absorbances were measured at 490 nm using a Victor Multilabel Counter.

2.4 Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in MC3T3-E1 cells was detected by LCSM using Fluo-3/AM. Briefly, MC3T3-E1 cells cultured on cover glass were loaded with Ca^{2+} indicator Fluo-3/AM (5 μM) dissolved in loading buffer (10 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM pyruvate, 11 mM dextrose, and 1 mM $CaCl_2$, pH 7.3) containing 1% bovine serum albumin at 37℃ for 30 min, and additional 30 min were allowed to hydrolyze Fluo-3/AM. Changes of $[Ca^{2+}]_i$ in osteoblast-like cells were measured at 488 nm excitation/530 nm emission by LCSM.

2.5 Wound-healing Assay

MC3T3-E1 cells were cultured with the MEM medium containing 10% FCS for 24h, and mimicking wounding was made in the cell monolayer with a pipette tip. The cells were washed three times with PBS. Afterwards, the flasks were filled with medium and incubated in RPM. Phase-contrast images of migrating cells were captured by a contrast microscope (Nikon, Japan).

2.6 Statistical Analysis

All data are given as mean ± standard deviation (SD). Statistical significance was performed by Student's *t* test, and differences were considered significant at $P<0.05$.

3. Results:

3.1 Simulated Weightlessness Decreased the Cell Area of MC3T3-E1 Cells

MC3T3-E1 cells cultured in either a normal gravity environment or in a simulated weightlessness environment were stained with HE staining (**Fig. 1**). The control cells

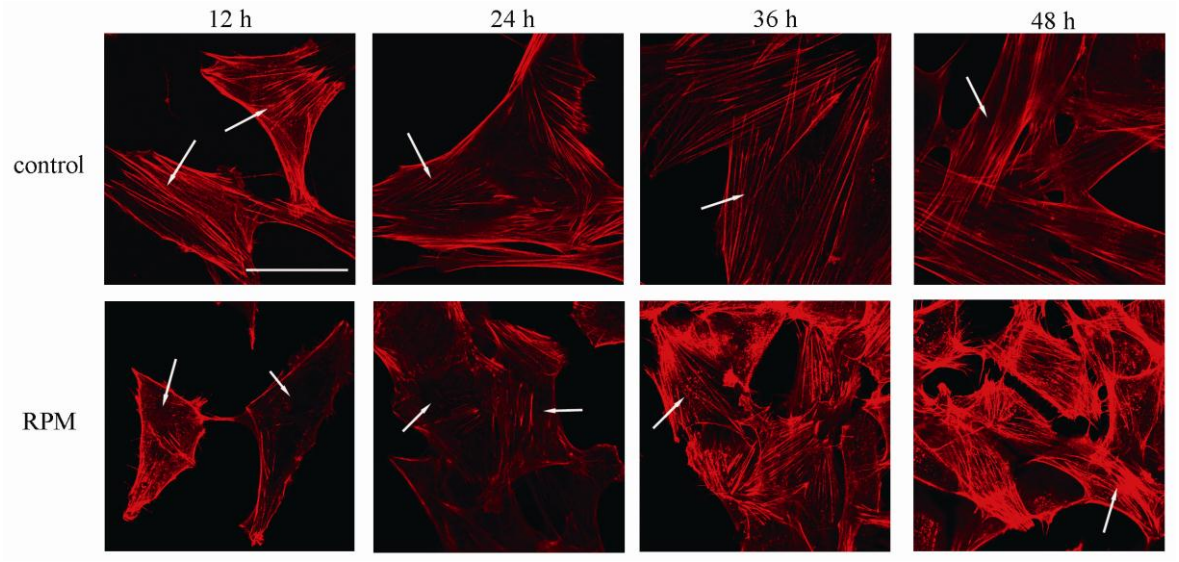

Fig. 3 The ventral stress fiber disassembled of MC 3T3-E1 cells cultured in RPM **(Bar=50 μm)**

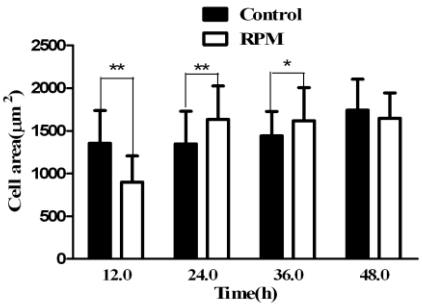

Fig. 2 The cell area change of MC3T3-E1 cell cultured in RPM.

