The Growth of Mouse Osteoblast Cell under Vibration Wave in Vitro

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Abstract: Osteoporosis is gradually becoming the number one invisible killer in this current aging society. Along with the extension of human life-span, osteoporosis turned into the second major epidemic disease which is lower to cardiovascular diseases. Chemical medicine is the usual prescription, but its side effect should not be overlooked. Therefore, some researchers discuss the treatment of osteoporosis by using physical stimulation. Low capacity supersonic stimulation to the bone cell has been widely studied and it has been in the stage of clinical application. But few report discussed about the best range of energy impulses to the bone cell. According to the literature, when supersonic energy act on the organism, the propagation of the sound wave will create heat, vibration, and massage effect to the tissue and will provide both constructive and destructive physical therapy affects. Osteoblast cell is a mononuclear cell which can cooperate with other cells to generate or rebuild osteoid of the skeleton. This research studies the culture of mice MC3T3 osteoblast cell in vitro, which stimulates the growing cell with mechanical broad range of frequency (20 KHz to 10 MHz) without temperature factor and investigates the effect of different frequency, amplitude, excitation duration, and wave form of the stimulation. The results show that several different parameters of the vibration excitation have the positive effect onto the osteoblast cell proliferation, and the density of the bone is also increased remarkably.
[P.P. Yu, C.M. Hsu, B.W. Huang, J.-G. Tseng, K.T. Yen, J.-Y. Ko, F.-S.Wang, W.H. Jhong. **The Growth of Mouse Osteoblast Cell under Vibration Wave in Vitro** *Life Sci J* 2013;10(3):1721-1726]. (ISSN:1097-8135). http://www.lifesciencesite.com. 259

Keywords: Osteoporosis, Supersonic, Vibration Wave, Osteoblast Cell

1. Introduction

Rapid heal over and effective grow up of the injured bone are urgent issues and also the goals to be accomplished. The former decreases the growth probability of bone scab and the latter shortens the rehabilitation period after bone operation for the patient. Many medical researches prove that the patients who are stimulated by low level supersonic energy (Oh, et al. 2011; Choi, et al., 2012; Li, et al., 2012) for 20 minutes a day within three month period will have more heal over probability than those who do not take the same treatment.

Osteoblast like cell (Adachia et al., 2008; Kodama et al., 1983; Quarles et al., 1992) is usually employed to study the cell proliferation. Supersonic therapy is a popular treatment nowadays, but why it is used for certain specific energy level is still obscured. Bassett et al. (1964) first used non-invasion Pulsed Electromagnetic Fields (PEMF) to stimulate the growth of the bone. Furthermore, Bassett et al. (1982) found that to stimulate maternal mouse by PEMF with frequency of 5 Hz for 14 days would get the best bone growing condition and proved that the single electromagnetic stimulation would have the positive curative effect for osteoporosis patients. Mishima (1988) obtained the similar result of increasing the activity and volume of bone formation of osteoporosis rat's back leg by certain period of PEMF stimulation.

Chang, et al. (2001) cultured osteoclasts cell from matured bone marrow of femur and tibia of female Wistar rat. Then, those cells were stimulated by different single electromagnetic field (sPEMF) strength and period. The result showed that the inhibition of osteoclasts cell proliferation was taken place under the stimulation strength of 0.2 mV/cm and period of 0.5 hr, 2 hr, and 8 hr, respectively.

Tanaka et al. (2003) concluded that the osteoblasts cells (MC3T3-E1) are more sensitive to low amplitude, broad frequency (0 to 50Hz) strain, and this kind of strain could sensitize osteoblasts to high amplitude, low frequency strain, which also could imply a potential contribution of stochastic resonance to the mechanical sensitivity of osteoblasts. Huang et al. (2009) studied the culture of mice MC3T3 osteoblast cell in vitro, stimulated the growing cell with mechanical broad range subsonic frequency (Oh, et al., 2011) with or without temperature factor and investigated the effect of different amplitude, repeated number of times, and excitation durations of the stimulation. Sum up, it concluded that some frequencies (500Hz, 1000Hz) would suppress the proliferation of the cell, while

others (1500Hz, 2000Hz) would increase the number of cell.

