The Regeneration of the Bone Cells Under the Effect of Vibration Waves

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Abstract: The morbidity of osteoporosis is increasing fast recently due to people change their daily habits from dynamic events to sedentary activities based on the progress of technology. Osteoporosis makes bones become fragile loose, which cause back pain, loss of height, kyphosis, or even very prone to fracture, fractures and other symptoms. Osteoporosis is already one of the world's largest epidemic, second only to vascular disease. And it mostly depends on chemically treatments nowadays. However, the properties of chemical drugs will lead to many problems of the cells in human body. For examples, the body will produce drug exclusion, drug resistance, and many other side effects, which cannot be ignored. Therefore many scholars explore the physical stimulation to combat osteoporosis. Non-invasive physical stimulating of the biological cells or tissues through vibration is currently potential areas of research. There are many physical therapy scholars to conduct the research of high frequency vibration. This study employs low-frequency vibration wave generated by the piezoelectric sheet to stimulate the fetal calvarias cells by different sets of durations and repeated of times. The experimental results show that different duration and number of repeat stimulation period will indeed affect the proliferation of bone cells.
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1. Introduction

Several scholars focused on low magnitude, high frequency vibration (LMHFV) stimulation on the mouse cell and observed quite promising results. Kono et al. found that low magnitude; high frequency (LMHF) vibration stimuli had a positive effect on bone at the early stage of bone healing, particularly in trabecular thickness, at the incisor extraction socket [1]. Chow et al. indicated that LMHFV accelerated fracture healing by enhancing bone remodeling but the administration of ibandronate can impair this enhancement [2].

Shi et al. also showed that LMHFV was effective in promoting the fracture healing in ovariectomy group in all measured parameters, particularly in the early phases of healing, with the outcomes comparable to that of age-matched normal fracture healing [3]. Lau et al. concluded that osteocytes were able to sense LMHF vibration and respond by producing soluble factors that inhibit osteoclast formation [4]. LI adopted Pulsed Electromagnetic Field (PEMF) and ultrasound stimulation on osteoblasts and found that there are different transduction pathways for PEMF and ultrasound stimulation which enhance osteoblast proliferation [5]. Huang et al. studies the culture of mice MC3T3 osteoblast cell in vitro, stimulate the growing cell with mechanical broad range subsonic frequency with or without temperature factor and investigate the effect of different amplitude, repeated number of times, and excitation durations of the stimulation [6]. Shigeo et al. suggested that mechanical vibration at certain frequencies may modulate biosynthetic response of articular chondrocytes [7].

Jeng Sen et al. found that the vibration stimulated on the periosteal cell in horizontal direction will help its proliferation while in vertical direction will inhibit the growth of cells [8]. Wang et al. suggested that short waves could increase proliferation in human chondrocytes through activation of the ERK pathway, which was also involved in maintaining normal cell proliferation under physiological conditions [9]. Chen showed that the PEMF could enhance the osteoblast cells proliferation when the stimulation time was 4 hrs/day, and 5 days exposure [10]. Wu showed that the PEMF could enhance and inhibit the osteoclast proliferation when the induced electric field was 0.8 mv/cm and 0.2mv/cm, respectively [11]. Akira et al. showed that bone regenerative effect of low-intensity pulsed ultrasound (LIPUS) treatment on rat noncritical calvarias defects, as confirmed with in vivo micro-CT [12]. Castillo et al. proposed that low amplitude, broad frequency vibration superimposed onto an estrogenic waveform or vibration alone does not enhance cortical bone adaptation at the frequencies,

amplitudes and loading periods tested [13]. Wenger et al. evaluated the efficacy of whole body vibration (WBV) in an aging mouse model. WBV was found can improve measures of bone health for certain clinical conditions and ages [14].

Yu et al. The use of 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 [15].

