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Effects of Clinostat Culture on Morphology and Gene Expression of MLO-Y4 Osteocyte-Like Cells

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Abstract

The aim of this study is to investigate the effects of 2D clinostat-simulated weightlessness on biological characteristics of MLO-Y4 osteocyte-like cells. MLO-Y4 cells were incubated for 24 h and rotated using a 2D clinostat for 2 h. The bioeffects of clinostat culture on cellular morphology, cytoskeleton, and gene expression were investigated. The results show that 2D clinostat-simulated weightlessness induce actin cytoskeleton rearrangement, but unaffected the cellular morphology and number of processes/cell. Also, after 2 h of clinostat culture, expression of RANKL and IL-6 decreased by 19%±5% and 20%±4%, respectively, while cox-2 level increased by 65%±8%. These results provide some clue to explore the cellular mechanism of bone loss caused by weightlessness.

1. Introduction

After long-term space flight, some physiological changes have taken place to astronauts, such as bone loss, anaemia, muscle atrophy and immune alterations. Among them, bone loss is the most common physiological change during space flight. Previous studies showed the bone loss rate was 3% per month[1)]. And many studies have been done on the cellular mechanism of bone loss caused by weightlessness in real space and ground-based experiments. But the exact cellular mechanism is not clear yet.

Osteocytes, the most abundant cells in bone, have been paid more attention in recent years. Bonewald et al. (2006,2007,2008) thought osteocytes might be main mechanosensing cells in bone tissue[2-4)], which could sense the unloading caused by weightlessness, and transfer mechanical signal to osteoblasts and osteoclasts, influence the balance of bone formation and resorption, lead to bone loss at last.

Some studies have shown morphological changes of osteocytes in vivo happen after real space and simulated weightlessness. Rodionova et al. (2002) have reported that after space flight (Bion-11), the activity of some young osteocytes increase in the iliac crest of monkeys. The destruction of osteocytes leads to an increasing quantity of empty osteocytic lacunae in bone tissue[5)]. Aguirre et al. (2006) have shown tail suspension promotes the prevalence of osteocyte apoptosis, followed by bone resorption and loss of mineral and strength[6)]. Also, Heino et al. (2009) have shown that damaged osteocytes locally affect osteoclast precursors by secreting osteoclastogenic factors, and thus play a role in the initiation of resorption in bone remodeling[7)].

But what happened to osteocytes in vitro under weightlessness conditions. The studies are still limited. In previous studies, magnetic levitation produced by superconducting magnet was used to simulate weightless environment, and the results have shown that simulated weightlessness causes the changes of morphology, cytoskeleton arrangement and gene expression in MLO-Y4 cells[8)]. Also, parabolic flight resulted in the changes of cytoskeleton architecture, but not morphology and area of MLO-Y4 cells[9)]. The objective of this study is to investigate the responses of osteocyte-like cells to 2D clinostat-simulated weightlessness including morphology, cytoskeleton and gene expression.

2. Methods and Materials

2. 1 Cell culture

The MLO-Y4 cell line was donated by Professor Lynda Bonewald (University of Missouri-Kansas City). The cells were cultured in MEM (GIBCO) supplemented with 5% fetal bovine serum (FBS) and 5% calf serum (CS) including 1% penicillin/streptomycin. Cells were plated on glass slides (2.6 cm×2.2 cm) at the density of 2.5×10^5 per slide, then cells were cultured at 37 °C, 5% CO_2 in a humidified atmosphere for 24 h. The slides were randomly divided into 3 groups: stationary control group (SC), horizontal rotation control group (RC) and experimental group (E).

