Effect of Excess Gravitational Force on Cultured Myotubes in Vitro

Shigehiro HASHIMOTO, Haruka HINO
Biomedical Engineering, Department of Mechanical Engineering, Kogakuin University
Tokyo, 163-8677, Japan
shashimoto@cc.kogakuin.ac.jp http://www.mech.kogakuin.ac.jp/labs/bio/

and

Takeshi IWAGAWA
Department of Biomedical Engineering, Osaka Institute of Technology,
Osaka, Japan

ABSTRACT

An effect of an excess gravitational force on cultured myoblasts has been studied in an experimental system with centrifugal force *in vitro*. Mouse myoblasts (C2C12) were seeded on a culture dish of 35 mm diameter, and cultured in the Dulbecco's Modified Eagle's Medium until the sub-confluent condition. To apply the excess gravitational force on the cultured cells, the dish was set in a conventional centrifugal machine. Constant gravitational force was applied to the cultured cells for three hours. Variations were made on the gravitational force (6 G, 10 G, 100 G, 500 G, and 800 G) with control of the rotational speed of the rotator in the centrifugal machine. Morphology of the cells was observed with a phase-contrast microscope for eight days. The experimental results show that the myotube thickens day by day after the exposure to the excess gravitational force field. The results also show that the higher excess gravitational force thickens myotubes. The microscopic study shows that myotubes thicken with fusion each other.

Keywords: Biomedical Engineering, Myotube, Gravitational Force, Cell Culture, Fusion and Differentiation.

1. INTRODUCTION

Several effects of a gravity free condition on a biological system have been studied with various experimental systems [1-3]. Keeping a muscle condition is important for an astronaut in a micro gravity condition. The gravitational force might affect differentiation or on multiplication of muscle cells. The cell culture technique enables observation of the cell behavior in a designed condition about gravitation.

Control methodology for the cell culture would be applied to regenerative tissue technology. For example, myoblasts have been clinically applied to ischemic cardiomyopathy in the field of regenerative medicine. Behavior of biological cells depends on various environmental factors, such as electric, magnetic and mechanical fields [4-6].

A sophisticated experimental instrumentation is necessary to realize zero gravity condition on the earth [7, 8]. In the present study, a conventional centrifugal machine has been applied to the control method of the gravity condition, and the effect of an excess gravitational force on cultured muscle cells has been studied in an experimental system with the centrifugal force *in vitro*.

2. METHODS

Cell Culture

Mouse myoblasts (C2C12: mouse myoblast cell line originated with cross-striated muscle of C3H mouse) were suspended in the High-Glucose-Dulbecco's Modified Eagle's Medium (DMEM) with fetal bovine serum (FBS) at the density of one million cells per milliliter. Two milliliter of the suspension was poured into a culture dish of 35 mm diameter, and cultured for 24 hours in the incubator (MCO-18AIC, Sanyo, Osaka, Japan) on the first day. In the incubator, the temperature of 310 K and the carbon dioxide partial pressure of 5 percent are maintained.

Application of Excess Gravitational Force

The excess gravitational force was applied to cultured cells with a centrifugal force. The culture dish of 35 mm diameter was set on the top of a centrifugal tube of 50 mL. The tube was put on a swing rotor in the centrifugal machine (2800, Kubota, Tokyo, Japan) (Fig. 1).

Fig. 1: Four dishes on the rotor in the centrifugal machine.

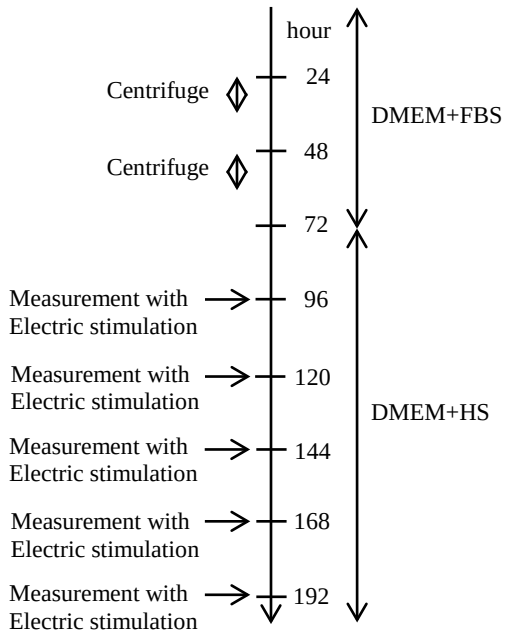

Fig. 2a: Protocol of cell culture.

The rotor was rotated with the speed of <300 rad/s, which makes an excess gravitational force of <800 G at the bottom of the culture dish. Variation was made about the excess gravitational force: 6 G, 10 G, 100 G, 500 G, and 800 G. To keep balance of the rotator in the centrifugal machine, two culture dishes were placed in the counter position each other.

The constant excess gravitational force was applied on cells for three hours at 298 K on the early part of the second day (Fig. 2). For the concurrent control study, a culture dish was kept at 298 K for three hours without the centrifugal force. After morphological observation, the cells were cultured in the incubator again for 21 hours on the rest part of the second day.

The second term stimulation with the excess gravitational force for another three hours was performed on the early part of the third day. The rest of the cultivation term, cells were cultured in the incubator. Cells are proliferated to sub-confluent condition in 72 hours.