Lambers et al. investigated mouse tail vertebrae adapt to mechanical loading by increasing the surface of formation sites and decreasing the surface of resorption sites, leading to an overall increase in bone strength. This new imaging technique will provide opportunities to investigate in vivo bone remodeling in the context of disease and treatment options, with the added value that both bone formation and bone resorption parameters can be nondestructively calculated over time [16]. Vanleene concluded that vibration therapy resulted in a significant increase in the cortical bone area and cortical thickness in the femur and tibia diaphysis of both vibrated oim and wild type mice compared to sham controls [17]. Cherng et al. The use of signal at 14Hz through algorithm analysis of the measured near field magnetic fluctuation and the modulation of gap junction intercellular communication (GJIC) within mouse osteoblast cells (MC3T3-E1) under RF-EMF power density at 0.3mWatt/cm2 [18].

Judex identified of the precise physical mechanisms by which cells responds to WBV will ultimately enable the optimization of the no pharmacologic means of controlling tissue mass and morphology in spite of detrimental systemic pressures toward the resorption of bone [19].

Prè showed the estrogenic effect of high frequency vibration treatment in the early stages of hASC differentiation (after 14 and 21 days). All together, results demonstrate the effectiveness of high frequency vibration treatment on hASC differentiation toward osteoblasts [20]. Dumas identify low amplitude, high frequency strain regimen able to increase major matrix proteins of bone tissue and to regulate the expression of VEGF variants, showing that an appropriate combined loading has the potential to functionalize cellularized bone-like constructs [21]. Sani et al. showed that relative absorption rate of electromagnetic radiation by bone marrow of rats are maximum in frequency of 500 Hz where as there was no absorption in 100 Hz frequency [22].

2. Experimental procedure for cell culture

Skull cells (fetal calvarias cells) of mouse are used in this study. Fetal calvarias cells belong to osteoblasts are important in the process of bone formation functional cells. Main function of the osteoblasts is to secrete bone matrix and synthesized. Osteoblasts can be seen in mouse, chicken and human embryonic. Osteoblasts also involve in the resorption and regulation, both of which are important core cells in the process of bone metabolism.

Experimental procedure for cell culture is as followed:

1. Fetal calvarias cells were removed from very low temperature liquid nitrogen bucket. And rapidly thawed in a water bath at 37 ℃.

2. Put the cell in 800rpm centrifuge for 5 minutes. Carefully siphon off the upper medium containing dimethyl sulfoxide DMSO Antifreeze.

3. Move the suspended cells from a little culture medium (L-G medium +10% FBS) to T75 type culture flask which containing 15ml medium, and place them in 37 ℃ incubator (with 5% CO2) to culture the cell.

3. MTT Assay and experimental procedure

The colorimetric assay using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is adopted in this study.

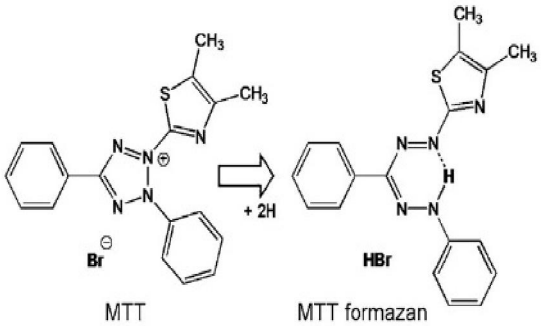


Fig. 1 MTT and its reduced formazan product [24]

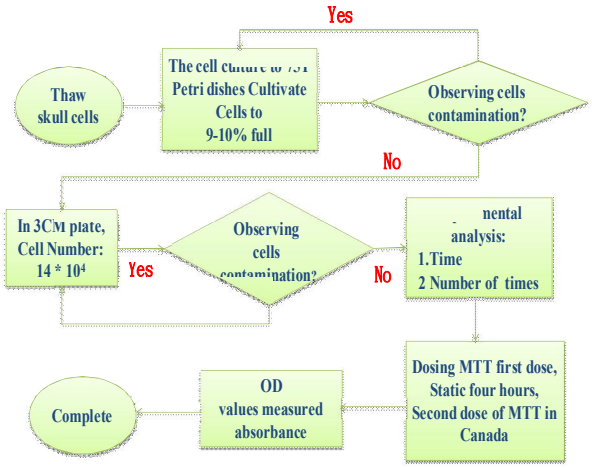


Fig. 2 MTT assay analysis procedure

MTT is a water-soluble yellow dye and can be reduced to water-insoluble purple formazan crystals by the dehydrogenase system of active cells [23].

