Effect of Ultrasonic Vibration on Proliferation of Cultured Cell

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ABSTRACT

The effect of mechanical stimulation of vibration on proliferation 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 sinusoidal alternating voltage at 1.0 MHz generated by a function generator. Three kinds of cells were used in the experiment: C2C12 (mouse myoblast cell), L929 (fibroblast connective tissue of mouse), Hepa1-6 (mouse hepatoma cell). After 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 the microplate photometer. The experimental results show that proliferation of cells is enhanced with mild vibration, and that the optimum vibration depends on the kind of cells.

Keywords: Biomedical Engineering, C2C12, L929, Hepa1-6, Cell Culture and Vibration.

1. INTRODUCTION

Ultrasonic vibration has been applied to human body in several cases: measurements of deep structure of body, and lithotrity [1, 2].

The cell culture technique has been developed, and cells have been cultured in the controlled environment. The effect of vibration on cell culture was studied in previous studies [3]. In most cases, the vibration with low frequency was applied to cell culture: shaking the scaffold, or vibrating the scaffold at the audible frequency.

Several methodologies have been clinically applied to regenerative medicine. The acceleration technique for proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro* [3-7]. Control methodology for proliferation and differentiation of cells would be applied to the regenerative tissue technology.

The mechanical stress is one of the interested points 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 [3, 5-7].

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

2. METHODS

Experimental System

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 diameter and 1 MHz resonance frequency, is used for vibrator (Fig. 1). A plate of six wells with a flat bottom of 35 mm internal diameter (Falcon) was used for the cell culture. The area of the bottom of each well for the cell culture is 9.6 cm^2. A polydimethylsiloxane (PDMS) disk, which contains the piezoelectric element, is attached on the outside surface of one of the bottom of the well (Fig. 2). The corner well is selected for attachment, so that the distance from the piezoelectric element varies at each well. The wells for control group are located at the separated plate (Fig. 3).

Fig. 1: Piezoelectric element.

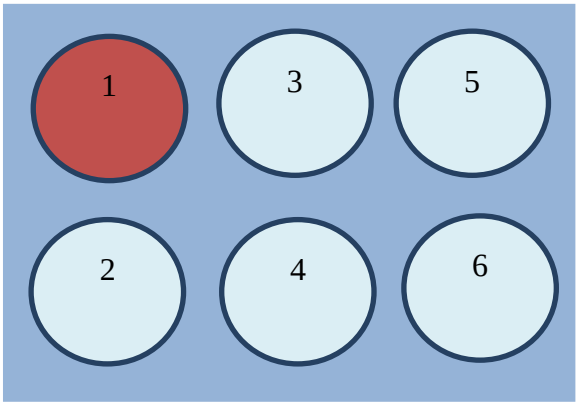


Fig. 2: Position of each well in the culture plate. Piezoelectric element is attached on the back of the well "number one".

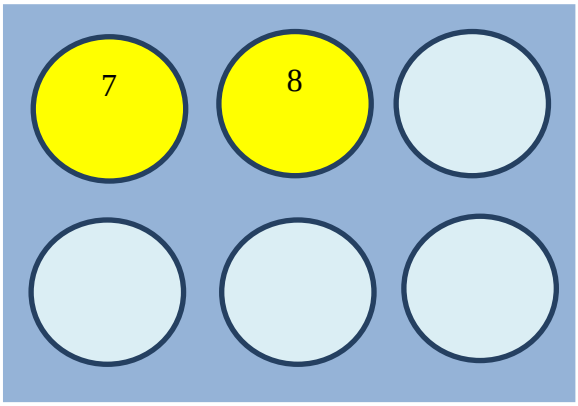


Fig. 3: Wells in the separated plate for control group (No. 8 & 9).

The contact between the disk and the bottom of the plate is kept by affinity between them without adhesive. To keep acoustic contact between the outside surface of the bottom of the dish and the piezoelectric element, a porous sheet filled with water is sandwiched between them.

