以物理刺激與藥物或細胞激素擷抗劑防治骨質疏鬆機轉之研究 子計畫二:不同低強度超音波刺激對骨細胞之影響

計畫編號:NSC 90-2213-E-002-130 執行期限:90 年8 月1 日至91 年7 月31 日 主持人:台大醫學院骨科 孫瑞昇醫師 共同主持人:中原大學醫工所 張恆雄教授 計畫參與人員:洪瑞千、林政鞍

Abstract

This study compares the effects of different intensity ultrasound on bone cell in the far field model with effects of the near field model from the literature, in order to understand the relations between prostaglandin E_2 and osteoblast growth. We use an in vitro model to investigate the effects of 1 MHz, pulsed 1:4, and five different spatialaverage temporal-peak intensity (150, 300, 600, 1200, 2400 mW/cm²) ultrasound stimulations in far field exposure (240 mm) on osteoblasts for 15 minutes. Optimum intensity in this study was 600 mW/cm^2 , and cell density and prostaglandin E_2 (PGE₂) secretion could be significantly stimulated at this intensity. This research may indicate that growth of osteoblasts by the ultrasound stimulation was at least partly due to increases in the synthesis and secretion of PGE₂. This well controlled model can lead to further research on the biological mechanisms for ultrasound.

Keywords: ultrasound \ osteoblast \ osteoclast \ osteoporosis \ cell culture

摘要:

台灣已漸邁入老年化社會,骨質疏鬆症影響層 面將日益擴大,提出一些解決之道勢在必行。 近二十年來,臨床上發現超音波對於骨折的癒 合有很大的療效,大大提高了骨折癒合過程中 骨細胞的活性,本研究希望利用探討不同強度 超音波刺激骨細胞之影響的方式,進一步提出 如何利用超音波抑制骨質流失的模式。本計畫 成功建立體外超音波刺激模式,針對骨母細胞 與蝕骨細胞施予不同強度超音波刺激並進行相 關生化分析,發現體外骨細胞刺激實驗中600 mW/cm^{2 (SATP)}對於骨細胞活性較有幫助,且反 應中PGE2的分泌有可能是加速造骨的表現的指 標。 **關鍵字:**超音波、骨母細胞、蝕骨細胞、骨質 疏鬆症、細胞培養

RESULTS

Cell Count

The changes of cell population when osteoblasts were treated with different intensities $(150, 300, 600, 1200, 2400 \text{ mW/cm}^2)$ of ultrasound stimulation or sham-exposure for 15 minutes are summarized in Table 1. The difference between the treated group and the paired sham group was significant in the group of osteoblasts that was treated with a dose of 600 mW/cm^2 (p<0.05, paired t test) and cell density of stimulation group treated by 600 mW/cm² elevated about 10 % from the sham group. The other groups of larger or smaller intensity treatment had no apparent difference from their paired sham groups, however there was an ascending tendency from150 to 600 mW/cm² and a descending tendency toward from 600 to 2400 mW/cm² (Table 1).

Analysis of prostaglandin E_2 (PGE₂) in culture medium

Steady-state levels of PGE₂ concentration in culture medium (about 48 pg/ml) of paired sham group were lower than which in the medium of ultrasound-treated group (about 54 pg/ml) statistically (p<0.05). Furthermore, PGE₂ concentrations of the well supernatants stimulated by 600, and 1200 mW/cm² ultrasound clearly rose to 67.02 ± 12.14 , and 64.05 ± 10.68 pg/ml from 45.06 ± 11.41 , and 50.8 ± 9.37 respectively (p<0.05) (Fig. 5). After 15 minutes of ultrasound stimulation at 600 mW/cm² intensity, PGE_2 concentration was clearly increased to nearly 50 % from the paired sham group, and PGE2 concentration in the 1200 mW/cm² group was increased about 28 % from the paired sham group.

Differences of PGE_2 concentration in other intensity stimulation groups were smaller than these two groups, although they were not divergent statistically between their own paired sham groups. PGE_2 concentration difference between stimulation group and paired sham group also had an ascending tendency from150 to 600 mW/cm² and a descending toward from 600 to 2400 mW/cm² (Fig. 5).

DISCUSSION

The multiple reflections of ultrasound signal were the major problems of dose investigation of ultrasound in vitro. We have tried to use the absorption models in the literature (Ito et al. 2000; Maxwell et al. 1994; Reher et al. 1998), but they didn't absorbing the signal well. After the absorption rubber was dismembered into 5 mm² and we put these particles into the absorption tube as shown in Fig. 2. The reflection of ultrasound signal was eliminated and the osteoblasts didn't be damaged.