This study uses purple-blue formazan crystals (Fig. 1) in the OD 550nm which is proportional to the product. Higher MTT metabolic yields (OD value) indicates better cell growth rate. The experimental procedure for cell culture (Fig. 2) is as followed:

1. Seed the cells in 3 cm dish (cell concentration is $14*10^4$ in each dish) and place them in incubator (37℃, 5% CO_2) for 24 hours.
2. Place the cells into the incubator for one night after the vibration tests.
3. Absorb the culture medium from each disk in the cells, adding fetal bovine serum (FBS) medium to 500μl.
4. Add 25μl MTT-1 reagent and stand for 4 hours.
5. Cells will turn into blue formazan crystal violet and attach above the dish (Fig. 3). Draw MTT-1 reagent.
6. Dry the dish, add 250μl of Sodium lauryl sulfate (SDS), and stand for 40 minutes.
7. Move 100 μl from every dish, transfer to 96 well dish, and use ELISA reader to analyze at 550 nm

4. Experimental Setup

A high-performance, small size, light weight piezoelectric box (Fig. 4) is built and non-intrusive low-frequency vibration tests for the cells are performed to investigate the growth situation of the cell.

The cells used in this study are cranial osteoblasts (Fetal calvarias cells) of mouse. The experimental setup includes function generator, displacement sensors, laser displacement meter, power amplifiers, spectrum analyzers, power supplies, piezoelectric box, etc. as shown in Figure 5. Two sets of low-frequency, in vitro, vibration tests are conducted: duration test and number of repeated duration tests. The temperature effects are eliminated for all the tests, i.e. all dishes are taken out of the incubator and placed in room temperature during the tests.