The piezoelectric element was vibrated by sinusoidal 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. 7).

Measurement of Vibration

Before the test for cell culture, the vibration in the medium was measured with a piezo probe (Figs. 4&5). 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 phosphate buffer solution of 2 cm^3 in a well to a piezo probe, which is dipped in the center of the solution in the well. The piezo probe translates the vibration to the electric oscillation (the piezoelectric effect). The oscillation is monitored by an oscilloscope.

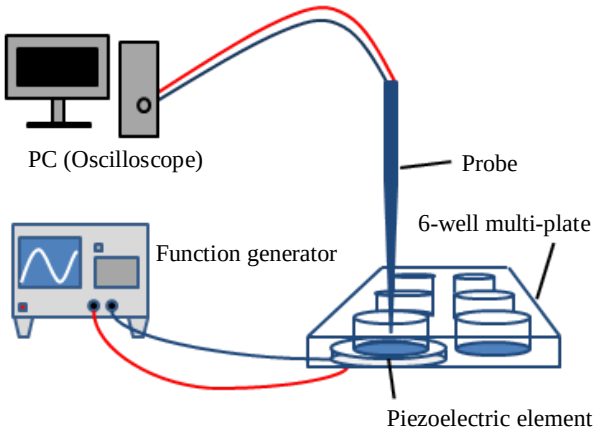


Fig. 4: Diagram of measurement of vibration in the medium.

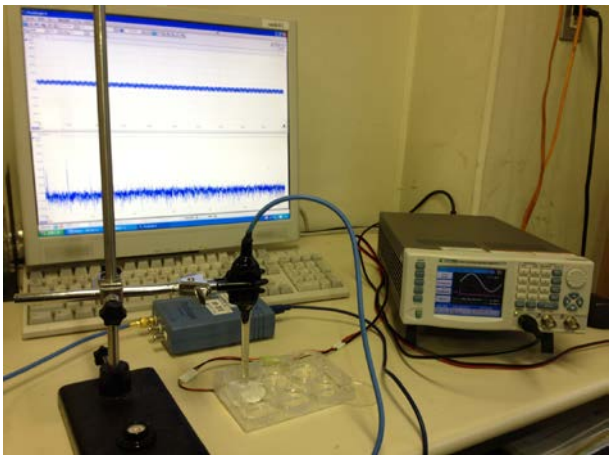


Fig. 5: Measurement of vibration in the medium.

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 is 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 three kinds of cells.

Before the suspension of 0.1 cm^3 was added into one of the wells, 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 a well, the suspension of 0.1 cm^3 in the well was collected and poured into the medium of the next well for mixing. The repetitive process makes dilution doubled of the density of cells in the suspension of successive wells.

Dojindo's Kit-8 (CCK-8), which includes highly water-soluble tetrazolium salt (WST-8), was added to every well, and incubated for several hours: 120 minutes for Hepa1-6, and 150 minutes for C2C12 and for L929. 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.) (Fig. 6).

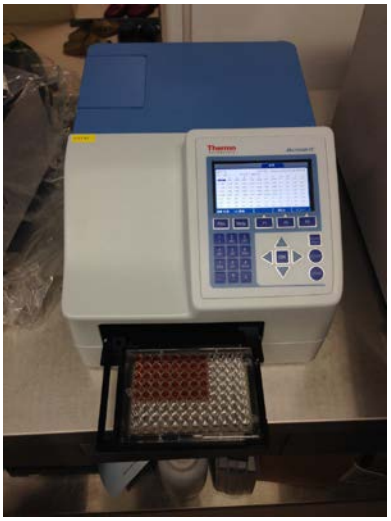


Fig. 6: Mounted samples in microplate photometer.