2. 2 Clinorotation

Clinorotation was performed at 30 rpm according to previous

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Table 1 Primer sequences

Gene name	Primer sequences	Annealing temperature(°C)	Amplication length (bp)
OPN	TGACATAGCCATAGGTGAGG	59	235
	TGCCCTTTCCGTTGTTGTC		
integrin	GTTCGGAGCAACAGTTCG	58	488
	TGACATAGCCATAGGTGAGG		
RANKL	TGACATAGCCATAGGTGAGG	70	300
	TGACATAGCCATAGGTGAGG		
IL-6	TGGCTTCGGGAGCACAAC	55	380
	CTGGCTTTGTCTTTCTTGTT		
Cox-2	TGGCTTCGGGAGCACAAC	57	437
	TGGCTTCGGGAGCACAAC		
18S	AATCAGGGTTCGATTCCGGA3	55	257
	AATCAGGGTTCGATTCCGGA3		

studies[10,11], with 3 specially designed flasks having an internal diameter of 4 cm in each group. Slides were inserted into a specially designed shelf in every flask. In E group, 4 slides were placed in the shelf vertically to the bottom of the flask, while in SC and RC group 8 slides were placed parallel to the bottom of the flask. Then the flask were filled with culture media and sealed with a silicon cushion and a plastic lid. Two short needles were inserted into the culture flask through two holes on each edge of lid, and air bubbles in the flask could be evicted from a needle by adding culture medium through another needle. After that, 3 flasks of E group were placed on the clinostat and rotated around a horizontal axis, while RC group around a vertical axis. The flasks of SC group were statically placed on the clinostat.

2. 3 Haematoxylin–eosin (HE) staining

Cells were collected and washed twice with PBS, pH 7.4 and fixed in 0.5% glutaraldehyde solution in PBS for 10 min. The coverslips were placed in 0.5% haematoxylin for 10 min and 0.5% eosin for 7 min, respectively. Then the cells were dehydrated by a series of ethanol solutions with increasing concentrations, and made the transparent with dimethylbenzene. The cell area, length-width ratio and number of processes/cell (the number of dendrite processes per cell) were observed and quantified under microscope using simple PCI software. Five random fields of vision were observed, and 20-40 cells were detected under one field. Statistical analysis was done by Graphpad Prism software (La Jolla, CA). The independent experiments were repeated three times.

2. 4 Confocal Imaging

After washing with PBS, cells were fixed for 10 min with 0.5% glutaraldehyde solution in PBS at room temperature. Then cells were permeabilized using PBS containing 0.1% Triton X-100 solution and blocked with blocking buffer (PBS+0.1% Tween +1% serum) for 10min.

Cells were probed with antibody of rhodamine-phalloidin labeled F-actin (1:40, Invitrogen) and α-tubulin (1:2000, Sigma) overnight. After washed by TBS with 0.1% Triton, cells were incubated for 1 h with FITC labeled (1:10, KPL) secondary antibody. Then the cells were washed and observed by confocal microscopy (Leica TCS SP5). The images were captured by LAS AF Imaging System and further analyzed by NIH image software Image J. The independent experiments were repeated three times.

2. 5 RT-PCR

Cells were lysed by Trizol (Invitrogen, USA), total RNA was extracted, and PCR was performed according to the protocol provided by RT-PCR reaction kit (TaKaRa, Japan). These primer sequences were shown in Table 1. PCR production was quantified and analyzed by NIH image software Image J and Quality One software. 18S rRNA was used as an internal control gene for normalization. The independent experiments were repeated three times by different RNA sample.

2. 6 Statistical Analysis

Statistical analysis is performed using GraphPad Prism 5 statistics software (San Diego, USA). All data are expressed as means ± standard deviation (SD). One way ANOVA is used for statistics analysis. $P<0.05$ is considered statistically significant.

3. Results

3. 1 Cell Morphology

There was not significant difference between three groups on cell area, length-width ratio (data not shown), and number of processes/cell after 2 h of clinostat culture. Also the cell morphology wasn't influenced (**Fig. 1**, **2**).

