# Effect of Excess Gravitational Force and Electric Pulse Field on Myoblast

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## ABSTRACT

The effects of mechanical and electric stimulations on cell culture have been studied *in vitro*. C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was cultured in Dulbecco's Modified Eagle's Medium with 10 percent of fetal bovine serum on a polystyrene dish for 12 days, while the mechanical force field or the electric field is intermittently applied, alternatively. To apply the mechanical force field to the cells on the bottom of the dish, the dish was set on a swing-rotor. The rotor was placed in a conventional centrifugal machine, to set the surface of the culture plate in the perpendicular position to the centrifugal force. Through electrodes of platinum wire, the electric pulse (width of 0.0025 s, period of 0.5 s) was applied to the medium, where cells were cultured. The experiment shows following results: both the excess gravitational force and the electric pulse fields decelerate proliferation of myoblasts, and accelerate differentiation of myoblasts, and mild gravitational force field is effective to accelerate differentiation of myoblast.

**Keywords:** Biomedical Engineering, Cell Culture, C2C12, Excess Gravitational Field and Electric Field.

## 1. INTRODUCTION

The acceleration technique for orientation, proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro* [1-23]. Control methodology for orientation, proliferation and differentiation of cells would be applied to the regenerative tissue technology.

The mechanical stress is one of the interested factors in the environment of cells, because they receive mechanical force *in vivo*. The mechanical stress on cells might induce various responses: deformation, migration, proliferation, and differentiation. Several methods have been designed to apply the mechanical stress to cells [1-17].

A transmission point of the stress to a specimen is important. In many studies, the stress is applied to a scaffold [8, 10, 11]. When fixation between the cell and the scaffold is not enough, the stress is not transmitted to the cell. A mechanical field, on the other hand, can be used to apply a continuous stress to a specimen [1-6, 12-17]. The specimen fixed at a position receives the shear stress in the mechanical field. The gravitational field is one of the mechanical fields [1-6].

The biological cells receive gravitational force on the earth. An astronaut needs exercise before standing, when he comes back from the space station. The characteristic of muscle tissue might change in the space station, where the tissue does not receive gravitational force [3, 4].

The electromagnetic stress is another factor in the environment of cells [18-23]. The muscle tissue is exposed to electric pulses in the biological body. The movement is also controlled with the electric pulses. The biological systems have ability to optimize themselves to their environment. The electric stimulation has also been applied to a body in some medical treatments for rehabilitation. The optimum electric stimulation has a potential to control growth of the muscle tissue, which might contribute to regenerative medicine.

In the present study, the effects of mechanical and electric stimulations on cell culture have been studied *in vitro*.

## 2. METHODS

### Excess Gravitational Force Field

The excess gravitational force was applied to cultured cells with the centrifugal force. A swing rotor has been manufactured to apply the vertical force to cultured cells (Figs. 1-3). The rotor consists of a fixing bracket, an arm, a shaft and a dish. The fixing bracket and the shaft are made of the steel (SS400). The arm and the dish are made of aluminum alloy (A7075).

When the rotor swings, the culture dish swings. During the rotation, the centrifugal force acts perpendicularly to the bottom plane of the culture dish. The bottom of the dish has a line on the outside of the culture surface to mark the observation point. The dish was set on the swing rotor in the centrifugal machine (CN-1040, Matsuuraseisakusyo. Ltd, Tokyo, Japan).

The rotor (turning radius of 8.5 cm) rotates with the constant speed of 1025 revolutions per minute, which makes the excess gravitational force of 100 G at the surface of the culture plate. The centrifugal machine was placed in an incubator to keep the partial pressure of bicarbonate of 5 % at 310 K. Ethanol is filled in the counter dish to make balance the mass (approximately 0.12 kg) between two dishes on the both ends of the arm of the rotor.

