Behavior of Cells in Excess Gravitational Field: Using Centrifuge

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ABSTRACT

The effect of mechanical field on orientation and extension of cells has been studied using centrifuge *in vitro*. Four kind of cells were used in the test: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), L929 (fibroblast connective tissue of C3H mouse), HUVEC (Human Umbilical Vein Endothelial Cells), Neuro-2a (a mouse neural crest-derived cell line). To apply the mechanical force field for 24 hours to the cells adhered on the glass plate, the plate was set in the tube in a conventional centrifugal machine, which was placed in an incubator. Variation was made at the angular position of the plate in the tube to make variation at the direction of the force on the surface of scaffold: normal and tangential. The experiment shows following results. The elongation of neurites of Neuro2a is accelerated in the excess gravitational force field. L929 tends to tilt to the direction of the tangential force. C2C12 tends to make orientation at diagonal direction of the tangential force. The cell tends to extend the area of adhesion at the excess gravitational force field. C2C12 tends to change the shape at the excess gravitational field.

Keywords: Biomedical Engineering, Cell Culture, Excess Gravitational Field, C2C12, L929, Neuro-2a and HUVEC.

1. INTRODUCTION

A biological cell is surrounded by mechanical force field *in vivo*. The cell is sensitive to the mechanical stimulation, and shows several responses: deformation, and migration. The response includes passive one and active one. The cell has compliance, and is deformed by force. The cell deforms, on the other hand, to minimize the intra force. The cell is moved by the force. The cell moves in response to the force.

The object is exposed to the force at the surface with two kinds of directions: normal, and tangential. The object is pressed by the normal force. The object is sheared on the surface with the tangential force.

The muscle tissue might decrease in the micro gravitational field [1, 2]. The muscle tissue might increase, on the other hand, in the hypergravity. The previous study shows that the excess gravitational field thicken the myotubes *in vitro* [3].

The acceleration technique for orientation, proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro* [3-14]. Control methodology for orientation, proliferation and differentiation of cells would be applied to the regenerative tissue technology.

In the present study, the effect of mechanical field on orientation and extension of cells has been studied using centrifuge *in vitro*.

2. METHODS

Hyper Gravitational Force Field

The hyper-gravitational force was applied to cultured cells with the centrifugal force.

A glass plate of 50 mm ×10 mm was used for the scaffold for cell culture. The glass plate is inserted in the tube, which is contained in the rotor. The angle between the radial direction of the rotation of the rotor and the axial direction of the tube in the rotor is 1 rad. The variation was made on the direction of the glass plate in the tube. In the group X the glass plate was set so that the culture surface is parallel to the radial direction of the rotation. The centrifugal force is applied parallel to the culture surface in the group X. In the group Y, the glass plate was set in the perpendicular position to the former group rotated at the longitudinal axis of the tube. Both the tangential fore (F_t) and the normal force (F_n) at the culture surface are applied by the centrifuge in the group Y (Fig. 1).

The centrifugal force (F_c) is calculated by Eq. 1.

$$F_c = m\, r\, \omega^2 \qquad (1)$$

In Eq. 1, m is mass, r is radius of the rotation, and ω is angular velocity.

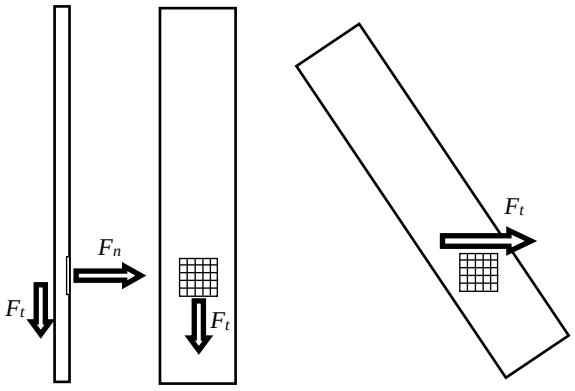

Fig. 1: Glass plate with mark: group X (right), group Y (left).

