Effect of Ultrasonic Vibration on Proliferation and Differentiation of Cells

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ABSTRACT

The effect of mechanical stimulation of vibration on proliferation and differentiation of cells has been studied *in vitro*. To apply the vibration on the cells, a piezoelectric element was attached on the outside surface of the bottom of the culture plate of six wells. The piezoelectric element was vibrated by sinusoidally alternating voltage at 1.0 MHz generated by a function generator. Five kinds of cells were used in the experiment: C2C12 (mouse myoblast cell), L929 (fibroblast connective tissue of mouse), Hepa1-6 (mouse hepatoma cell), HUVEC (human umbilical vein endothelial cell), and Neuro-2a (mouse neural crest-derived cell line). After the incubation for 24 hours, cells were exposed to the ultrasonic vibration intermittently for three days: for thirty minutes per day. At the end of the experiment, the number of cells was counted by colorimetric method with a microplate photometer. In the case of Neuro-2a, the total length of the neurite was calculated at the microscopic image. The experimental study shows following results. Cells are exfoliated by the strong vibration. Proliferation and differentiation of cells are accelerated with mild vibration. The optimum intensity of vibration depends on the kind of cells.

Keywords: Biomedical Engineering, Vibration, C2C12, L929, Hepa1-6, HUVEC and Neuro-2a.

1. INTRODUCTION

Biological cells are sensitive to the several kinds of stimulations [1, 2]. Acceleration techniques for proliferation and differentiation of cells *in vitro* might be applied to regenerative medicine.

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 [3-5].

The ultrasonic vibration has been applied to a human body in several cases [6]: measurements of deep structure of the body, and lithotrity.

The cell culture technique has been developed, and cells have been cultured in the controlled environment. The effect of the vibration on cell culture was studied in previous studies [7-13]. In most cases, a vibration with a low frequency was applied to the cell culture: shaking the scaffold, or vibrating the scaffold at an audible frequency.

In the present study, the effect of the ultrasonic vibration on proliferation and differentiation of cells has been studied *in vitro*.

2. METHODS

Experimental System

The mechanical vibration was applied to cultured cells with the following experimental system. A piezoelectric element (1Z28D-SYX, Fuji ceramics Corporation, Tokyo), which has 28 mm in diameter (6.2 cm^2) and 1 MHz of a resonance frequency, is used for a vibrator [7]. The piezoelectric element consists of piezoelectric materials and electrodes. A piezoelectric disk is sandwiched by a pair of electrode disks.

A plate of six wells with the flat bottom of 35 mm in internal diameter (Falcon) was used for the cell culture (Fig. 1). The area of the bottom of each well for the cell culture is 9.6 cm^2. The plate is made of polystyrene. The surface for the scaffold is coated with collagen.

A gel-pad of SONAGEL (Takiron Co., Ltd., Osaka, Japan), which is used at the ultrasonic diagnosis, was attached between the piezoelectric element and the outside surface of the bottom of the well (Fig. 2). The pad is transparent and adherent. The pad has acoustic impedance, which is close to that of the biological tissue.

The diameter of the pad (5 mm of the thickness) was adjusted to the diameter of the well of 35 mm to transmit the vibration to the one of the well in the plate of six wells. The corner well is selected for the attachment, so that the distance from the piezoelectric element varies at each well (Fig. 1).

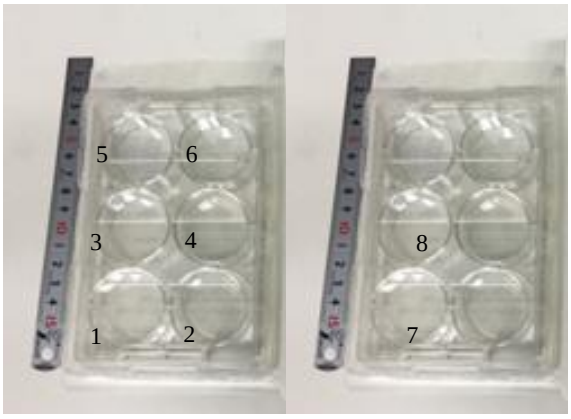

Fig. 1: Well number. Piezoelectric element was attached at No. 1. No. 7 and No. 8 are for control.

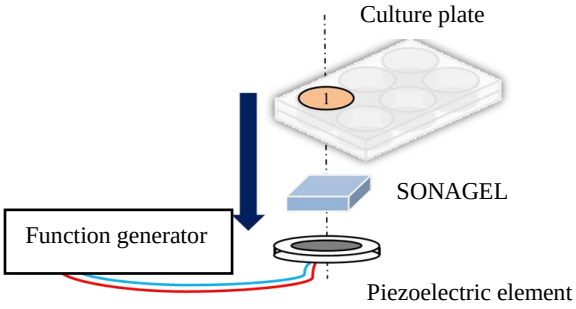

Fig. 2: How to apply vibration.