At the end of the third culture day, FBS was replaced with horse serum (HS) for differentiation. The ratio of serum in the medium is ten percent in FBS, and seven percent in HS. The medium was refreshed every two days.

A polystyrene culture dish with type I collagen coating (Iwaki, 3010-060, Asahi Glass, Tokyo, Japan) was used.

Morphological Study

Morphology of cells was observed with an inverted phase-contrast microscope (IX71, Olympus, Tokyo, Japan) every 24 hours for eight days. The same area was traced in the serial cultivation, and the maximum width of every myotube was measured after the sixth day of culture.

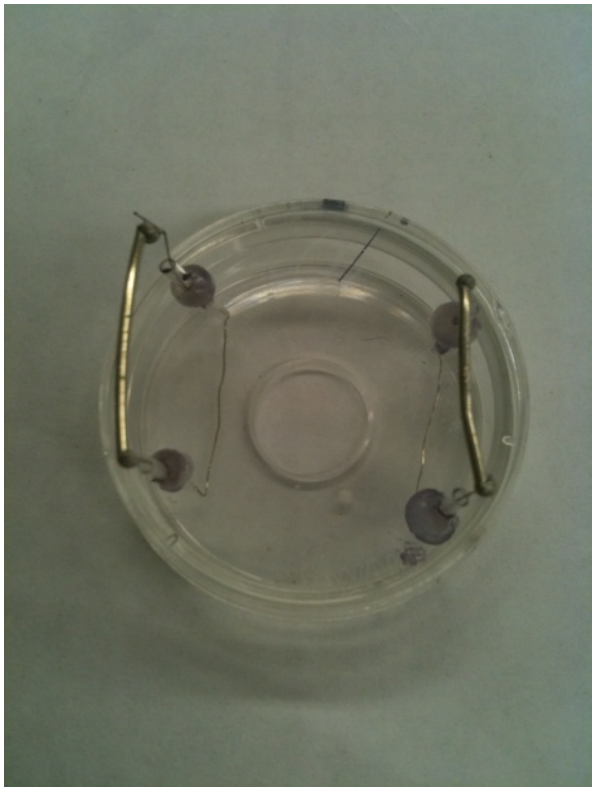

Fig. 2b: A pair of electrodes attached on the cover of the culture dish.

To confirm differentiation of muscle cells, the electric stimulation was applied to the culture medium after the fourth day, and the contractive movement was observed under the microscope. The electric pulses (the pulse width of one millisecond, the period of one second, and the amplitude of 30 V) were applied through a pair of electrodes of platinum wire (diameter of 0.2 mm), which are attached on the cover of the culture dish (Fig. 2).

3. RESULTS

Figs. 3a-8b exemplify typical microscopic appearance of cells. The bars in those figures indicate 0.050 mm. Fig. 3a shows cells before stimulation of the excess gravitational force for three hours on the second day. Myocytes adhere to the bottom of the culture dish in 24 hours (Fig. 3). Fig. 3b shows cells after stimulation of the gravitational force of 800 G for three hours on the second day. The figure shows that most of cells extend and some cells exfoliate after the excess gravitational stimulation. Figs. 3c & 3d show cells of the simultaneous control study on the second day. Any remarkable morphologic change has not been observed for three hours. Some cells also exfoliate in three hours of the control study.

Fig. 4a shows cells before stimulation of the excess gravitational force for three hours on the third day. Fig. 4b shows cells after stimulation of the gravitational force of 800 G for three hours on the third day. The results show that cells become slim after stimulation of 800 G.

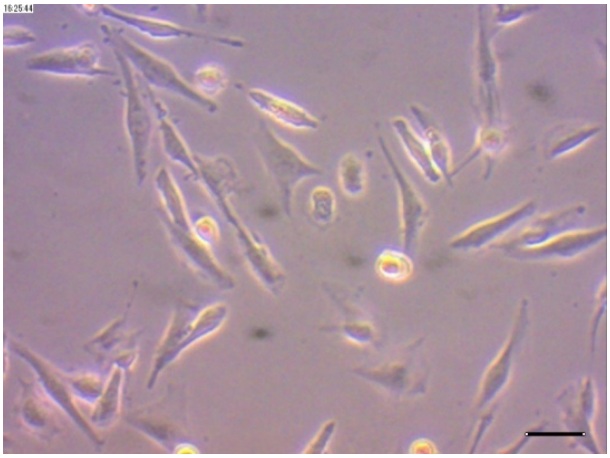

Fig. 3a: Cells before gravitational stimulation on the second day. The bar indicates 0.05 mm.

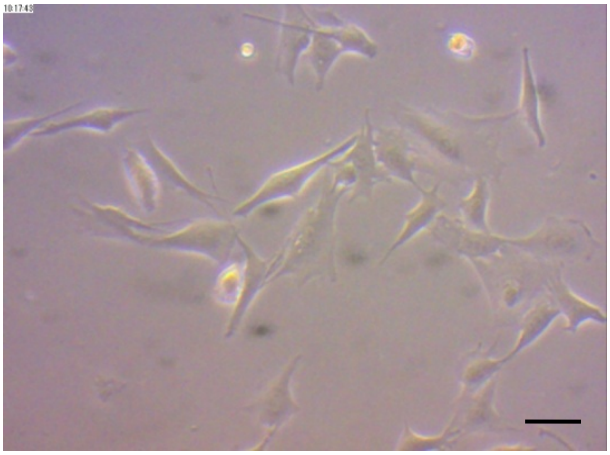

Fig. 3b: Cells after gravitational stimulation of 800 G for three hours on the second day. The bar indicates 0.05 mm.