Kim et al. (2009) reported that the specific levels of mechanical static stretching force increase cell proliferation and effectively stimulated the osteoblast differentiation of C2C12 cells in conjunction with BMP-2 stimulation, thus it indicated a synergistic interaction between mechanical strain and cytokine signaling.

Lau et al. (2010) conclude that osteocytes were able to sense low-magnitude, high-frequency vibration and to respond by producing soluble factors that inhibited osteoclast formation (Prè, et al., 2011). Wenger et al. (2010) employed whole-body vibration on bone properties in aging mice. Pan et al. (2010) used ultrasonic power to increase the extraction productivity of Yunzhi polysaccharides which could enhance alkaline phosphatase (ALP) activity in osteoblast cell.

Suzuki et al. (2007) developed a goldfish scale in vitro model system that contained osteoblasts, osteoclasts, and bone matrix all of which were similar to those were found in human bones. They found that in this co-culture system, osteoblasts and osteoclasts in the scale sensitively responded to several degrees of acceleration (0.5G to 6G) which was useful for the analysis of bone metabolism under loading or unloading.

Since a lot of preliminary studies indicated that low-magnitude mechanical stimuli might assist in implant osseointegration, so collectively, hypothesize that low-magnitude mechanical vibration might be applied clinically to strengthen and accelerate osseointegration of dental implants (Zhao et al., 2009; Zhang, et al., 2012).

When the vibrating wave propagates through a biological tissue, bioeffects including those induced by heat and vibration could result constructive and destructive effect for physiotherapy according to previous study. Also, the study of microscopic cell can assist to more understanding the macroscopic dynamic characteristic of bone structure (Huang, et al, 2009).

The purpose of this research is to find the influence of vibration wave which stimulus over high frequency broad range onto mice MC3T3 osteoblast cell in vitro with different parameters of amplitude, time, excitation durations, etc.

2. Experimental Analysis

The experiment is designed for MC3T3 osteoblast cell in vitro under high frequency vibration wave excitation by changing of amplitude, frequency range, time duration, and wave form. The response of the osteoblast cell is verified by the variation of mRNA (Messenger RNA).

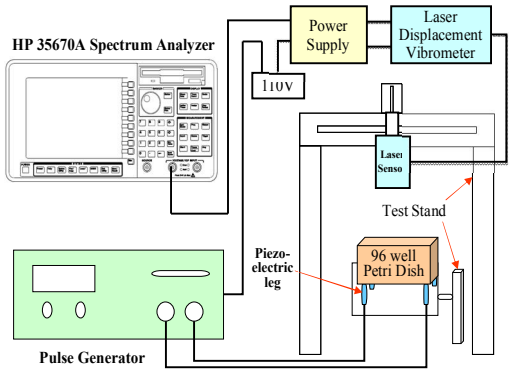

Figure 1. Experimental set up diagram

Figure 2. Experimental set up physical picture

2.1 Experimental Setup

A tinfoil paper is paved under the Petri dish and 75% alcohol is sprayed around it before the experiments to eliminate the temperature effects or bacteria infection possibility. The diagram and physical picture of experimental set up is shown in Figure 1 and 2, respectively, to investigate the proliferation effects of osteoblast cell (MC3T3) under high frequency vibration wave stimulation with different parameters: broad range of frequencies, amplitude, duration, repeated times, etc.

The 6 or 96 MC3T3 cell culture well is fixed at the platform supported by four Piezoelectric (PZT) legs which are connected and are excited by 110V pulse generator simultaneously. These PZT legs can drive the object under test from 0 Hz to 10 MHz which pass through the high or ultra-high frequency range. An optical sensor of the Laser Displacement Vibrometer is hanged on the test stand and is placed around 3 cm apart from the top of the cell culture plate to measure actual cell vibration signal. This Laser Displacement Vibrometer (LDV) is connected to one channel of HP 35670A spectrum analyzer through 110V power supply in order to measure the displacement of the platform in time response.