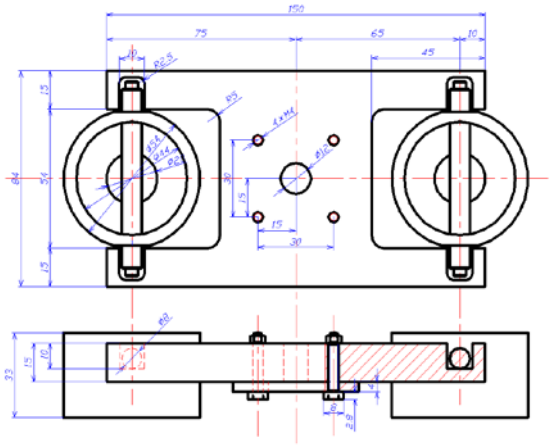

**Fig. 1:** Dimension (mm) of designed swing rotor.

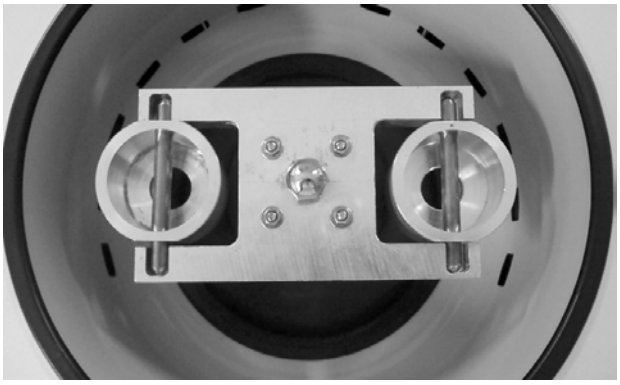

**Fig. 2:** Manufactured swing rotor in centrifugal machine.

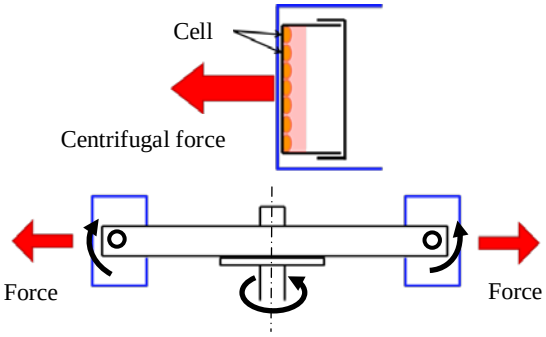


**Fig. 3:** Normal force on cell culture plane during centrifuge.

**Electric Field**

To apply electric pulse to culture cells, a pair of electrode has been made. The electrode is made of a platinum wire of 0.2 mm diameter. To keep the position of the electrode, the wire is fixed on a cap made of polydimethylsiloxane (PDMS). To decrease concentration of electric current around the wire, the wire is bended to make a square of 15 mm × 10 mm (Figs. 4&5). The distance between two electrodes is 30 mm.

The electric pulse (interval of 0.5 s, amplitude of 0.1 V, duration of 0.0025 s) was generated by an electric stimulator (SEN5201, Nihon Kohden Corporation, Japan). The pulses were applied through a couple of electrodes of platinum wire, which was dipped into the medium of the culture dish (Fig. 6). A resistance of thirty ohms ($r$) is serially connected in the electric circuit (Fig. 7). The electric signal was monitored with an oscilloscope. From the monitored potential difference of 5 mV ($V_2$) between the terminals of the resistance, the electric current is calculated as 0.17 mA ($I$).

**Cell Culture**

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) of the passage four was used in the test. D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin was used for the medium.

The cells were seeded on the culture dish of 35 mm diameter at the density of 2000 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 and to the electric pulse field.