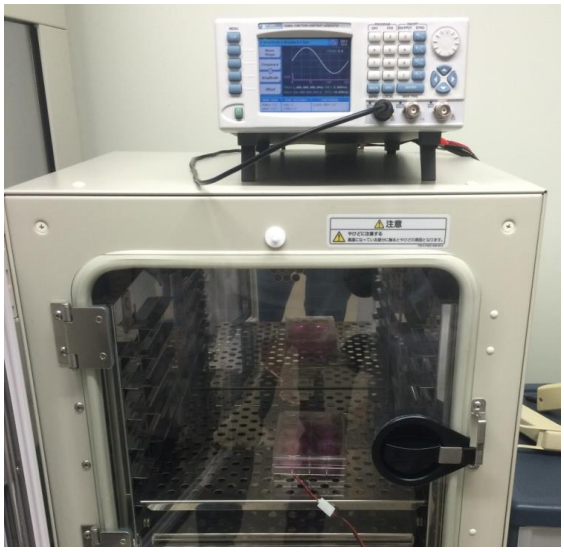

Fig. 3: Experimental system.

The wells for control group (without vibration) are located at the separated plate (Fig. 1). During the application of the vibration, the plate for control group is kept in the separated incubator.

The piezoelectric element was vibrated by the sinusoidally alternating current at 1.0 MHz generated by a function generator (PM8572A, Tabor Electronics Ltd.). The amplitude (peak to peak) of the sinusoidal voltage (*Vp-p*) was adjusted to 16 V.

During incubation, the sinusoidal voltage is transmitted to the piezoelectric element from the function generator placed outside of the incubator (Fig. 3).

Measurement of Vibration

Before the test for the cell culture, the vibration in the medium was measured with a piezo probe [7]. The piezo electric element attached on the bottom of the culture dish is vibrated by a function generator (the inverse piezoelectric effect). The vibration propagates through the ultrapure water of 5 cm^3 in a well to a piezo probe, which is dipped in the center of the water in the well. The piezo probe translates the vibration to the electric oscillation (the piezoelectric effect). The electric oscillation is monitored by an oscilloscope.

The electric power of the piezoelectric element (P) is calculated by Eq. 1.

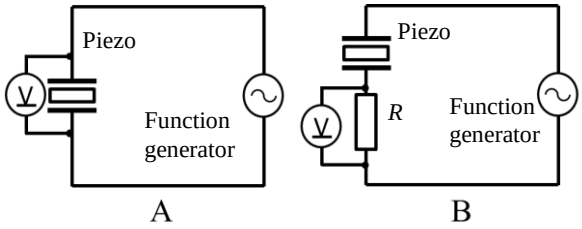

Fig. 4: Electric circuit. During stimulation to cell culture (A), Measurement with resistance (B).

$$P = V^2 / R \quad (1)$$

In Eq. 1, V is the voltage between the ends of the element, and R is electric resistance of the element.

The power density of the element (D) is calculated by Eq. 2.

$$D = P / S \quad (2)$$

In Eq. 2, S is the transmission area of the element. D is related to the energy passing through the unit area in the unit time.

When the electric resistance of 50 Ω is inserted in serial to the piezoelectric element connected to the sine wave of $V_{p\text{-}p}$ (16 V, generated by function generator), the voltages are 5.6 V and 1.0 V, respectively (Fig. 4 (B)). Thus, the resistance of the element corresponds to 9.1 Ω, when the reactance is very small.

V = 1.9 V in the present study, when the piezoelectric element is vibrated by sine wave of $V_{p\text{-}p}$ (= 16 V) with the function generator (Fig. 4 (A)). When V = 1.9 V and R = 9.1 Ω, P = 0.40 W. When S = 6.2 cm^2, D = 0.065 W/cm^2.

Calibration of cell Count

Cells were pre-cultured in a polystyrene dish, and exfoliated from the bottom of each well with trypsin. The number of cells was counted with Burker-Turk hemocytometer under a phase contrast microscope (IX71, Olympus, Tokyo). The suspension at the density of 500000 cells/cm^3 was prepared for five kinds of cells.

Before the suspension of 0.1 cm^3 was added into the first well, the medium of 0.1 cm^3 was poured into every well of the 96-wells-plate. D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (fetal bovine serum, after decomplementation) and 1% penicillin/ streptomycin was used for the medium. After mixing the suspension with the medium in the first well, the suspension of 0.1 cm^3 in the first well was collected and poured into the medium of the second well for mixing. The repetitive process makes dilution doubled of the density of cells in the suspension of successive wells.

