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doi: 10.1016/S0273-1177(03)00497-6

THE EFFECTS OF SIMULATED MICROGRAVITY ON CULTURED CHICKEN EMBRYONIC CHONDROCYTES

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ABSTRACT

Using the cultured chicken embryonic chondrocytes as a model, the effects of simulated microgravity on the microtubular system of the cellular skeleton, extracellular matrix, alkaline phosphatase activity, intracellular free calcium concentration and mitochondrial ATP synthase activity with its oligomycin inhibition rate were studied with a clinostat. The microtubular content was measured by a flow cytometer. The decrease of microtubular content showed the impairment of the cellular skeleton system. Observation on the extracellualr matrix by the scanning electron microscopy showed that it decreased significantly after rotating, and the fibers in the extracellular matrix were more tiny and disorderly than that of the control group. It can be concluded that the simulated microgravity can affect the secreting and assembly of the extracellular matrix. In contrast to the control, there was a time course decrease in alkaline phosphatase activity of chondrocytes, a marker of matrix mineralization. Meanwhile a significant drop in the intracellular calcium concentration happened at the beginning of rotation. These results indicate that simulated microgravity can suppress matrix calcification of cultured chondrocytes, and intracellular free calcium may be involved in the regulation of matrix calcification as the second signal transmitter. No significant changes happened in the mitochondrial ATP synthase activity and its oligomycin inhibition rate. Perhaps the energy metabolism wasn't affected by the simulated microgravity. The possible mechanisms about them were discussed.

INTRODUCTION

The decalcification and strength decreasing of weight-bearing bones were found in the astronauts under long-time microgravity condition. Caillot-Augusseau et al. (Caillot-Augusseau et al. 1998, 2000) measured the serum concentrations of bone alkaline phosphatase (BAP), intact osteocalcinand (iBGP), type 1 procollagen propeptide (PICP) and other element. They found that BAP, iBGP, and PICP were decreased during the flight. As the current popular field in the space medicine and space life science, the investigation of these problems is of great significance. It's obvious that the bone loss and the decomposing of the collagens are related to the microgravity effects on calcification, energy metabolism, cellular signal system, cellular skeleton and extracellular matrix. Since it's difficult to study the biochemical mechanism of above problems in vivo, the cultured cells which metabolism were similar with the bone cells were used as research models to remove the interference of some factors, such as hormone regulating, neural regulating, the interactions between different cells, and so on. Decreased chondrogenesis was seen in cultures of embryonic limb bud cells flown in space on IML-1 (Duke, et al. 1992). In spaceflown rats with fractured fibula, formation of cartilage callus was minimal (Kaplansky, et al. 1991). Freed et al. (Freed, et al. 1997) studied the reconstructed cartilage under microgravity and found that the structure

Adv. Space Res. Vol. 32, No. 8, pp. 1577–1583, 2003

Printed in Great Britain
0273-1177/$30.00 + 0.00

of this reconstructed cartilage as well as the morphology of the chondrocytes had changed. In our experiment, the cultured chicken embryonic chondrocytes were used as the experimental material. We studied the microgravity effects on the extracellular matrix where the calcification happens, the microtubulin of the cell skeleton, alkaline phosphatase which is the marker of chondrocyte calcification, the intracellular free calcium concentration and the mitochondrial ATP synthase which is the key enzyme of energy metabolism and tried to investigate the common rules. It will be of important help to understand the problems of the bone loss and the changes of bone tissues under the microgravity.

MATERIALS AND METHODS

Cell Culture of Chicken Embryonic Chondrocytes

Refer to the document (Wang, et al. 1986). Chicken embryos were developed for 14 days at 38°C. Their limb bones were cut, immersed in penicillin G (1000unit/ml) and streptomycin sulphate (1000unit/ml) for 5 min stripped the bone membranes and other connective tissues and obtained the cartilages. The cartilages were digested by 0.25% trypsin. The trypsinized cells were seeded into cultural bottles.