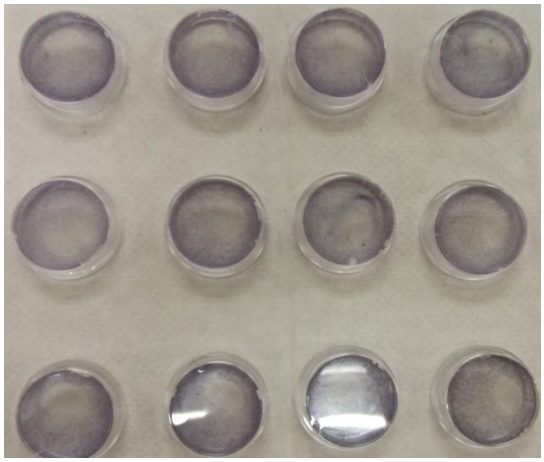


Fig. 3 MTT turned into purple blue crystals

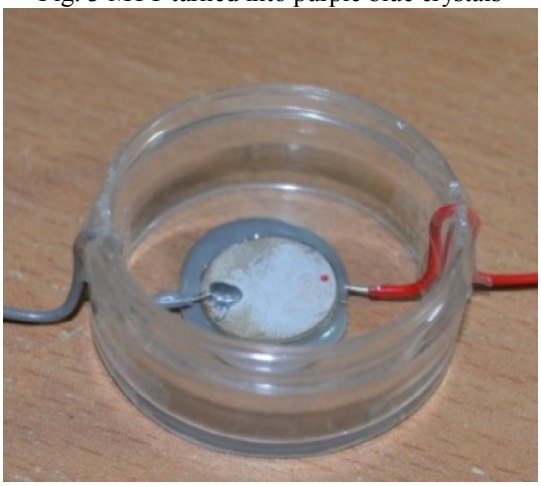


Fig. 4 homemade non-invasive low-frequency piezoelectric Box

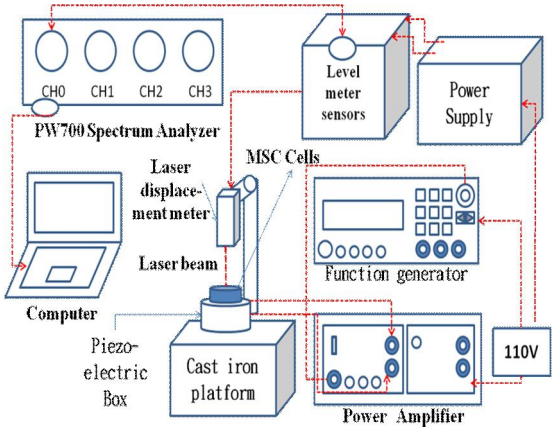


Figure 5 Schematic diagram of the experimental set up

Place 3 cm petri dish on a sheet of foil, and spray 75% alcohol around the dish to disinfect the test platform in order to isolate any bacterial contamination.

5. Results and Discussions

(1) Duration effect:

There are six cell culture dishes divided into control_1, control_2 and four different excitation durations: 20 min, 30 min, 40 min and 60 min. The stimulation parameters include: SIN waveform, fixed frequency of 100 Hz, fixed amplitude of 1 VP.

All the cell culture dishes are taken from incubator (37°C) to vibration test environment at room temperature 27°C, humidity 56%, total of four different test durations, the minimum working time of a set of experiment is 80 minutes. Since MTT assay process will kill the cell, it requires two control groups (for all experiments in this study) without any external stimulation in order to assess the initial and final natural growth of cell proliferation to compare with that of the test groups.

Each duration test is repeated for ten times and the highest and the lowest value are discarded to prevent odd data. Hence, only eight groups of duration data are shown in Fig. 6. The average value of the eight groups represents the cell activity of mitochondria as shown in Fig. 7. The excitation duration of 30 minutes possess the highest cell activity. The groups undergo 40 and 60 minutes of stimulating duration have slow down the proliferation.

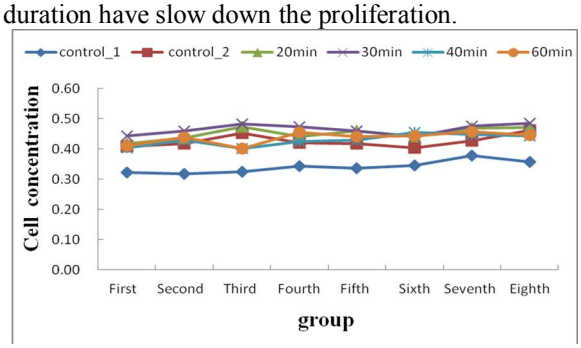


Fig. 6 Eight groups of cell concentration undergo different duration of stimulation.