Cell Count Kit-8 (CCK-8) of 0.01 cm^3, which includes highly water-soluble tetrazolium salt (WST-8), was added to every well, and incubated for 150 minutes. The intensity of absorbance at the wave length of 450 nm was measured with a microplate photometer (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, Inc.) [7].

Cell Culture

Five 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), Hepa1-6 (mouse hepatoma cell line of C57L mouse), HUVEC (human umbilical vein endothelial cells), and Neuro-2a (mouse neural crest-derived cell line). The passage of each cell is as follows: eighth of C2C12, sixth of L929, fifth of Hepa1-6, fourth of HUVEC, and second of Neuro-2a.

D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (fetal bovine serum, after decomplementation) and 1% penicillin/ streptomycin was used for the medium. In the case of HUVEC, EBM-2 was used instead of D-MEM. In the case of Neuro-2a, Retinoic Acid was added to the medium to induce differentiation. The concentration of Retinoic Acid in the medium was adjusted to 20 μM. The medium of 2 cm^3 in the well was refreshed every two days. Cells were cultured at 310 K with 5 % of CO_2 in an incubator throughout the test including exposure to the vibration.

Cells were seeded on the bottom of each well at the density of 1000 cells/cm^2. After incubation for 24 hours, cells were exposed to the ultrasonic vibration intermittently for three days: for thirty minutes per day. After the last exposure to the vibration for thirty minutes, cells were incubated for another 24 hours. At the end of every test, the cells were observed by a phase contrast microscope (IX71, Olympus, Tokyo), and exfoliated from the bottom of each well with trypsin. Each suspension of cells was centrifuged, and the supernatant was discarded. After the medium of 3 cm^3 was mixed, the suspension of 1 cm^3 was collected. WST-8 of 0.1 cm^3 was added to the suspension. After incubation for 150 minutes, the number of cells at each well was counted by colorimetric method with the microplate photometer.

In the case of Neuro-2a, the outline of each cell was traced, and the total length of the neurite was calculated from each length of the neurite at the microscopic image (Fig. 5).

During the exposure to the vibration for thirty minutes, the cells for control test were cultured in the separated compact incubator. After the exposure for thirty minutes, cells were returned to the original incubator, and cultured for the rest of the day. In the control group, cells were continuously cultured in the original incubator.

To confirm the distribution of cells adhere on the bottom of the well, Giemsa stain treatment was applied at the end of the cell culture.

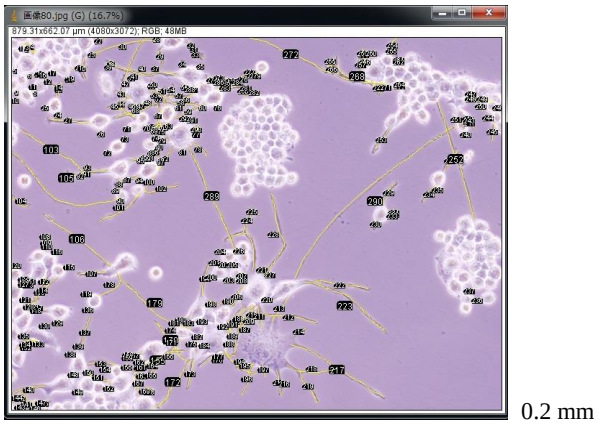

Fig. 5: Trace of neurite on microscopic image of Neuro-2a.

3. RESULTS

Table 1 shows the results of the measurement of the vibration in the medium at the center of each well. The distance is measured by a scale between the centers of the wells. The well "from No. 1 to No. 6" corresponds to that of Fig. 1. The level of -20 dB means one tenth by attenuation. In Table 1, the well number one is the origin, where the piezoelectric element is attached. The result shows that the vibration decreases with the distance from the piezoelectric element.

Table 1: Vibration in the medium in each well. Well number corresponds to that of Fig. 1.

Well	Distance [mm]	Level [dB]	Power [W/cm^2]
1	0	0	0.065
2	39	-13	0.015
3	39	-10	0.021
4	55	-14	0.013
5	78	-43	0.0005
6	89	-33	0.0015

Figs. 6A-6E show the relation between the absorbance of light at the wave length of 450 nm and the density of cells [/well] for suspension of five kinds of cells, respectively. The absorbance is proportional to the density of cells. The approximated line is used to calculate the number of the cells from the absorbance of the light.