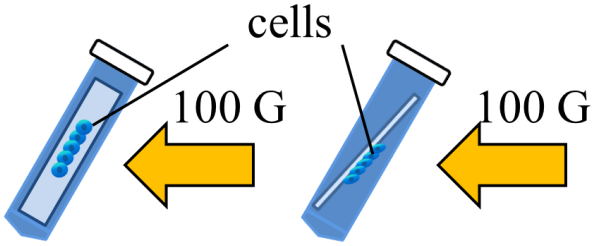

Fig. 2: Group X (left), and Y (right).

Fig. 3: Centrifuge in incubator.

In the gravitational field, gravitational force (F_g) is calculated by Eq. 2, where g is gravitational acceleration.

$$F_g = m\,g \qquad (2)$$

In the present study, the centrifugal acceleration of 100 G (1 G is equal to the gravitational acceleration) is generated at r = 0.07 m by ω = 120 rad/s with the centrifugal machine (CN-1040, Matsuuraseisakusyo. Ltd, Tokyo, Japan).

In the group X, the tangential force (F_t) of 100 G is applied to the surface of the glass plate. In the group Y, both the normal force (F_n) of 87 G and the tangential force (F_t) of 50 G are simultaneously applied to the surface of the glass plate (Fig. 2).

Perpendicularly crossing lines of grooves, which make 25 squares (5 × 5) of 1 mm × 1 mm, are marked on the rear surface of the glass plate to trace the cell behavior (Fig. 1). One of the directions of the lines corresponds to the direction of the tangential force by the centrifuge (Fig. 1). The grooves (0.016 mm width, 0.002 mm depth) were machined by the ultrashort pulse laser (IFRIT, Cyber Laser Inc., Tokyo, Japan).

During the stimulation test to cells, the centrifugal machine was placed in an incubator to keep the content of carbon dioxide of 5 % at 310 K (Fig. 3).

Cell Culture

Four kinds of cells were used in the experiment: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), L929 (fibroblast connective tissue of C3H mouse), HUVEC (human umbilical vein endothelial cells), and Neuro-2a (a mouse neural crest-derived cell line).

C2C12 of the passage between three and nine was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. The medium was changed to D-MEM (Dulbecco's Modified Eagle's Medium) containing 2% decomplemented HS (horse serum) and 1% penicillin/ streptomycin for differentiation.

In the test with C2C12, the experiment of 150 G (r = 0.07 m by ω = 140 rad/s) was added instead of 100 G.

L929 of the passage between three and nine was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin.

HUVEC of the passage between three and eight was cultured in EBM-2 (Endothelial Cell Basal Medium) containing 2% decomplemented FBS (fetal bovine serum).

Neuro2a of the passage between two and nine was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. For differentiation, the medium was changed to D-MEM (Dulbecco's Modified Eagle's Medium) containing 2% decomplemented FBS (fetal bovine serum) and of Retinoic Acid for differentiation. The concentration of Retinoic Acid in the medium is adjusted to 20 μM.

The cells were seeded on the glass plate at the density of 1000 cells/cm^2. After the cells were cultured in the incubator for 24 hours without stimulation, the cells were exposed to the excess gravitational field by centrifuge for 24 hours. The glass plate, on which cells adhered, was set in the medium in the tube. The angle of the glass plate in the tube was adjusted to that of group X or Y (Fig. 2). Several tubes with the glass plate were set in the rotor to cultivate cells of group X and Y simultaneously. Each tube was capped with a film of gas permeable paraffin [4]. After the centrifuge, the cells were cultured successively in the incubator for another 24 hours.

To keep the content of carbon dioxide of 5 % at 310 K, the cells were incubated in an incubator through the entire experimental term including the term of exposure to the excess gravitational field. Namely, the centrifugal machine was placed in the incubator during the centrifugation (Fig. 3). In the control group (normal force of 1 G), the cells were cultured without centrifuge on the glass, which was placed in the polystyrene dish (50 mm diameter) without collagen coating.

Morphological Study

The morphology of each cell was observed with an inverted phase-contrast microscope (IX71, Olympus, Tokyo) every 24 hours during the test: after 24 hours of culture, in three minutes after exposure to the excess gravitational field, after additional 24 hours. In the same area of the marked square, the behaviors of the cells were traced. The outline of each single cell

(except cells in the colony) is traced with "Image J" to calculate parameters: the orientation, the projected area, and the shape index (Fig. 4).