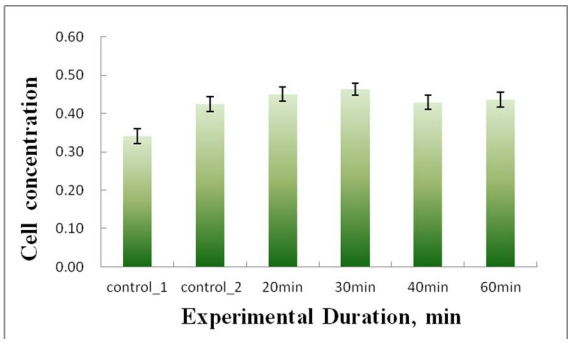
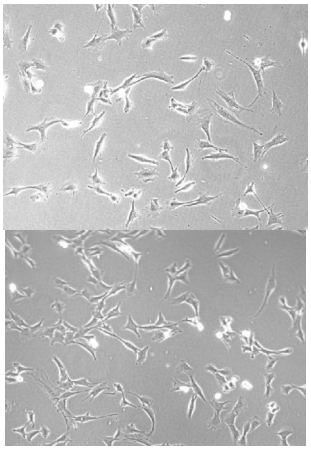
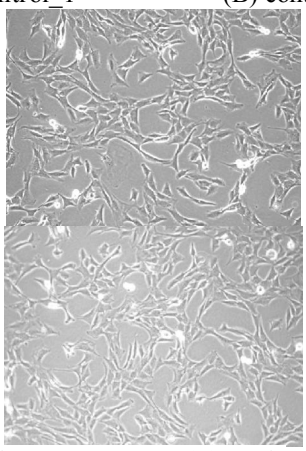


Fig. 7 Average cell concentration values with different excitation durations

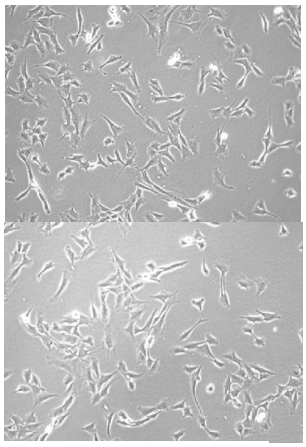
Cell micrographs taken after different stimulation durations are shown in Fig. 8. The highest density of the cell in the picture is 30 minutes stimulation duration and the results are confirmed and shown in Fig. 7.



(A) control_1 (B) control_2



(C) 20min (D) 30min



(E) 40min (F) 60min

Fig. 8 Cell micrographs taken after different stimulation durations.

(2) Number of duration effect:

There are seven cell culture dishes divided into control_1, control_2 and five different excitation times of the same duration: one through five times. The stimulation parameters include: duration of 30 minutes, SIN waveform, fixed frequency of 100 Hz, fixed amplitude of 7 VP, temperature 28.3°C, humidity 48%. The minimum working time of each set of experiment is 150 minutes.

Each duration test is repeated for seven times. The highest and the lowest value are discarded to prevent odd data. Hence, only five groups of duration data are shown in Fig. 9. The average value of the five groups represents the cell activity of mitochondria as shown in Fig. 10. Three times excitation for 30 minutes duration has the highest cell activity, i.e. the highest proliferation of cell. The groups which undergo for four and five times and stimulate for 30 minutes duration have slow down the proliferation. While the group which undergoes for one time and stimulates for 30 minutes has the lowest cell activity.

Cell micrographs taken after different stimulation durations are shown in Fig. 8. The highest density of the cell in the picture is 30 minutes stimulation duration and the results are confirmed s shown in Fig. 11.

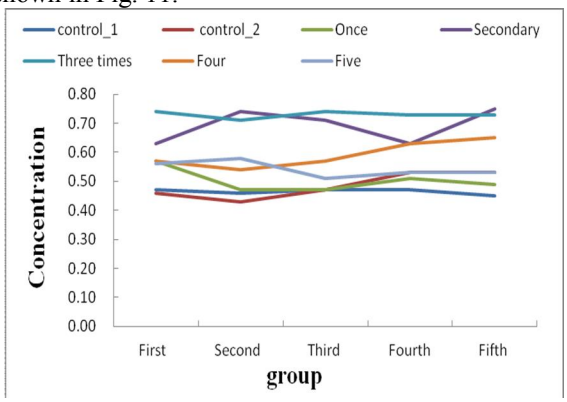


Fig. 9 Eight groups of cell concentration undergo different duration of stimulation.