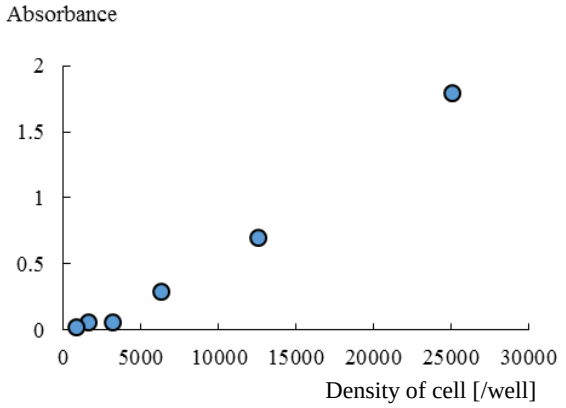

Fig. 6A: Absorbance of light and density of C2C12 [/well].

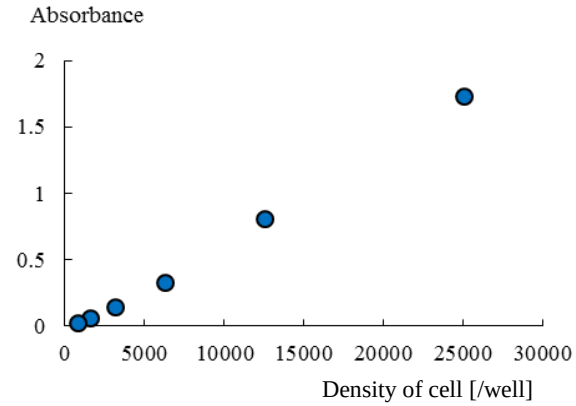

Fig. 6B: Absorbance of light and density of L929 [/well].

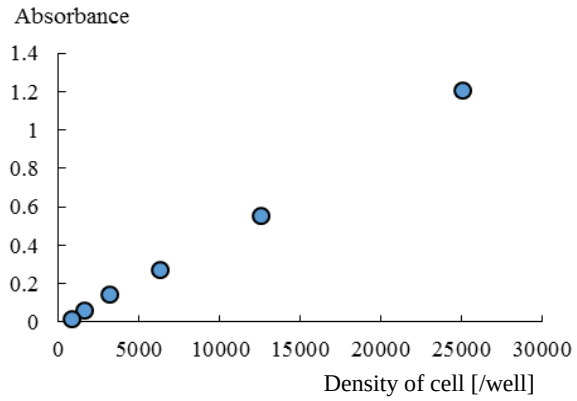

Fig. 6C: Absorbance of light and density of Hepa1-6 [/well].

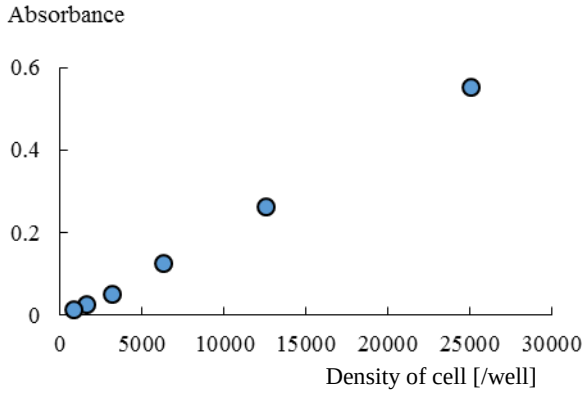

Fig. 6D: Absorbance of light and density of HUVEC [/well].

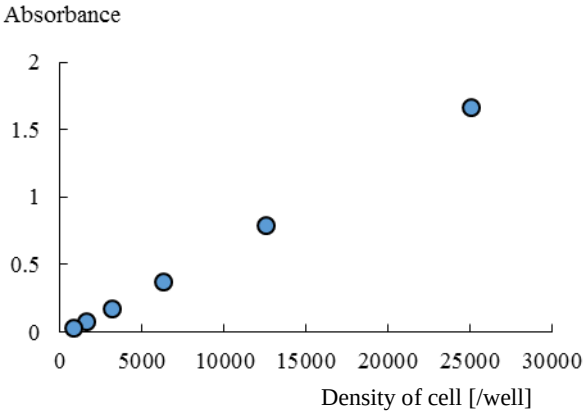

Fig. 6E: Absorbance of light and density of Neuro-2a [/well].

Fig. 7 exemplifies the cells observed during the experiment. The dimension of left to right at each figure is 2 mm. The rounded particles are floating cells. C2C12 proliferates to the confluent manner even in the vibration group.

Figs. 8A-8E show the number of cells in each well. In Fig. 8, data are plotted at the power density of each well, which is listed at Table 1. The number of cells of 9600 (initial density 1000 cells/cm^2 × 9.6 cm^2) corresponds to the same number of seeding.