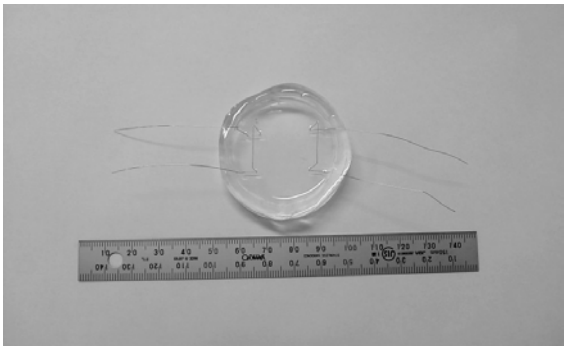

**Fig. 4:** Electrodes on cap.

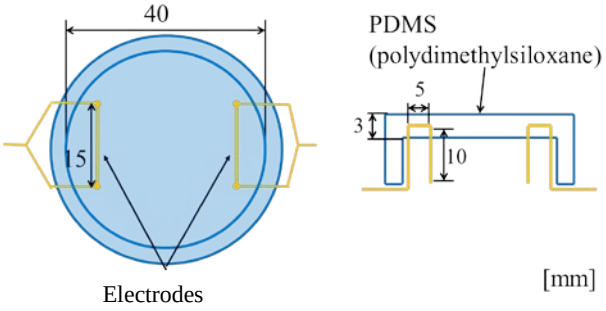


**Fig. 5:** Dimension (mm) of electrodes on cap.

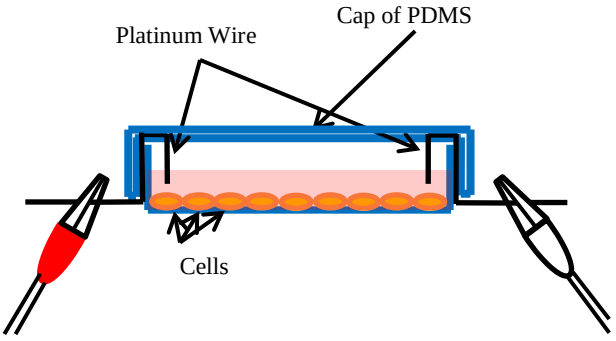


**Fig. 6:** Electrodes on cap of culture dish.

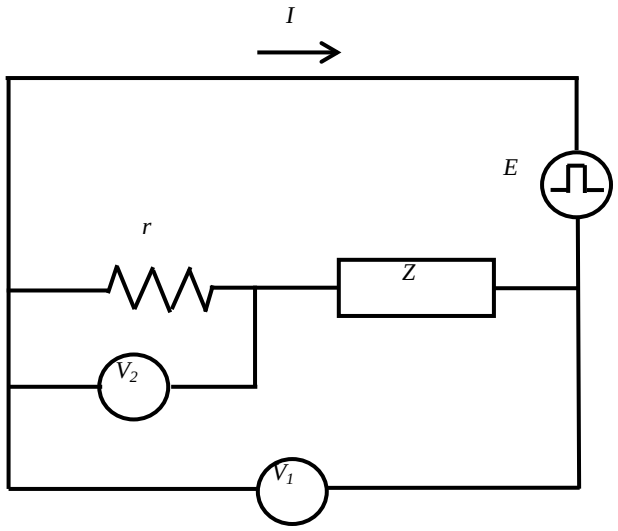


**Fig. 7:** Electric circuit: *E*, pulse generator; *Z*, medium; *r*, resistance; *I*, current; *V*, voltage.

The cell culture tests for 12 days were divided into four groups: excess gravity for one hour per day and electric pulse for one hour per day (A), excess gravity for two hours per day (B), electric pulse for two hours per day (C), and control without any stimulation (D) (Figs. 8-15).

The medium was refreshed every two days. The dish was placed in an incubator through the entire experimental term including the term of stimulation to keep the partial pressure of bicarbonate of 5 % at 310 K.

**Morphological Study**

Density and morphology of cells was observed with an inverted phase-contrast microscope (IX71, Olympus, Tokyo) every 24 hours during the test. During the microscopic observation, the culture dish with cells was taken out from the incubator. On the eleventh day, the width of each myotube was gauged, and the maximum value was compared among the groups. The data in the position, where the multiple myotubes are superimposed, were not counted for the maximum value.

**Electric Stimulation to Confirm Differentiation**

At the end of tests, myotubes were stimulated with electric pulses to confirm differentiation of C2C12. The electric pulse (interval of 0.5 s, amplitude of 10 V, duration 0.003 s) was generated by an electric stimulator (SEN5201, Nihon Kohden Corporation, Japan). The pulses were applied through a couple of electrodes of platinum wire of 0.2 mm diameter, which was dipped into the medium of the culture dish (Fig. 6). The movement of the myotubes was observed with the inverted phase-contrast microscope.