To evaluate the orientation, the angle between the direction of tangential force by centrifuge and the longitudinal direction of each cell was measured. To evaluate the deformation, the projected area was calculated as the area surrounded by the outline of each cell. The shape index was calculated by the ratio between the major axis and the minor axis of each cell projected to the plate of the scaffold. The shape index becomes unity at the circle.

To evaluate differentiation of Neuro-2a, the extension of neurite was observed.

3. RESULTS

Fig. 5 shows the angle between the direction of the tangential force (on the surface of the scaffold by centrifuge) and the longitudinal direction of each cell. The degree of 90 shows that the longitudinal direction of the cell is parallel to the direction of the tangential force. In fig. 5, zero degree means the longitudinal direction of each cell is perpendicular to the direction of tangential force. In Fig. 5, data are arranged in the ascending order. When the angles are distributed at random, the data align on the linear line. In the range of dense population, the data align on the gentle inclination line. In the range of sparse population, on the other hand, the data align on the steep inclination line.

Fig. 5 shows the following results. In the control study, the angles distribute at random. C2C12 shows tendency to align on the direction to the tangential force of 50 G with normal force of 87 G by centrifuge. C2C12 shows tendency to align on the diagonal oblique direction to the tangential force of 150 G by centrifuge (to 70 and 130 degrees). L929 shows tendency to align on the perpendicular direction to the tangential force of 50 G with normal force of 87 G by centrifuge. L929 shows slight tendency to align on the direction perpendicular (zero and 180 degrees) to the tangential force of 100 G by centrifuge. HUVEC shows slight tendency to align on the direction to the tangential force of 50 G with normal force of 87 G by centrifuge.

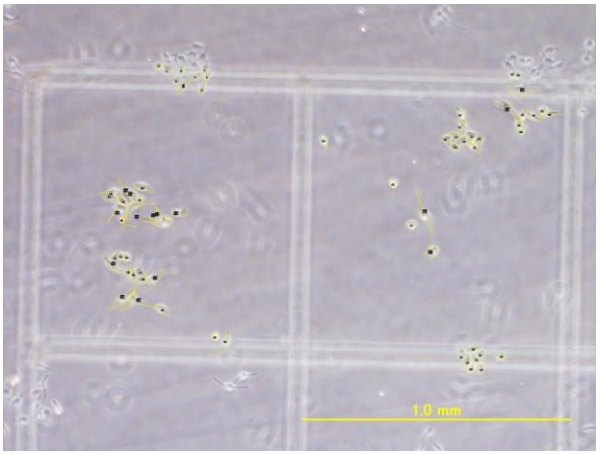

Fig. 4: Outline of each single cell is traced. Square mark shows 1 mm × 1 mm.

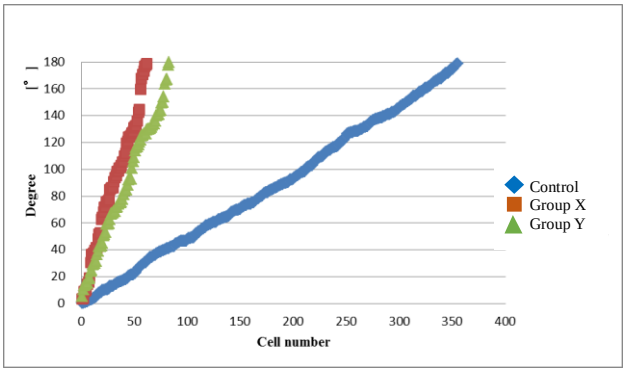

Fig. 5a: Angle of C2C12. 90 deg : centrifuge.

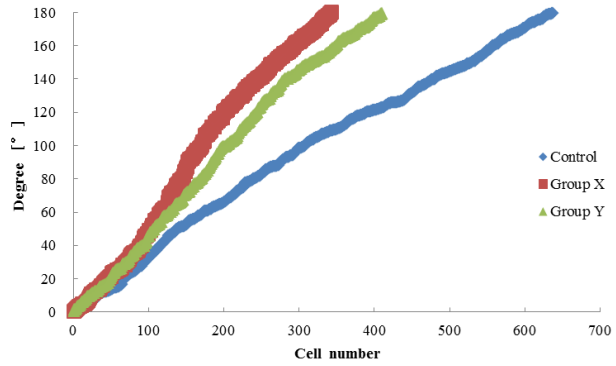

Fig. 5b: Angle of L929.