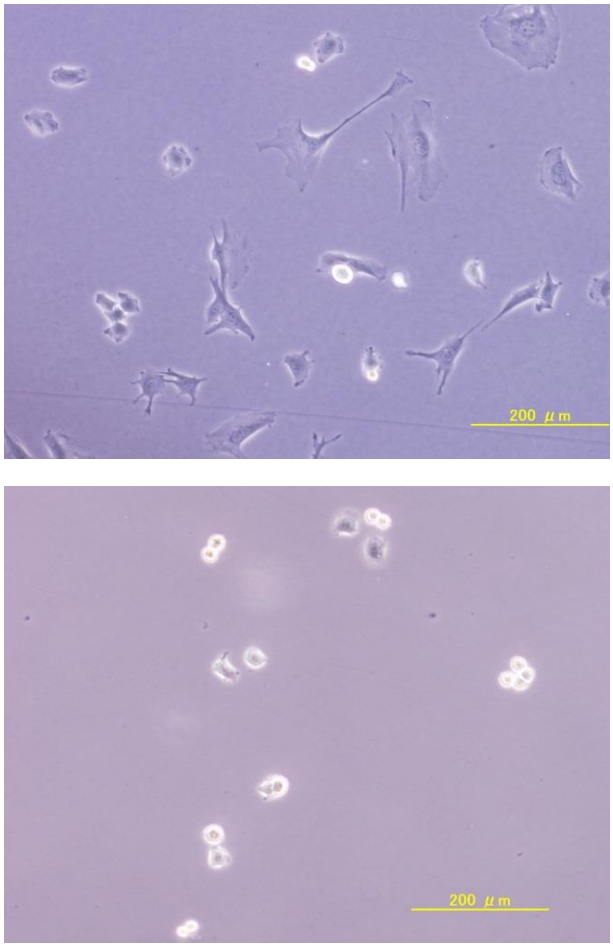

Fig. 7: C2C12 (upper) and Neuro-2a (lower).

Fig. 8A shows that C2C12 proliferates higher than 150000 cells, which corresponds to the confluent state in every well in four days. Fig. 8C shows that the number of Hepa1-6 increases in the well distant from the source of vibration (at the small power density), although the number of Hepa1-6 is very small in the well at the source of vibration (at 65 mW/cm^2).

Fig. 5 exemplifies microscopic image of Neuro-2a after the experiment.

Although the vibration tends to decrease the number of cells of Neuro-2a, the vibration tends to increase the total length of neurites of Neuro-2a (Fig. 9).

Fig. 10 shows the microscopic image of cells after Gimsa stain treatment. The stained cells decrease at the well No.1, where the vibration power is strong (65 mW/cm^2).

At the strong vibration (65 mW/cm^2), C2C12 and Hepa1-6 exfoliates. At the mild vibration (20 mW/cm^2), proliferation of C2C12 accelerates. The total length of the neurites extends at the vibration, although the number of Neuro-2a decreases at the vibration. Proliferation of L929 and HUVEC do not depend on the power density of vibration lower than 0.065 W/cm^2.

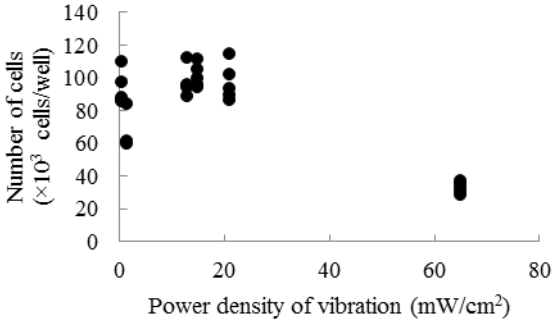

Fig. 8A: Number of C2C12 vs. power density of vibration [mW/cm^2].

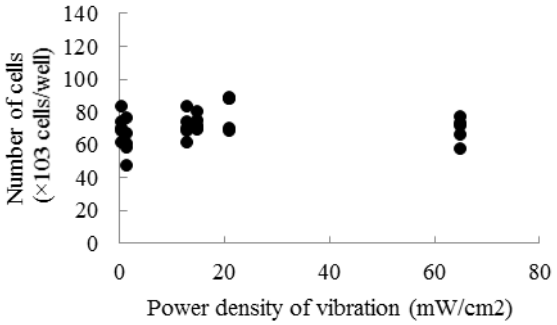

Fig. 8B: Number of L929 vs. power density of vibration [mW/cm^2].

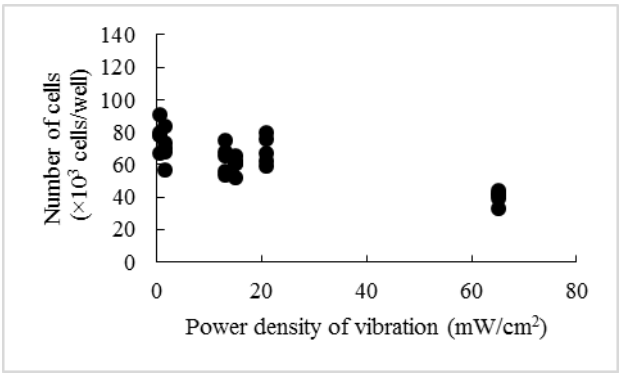

Fig. 8C: Number of Hepa1-6 vs. power density of vibration [mW/cm^2].