## 3. RESULTS

Centrifugal force is normal direction in the following figures (Figs. 8-15). Figs 8-13 show cells of each group: A (upper right), B (lower left), C (lower right), and D (upper left). The experiments show the following results.

In every group, C2C12 proliferates to the confluent state and differentiates into myotubes. In the group C, proliferation of cells to the confluent state delays one day compared with the other groups.

Several cells exfoliate from the bottom of the dish and are floating in the medium in group A and B (Figs. 9-13).

The maximum value of the width of the myotube (bars in Figs. 14&15) in each group (A, B, C, D) on the twelfth day is 0.096 mm, 0.103 mm, 0.087 mm, 0.089 mm, respectively (Figs. 13-15). The value is highest in the group B.

In every group, differentiation into myotube is confirmed by the electric stimulation under the microscopic observation. The repetitive cyclic contraction of the myotube is observed on the eleventh day of culture, when the electric pulses of the amplitude higher than 5 V are applied to the medium. The number of the contractive myotubes is small in the group C. Several myotubes exfoliate from the bottom of the dish, when they repeat cyclic contraction.

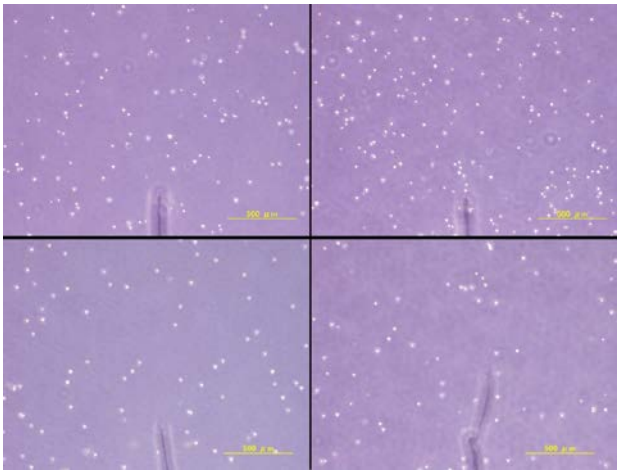

**Fig. 8:** C2C12 on the first day before stimulation. Dimension from left to right is 1 mm in each section.

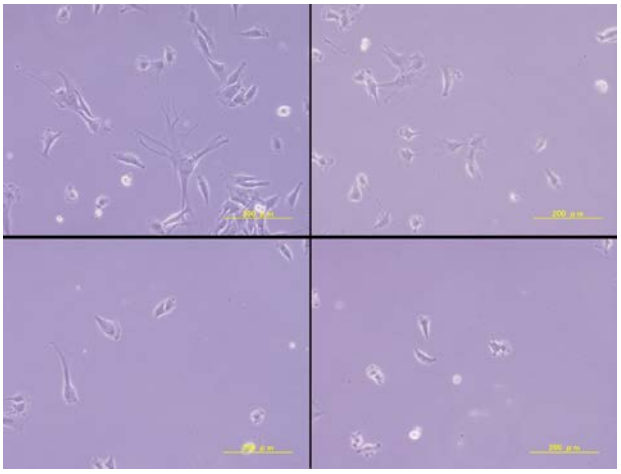

**Fig. 9:** C2C12 on the second day (extends pseudopod). Dimension from left to right is 1 mm in each section.

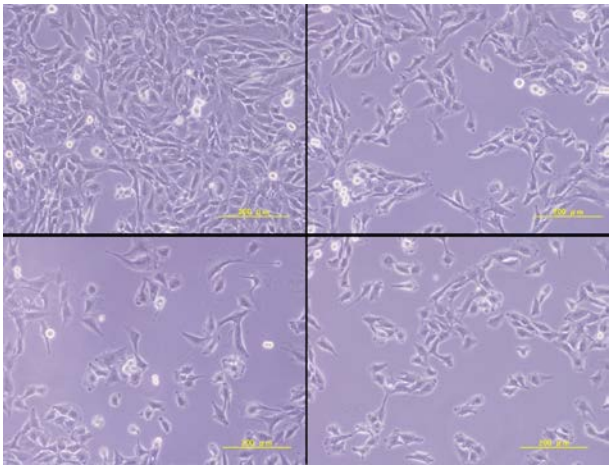

**Fig. 10:** C2C12 on the fourth day. Dimension from left to right is 1 mm in each section.

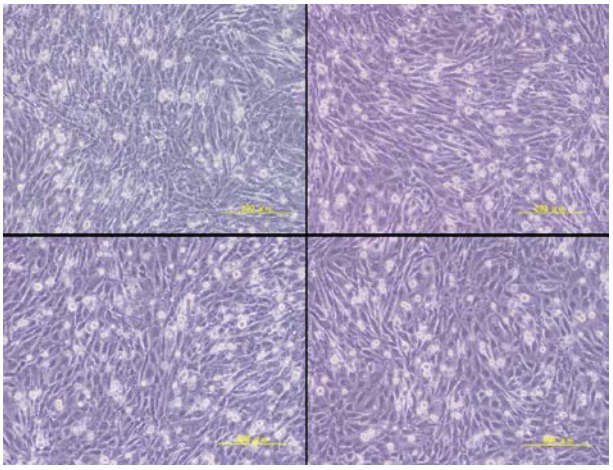

**Fig. 11:** C2C12 on the sixth day (confluent). Dimension from left to right is 1 mm in each section.

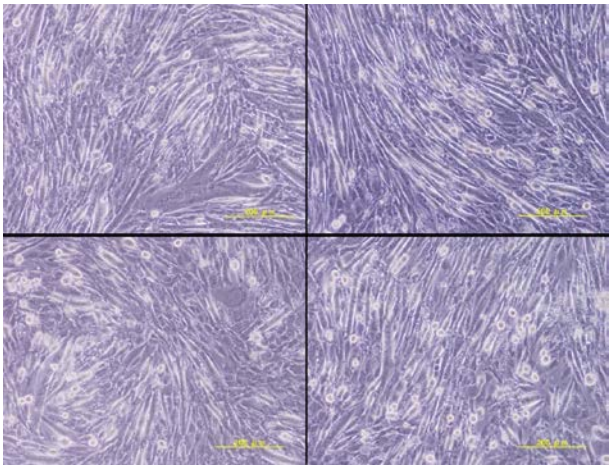

**Fig. 12:** Myotubes on the eighth day. Dimension from left to right is 1 mm in each section.

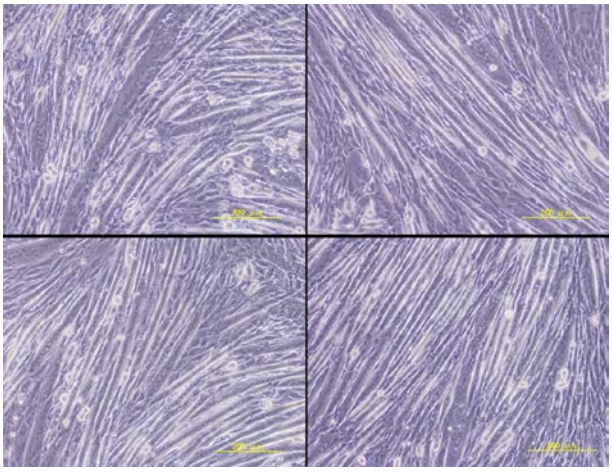

**Fig. 13:** Myotubes on the twelfth day. Dimension from left to right is 1 mm in each section.

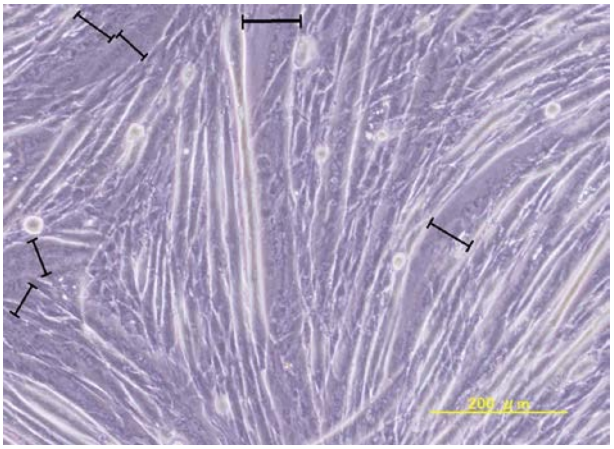


**Fig. 14:** Width of myotubes on the twelfth day in group A. Dimension from left to right is 1 mm. Bars show width of myotube.