The results in Table 1 evidenced that osteoblasts cell density can be increased significantly by the 600 mW/cm² ultrasound exposure, although it seemed that there were large differences between the five sham groups. For example: the cell density of the 600 mW/cm^2 sham group was 186.82 cells/mm² with 7.28 standard deviation, and the 1200 mW/cm² sham group was 201.3 cells/mm² with 12.26 standard deviation. The value of 1200 mW/cm^2 sham group was almost the same as the cell density of the 600 mW/cm^2 stimulation group (203.37 cells/mm²) with 8.14 standard deviation). It was because we only have two ultrasound stimulators and we just can stimulate two dishes accompanying two sham dishes at the same time. Although we changed the culture medium to the 1% serum one after the 48 hours incubation with 10% serum one, cell density still increased (as you can see from 162.93 to 188.24 and finally to 213.62 cells/mm²) before the exposure. It means that the beginning time of the 150 mW/cm^2 exposure was at least 75 minutes earlier than the beginning time of the 300 mW/cm^2 exposure and almost 7 hours earlier than the beginning time of the 2400 mW/cm² exposure. But each group was independent with each other and corresponding to their own sham group data. Similarly, PGE₂ concentrations at 1 h postinsonation also changed significantly at 600 (and

1200 mW/cm²) compared with its specific sham group in Fig. 5.

There was a clear consistency of effect from different ultrasound intensity treatments in increasing cell density and PGE₂ concentration. After 24 hours incubation of osteoblasts exposed to 600 mW/cm² ultrasound for15 minutes, the cell density of this group increased (Table 1) and PGE₂ concentration in the medium with this stimulation was also distinctly increased after 60 minutes incubation (Fig. 5). The smallest (150 mW/cm^2) largest (2400 mW/cm²) intensities of and ultrasound stimulation both had no significant difference from each of their paired sham groups in either cell density or PGE₂ concentration. Although there was also no evident variation of 300 mW/cm^2 ultrasound stimulation by any test here, it seems that test values lay between the 150 mW/cm^2 and 600 mW/cm^2 stimulation group values (Table 1, Fig. 5).

Researchers have studied therapeutic ultrasound in animal and clinical trials with near field exposure, in which a transducer contacts directly with the skin. However, they have investigated in vitro tissue or cell culture systems with both near field and far field exposure. Reher et al. (1997, 1998, 1999) showed that optimum intensity of mouse calvaria system was 100 mW/cm^2 with 1 MHz ultrasound, and the optimum intensities of osteoblasts system were 100 and 400 mW/cm² with 1MHz ultrasound, and 15 and 30 mW/cm² with 45 kHz (long wave) ultrasound, but all of these exposures were in the near field (5 mm from transducer to culture well) with pulse rate 1:4. Kokubu et al. (1999) and Ito et al. (2000) investigated 150 mW/cm² ultrasound stimulation (optimum intensities in animal and clinical model) on osteoblastic cell lines using far field exposure, finding that the optional distances were 200 mm and 130 mm respectively. It appeared that the in vitro cell culture model should use lower intensity received directly by cells than either the animal or clinical model.

Optimum intensity of our exposure system was 600 mW/cm^{2(SATP)} with repetition rate: 100 Hz; duty cycle: 1:4; frequency: 1 MHz; stimulation time: 15 min; and exposure distance from transducer to culture well of 240 mm. The optimum intensity of long bone rudiment system evinced by Wiltink et al. (1995) was 770 mW/cm^{2(SATP)} for the far field treatment (180 mm), which was similar to our optimum intensity (600 $mW/cm^{2(SATP)}$) of osteoblasts system for the exposure distance 240 mm. Parvizi et al. (1999) also showed that the optimum intensity of chondrocyte culture system was 600 $mW/cm^{2(SATP)}$, which was identical to our results, although their ultrasound exposure was with near field (5 mm) and at different repetition rate (1 kHz).

ultrasound of We evinced that 600 mW/cm^{2(SATP)} intensity with 240 mm exposure distance clearly increased both the number of osteoblasts and the PGE₂ secretion clearly. These results seem to indicate the effects of increasing of DNA synthesis and expression of PGE₂ through cyclooxygenase-2 (COX-2) mRNA (Kokubu et al. 1999; Reher et al. 1998). Kokubu et al. (1999) showed that the production of PGE_2 in mouse osteoblasts was augmented by ultrasound exposure (200 microsecond burst sine wave of 1.5 MHz repeating at 1 kHz, 30 mW/cm^{2(SATA)}, 20 minutes), which was threefold at 60 minutes in comparison with unexposed samples. We also proved that PGE₂ secretion could be stimulated by ultrasound exposure (2000 microsecond burst sine wave of 1 MHz repeating at 100 Hz, 120 mW/cm^{2(SATA)}, 15 minutes), which was 50 % increased at 60 minutes in comparison with the sham exposure. The PGE_2 concentration in Kokubu et al. (1999) experiment was about 200 to 1000 pg/ml, which was almost 10 times the value of our experiment (See Fig. 5). Although the PGE₂ analysis point (after 60 minutes to the exposure) was the same, the exposure time (20 minutes) of Kokubu et al. (1999) was longer than ours. Numbers of stimulation pulse of them was also more than ours. The most important reason of this result was the culture medium in Kokubu et al. (1999), which had 10% serum, and our culture medium at exposure period was just 1% serum adding.