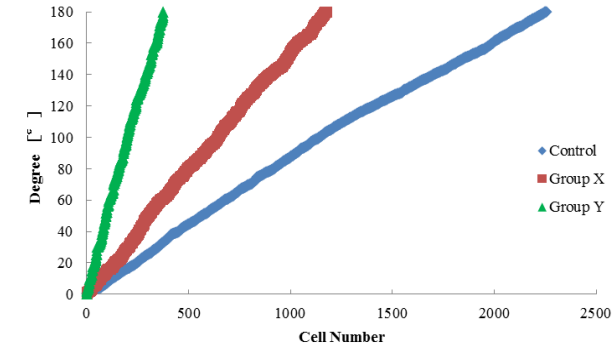

Fig. 5c: Angle of HUVEC.

In Figs. 6-7, the column shows the mean value, and the bar shows the range (±) of the standard deviation. The mean value in Fig. 6 shows that the area of each single cell (C2C12, L929, and HUVEC) tends to increase under the mechanical stimulation.

Fig. 7 shows that there is not statistically significant difference at the shape index of cell between control study and centrifuge study. Only the shape index of C2C12 tends to increase. The number of Neuro-2a with neurites increases after centrifugation. Fig. 8 shows that the differentiation of Nero-2a [15] is accelerated by the mechanical stimulation by centrifuge.

L929 changes the shape at the excess gravitational field (Fig. 9a). Some cells of HUVEC are elongated in the excess gravitational force field (Fig. 9b). The direction of the centrifugal force is vertical in Fig. 9.

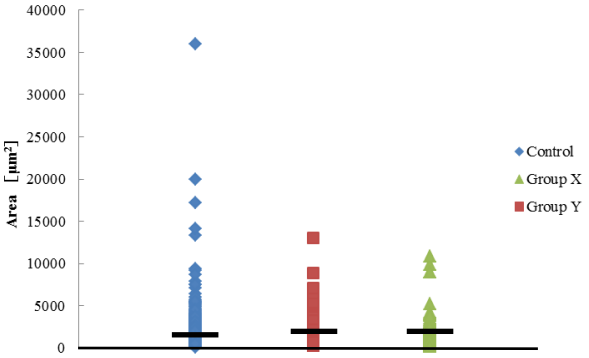

Fig. 6a: Area of C2C12. Mean: 1170, 1810, 1883 μm^2.

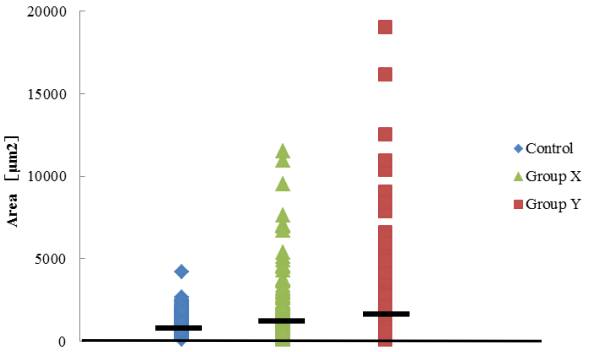

Fig. 6b: Area of L929. Mean: 876, 1371, 1696 μm^2.

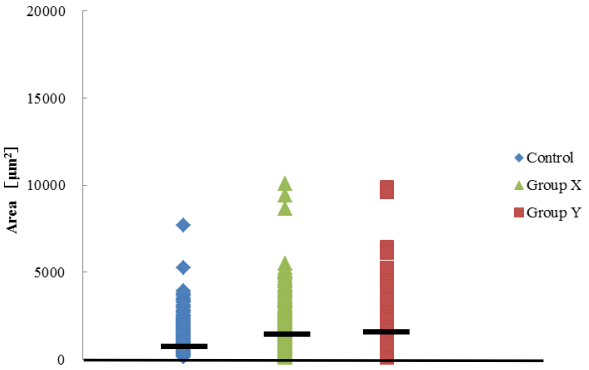

Fig. 6c: Area of HUVEC. Mean: 775, 1204, 1266 μm^2.