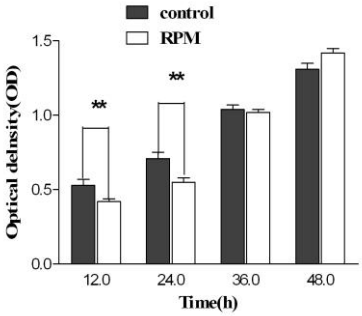

Fig. 4 The proliferation was decreased of MC3T3-E1 cells cultured in RPM

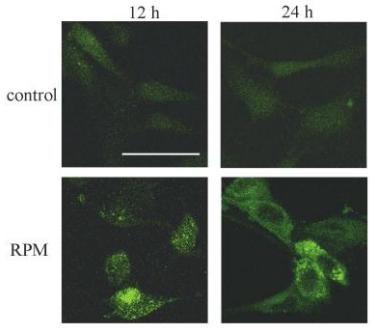

Fig. 5 $[Ca^{2+}]_i$ of MC3T3-E1 cultured in RPM increased (Bar=100 μm)

attached to the substrate showed their fibroblast-like shape, but cellular morphology cultured in RPM changes dramatically. After 12 h, MC3T3-E1 cells cultured in RPM lost their shape, taking a retracted aspect. But cells cultured in RPM for 24, 36 and 48 h had the same morphology as compared with control cells.

The cell area was estimated using Image Pro Plus software 6.0. Morphometric analysis illustrated that there was a significant cell area difference between cells cultured conventionally and in RPM for 12 h and 24 h (**Fig. 2**).

3.2 Stress Fiber Disassembled of MC3T3-E1 Cells Cultured in the RPM

In our experiments, the microfilaments were sensitive to RPM. The control cells of MC3T3-E1 (**Fig. 3**) had a dense sub plasma membrane layer of F-actin, and displayed abundant ventral stress fibers. The ventral stress fiber was disrupted for 12 h and 24 h, and only a few fibers could be detected.

3.3 Simulated Weightlessness Decreased the Proliferation of MC3T3-E1 Cells

The proliferation of MC3T3-E1 cell cultured in normal gravity and in RPM for 12 h, 24 h, 36 h and 48 h was detected by MTT method. Our studies showed that the cell activity of MC3T3-E1 cells in 12 h and 24h decreased significantly. But when the time elongated to 36 h and 48h, the cell activity had no difference between control and RPM (**Fig. 4**).

3.4 Calcium Concentration Increased of MC3T3-E1 Cells Cultured in the RPM

To examine whether RPM affected the $[Ca^{2+}]_i$ of MC3T3-E1 cells or not , we analyzed calcium concentration change by specific calcium indicator(Fluo-3/AM) (**Fig. 5**). $[Ca^{2+}]_i$ in

MC3T3-E1 cells for 12 h was 4.66 ± 1.11 in control group, and 13.44±7.10 (P<0.05) in RPM group. The calcium concentration is 3.76±1.39 in control group, and 13.52±7.20 in control group (P<0.05) (**Fig. 6**).

3.5 Cell Migration of MC3T3-E1 Cultured in RPM was Inhibited

To investigate the cell migration of MC3T3-E1 cells cultured in RPM, the Wound-healing assay was used in the experiment. After creating a scratch wound, MC3T3-E1 cells were exposed to RPM for 12, 24, 36 and 48 h. The results showed that the cell motility was inhibited by RPM when compared to the control cells (**Fig.7**).

4. Discussion

As we know, gravity acts permanently on cells as stimulation. Understanding the effect of gravity on cell function is an intriguing scientific problem. Microgravity causes bone loss in humans, and the underlying mechanism is thought to be at least partially due to a decrease in bone formation by osteoblasts, which are the key components of the bone unit and playing an essential role in bone formation[10].

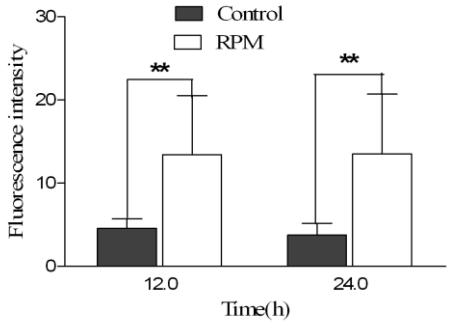

Fig.6 The morphometric analysis results of $[Ca^{2+}]_i$ in MC3T3-E1 cells cultured in RPM.

It has been reported that the weightlessness environment can cause reduction in the secretion of alkaline phosphatase and Runx2[11], which affect the morphology and cytoskeleton[12]. In this study, we found that the cells exposure to RPM retracted in 12 h, and the cell area decreased significantly.

Cell migration is an important function for osteoblast in bone formation after bone loss. Investigations performed in space have shown that gravity changes affect cellular migration [13].The results in this paper indicated that the cell motility was inhibited when exposed to RPM for 12-48 h.