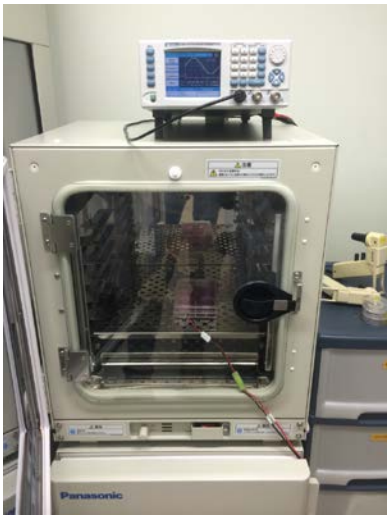


Fig. 7: Piezoelectric element in incubator is vibrated by function generator.

Cell Culture

Three kinds 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), and Hepa1-6 (mouse hepatoma cell line of C57L mouse).

D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (fetal bovine serum, after decomplementation) and 1% penicillin/ streptomycin was used for the medium. 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.

In the case of C2C12, fourth passage was used. 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 L929, tenth passage was used. 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 24 hours. At the end of the test, cells were 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, and WST-8 of 0.1 cm^3 was added. After incubation for 150 minutes, the number of cells at each well was counted by colorimetric method with the microplate photometer.

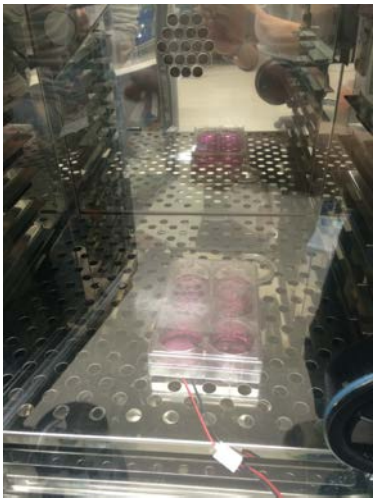


Fig. 8: Cells in wells exposed to vibration in incubator.

In the case of Hepa1-6, sixth passage was used. Cells were seeded on the bottom of each well at the density of 2000 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 24 hours. At the end of the test, cells were 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, and WST-8 of 0.1 cm^3 was added. After incubation for 120 minutes, the number of cells at each well was counted by colorimetric method with the microplate photometer.

During the exposure to the vibration for thirty minutes, the cells were cultured in the separated compact incubator (Figs. 7&8). 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.

3. RESULTS

Table 1 shows the results of the measurement of vibration in the medium at the center of each well. The distance is measured by a scale between the centers of the wells. The number of the well corresponds to that of Fig. 2. The level of -20 dB means one tenth by attenuation. The well number one is the origin in Table 1. The result shows that the damping of the vibration is proportional to the distance from the piezoelectric element.

Table 1: Vibration in the medium in each well.

Well	Distance [mm]	Voltage [mV]	Level [dB]
1	0	3.88	0
2	39	0.245	-24
3	39	0.195	-26
4	55	0.155	-28
5	78	0.0489	-38
6	89	0.0388	-40

In Figs. 9-11, relation between the absorbance of light at the wave length of 450 nm and the density of cells is shown for suspension of three 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.

Figs. 12-14 show the cells observed at the end of the experiment. The dimension of left to right is 2 mm. The rounded particles are floating cells. C2C12 proliferates to the confluent manner even in the vibration group.

Figs. 15-17 show the number of cells in each well. Each column shows mean value of five samples from each well and the vertical bar shows the range between maximum and minimum data of the five samples. The number of well correspond to that of Fig. 2 and Fig. 3.

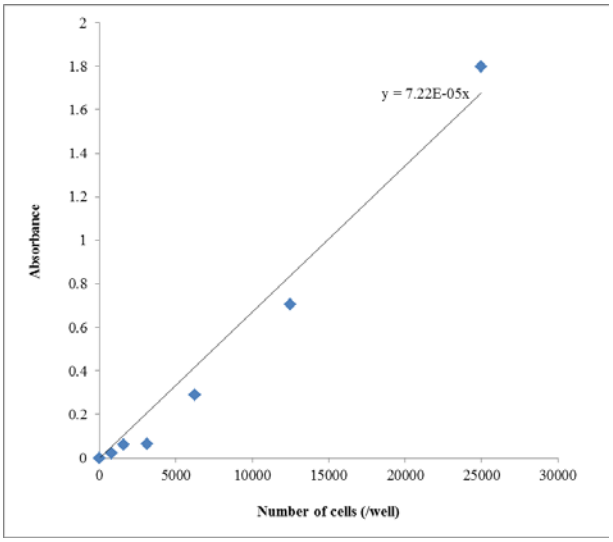


Fig. 9: Relation between absorbance and the number of C2C12 in a well of microplate.