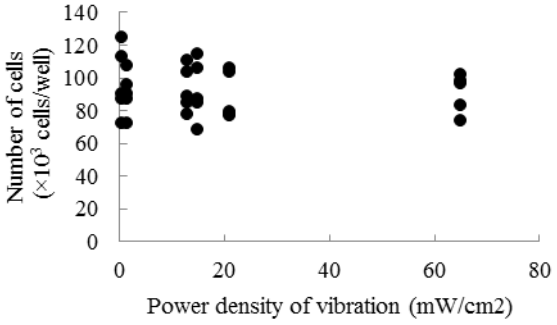

Fig. 8D: Number of HUVEC vs. power density of vibration [mW/cm^2].

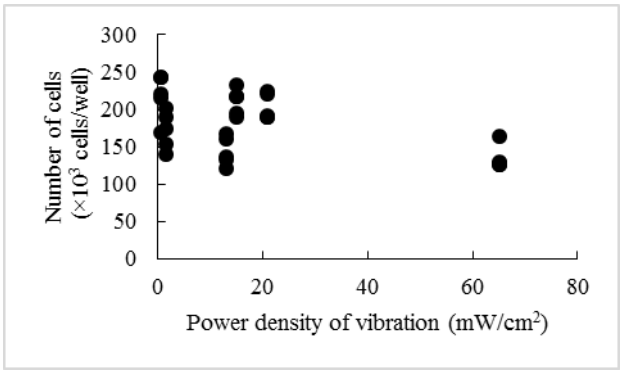

Fig. 8E: Number of Neuro-2a vs. power density of vibration [mW/cm^2].

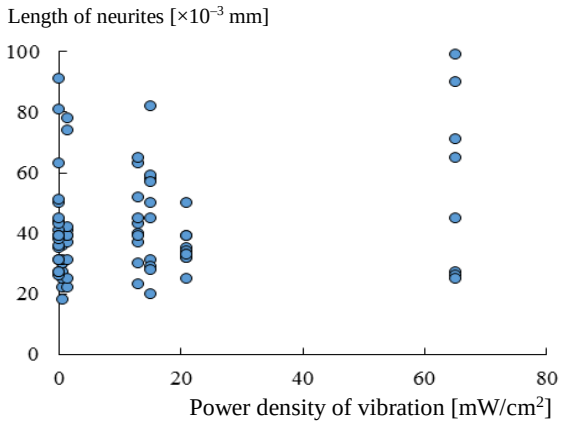

Fig. 9: Total length of neurites of Neuro-2a [$\times 10^{-3}$ mm] vs. power density of vibration [mW/cm^2].

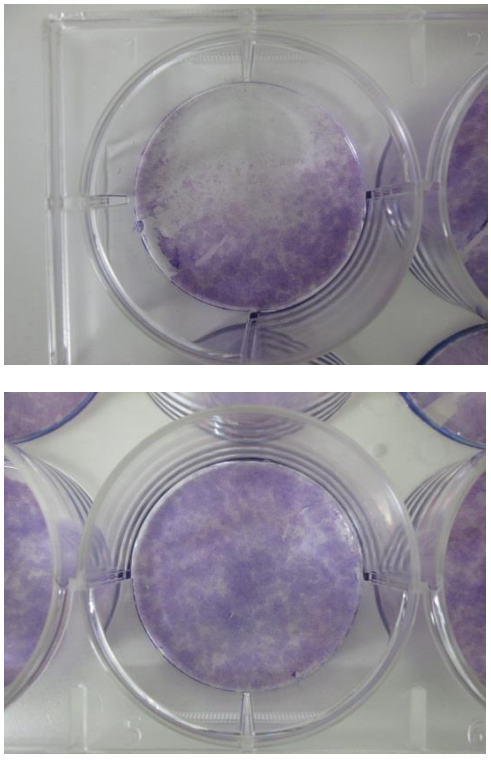

Fig. 10: Gimsa Stain of C2C12 at well No.1 (left) and No.5 (right) wells of 35 mm diameter.

4. DISCUSSION

The vibration has effects of heating and stirring in the fluid. The stirring is visualized by the wavy movement of the surface of fluid [8]. The temperature of the surface can be measured by a thermography.