The medium is F12 (GIBCO, USA) containing 10% fetal calf serum, penicillin G (100unit/ml) and streptomycin sulphate (100unit/ml). The cell density was 500,000/ml. After the cells grew for 40h and adhered to the wall of the bottles, all the bottles were full filled with the growth medium to minimize the shear force when cultured on the clinostat. And then half of the bottles were put on the clinostat to simulate microgravity, the other bottles were used as the control group lay aside under the same temperature without rotation. The growth medium was refreshed every other day. All the cells were cultured at 37°C.

The clinostat was designed and manufactured in the Department of Biophysical Technology, Institute of Biophysics, Chinese Academy of Sciences. The rotating speed was 30 rpm. The minimum rotation radius was1.6 mm, that the minimum centrifugal force was 1.6×10^{-3}g; the maximum rotation radius was 6 mm, that the maximum centrifugal force was less than 6.0×10^{-3}g.

Measurement of the Microtubule Content

Rotated for 48h, the cells were washed with Simms buffer (NaCl 0.274mol/L, KCl 0.054mol/L, Na_2HPO_4 0.030mol/L, Glucose 0.111mol/L, pH 7.2) and then were digested with 0.25% Trypsin in Simms buffer for 2 min. Collected the cells into the medium, fixed them in 4% glutaraldehyde for 5min, then the cells were lysed in 0.1% TritonX-100 and centrifuged at 1000rpm for 10min at room temperature. After one wash and another centrifugation, cells were incubated at 37°C for one hour with the anti-microtubulin antibody(SIGMA, USA). Washed and centrifuged again, the cells were allowed to react for 1 hour with FITC-conjugated goat immunoglobulins specific for rabbit immunoglobulin. The mean fluorescent intensity was measured at excitation 488nm and emission 530nm on a flow cytometer (Coulter Co. USA, Type: ETICS XL). The average fluorescent intensity means the relative microtubulin content.

Preparation of the Specimens for the Scanning Electron Microscope

Cut cover glasses into pieces and pasted the pieces into the culture bottles. The cells grew on the pieces. Rotated for 48h, the small pieces were taken from the bottles and immersed in PEM buffer solution (containing PIPES100mM, EGTA1mM, $MgCl_2$ 0.5mM, pH6.9) containing 0.5% TritonX-100 for 15min, fixed in 2% glutaraldehyde for15min, 1% osmic acid for 30min, then hydrated in a series of ethanol, and desiccated at the critical point. After spray-golded, the specimens were observed and photoed by a JSM-35CF (JEOL, Japan) scanning electron microscope.

Determination of Specific Activity of Alkaline Phosphatase

The cultured cells were collected into Simms buffers after 48h rotation, and then homogenized and sonicated at 0-4°C (50W×2min×2) for the measurement of the specific activity.

Alkaline phosphatase activity was measured as described in (Hatori, et al. 1995). Briefly, 100μl sample was mixed with 1.9ml substrate containing p-nitrophenyl phosphate disodium (pNPP, Sigma 104) 5mmol/L, Tris-HCl 1.5mol/L, $ZnCl_2$ 1mmol/L, $MgCl_2$ 1mmol/L, pH9.0. The absorbance at 410nm was measured for 30min at room temperature. $1A_{410}$=64nmol product (pNP).

DNA content was determined by a modification of the method of Labarca C and Paigen K (Labarca, et al. 1980). 0.5ml homogenate was incubated at room temperature in DNA assay buffer containing Na_3PO_4 0.05mol/L, NaCl 2.0mol/L, EDTA 2mmol/L, Hoechst H 33258 1μg/ml, pH 7.4 in dark for 20 hours. Then the sample was centrifuged at 1000g for 2 min. The fluorescence intensity of the supernatant was measured at excitation 356nm and emission 458nm on F4010 Hitachi Fluophotometer. Calf thymus DNA was used for calibration curve.