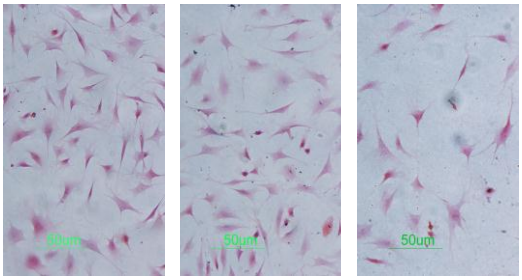

Fig. 1 Analysis of the effects of clinostatation on MLO-Y4 cell morphology by HE staining. Simple PCI software was used to detect cell area, length-width ratio and number of processes/cell. SC: stationary control, RC: horizontal rotation control, E: experimental

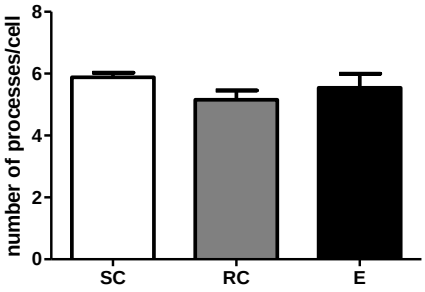

Fig. 2 The number of processes/cell was analyzed by simple PCI. There was no significant differences among SC, RC and E groups.

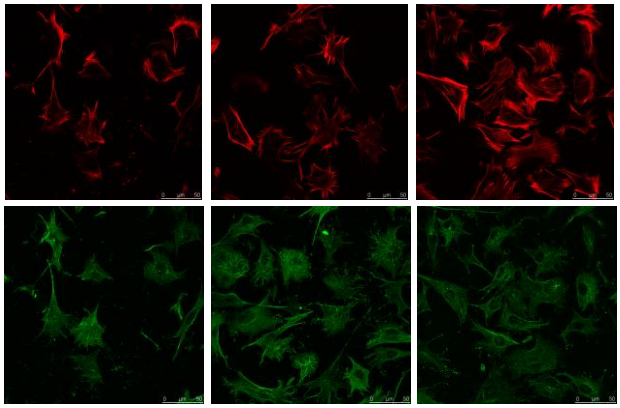

Fig. 3 Confocal microscopy analysis of the effects of clinostatation on MLO-Y4 actin fibers(red) and microtubules(green. Cells were incubated by antibody of rhodamine-phalloidin labeled F-actin (1:40), α-tubulin (1:2000) and FITC labeled (1:10) secondary antibody

3. 2 Cell Cytoskeleton

In SC and RC groups, F-actin was located mainly at the cell periphery or passed through the cytoplasm in the silk structure. In E group, actin fibers became thicker than that in SC and RC groups. Microtubules distributed in the whole cytoplasm, there was not obvious differences among three groups (**Fig 3**).

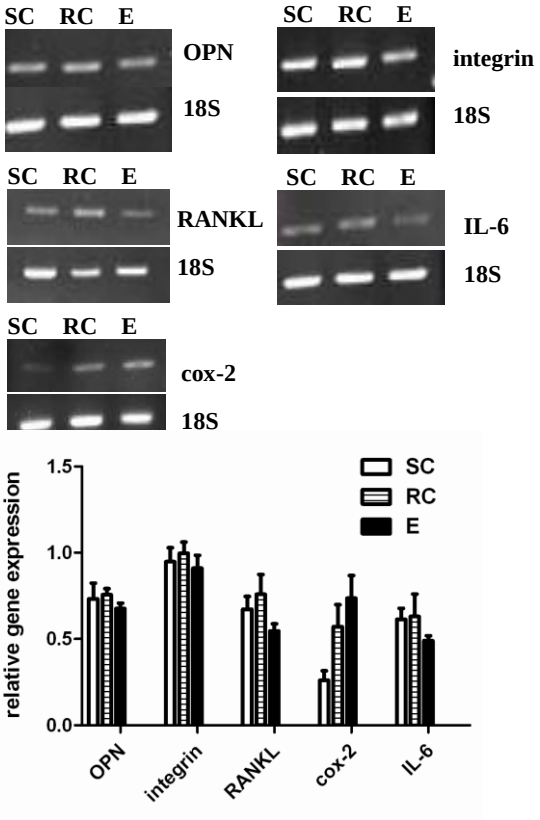

Fig. 4 RT-PCR analysis of expression of some genes in clinorotated MLO-Y4 cells. The bar graph shows the quantified results of three independent experiments.