The strong vibration has thermo-effect. The thermo-effect might be applied to decrease the proliferation of cancer cells at 315 K. Thermography shows that the surface of the medium is around 310 K during application of vibration in the present study. The temperature of 310 K is body temperature, and might have minor effect on the proliferation of cells.

When the voltage, which is applied to the probe, increases, the surface of the medium becomes convex and vibrates (Vp-p > 16 V). The prominent vibration might generate the macroscopic flow, which has the stirring effect. In the present study, Vp-p is 16 V, although the micro vibration might have the local stirring effect.

In the vibrating system, distribution of the intensity depends on the property of the vibrating substance for refraction and reflection. That is the reason why the intensity of the vibration is not simply governed by the distance from the source of the vibration (Table 1).

The intensity of the vibration applied on the cells might be smaller than 0.065 W/cm^2 by the damping effect in the present experimental system. The slight rise of temperature in the medium during the vibration means the energy loss.

The frequency of 1 MHz has been applied to the fractured bone in the clinical treatment [14]; e.g. thirty minutes per day.

The wave length of 1 MHz corresponds to the wave length of 0.0015 m in the water (velocity of 1500 m/s), which is longer than the diameter of the cell in the present study.

The proliferation of cells depends on the density of cells in the well [7]. The density of each well is carefully controlled in the present study.

The acceleration of the proliferation of cells might mean the oncogenic transformation (canceration) of cells. For comparison, Hepa1-6 is cultured in the vibration. The proliferation of the cell might be accelerated with the shortage of the resting state of the cell cycle [15].

The calibration line is convenient to be used for calculation of the number of cells in the present study, because the absorbance is proportional to the density of cells of five kinds: C2C12, L929, Hepa1-6, HUVEC, and Neuro-2a.

In the previous study, the mild vibration tended to accelerate the proliferation of C2C12 [7]. In the present experiment, the number of cells in vibration group is almost equal to that of control group, and cells proliferate to confluent state in four days. Most of cells of C2C12 are not exfoliated in the present study, although several cells of C2C12 were exfoliated during exposure to the vibration in thirty minutes in the previous study. The difference of the dimension of the dish might make variation of the flow in the culture dish, which governs the exfoliation of cells. The behavior of the cell depends on adhesion to the scaffold [16, 17].

The effect of the low-frequency ultrasound on the neuronal activity was studied in the previous study [3, 18-22]. The low-intensity ultrasound treatment might increase mass transport, and enhance proliferation, metabolic activity, and differentiation of cells [7].

The previous study showed that both the number of C2C12 and contractile protein increase under the ultrasonic vibration at 22 kHz [11]. In several clinical treatments, the ultrasonic vibration (>1 MHz) is applied to the human body.

5. CONCLUSION

The effect of mechanical stimulation of vibration at 1.0 MHz on proliferation of cells has been studied *in vitro*. Five kinds of cells were used in the experiment: C2C12 (mouse myoblast cell), L929 (fibroblast connective tissue of mouse), Hepa1-6 (mouse hepatoma cell), HUVEC (human umbilical vein endothelial cells), and Neuro-2a (mouse neural crest-derived cell line). The experimental results show that proliferation and differentiation of cells accelerate at the mild vibration, and that the optimum intensity of the vibration for proliferation and differentiation depends on the kinds of cells.

6. ACKNOWLEDGMENT

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REFERENCES

[1] S. Hashimoto and K. Tachibana, "Effect of Magnetic Field on Adhesion of Muscle Cells to Culture Plate", **Journal of Systemics Cybernetics and Informatics**, Vol. 11, No. 4, 2013, pp. 7-12.

[2] 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.

[3] S. Hashimoto, H. Hino and T. Iwagawa, "Effect of Excess Gravitational Force on Cultured Myotubes in Vitro", **Journal of Systemics, Cybernetics and Informatics**, Vol. 11, No. 3, 2013, pp. 50-57.

[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] S. Hashimoto, F. Sato, H. Hino, H. Fujie, H. Iwata and Y. Sakatani, "Responses of Cells to Flow in Vitro", **Journal of Systemics Cybernetics and Informatics**, Vol. 11, No. 5, 2013, pp. 20-27.

[6] A. Khanna, R.T.C. Nelmes, N. Gougoulias, N. Maffulli and J. Gray, "The Effects of LIPUS on Soft-tissue Healing: a Review of Literature", **British Medical Bulletin**, Vol. 89, No. 1, 2009, pp. 169-182.

[7] H. Nakajima, H. Hino, S. Hashimoto, Y. Takahashi and T. Yasuda, "Effect of Ultrasonic Vibration on Proliferation of Cultured Cell", **Proc. 19th World Multi-Conference on**

Systemics Cybernetics and Informatics, Vol. 2, 2015, pp. 276-281.