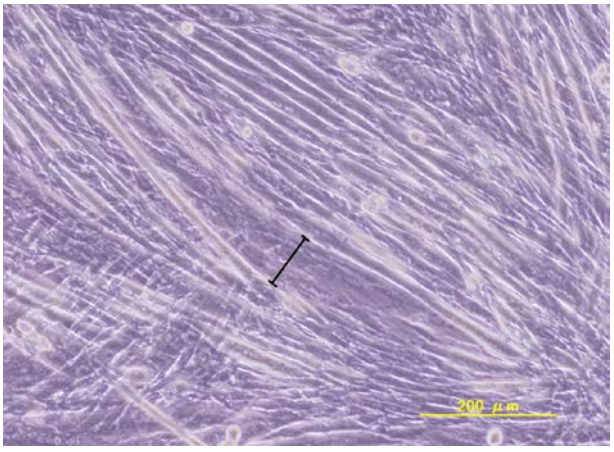


**Fig. 15:** Maximum width of myotube on the twelfth day in group C. Dimension from left to right is 1 mm. Bar shows width of myotube.

## 4. DISCUSSION

Although the serum in the medium is usually decreased to induce differentiation from myoblasts to myotubes *in vitro*, the contents of the medium were not changed throughout the present study. The myoblasts tends to differentiate into myotubes, when the cells proliferate to the confluent state. In the most of the cell culture experiments, contents of the medium are changed to that for differentiation such as the medium includes horse serum [1].

Several electric pulses are generated in the biological tissue. The electric pulses might have multiple effects on biological activities [18-23]. The electric pulse might decelerate proliferation of cells. The electric stimulation might accelerate differentiation of myoblast to myotubes. Because of the multiple effects, myoblast does not show maximal differentiation in the present study. To apply electric stimulation on myoblasts for differentiation, the optimum intensity should be chosen: the frequency, the amplitude, and the exposure time.

The excess gravitational force field, on the other hand, shows maximal effect on differentiation of myoblast in the present study. The mechanical stimulation might have more direct relation to differentiation of myoblast than electric stimulation, because the muscle operates against forces.

The culture dish without treatment was used in the present study, to emphasize mechanical and electrical effects.

The response of biological system to the microgravity field has been studied using a space satellite. The cell cycle might extend in the space [3]. C2C12 differentiation might be accelerated and the myotube might align to the direction perpendicular to the centrifugal field [2].

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 [8, 10, 11]. To apply continuous uniform mechanical stimulation to the cells, centrifugal force is used in the present study [1].

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 [18-22] and magnetic stimulation [23]. Another study shows that mechanical stimulation improves a tissue-engineered skeletal muscle [7]. 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 [1-17]. The shear flow governs the orientation of endothelial cells [15-17]. The shear stress affects the orientation of the smooth muscle cells in the biological tissue [8]. The direction of the mechanical field affects fibroblasts [10]. The effect of shear flow on orientation of cells depends on the kinds of cells [12]. 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 [9].

Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [1]. The mechanical stimulation can decrease proliferation of cells [1]. 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 [1].

## 5. CONCLUSION

Effects of intermittent application of the excess gravitational force and the electric pulse fields on differentiation of myoblast have been studied *in vitro*. To apply the excess gravitational force field on cells, the cell culture dish was set in the manufactured swing rotor set in a conventional centrifugal machine. To apply the electric pulse field on cells, a pair of electrodes was dipped in the medium. The experiment shows following results: both the excess gravitational force and the electric pulse fields decelerate proliferation of myoblasts, and accelerate differentiation of myoblasts, and mild gravitational force field is effective to accelerate differentiation of myoblast.

## 6. ACKNOWLEDGMENT

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