Measurement of Intracellular Ca^{2+} Concentration

The method referred to (Wang, et al. 1996; Grynkiewicz, et al. 1985) and was modified. When the cells were cultured on the clinostat for one and two days respectively, the cells as well as the control ones were incubated with Fura-2/AM (5μmol/L) for 45 min at room temperature in a dark place. Then the cell culture was digested with 0.25% Trypsin in Simms buffer for 5 min. To separate cells, it was centrifuged at 400g for 10min at room temperature. The cells were then washed twice with Simms buffer and were suspended into the same buffer at concentration of 5×10^5 cells/ml. For fluorescence measurement, a F4500 Hitachi Fluophotometer was used. Intracellular free calcium concentration was calculated by using the following equation :

$$(Ca^{2+})_i \text{ (nmol/L)} = Kd\times(R-R_{min})/(R_{max}-R)\times\beta \quad (1)$$

Where R was the ratio of the 510nm fluorescence at the two excitation wavelengths 340nm and 380nm. R_{max} was the fluorescence ratio determined by adding $CaCl_2$ (1mmol/L) and Triton X-100 (final concentration 1%). R_{min} was determined by subsequent addition of 50 mmol/L EGTA (ethylene glycol bis (β-aminoethyl ehter) -N, N'-tetraacetic acid). β was the ratio of fluorescence of Fura-2 when excited at 380nm in the zero and the saturating Ca^{2+}. Kd was the dissociation constant of Fura-2 for Ca^{2+}, assumed to be 224nmol/L (Grynkiewcz, et al. 1985).

Measurement of the Mitochondrial ATP Synthase Activity

After rotation for 2 days, washed the cells for two times using a Simms buffer, then scraped the cells into 1.5ml Simms buffer. The cells were sonicated at 0-4°C (50W×2min×2), and measured the mitochondrial ATP synthase activity and oligomycin inhibition rate by the enzyme-linked method (Li, et al. 1983).

RESULTS AND DISCUSSION

Effect of the Simulated Microgravity on Cellular Microtubules

The average fluorescence intensities were shown in Table 1, which shows a notable decrease in the microtubulin content after rotating.

Tab 1 Data of the microtubular fluorescence intensity

Experiment No.	1	2	3	4	Average
Control Group	180.7	158.3	218.0	80.8	159.5±57.9
Rotation Group	155.5	152.9	195.1	63.8	141.8±55.5

The data are relative intensities. $P<0.02$, n=4.

The cellular microtubular system has many functions. Besides cell-supporting function, transportation of substances, cellular secretion and signal transduction are all relative to the microtubular system. Tabony J. et al. (Tabony J. et al. 1992, 2001; Papaseit, P. 2000) suggested that the microtubule self-organisation depends on gravity. The above data showed that the microtubular system was impaired by the simulated microgravity. The microtubule is a polymer consisted of microtubulin dimers and it is in a dynamic balance. Since the structure of the microtubule is polar and its polymerization and depolymerization are along with the "head-tail" direction (Hackney, 1994), we believed that the continuous change of gravity direction caused by rotation affected the dynamic balance of the microtubule. So the microtubular system was impaired. We know that the kinesin and dynein proteins are always moved along the microtubule for transportation of substances (Koonce, et al. 1997). The impairment of the microtubular system must affect the transportation of many important substances.

Effect of Simulated Microgravity on the Extracellular Matrix

The scanning electron microscope photos were represented in Figure 1. It was shown that the extracellular matrixes of the rotated group were notably fewer than that of the control group. The fibers in the extracellular matrix of the control group were thicker and clearer than those of the rotated group.