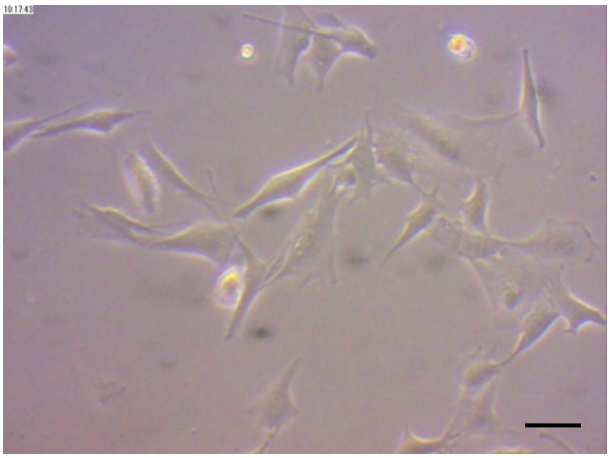

Fig. 3c: Cells before control three hours on the second day. The bar indicates 0.05 mm.

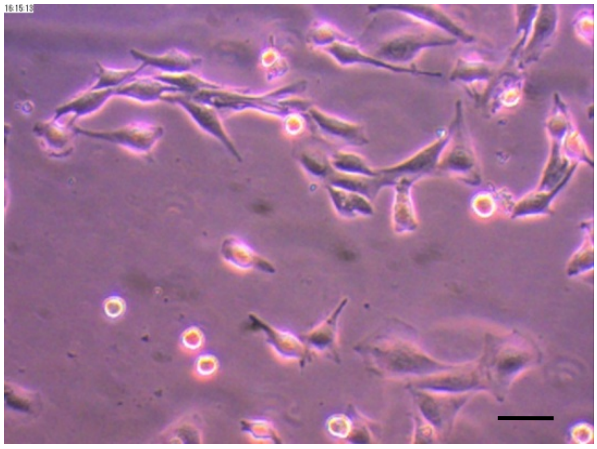

Fig. 3d: Cells after control three hours on the second day. The bar indicates 0.05 mm.

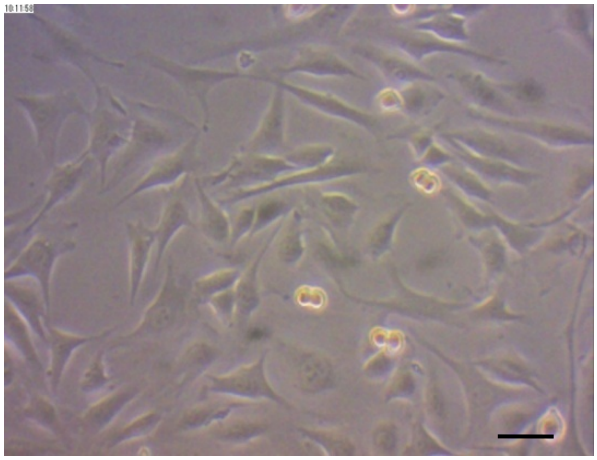

Fig. 4a: Cells before gravitational stimulation of 800 G for three hours on the third day. The bar indicates 0.05 mm.

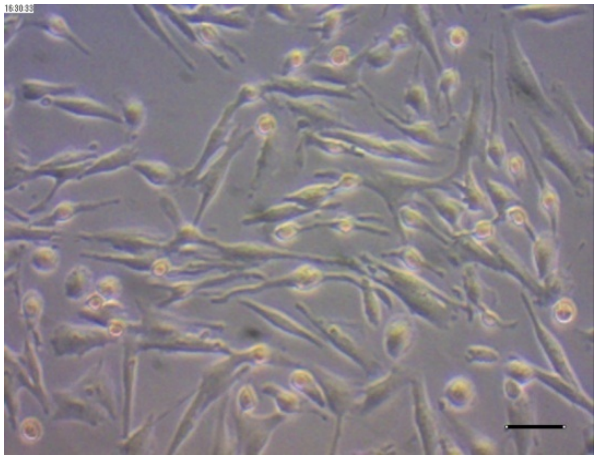

Fig. 4b: Cells after gravitational stimulation of 800 G for three hours on the third day. The bar indicates 0.05 mm.

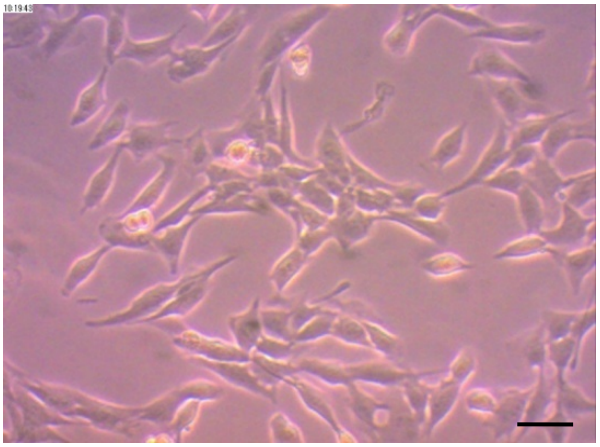

Fig. 4c: Cells before control three hours on the third day. The bar indicates 0.05 mm.