[8] H. Nakajima, S. Hashimoto and T. Yasuda, "Effect of Ultrasonic Vibration on Culture of Myoblast", **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 144-149.

[9] C.Z. Wang, G.J. Wang, M.L. Ho, Y.H. Wang, M.L. Yeh and C.H. Chen, "Low-magnitude Vertical Vibration Enhances Myotube Formation in C2C12 Myoblasts", **Journal of Applied Physiology**, Vol. 109, No. 3, 2010, pp. 840-848.

[10] H. Park, M.C. Yip, B. Chertok, J. Kost, J.B. Kobler, R. Langer and S.M. Zeitels, "Indirect Low-Intensity Ultrasonic Stimulation for Tissue Engineering", **Journal of Tissue Engineering**, Vol. 2010, 2010, Article ID 973530, 9 pages.

[11] N. Suzuki, K. Kitamura, T. Nemoto, N. Shimizu, S. Wada, T. Kondo, M.J. Tabata, F. Sodeyama, K. Ijiri and A. Hattori, "Effect of Vibration on Osteoblastic and Osteoclastic Activities: Analysis of Bone Metabolism Using Goldfish Scale as a Model for Bone", **Advances in Space Research**, Vo. 40, No. 11, 2007, pp. 1711-1721.

[12] K. Ninomiya, R. Yamada, H. Meisaku and N. Shimizu, "Effect of Ultrasound Irradiation on Bacterial Internalization and Bacteria-mediated Gene Transfer to Cancer Cells", **Ultrasonics Sonochemistry**, Vol. 21, No. 3, 2014, pp. 1187-1193.

[13] G. Khayat, D.H. Rosenzweig, Z. Khavandgar, J. Li, M. Murshed and T.M. Quinn, "Low-Frequency Mechanical Stimulation Modulates Osteogenic Differentiation of C2C12 Cells", **International Scholarly Research Notices: Stem Cells**, Vol. 2013, 2013, Article ID 138704, 9 pages.

[14] F. Padilla, R. Puts, L. Vico and K. Raum, "Stimulation of Bone Repair with Ultrasound: A Review of the Possible Mechanic Effects", **Ultrasonics**, Vol. 54, No. 5, 2014, pp. 1125-1145.

[15] S. Hashimoto, M. Toda, M. Mizobuchi and T. Kuromitsu, "Simulation of Cell Group Formation Regulated by Coordination Number, Cell Cycle and Duplication Frequency", **Journal of Systemics Cybernetics and Informatics**, Vol. 11, No. 4, 2013, pp. 29-33.

[16] 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.

[17] S. Hashimoto, T. Nakamura, O. Hasegawa and M. Yasaka, "Thrombus Formation on Ultrasonically Vibrating Surface", **Proc. 5th World Multiconference on Systemics, Cybernetics and Informatics and 7th International Conference on Information Systems Analysis and Synthesis**, Vol. 10, 2001, pp. 448-451.

[18] A. Suzuki, T. Takayama, N. Suzuki, M. Sato, T. Fukuda and K. Ito, "Daily Low-intensity Pulsed Ultrasound-mediated Osteogenic Differentiation in Rat Osteoblasts", **Acta Biochimica et Biophysica Sinica**, Vol. 41, No. 2, 2009, pp. 108-115.

[19] T. Inubushi, E. Tanaka, E.B. Rego, M. Kitagawa, A. Kawazoe, A. Ohta, H. Okada, J.H. Koolstra, M. Miyauchi, T. Takata and K. Tanne, "Effects of Ultrasound on the Proliferation and Differentiation of Cementoblast Lineage Cells", **Journal of Periodontology**, Vol. 79, No. 10, 2008, pp. 1984-1990.

[20] K. Sena, S.R. Angle, A. Kanaji, C. Aher, D.G. Karwo, D.R. Sumner and A.S. Virdi, "Low-intensity Pulsed Ultrasound (LIPUS) and Cell-to-cell Communication in Bone Marrow Stromal Cells", **Ultrasonics**, Vol. 51, No. 5, 2011, pp. 639-644.

[21] S.R. Angle, K. Sena, D.R. Sumner and A.S. Virdi, "Osteogenic Differentiation of Rat Bone Marrow Stromal Cells by Various Intensities of Low-intensity Pulsed Ultrasound", **Ultrasonics**, Vol. 51, No. 3, 2011, pp. 281-288.

[22] W.J. Tyler, Y. Tufail, M. Finsterwald, M.L. Tauchmann, E.J. Olson and C. Majestic, "Remote Excitation of Neuronal Circuits Using Low-Intensity, Low-Frequency Ultrasound", **PLoS ONE Ultrasonic Neurostimulation**, Vol. 3, No. 10, 2008, 11 pages, e3511.