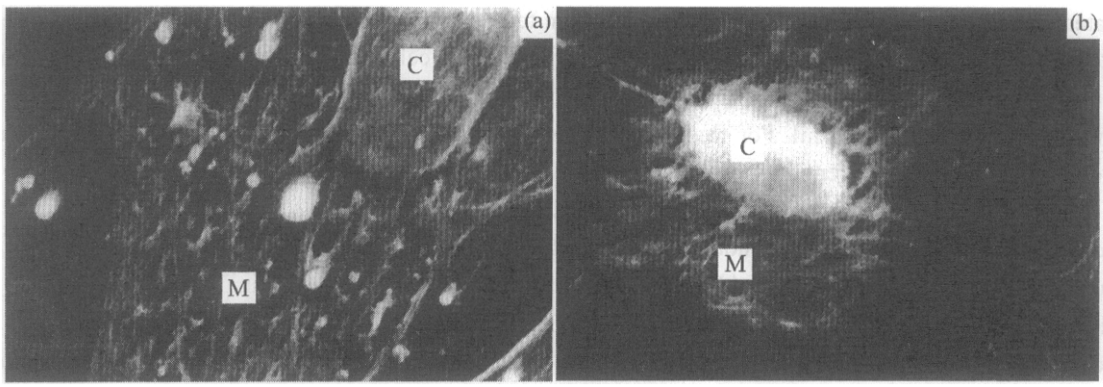

Fig 1 Scanning electron microscopic photos, showing the extracellular matrix of the chondrocytes. The size of cells in both photos was about 10μm. C: the cell body M: the extracellular matrixes a. the extracellular matrixes of the control cells were widely dispersed. The fibers were thick and clear. b. the extracellular matrixes of the rotated cells were much fewer. The fibers were tiny and disorderly

By the scanning electron microscope, it was shown that the extracellular matrixes of the cultured chondrocytes under the simulated microgravity were fewer than those under the normal condition. This result was consistent with the reduction of the formation of the cartilage callus under the microgravity which reported by Duke and Kaplansky et al. (Duke, et al. 1992; Kaplansky, et al. 1991). The result that the fibers of the rotated group are tiny, blur and disorderly was possibly involved with the balance of assembly and metabolism of collagen fibers in the extracellular matrix. Three interpretations were raised to explain this result. The first one is that the microgravity maybe induced the increasing of some substances, which functioned to de-aggregate the collagen fibers. Then the balance between the aggregation and de-aggregation of the collagen fibers trended to de-aggregation state, that is, the collagens were difficult to form big fibers. The second interpretation is that the balance between the aggregation and de-aggregation of the collagen fibers is related to the concentration gradient of collagens outside the cells. Normally, the gradient is gravity-dependent. The rotation group changed the gravity orientation continuously so that the gradient was disturbed. The fibers became disorder. The third interpretation is about the microtubular system. Because of the impairment of the microtubular system, perhaps the transportation

of substances and the cellular secretion would be blocked. If the secretion of the collagen decreased, the above result would be happened also. These three mechanisms maybe worked together.

Effect of Simulated Microgravity on Alkaline Phosphatase Specific Activity

From the calibration curve of DNA, measured by concentration of DNA verses fluorescence intensity (F), we obtained the following equation which was a liner estimation of the data of the curve with the correlation r=0.999.

$$(\text{DNA})\ (\mu g) = (F-2.853)/17.311 \tag{2}$$

Then alkaline phosphatase activity can be calculated by the following equation:

$$\text{ALPase specific activity (nmol/L pNP/min/}\mu\text{gDNA)} = 92.33\times\Delta A_{410}/(F-2.853) \tag{3}$$

Where ΔA_{410} is the change of the absorbance at 410nm in 30 minutes, F is the fluorescence intensity of the sample in DNA assay buffer.

After statistical analysis for the three experiments, the alkaline phosphatase activities were shown in Table 2, which shows a decrease in ALPase activity when the chondrocytes were cultured under simulated microgravity compared with the control.

Table 2 The effect of simulated microgravity on the specific activity of ALPase of the cultured chondrocytes

Experiment No.	1	2	3	Average
Control Group	0.821	1.187	0.655	0.888±0.272
Rotation Group	0.544	0.899	0.514	0.652±0.214

Unit: nmol product/min/μgDNA, n=3. $P<0.05$.

There may be two possible ways for alkaline phosphatase to be concerned with chondrocytes mineralization: 1) ALPase functions to hydrolyze organic phosphates and pyrophosphates. After removing pyrophosphates, a circulating inhibitor of calcification, and raising the local levels of Pi, the calcification was initiated (Zhang, et al. 1995). 2) ALPase is a membrane bound enzyme that becomes phosphorylated itself at low pH. The changes in the phosphoryl status may facilitate vesiculation or serve as a nucleating site for mineralization. And the phosphorylated ALPase itself may also be a nucleating site for mineralization (Shapiro, et al. 1981). So the ALPase activity was considered as a maker for calcification. After statistical analysis at 95% confidence of our results, the ALPase activity of the chondrocytes cultured under simulated microgravity was declined significantly in contrast to that of the control (Table 2). This means that simulated microgravity may impede the calcification of cultured chondrocytes.