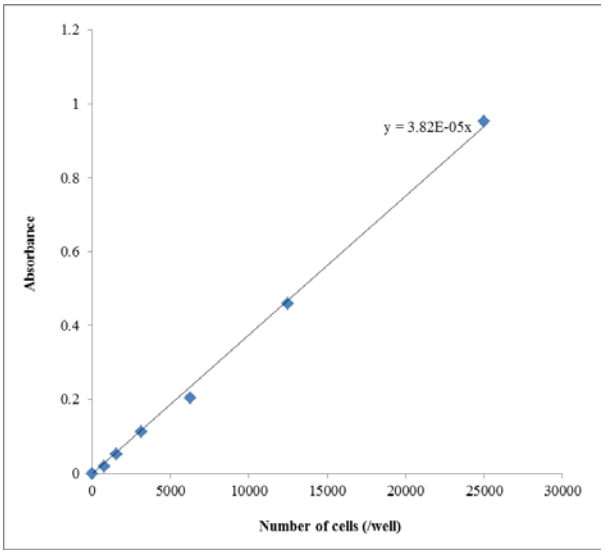


Fig. 10: Relation between absorbance and the number of L929 in a well of microplate.

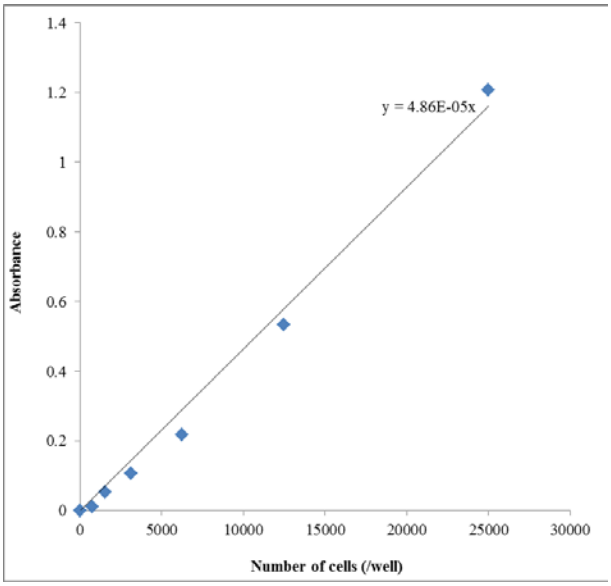


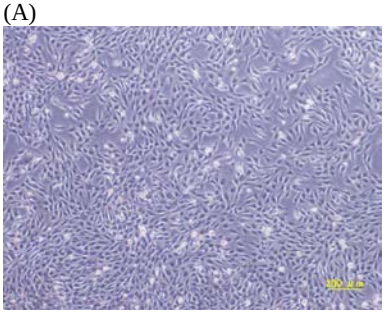
Fig. 11: Relation between absorbance and the number of Hepa1-6 in a well of microplate.

Fig. 15 shows that C2C12 proliferates higher than 15000 cells, which corresponds to the confluent state in every well in four days (Fig. 12). The number of cells of 9600 corresponds to the same number of seeding.

Fig. 16 shows that the number of L929 increases in the well distant from the source of vibration (number one), although the number of L929 is very small in the well at the source of vibration (Fig. 13(B)). The number of cells of 9600 corresponds to the same number of seeding.