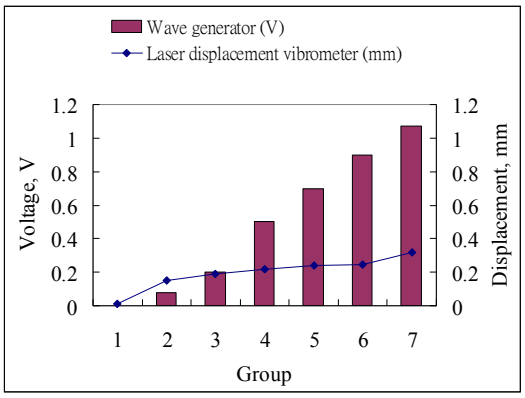

Figure 3. Displacement measurement

The temperature at the time of each experiment is recorded to avoid its influence to the cell. The Scanning Electron Microscope (SEM) photos of the growing cell patterns are taken before and after each experiment. The Osteocalcin (OCN) and Alkaline phosphatase (ALP) mRNA are extracted to verify the increasing of the osteblasts cell.

2.2 Displacement Measurement and Validation

To find out the movement of pulse generator which provides 20 times amplified of voltage output, the corresponding displacement of LDV is measured, and the relationship between them is first verified. Seven different voltage amplitude groups from 0 V to 1.07 V of the pulse generator are used with 500 Hz excitation frequency and 10 seconds to acquire data. The corresponding seven displacements of Petri dish which are detected by LDV from 0.012 mm (closed to 0) to 3.17 mm are obtained as shown in Figure 3. It can be seen that both voltage and displacement curve have the linearly ascending trend.

3. Results and Discussions

The results are discussed in the following subsections: frequency influence, experimental duration influence, amplitude influence, and wave form influence.

3.1 Frequency Influence

There are nine sets of cell culture plates which are divided to control_1, control_2 and seven different excitation frequencies: 2K to 10 MHz with same fixed sine wave form, amplitude (1.07 Vpk, peak to peak), and experimental duration (15 min). All the culture plates are taking out of the 37°C incubator when one set of culture plate is stimulated in room temperature. The total working time is 105 min and the average temperature is 25.78 °C during the experiments. Since MTT and RNA assays will cause the death of the cell, hence, it needs two control sets (used in all experiments of this study) to evaluate the initial and final cell concentration under nature growth without any external stimulation. The cell proliferation which is increasing with frequencies and 10 MHz causes the highest cell concentration as shown in Figure 4. Frequency does not effected OCN mRNA (red line, square markers) much, while high value of ALP mRNA (green line, triangle markers) at 10 MHZ shows the increasing effect of early bone metabolism. Please note that Cell concentration is shown by the left coordinate, brightness ratio for both OCN mRNA and ALP mRNA is shown by the right coordinate in Figure 4, 5, 7, 8, respectively.

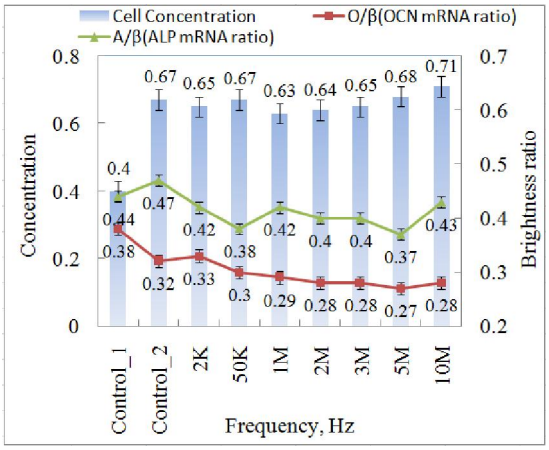

Figure 4. Cell growth, OCN mRNA ratio and ALP mRNA ratio variation by stimulation frequency