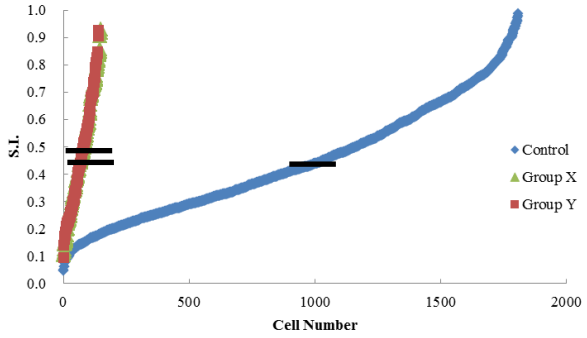

Fig. 7a: Shape index of C2C12. Control: n=1810, Group X: n=140, Group Y: n=149. Mean: 0.44, 0.49, 0.45.

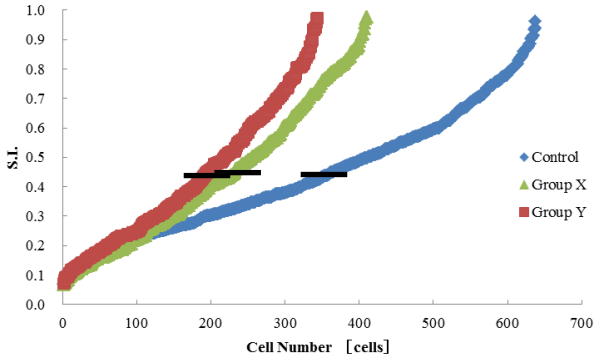

Fig. 7b: Shape index of L929. Control: n=638, Group X: n=411, Group Y: n=344. Mean: 0.43, 0.44, 0.43.

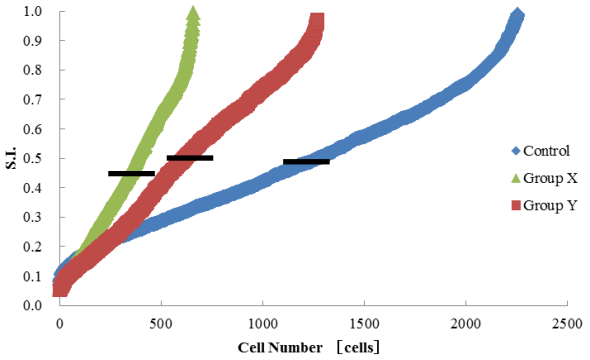

Fig. 7c: Shape index of HUVEC. Control: n=2262, Group X: n=658, Group Y: n=1269. Mean: 0.48, 0.45, 0.50.

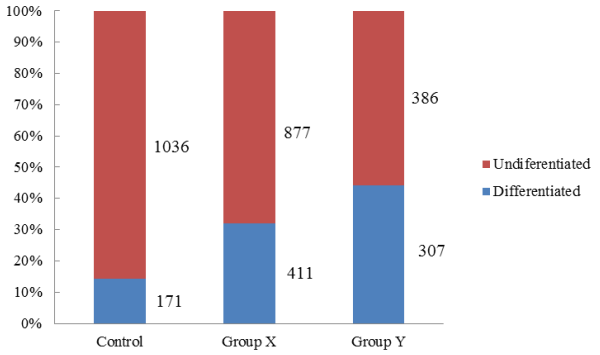

Fig. 8: Ratio of Nero-2a with neurites.

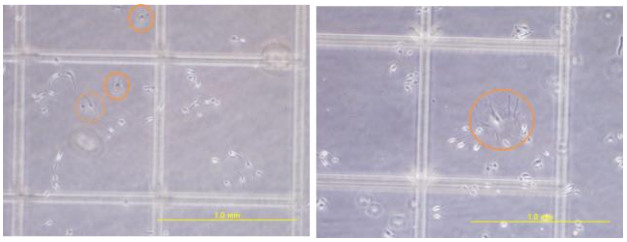

Fig. 9a: L929 after stimulation: group X (left), group Y (right). Square mark shows 1 mm × 1 mm.