Fig. 17 shows that vibration does not affect the number of Hepa1-6. The number of cells of 19200 corresponds to the same number of seeding.

(A)



(B)

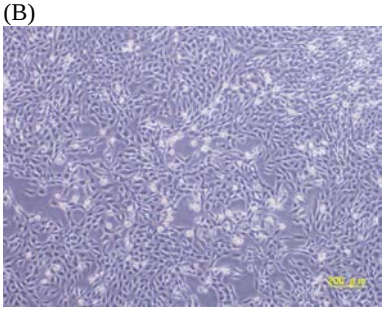
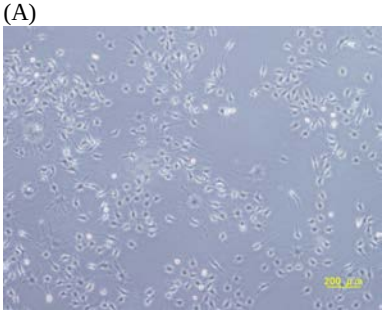


Fig. 12: C2C12 at the end of test in well: control (A), No. 1 (B).

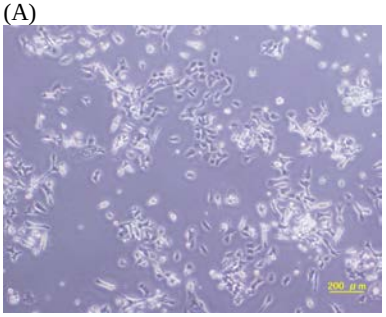
(A)



(B)

Fig. 13: L929 at the end of test in well: control (A), No. 1 (B).

(A)



(B)

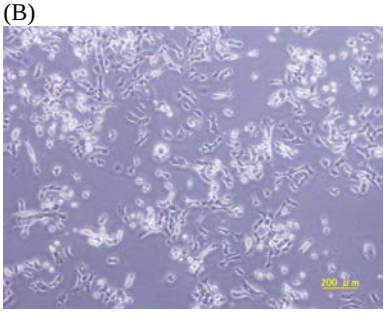


Fig. 14: Hepa1-6 at the end of test in well: control (A), No. 1 (B).

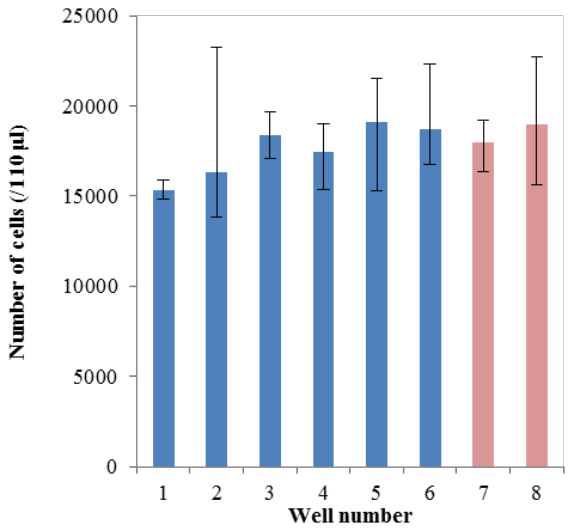


Fig. 15: Number of C2C12 in each well.

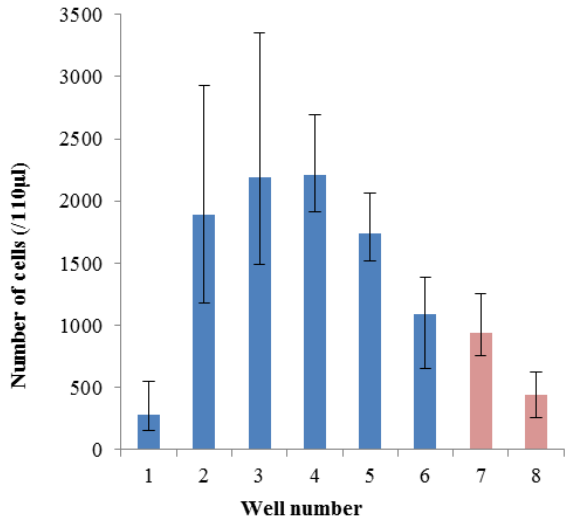


Fig. 16: Number of L929 in each well.