Effect of Simulated Microgravity on Intracellular Free Calcium Concentration

The results of the intracellular free calcium concentration are shown in Table 3. In the beginning period after rotation, the intracellular calcium concentration of the chondrocytes that were cultured under simulated microgravity was decreased much more rapidly than that of the control. And after that, the two groups have no significant difference.

Table 3 The data of the intracellular free calcium concentration(Unit: nmol/l)

Experiment No.		Before rotation	24 hours	48 hours
1	Control Group	113.99	102.40	99.25
	Rotation Group		72.30	72.42
2	Control Group	146.83	86.17	78.26
	Rotation Group		79.46	80.15

Since intracellular free calcium concentration is usually far lower (10^3-10^4 times) than extracellular free calcium concentration, it is less likely to directly take part in calcification. But, as the second signal transmitter, intracellular free calcium can affect cell functions and biological processes by enzyme or gene regulation. The decrease in intracellular free calcium concentration of the chondrocytes under simulated microgravity indicates that intracellular calcium may be concerned with the cellular effects of the simulated microgravity as the 2nd signal messenger.

Effect of the Simulated Microgravity on the Activity of Mitochondrial ATP Synthase

The results were shown in Table 4. From the data, we couldn't find a significant and regular difference between the control and rotation groups.

Table 4 The effects of the simulated microgravity on the mitochondrial ATP synthase and the oligomycin inhibition rate (Unit of specific activity: 1μM ATP hydrolyzed/min/mg protein)

Experiment No.		1	2	3
Control Group	Specific Activity	0.133	0.073	0.094
	Inhibition rate (%)	35.6	47.3	45.2
Rotation Group	Specific Activity	0.156	0.061	0.102
	Inhibition rate (%)	49.5	46.0	47.0

The measurement showed the simulated microgravity didn't significantly affect the activity of mitochondrial ATP synthase and its oligomycin inhibition rate. Because the mitochondrial ATP synthase plays a key role in the cell energy metabolism, it seems the cell energy metabolism is not affected by the simulated microgravity.

The experiment of Freed (Freed, et al. 1997) had reported the changes of the cartilage structure and the morphology of the chondrocytes under the real microgravity. Our result about the impairment of the microtubular system maybe is an earlier symptom of the cellular morphological change. It showed the simulated microgravity was exactly able to simulate the real microgravity effects. The results of Duke and Kaplansky et al. (Duke, et al. 1992; Kaplansky, et al. 1991) also proved it. Other results done by us, such as the decreased activity of ALPase, gave the possible mechanisms of the decreased calcification in the sub-cellular and molecular levels.

Although the mechanisms of cellular microgravity effects haven't been elucidated thoroughly, we can briefly analyze the possible pathway of the simulated microgravity cellular effects based on our results.

To a single cell, we believe that the first phase of the simulated microgravity effects is its direct physical effects, as follows: one is changing the concentration gradient of extracellular secreted substances. That will induce the negative balance of the extracellular matrix. The feedback on this effect will change the cellular environment and the cellular secreting rate, etc. Another one is directly affecting the cellular structures such as the microtubular system.

With the secondary response, the change of the concentration gradient of extracellular substances decreases the extracellular matrix, then directly affects the cartilage calcification. The impairment of the microtubular system will affect the transportation of the intra- and extra-cellular substances, the signal transduction and the cell shape-supporting. These procedures are related to the change of intracellular Ca^{2+} concentration. This change is the prelude of the posterior responses. As the 2nd messenger, Ca^{2+} can modify the activities of some enzymes such as ALPase or affect the gene expressions by the signal transduction. In the whole procedure, we think that the microtubular system and the extracellular matrix are important.

These results will be helpful to study the problem of bone loss under microgravity and find the effective methods to solve it. The advanced study is proceeding.

ACKNOWLEDGEMENT

This work was funded by Chinese State 863 High-Tech Project (Gant No.2-7-2-14).

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Manuscript received 11 November 2002; revised 26 March 2003; 31 March 2003.