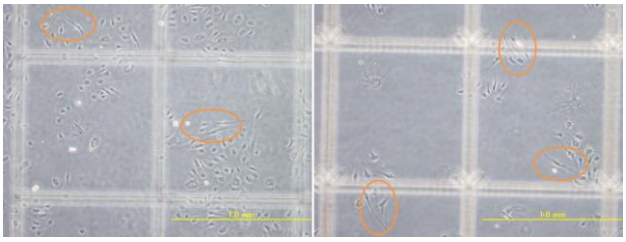

Fig. 9b: HUVEC after stimulation: group X (left), group Y (right). Square mark shows 1 mm × 1 mm.

4. DISCUSSION

Cells passively follow the direction in the strong tangential force field. Immediately after the centrifugation, cell might start to show active response to the mechanical stimulation. Mild tangential force field induces the active reflection of cells to tilt perpendicularly to decrease internal force of the cells.

The cells might change the orientation at the differentiation. C2C12 made perpendicular orientation of myotubes to the flow direction in the previous study [16]. The orientation of C2C12 at diagonal direction of flow might be preparation to make perpendicular orientation of myotubes in the successive cultivation. C2C12 might differentiate earlier on polystyrene dish than glass plate [3].

The response of biological system to the microgravity field has been studied using a space satellite. The cell cycle might extend in the space [1, 2]. C2C12 differentiation might be accelerated and the myotube might align to the direction perpendicular to the centrifugal field [4].

When mechanical stimulation is applied to the scaffold, the whole stimulation cannot always be transmitted to the cells. When the tension applied to a scaffold, the deformation of the scaffold generates compression and shear in the different direction simultaneously [17-19]. To apply continuous uniform mechanical stimulation to the cells, centrifugal force [3-5] is used in the present study.

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on the cultured biological tissue. The previous study showed that the behavior of cells depends on the electric [11, 21-23] and magnetic stimulation [12]. Another study shows that mechanical stimulation improves a tissue-engineered skeletal muscle [24]. The results of the study will contribute to acceleration technique in regenerative medicine

The previous studies showed that a mechanical field governs behavior of cells [3-10, 16-20, 24-29]. The shear flow governs the orientation of endothelial cells [28, 29]. The shear stress affects the orientation of the smooth muscle cells in the biological tissue [17]. The direction of the mechanical field affects fibroblasts [19]. The effect of shear flow on orientation of cells depends on the kinds of cells [16]. Although HUVEC (human umbilical vein endothelial cells) orients along the stream lines, C2C12 tilts from the stream lines to make myotubes. The previous study showed orientation of cells perpendicular to the stretch direction [18].

Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [3]. The mechanical stimulation can decrease proliferation of cells [3]. The mechanical stress also exfoliates several cells, which makes vacancy around the adhesive cell. The differentiation might be optimization of cells to the changing environment. The mechanical stress can accelerate differentiation of C2C12 to make myotubes [3].

The effect of mechanical field on orientation and extension of cells has been studied using centrifuge *in vitro*. The elongation of neurites of Neuro2a is accelerated in the excess gravitational force field, especially in the normal stress field. The normal force field has effect similar to adhesion to the scaffold. The results of three kinds of cells (C2C12, L929, HUVEC) show that the single cell tends to tilt to the direction of gravitational force. L929 extends the area of adhesion at the tangential force field. L929 changes the shape at the excess gravitational field. Some cells of HUVEC are elongated in the excess gravitational force field.

Exfoliation of cells tends to increase by the combination of excess normal force and shear force than pure shear force at the scaffold. The area of the cell projected to the scaffold tends to increase under the excess gravitational field. The shape of L929 and HUVEC tends to change under the excess gravitational field. The response of the cell depends on the direction of the gravitational field.

5. CONCLUSION

The effect of mechanical field on orientation and extension of cells has been studied using centrifuge *in vitro*. Four kind of cells were used in the test: C2C12 (mouse myoblast), L929 (mouse fibroblast), HUVEC (Human Umbilical Vein Endothelial Cells), Neuro-2a (a mouse neural crest-derived cell). The experiment shows the elongation of neurites of Neuro2a, the tilt of L929, the diagonal tilt of C2C12, extension of the area of adhesion of cells, and the change of the shape of C2C12 at the excess gravitational field.

6. ACKNOWLEDGMENT

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