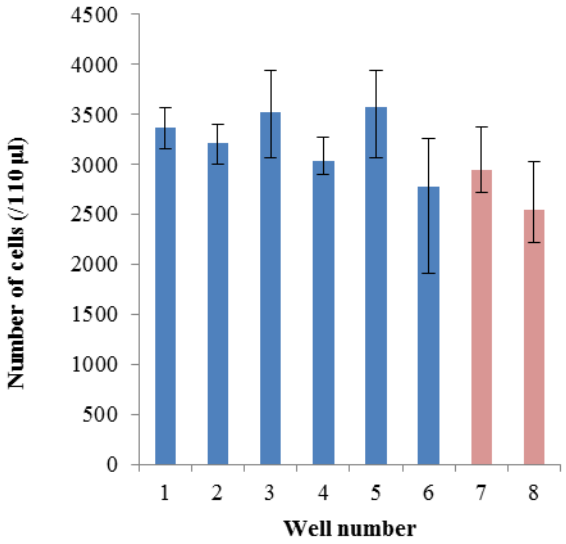


Fig. 17: Number of Hepa1-6 in each well.

4. DISCUSSION

Dojindo's highly water-soluble tetrazolium salt (WST-8) is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye. The formazan dye has the maximum point of absorption at the wave length of 450 nm. The dye is soluble in the media of the cell culture. The formazan dye is generated by the activities of dehydrogenases in cells, so that the amount of the formazan dye might be proportional to the number of living cells. The calibration line is convenient to be used for calculation of the number of cells in the present study, because the absorbance was proportional to the density of cells of three kinds: C2C12, L929, and Hepa1-6.

Fig. 12 shows that C2C12 proliferates to the confluent state in every well. In the previous study, the mild vibration tends to accelerate the proliferation of C2C12 [3]. 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 are exfoliated during exposure to the vibration in thirty minutes in the previous study. The difference of dimension of the dish might make variation of the flow in the culture dish, which governs the exfoliation of cells.

The proliferation of L929 is not so fast as C2C12 in the present study. Fig. 16 shows that the proliferation of L929 increases with mild vibration (No. 2-6 in Fig. 16), although the strong vibration (in the well number one) inhibits proliferation of L929. Fig. 17 shows that vibration does not affect proliferation of Hepa1-6, although cells do not proliferate to the confluent state. The numbers of cells are small in the group of L929 and Hepa1-6 including control tests. The small number of cells might be results of exfoliation of cells, or of little adhesion of cells. Although the culture condition might not be good for proliferation in the present experiments, the numbers of cells are larger in the mild vibration group than in control group. The experimental results show that the mild vibration might enhance 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 macroscopic flow, which has stirring effect. In the present sturdy, Vp-p is 16 V, although micro vibration might have local stirring effect.

The effect of low-frequency ultrasound on neuronal activity was studied in the previous study [1]. Low-intensity ultrasound treatment might increase mass transport, and enhance C2C12 proliferation, metabolic activity, and differentiation of cells [2].

5. CONCLUSION

The effect of mechanical stimulation of vibration at 1.0 MHz on proliferation of cells has been studied *in vitro*. Three kinds of cells were used in the experiment: C2C12 (mouse myoblast cell), L929 (fibroblast connective tissue of mouse), Hepa1-6 (mouse hepatoma cell). The experimental results show that proliferation of cells is enhanced with mild vibration, and that the optimum vibration depends on the kind of cells.

6. ACKNOWLEDGMENT

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REFERENCES

[1] 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**, Vol. 3, No. 10, 2008, e3511.

[2] 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.

[3] 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.

[4] 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.

[5] 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.

[6] 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.

[7] 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.