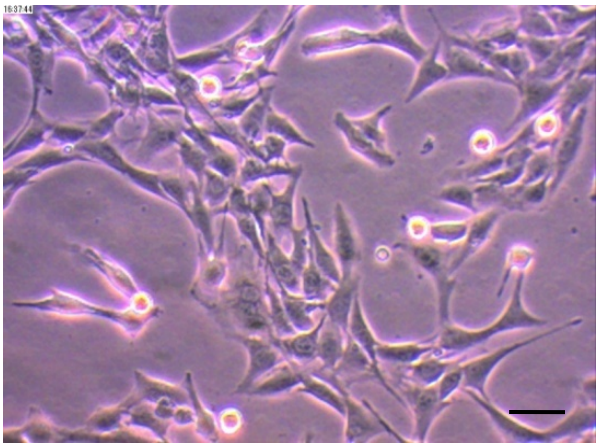

Fig. 4d: Cells after control three hours on the third day. The bar indicates 0.05 mm.

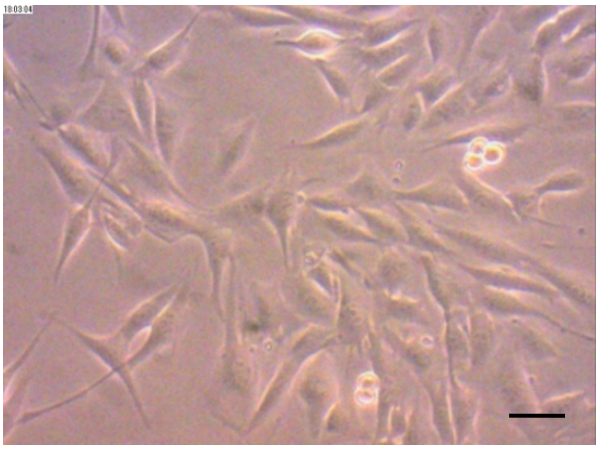

Fig. 5a: Cells in the circumferential area of gravitational stimulation of 800 G on the fourth day. The bar indicates 0.05 mm.

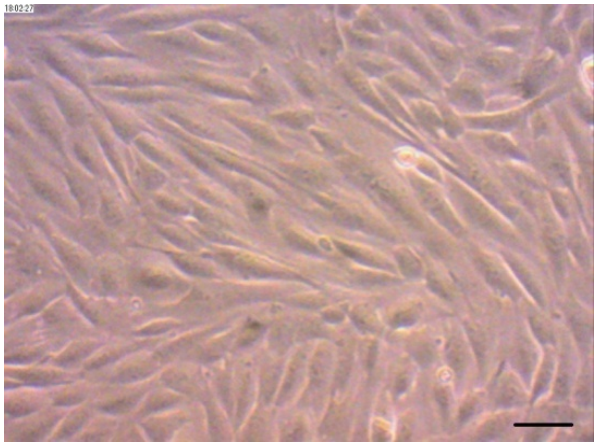

Fig. 5b: Cells in the middle area of gravitational stimulation of 800 G on the fourth day. The bar indicates 0.05 mm.

Figs. 4c & 4d show cells of the simultaneous control study on the third day. Any remarkable morphologic change has not been observed for three hours. Some cells also exfoliate in three hours of the control study.

Fig. 5a shows cells in the circumferential area of the stimulation (800 G) study on the fourth day (72 hours). Fig. 5b shows cells in the middle area of the stimulation (800 G) study on the fourth day.

Fig. 5c shows cells in the circumferential area of the control study on the fourth day. Fig. 5d shows cells in the middle area of the control study on the fourth day. The results show that cells are proliferated to sub-confluent condition in the middle area.

Fig. 6a shows cells of the stimulation group of 6 G on the eighth day. Fig. 6b shows cells of the simultaneous control study on the eighth day. Fig. 7a shows cells of the stimulation group of 800 G on the sixth day. Fig. 7b shows cells of the simultaneous control study on the sixth day. Fig. 8a shows cells of the stimulation group of 800 G on the eighth day. Fig. 8b shows cells of the simultaneous control study on the eighth day. The results show that cells start to fuse each other, and to form myotubes. Fig. 8b shows three lines of nuclei in a myotube.

The results at higher gravitational force show that cells aggregation decreases at the first stage. Several cells are floating in Fig. 3b. Cells are scattered and elongated in Fig. 3b.

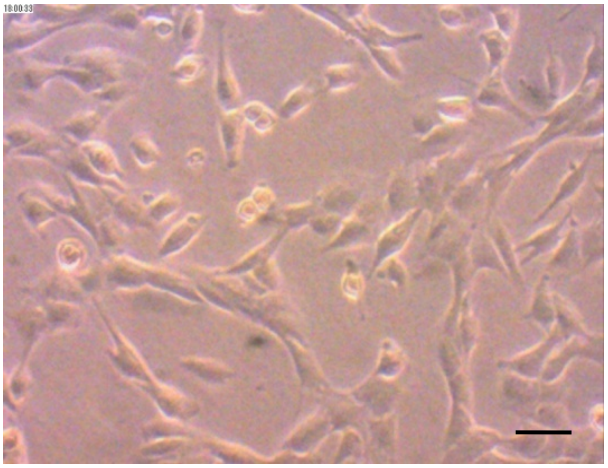

Fig. 5c: Cells in the circumferential area of control study on the fourth day. The bar indicates 0.05 mm.