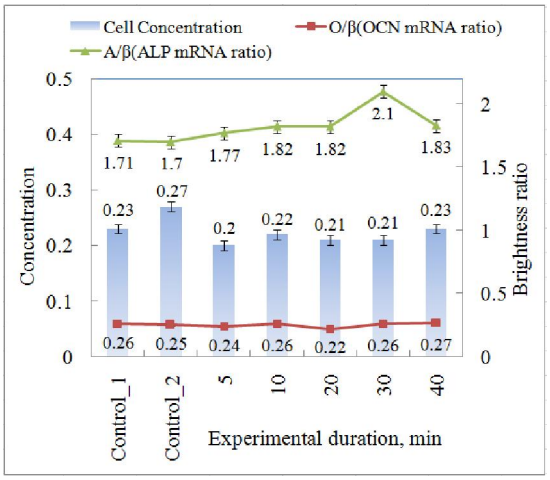

Figure 5. Cell growth, OCN mRNA ratio and ALP mRNA ratio variation by stimulation duration

3.2 Experimental Duration Influence

There are five sets of experimental duration from 5 min to 40 min with same fixed sine wave form, amplitude (1.07 Vpk, peak to peak), and frequency (10 MHz). The total working time is 105 min and the average temperature is 24.43°C during the experiments. The cell proliferation is better both at 10

min and 40 min of stimulation duration (Figure 5) and OCN mRNA (red line, triangle markers), respectively. While the increasing effect of early bone metabolism of ALP mRNA (green line, square markers) is at 30 min duration. The SEM photos of (a) cell proliferate naturally (control_2 group) and (b) 10 min stimulation duration is shown in Figure 6.

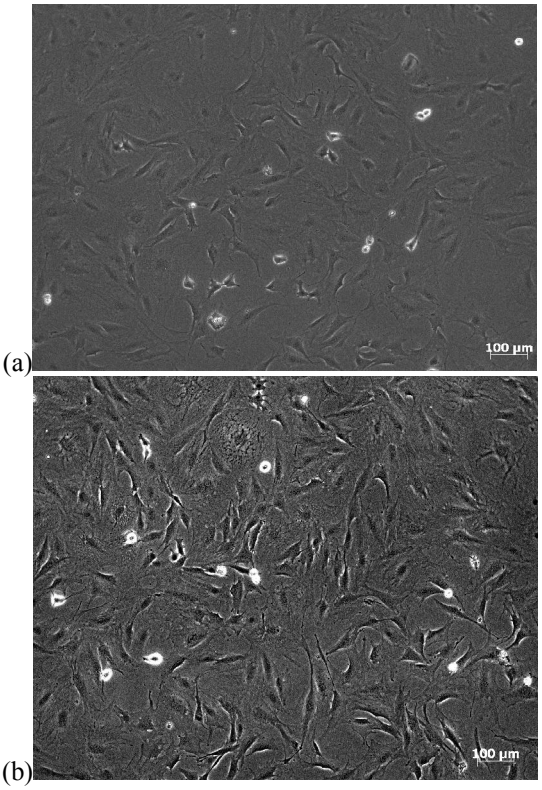

Figure 6. SEM photos: (a) cell proliferate naturally (control_2), (b) 10 min of stimulation duration