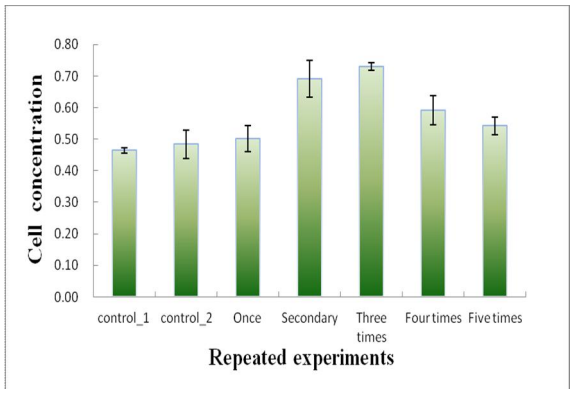
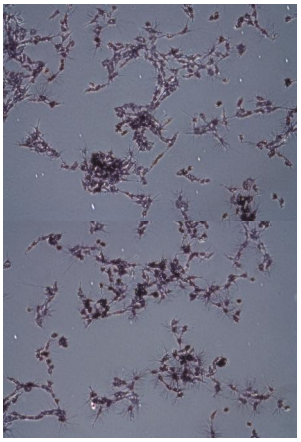
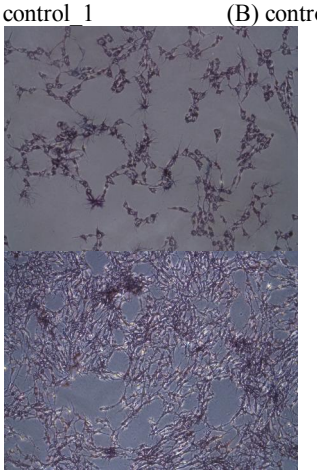


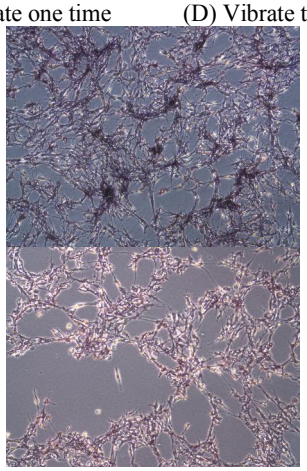
Fig. 10 Average cell concentration values with different excitation times for same duration



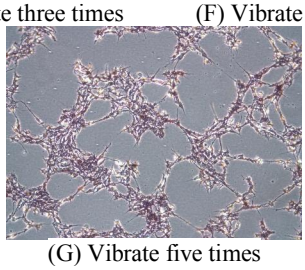
(A) control_1 (B) control_2



(C) Vibrate one time (D) Vibrate two times



(E) Vibrate three times (F) Vibrate four times



(G) Vibrate five times

Figure 11 The number of cells under the growth conditions MTT

6. Summary

In this study, low frequency vibration waves generated by the piezoelectric box are used to investigate the case of bone cell proliferation. Therefore, the time at low frequencies to stimulate bone cells caused the optimum range is 30 minutes for 3 times. And yet it has seldom been proposed on bone cell proliferation frequency effect. By investigation the best number for a case is found to be three times. Therefore, upon the physical treatment, the use of low-frequency vibration to treat osteoporosis will be the trend in future development.

This study investigates low frequency stimulation affects the proliferation of skull cells (fetal calvarias cells) of mouse. Different excitation parameters, duration and repeated times of duration with fixed excitation frequency, wave form, amplitude, etc., are compared for the cultivation of cells. Once the fetal calvarias cells of mouse are understood, it can be used to analyze the related parameters of real human osteoblast or other cells in the future. Therefore, the result of this research is quite useful for physician's reference.

Several aspects are discovered during this research and listed as followed:

1. Cell cultivation and MTT assay techniques are adopted in this research before execution mechanical stimulating process.

2. 30 minutes duration and three times of repeated excitation with frequency 100 Hz, amplitude 7 Vp etc., compose the best range to the proliferation of osteoblast cell.

3. The promising results of low frequency excitation for the cell give another evidence of reducing osteoporosis condition with physical therapy.

7. Acknowledgement

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