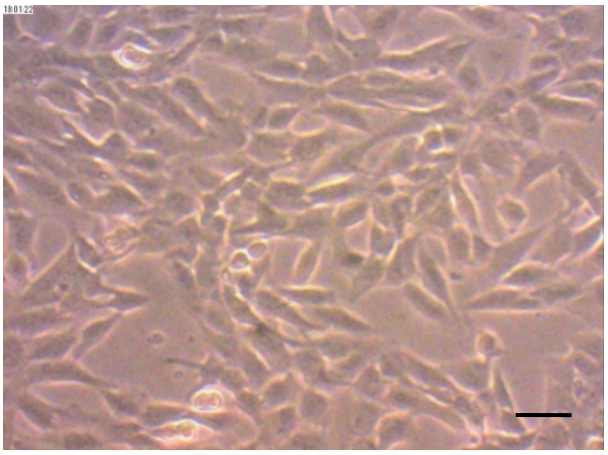

Fig. 5d: Cells in the middle area (d, lower) of control study on the fourth day. The bar indicates 0.05 mm.

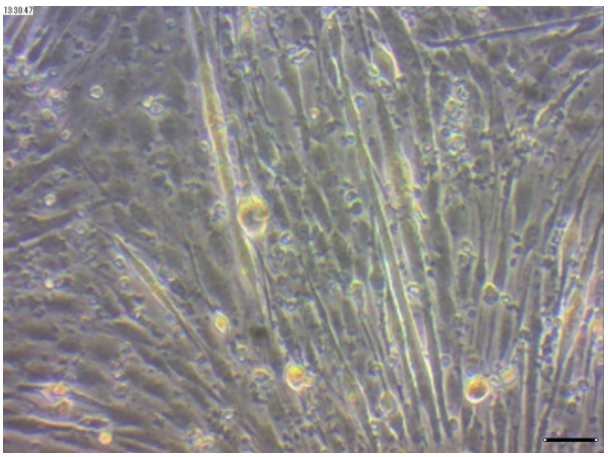

Fig. 6a: Cells of stimulation group of 6 G on the eighth day. The bar indicates 0.05 mm.

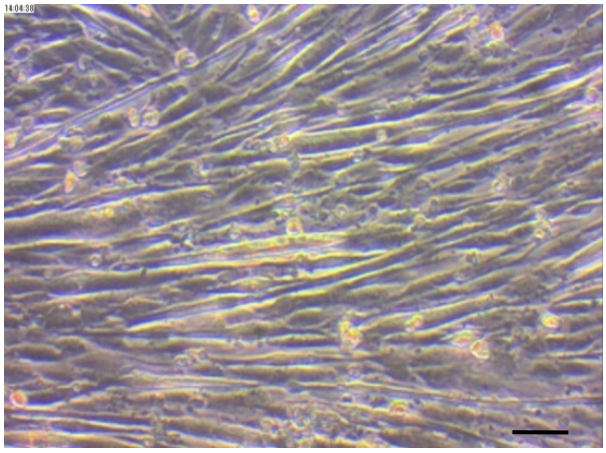

Fig. 6b: Cells of control group on the eighth day. The bar indicates 0.05 mm.

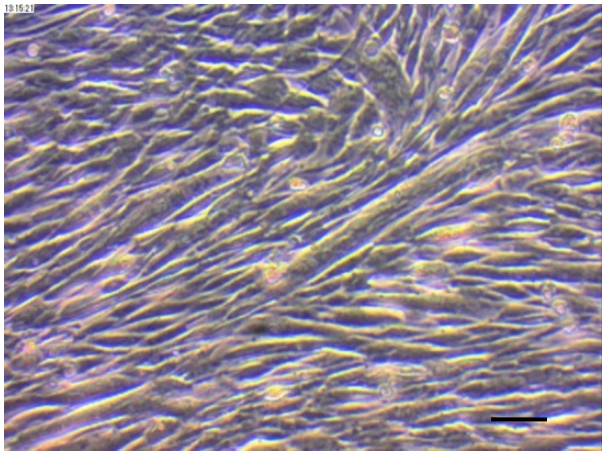

Fig. 7a: Cells of stimulation group of 800 G on the sixth day. The bar indicates 0.05 mm.

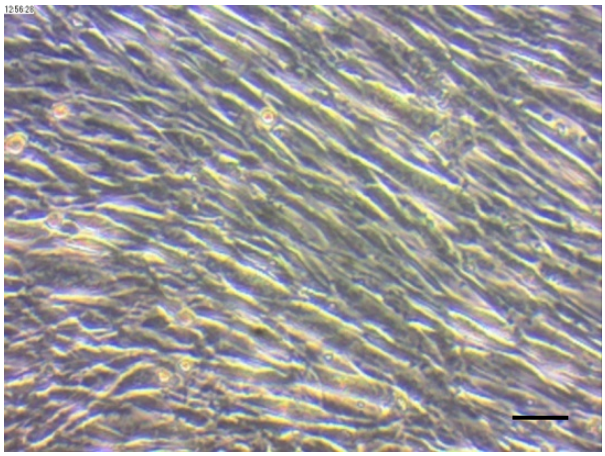

Fig. 7b: Cells of control group on the sixth day. The bar indicates 0.05 mm.