Cytoskeleton is involved in the cell migration. In this process, cells undergo a series of characteristic changes in shape: the extension of a lamellipodium or pseudopodium, the adhesion of the extended leading edge to the substratum, forward flow and retraction of the cell body, and all these phenomena are the results of cytoskeleton[14]. The microtubule of human lymphocytes (Jurkat) was diffuse, shortened and extended from poorly defined microtubule organizing centers induced by space flight for 4 and 48 h[15]. Gruener et al[16] carried out parallel experiments on the slow clinostat and then in space-flight to examine the effects of altered gravity on the structure of the cytoskeleton in cultured *Xenopus* embryonic muscle cells. Space-flown cells showed marked changes in the distribution and organization of actin filaments, and similar effects were found after clinostat rotation. The cytoskeleton of the flight MC3T3-E1 cells had a reduced number of stress fibers and a unique abnormal morphology[17]. Here, we reported that structural changes occur to the cytoskeleton of MC3T3-E1 cells exposed to RPM. We found that the ventral stress fiber disassembled in cells exposed to RPM for 12 and 24 h which was similar with the results of space flight. The results showed that reduced migration response in MC3T3-E1 cells cultured in

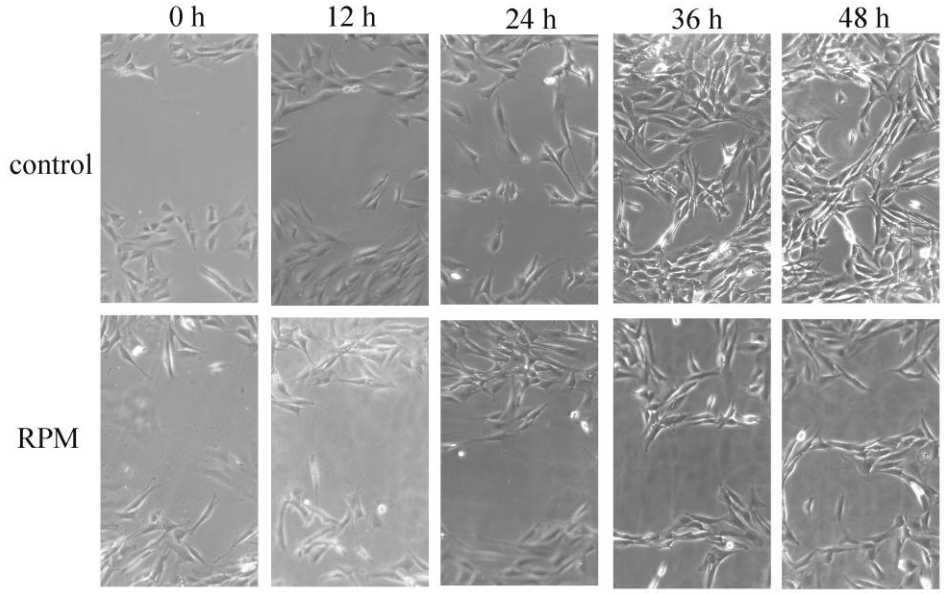

Fig. 7 The migration of MC 3T3-E1 cells cultured in RPM decreased **(**Bar=100 μm)

RPM is linked to changes of cytoskeleton.

Many regulator molecules can sense the gravity and regulate the function of osteoblast[18]. Ion channels[19] and cytoskeleton[20] may be the mechanoreceptors and, can sense the change of gravity. Higashibata et al[21] found that the disorganization of the actin fibers was caused by the inhibition of Rho activation by 3D clinorotation. In our experiment, $[Ca^{2+}]_i$ increased in the MC3T3-E1 cells cultured in RPM. The relationship between calcium and stress fiber is still unknown and will be investigated in the future. A number of studies in different cell types have reported that cytosolic calcium is involved in actin cytoskeleton [22]. We hypothesized that the osteoblast can sense the gravity by ion channel and lead to the increase of calcium concentration which can regulate the microfilament reorganization and affect cell migration.

RPM is a device for simulating the weightlessness effects on the earth; by controlling the simultaneous rotation of two axes, RPM cancels the cumulative gravity vector at the center of the device, producing an effect of weightlessness environment. Previous reports have demonstrated that the RPM could simulate the effect of weightlessness in the space flight. Makihira et al. had shown that the RPM inhibit the Runx2, Osterix, type I collagen aI chain, RANKL and OPG function of MC3T3-E1 osteoblast-like cells[23]. Our MTT assay results showed that RPM inhibited the proliferation of MC3T3-E1 cells at 12 and 24h, but for a long time for 36 and 48 h, the proliferation was promoted by RPM. The results showed that the short exposure of MC3T3-E1 to RPM can simulate the inhibition effect of weightlessness in space flight, but this change recovered in long time exposure which indicated that the cells may have adapted to the new mechanical environment.

We concluded that the RPM exposure can decrease the area, disrupt the stress fiber, increase the $[Ca^{2+}]_i$, inhibit the proliferation and migration of MC3T3-E1 osteoblast-like cells for a short time (such as 12 h and 24 h). But with the extension of time, the cells recovered. Those data would provide new insights into the effects of osteoblast exposed to RPM and into the mechanism of bone loss that is induced by spaceflight.

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