3. 3 Gene Expression

Compared to SC and RC groups, the expression of some genes in mRNA level changed in E group. After 2 h of clinostat culture, expressions of OPN and integrin slightly decreased, and RANKL and IL-6 decreased by 19%±5% and 20%±4%, respectively, while cox-2 level increased by 65%±8% (**Fig. 4**).

4. Discussion

Space weightlessness could cause bone loss. Bone cells change their structure and function to respond to the decrease of mechanical stress, which might be a primary cellular mechanism of bone loss[12]. Many studies have shown that osteocytes could be a central sensor in bone tissue, which could transfer mechanical signal to biochemical signal and influence the function of effector cells such as osteoblasts and osteoclasts[2, 13].

In this study 2D clinostat was used to simulate weightlessness, which rotate continuously and change the direction of gravity vector so that cells among it could not responsd to gravity. Some experiments have been done using clinostat in bone cells[14-15].

Cytoskeleton plays an important role in responding to external mechanical stimuli. Actin-based cytoskeleton may be

one of the targets for cells to respond to gravity, which is confirmed by previous studies in osteoblasts in space flight and ground-based experiments[15,16]. Also in MLO-Y4 cells, the change of cytoskeleton has been observed after parabolic flight and magnetic levitation by our group[8,9]. In this study, actin fibers became thicker, especially in the cytoplasm. Actin cytoskeleton rearranged to resist to unloading caused by 2 h of clinostat culture, which also proved that cytoskeleton be one of the targets for cells in responding to mechanical alteration. However, no significant change was detected in the overall morphology of osteocytes, including the number of processes/cell, cell area and length-width ratio.

Integrin could serve as a mechanosensitive molecule linking components of extracellular matrix (ECM) with intracellular actin cytoskeleton, and initiate intracellular signaling in bone[17]. In MLO-Y4 cells, high level of beta1 integrin was involved in cell adhesion to ECM[18]. Osteopontin (OPN) could interact with integrin, promote cell adhesion with ECM[19], and participate in the process of bone reconstruction and bone mineralization[20]. Simulated weightlessness could decrease OPN expression in osteoblasts[21]. In this experiment, the expressions of OPN and integrin in mRNA level both slightly decreased after 2 h clinostatation, to a certain extent, indicating reduction of adhesion ability of MLO-Y4 osteocyte-like cells with ECM.

In addition to regulating structure and appearance to adapt to mechanical unloading, osteocyte themselves also can influence bone remodeling through soluble factor paracrine or gap junction between effector cells[13].

Cox-2 is an inducible isozyme of synthesizing prostaglandins (PGs), which play a crucial role as a signal molecule in the process of bone metabolism[22,23]. Receptor activator of nuclear factor-κB ligand (RANKL) also is a critical molecule in osteoclast formation and activity[24,25]. Microgravity increase osteoblasts' regulation ability of initiating osteoclastogenesis by up-regulating of RANKL/OPG ratio[26], and fluid flow stress decrease RANKL/OPG ratio and down-regulated osteoclastogenesis in MLO-Y4 cells[27,28]. Interleukin-6 could promote osteoclast differentiation and bone resorption in vitro and in vivo[29]. Osteoblasts cultured in rotation wall vessel bioeffector (RWV) have shown an upregulation of IL-6 mRNA[30].

Most of previous researches have shown that after subjected to weightlessness, bone cells express more RANKL and IL-6 to increase bone resorption, while less cox-2 to reduce bone formation. But we got different results after 2 h culture of simulated weightlessness, which might be a stress reaction of MLO-Y4 cells in acute stage due to compensatory mechanism. After a short-time stress of simulated weightlessness, it is necessary to osteocytes to rapidly regulate structure and function, including gene expression and secretion of some soluble factors, to adapt to changed microenvironment.

In summary, our results show that gene expression and actin cytoskeleton arrangement are both sensitive to mechanical unloading, which are regulated rapidly to adapt to changed mechanical microenvironment in MLO-Y4 cells. These changes could be related to osteocytes' structure and regulation to effector cells. And 2 h of clinostat culture do not influence tubulin arrangement and overall morphology in MLO-Y4 osteocyte-like cells. These findings will provide some clue for studying the cellular mechanism of bone loss.

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