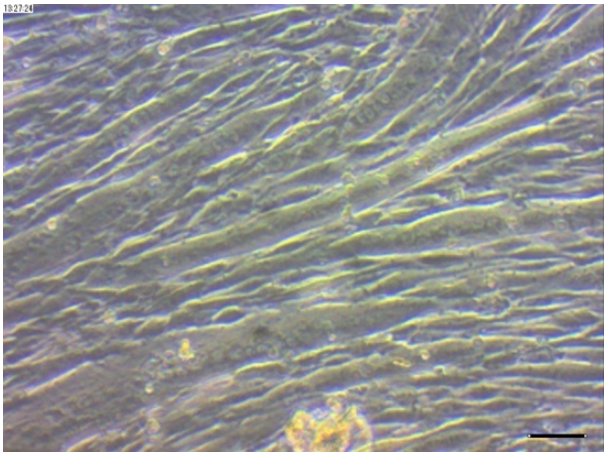

Fig. 8a: Cells of stimulation group of 800 G on the eighth day. The bar indicates 0.05 mm.

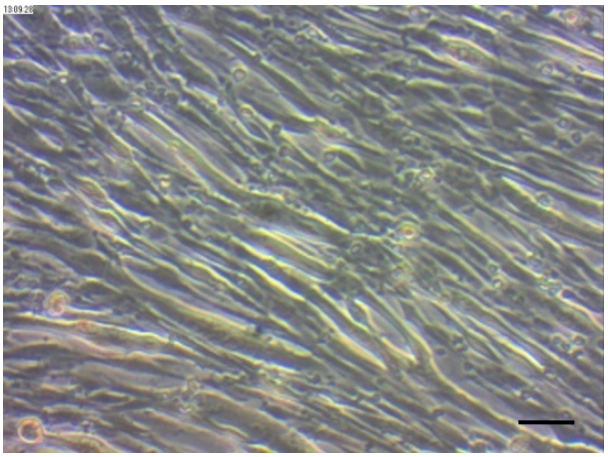

Fig. 8b: Cells of control group on the eighth day. The bar indicates 0.05 mm.

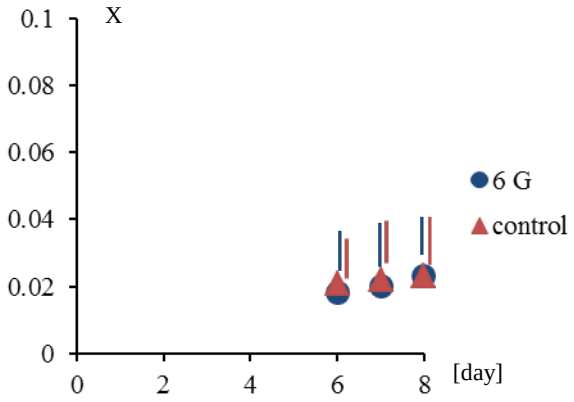

Fig. 9a: The relation between the maximum width of myotube (X, mm) and days of culture: 6 G.

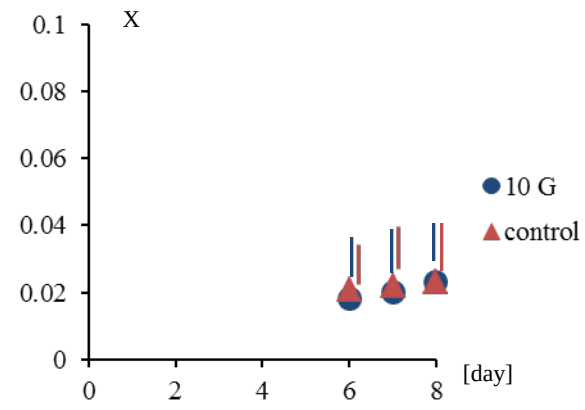

Fig. 9b: The relation between the maximum width of myotube (X, mm) and days of culture: 10 G.

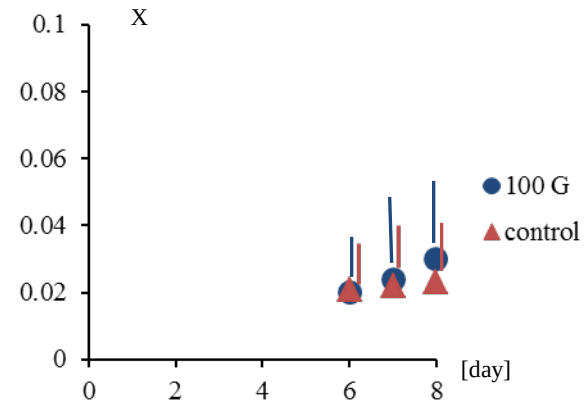

Fig. 9c: The relation between the maximum width of myotube (X, mm) and days of culture: 100 G.

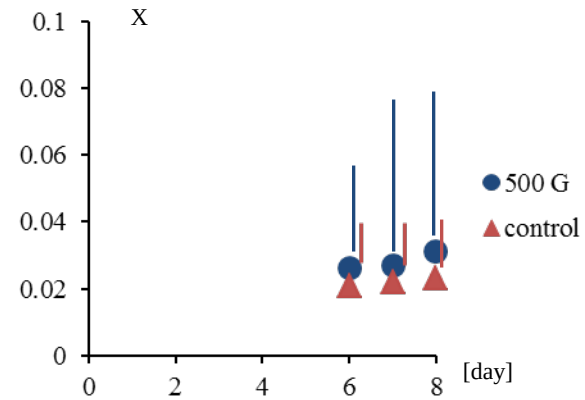

Fig. 9d: The relation between the maximum width of myotube (X, mm) and days of culture: 500 G.