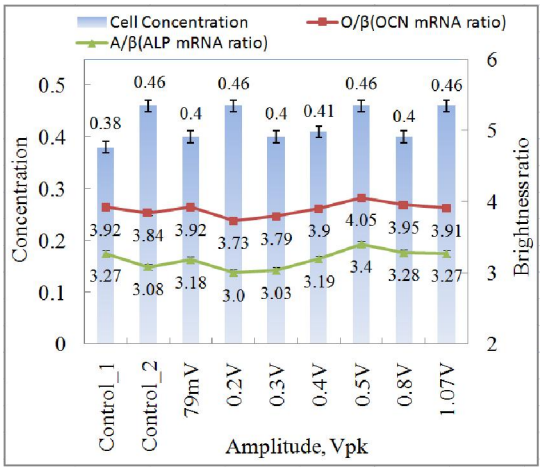

Figure 7. Cell growth, OCN mRNA ratio and ALP mRNA ratio variation by stimulation amplitude

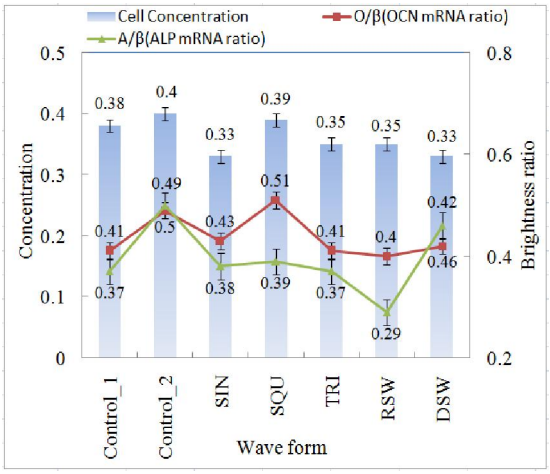

Figure 8. Cell growth, OCN mRNA ratio and ALP mRNA ratio variation by stimulation wave form

3.3 Amplitude Influence

There are seven sets of amplitude variation from 0.079V to 1.07V with same fixed sine wave form, duration (15 min), and frequency (10 MHz). The total working time is 75 min and the average temperature is 23.42oC during the experiments. The cell proliferation is better at 0.2V, 0.5V, and 1.07V of stimulation amplitude as shown in Figure 7. Both OCN (red line, square markers) and ALP mRNA (green line, triangle markers) have better performance at 0.5V amplitude.

3.4 Wave Form Influence

There are five sets of wave form: sine wave (SIN), square wave (SQU), triangle wave (TRI), rising sawtooth wave (RSV), and down sawtooth wave (DSW) with same duration (15 min), frequency (20 KHz), and amplitude (1.07 Vpk). The total working time is 75 min and the average temperature is 22.4°C during the experiments. The cell proliferation is better at square (SQU) stimulation wave as shown in Figure 8. The OCN (red line, square markers) and ALP mRNA (green line, triangle markers) have better performance under SQU and DSW excitation, respectively.

4. Conclusions

Supersonic and vibration wave stimulation to the tendon and osteoblast cell have been widely studied. But the effective range is still needed to be discussed. This research discussed the effect of different stimulation parameters, such as high frequency range, excitation duration, amplitude, and wave form to the osteoblasts MC3T3 cell. The results are compared with osteocalcin and ALP mRNA expressions.

The summary of this studied are listed as follows:

1. When stimulate the osteoblasts cell intensively, there is no positive or negative effect since the cell absorbed too much energy and it reached saturation status.
2. In frequency variation test, the better cell proliferation is occurred at 10 MHz. The ALP mRNA shows the increasing effect of early bone metabolism.
3. In excitation duration test, the cell proliferation for 10 min and 40 min are better than other cases. Similar result is found in OCN mRNA, but 30 min of stimulation is better in ALP mRNA.
4. In amplitude variation test, the cell proliferation is better at 0.2V, 0.5V, and 1.07V of stimulation amplitude. Both OCN and ALP mRNA have better performance at 0.5V amplitude.
5. In the wave form test, the cell proliferation is better at square (SQU) stimulation wave. The OCN and ALP mRNA have better performance under SQU and DSW excitation, respectively.

5. Acknowledgment

The authors would like to thank the National Science Council, Taiwan, Republic of China, for financially supporting this research through Grant NSC99-2632-E-230-001-MY3.
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8/12/2013