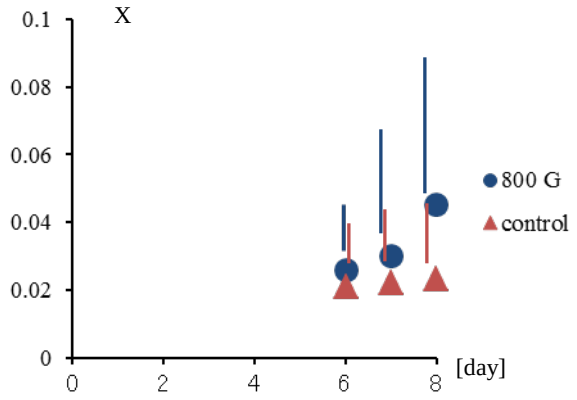

Fig. 9e: The relation between the maximum width of myotube (X, mm) and days of culture: 800 G.

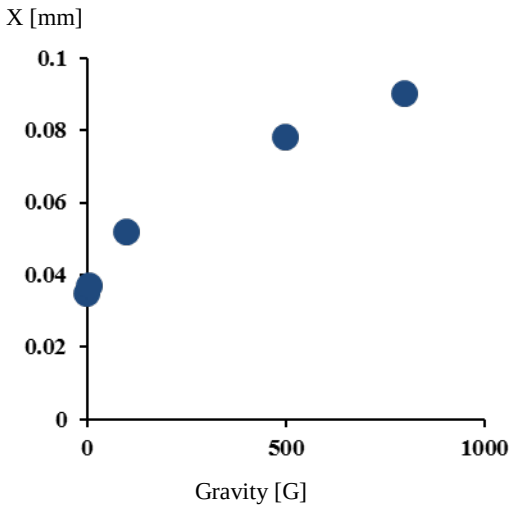

Fig. 10: Relation between gravity and the maximum width of myotube (X, mm) in eight days of culture.

The relation between the maximum width of myotube (X, mm) and days of culture is displayed in Fig. 9. Measured value of X scatters, so that both the mean value and the maximum value are displayed in Fig. 9.

The Data point shows the mean value and the top of the bar shows the maximum value.

The relation between the gravity and the maximum width of myotube is collected in Fig. 10. Measured value of the width scatters, so that maximum value is displayed in Figs. 10. The width of myotube becomes larger at the higher gravitational forces (500 G, 800 G).

When the periodical pulses were introduced in the culture medium through electrodes, repetitive contractions were observed at the myotubes both in the control study and in the excess gravitational force stimulation study. The contraction of the myotube was synchronized with the electric pulses.

4. DISCUSSION

The effect of gravitational forces on biological system has been discussed in several previous studies. Some of these studies have been extended to a biological reflection under condition with micro gravitational forces. The gravitational force at a space station, for instance, is smaller than a millionth part of that at the surface of the earth.

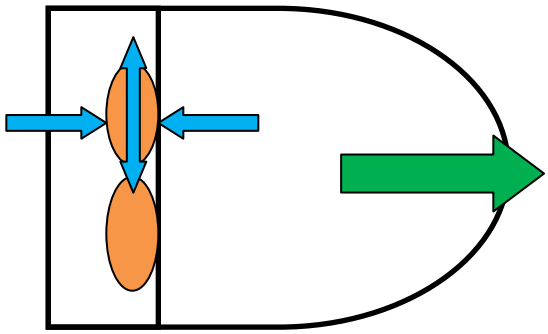

Fig. 11: Forces around a cell, during centrifugation.

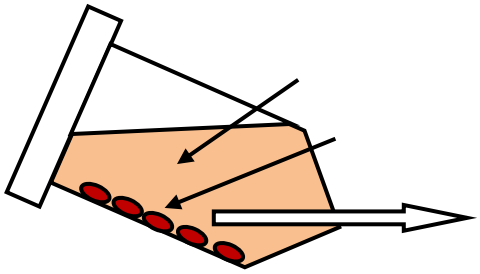

Fig. 12: C2C12 cultured on the lateral wall of centrifugal tube.

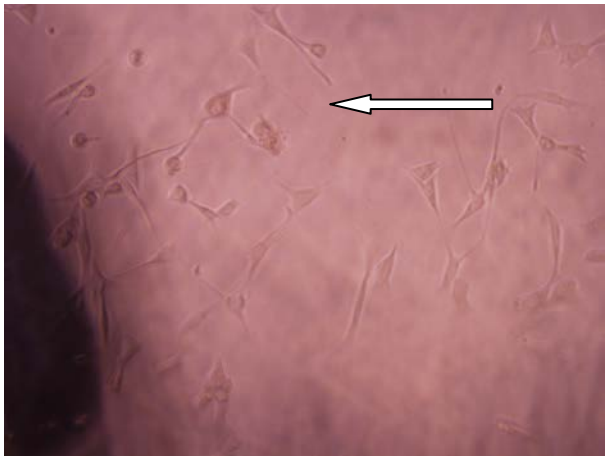

Fig. 13: C2C12, after centrifugation: 100 G, 3 min. Dimension from left to right is 1 mm. Direction of centrifugal force is from right to left (arrow).

An effect of the higher gravitational field on a mutation of a cell has been studied in the previous experiment *in vitro* [3]. At the higher gravitational force in the present study, several myocytes are exfoliated. That may controls aggregation of cells and the cells acquire space for elongation around themselves. These effects might accelerate differentiation to form myotubes.

The functional property of myotubes was confirmed by their repetitive contractions with electric pulses, which were introduced through the electrodes dipped in the medium.

The direction of the centrifugal force is perpendicular to the plane of cell culture (Fig. 11). In the gravitational field, several mechanical stresses might be generated around the cells: the compressive stress between the cell and the bottom of the dish, the compressive stress between cells, and the tensile stress in the perpendicular direction to the gravitational field (Fig. 11).

Some studies show that the electric stimulation enhances differentiation of muscle cells [9, 10]. Another study shows that the mechanical stimulation improves the tissue-engineered human skeletal muscles [5].

The mechanical stimulation might affect orientation of myoblasts. The effect of the centrifugal force on the orientation of myoblasts can be investigated, when the cell culture plane is set parallel to the centrifugal force (Fig. 12). Fig. 13 exemplifies C2C12 on the lateral wall of the tube after centrifugation of 100 G for three minutes. The direction of the centrifugal force is from right to left in Fig. 12. C2C12 tends to tilt to the direction perpendicular to the centrifugal force [11].

5. CONCLUSION

The experimental results show that the myotube thickens day by day after the intermittent constant excess-gravitational-force stimulation for three hours. The results also show that thickness of myotube increases with the excess gravitational force (<800 G). The microscopic study shows that myotubes thicken with fusion each other.

REFERENCES

[1] P. J. Rijken, R. P. De Groot, N. Van Belzen, S. W. De Laat, J. Boonstra, and A. J. Verkleij, "Inhibition of EGF-Induced Signal Transduction by Microgravity is Independent of EGF Receptor Redistribution in the Plasma Membrane of A431 Cells", **Experimental Cell Research**, Vol. 204, 1993, pp. 373-377.

[2] A. Villa, S. Versari, J. A. M. Maier and S. Braadamante, "Cell Behavior in Simulated Microgravity: A Comparison of Results Obtained with RWV and RPM", **Gravitational and Space Biology**, Vol. 18, No. 2, 2005, pp. 89-90.

[3] J. P. Hatton, F. Gaubert, M. L. Lewis, Y. Darsel, P. Ohlmann, J. P. Cazenave and D. Schmitt, "The Kinetics of Translocation and Cellular Quantity of Protein Kinase C in Human Leukocytes are Modified during Spaceflight", **Federation of American Societies for Experimental Biology Journal**, Vol. 13, Suppl, 1999, pp. S23-S33.

[4] S. Hashimoto and M. Okada, "Orientation of Cells Cultured in Vortex Flow with Swinging Plate in Vitro", **Journal of Systemics, Cybernetics and Informatics**, Vol. 9, No. 3, 2011, pp. 1-7.

[5] C. A. Powell, B. L. Smiley, J. Mills and H. H. Vandenburgh, "Mechanical Stimulation Improves Tissue-Engineered Human Skeletal Muscle", **American Journal of Physiology: Cell Physiology**, Vol. 283, 2001, pp. C1557-C1565.

[6] K. Sakiyama, S. Abe, Y. Tamatsu and Y. Ide, "Effects of Stretching Stress on the Muscle Contraction Protein of Skeletal Muscle Myoblasts", **Biomedical Research**, Vol. 26, No. 2, 2005, pp. 61-68.

[7] T. G. Hammond and J. M. Hammond, "Optimized Suspension Culture: the Rotating-Wall Vessel", **American Journal of Physiology - Renal Physiology**, Vol. 281, No. 1, 2001, pp. F12-F25.

[8] L. Yuge, I. Hide, T. Kumagai, Y. Kumei, S. Takeda, M. Kanno, M. Sugiyama and K. Kataoka, "Cell Differentiation and p38MAPK Cascade are Inhibited in Human Osteoblasts Cultured in a Three-Dimensional Clinostat", **In Vitro Cellular & Developmental Biology - Animal**, Vol. 39, 2003, pp. 89-97.

[9] J. Stern-Straeter, A.D. Bach, L. Stangenberg, V.T. Foerster, R.E. Horch, et al., "Impact of Electrical Stimulation on Three-dimensional Myoblast Cultures- A Real-time RT-PCR Study", **Journal of Cellular and Molecular Medicine**, Vol. 9, No. 4, 2005, pp. 883-892.

[10] S. Hashimoto, F. Sato, R. Uemura and A. Nakajima, "Effect of Pulsatile Electric Field on Cultured Muscle Cells in Vitro", **Journal of Systemics, Cybernetics and Informatics**, Vol. 10, No. 1, 2012, pp. 1-6.

[11] H. Hino, S. Hashimoto, M. Ochiai and H. Fujie, "Effect of Mechanical Stimulation on Orientation of Cultured Cell", **Proc. 17th World Multi-conference on Systemics Cybernetics and Informatics**, 2013, in press.