The increased synthesis and release of PGE_2 may act as an autocrine or paracrine factor. Also, the administration of PGE_2 in various in-vitro and in-vivo models led to increased bone remodeling and turnover (Shih and Norridin 1986; High 1987; Jee et al. 1990; Li et al. 1990; Welch et al. 1993). This is attributable to either increased bone resorption (Klein and Raisz 1970; Saffar and Leroux 1988; Collins and Chambers 1991) or bone formation (Chyun and Raisz 1984; Jee et al. 1985; Nagata et al. 1994) or both. It is known that the administration of PGE_2 at the concentration of 10^{-7} M has an osteogenetic effect by activation of the

osteoblasts and synthesis of collagen (Nagata et al. 1994). It has also been shown that PGE₂ in the concentration of 10⁻⁵ to 10⁻⁸ M can increase the concentration of Ca²⁺ and the synthesis of collagen protein (Nagai et al. 1993). In the presence of ultrasound stimulation, PGE₂ secretion of the experimental groups at 600 and 1200 mW/cm² intensities increased significantly after 15 minutes treatment compared to the sham-exposure group, and the concentrations of PGE₂ at these two intensities was located just about 10⁻⁸ M, corresponding to increasing of cell density. This research might indicate that the increase of osteoblasts growth by ultrasound stimulation was at least partly due to increases in the synthesis and secretion of PGE_2 . It seems that changes in PGE_2 in the supernatants are probably more likely to reflect membrane-mediated changes in secretion, which induced by ultrasound mechanical waves propagation.

Our study in 1991 (Tsai et al.) showed that growth and repair of fractured bone were enhanced after treatment with ultrasound, and activations of PGE₂ synthesis in bone and surrounding muscle were also observed. These results proved that PGE₂ might be involved in the stimulation of the vascular changes, bone resorption and the proliferation of osteogenic cells observed after trauma to bone. It also showed that enhanced growth and repair of fractured bone by ultrasound might be mediated partly by the release of PGE₂. In this study, we also evinced that PGE_2 secretion of osteoblasts in the culture medium could be increased by ultrasound stimulation. It means that the PGE₂ value which we got in vivo (Tsai et al. 1991), might partly consist of the secretion of osteoblasts themselves, and they probably promote the healing process by this way.

The results of this study support current findings in the literature suggesting that low level ultrasound treatment may have a stimulatory effect on the bone cell growth processes. The new absorbing method of ultrasound wave in this study is useful to the in vitro investigations. However, the appropriate ultrasound dose and treatment duration required to achieve maximal stimulation remain to be determined for both animal and patients. We believe that the observation of ultrasound stimulation of bone cell growth in a highly controlled, well-studied in vitro model will lead to further research on the biological mechanisms for this effect.

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Related Publication

- Jui-Sheng Sun, Rue-Chain Hong, Walter Hong-Shong Chang*, Li-Ting Chen, Feng-Huei Lin, and Hwa-Chang Liu: In Vitro Effects of Low-Intensity Ultrasound Stimulation on the Bone Cells. *J Biomed Mater Reseach* 5; 57(3): 449 - 456, 2001. (SCI)
- Li J, Chang WH, Lin A and <u>Sun JS</u>. "Different Intensities of 1:4 Pulsed Ultrasound Stimulation on Osteoblasts". *Ultrasound in Medicine and Biology*, 28(5): 683-690, 2002. (SCI)



Figure 1: Schematic diagram of the experimental arrangement for the ultrasound stimulation. AC = Absorption Chamber; AR = Absorption Rubber; D = Distance from transducer to cell flask, 240mm; DT = Degas Tank; DW = Distilled Walter; ES = Exposure Sample; GT = Glass Tank; R = x-y Roter; SM = Stepping Motor; T = Transducer; TC = Temperature Controller.



Fig. 2. Measurement of axial attenuation when the transducer transmits acoustic waves (1MHz, 1 W) through distilled water.



Fig. 3. Field plot of the beam intensity, which were 240 mm from the ultrasound transducer (1 MHz, 3W), after passing through the culture flask.



Fig. 3. Osteoclast Activity assay



Fig. 5. The effect of ultrasound of varying intensity from 150 to 2400 mW/cm $^2_{SATP}$, 1 MHz pulsed 2:8 ms, on PGE2 synthesis in osteoblasts. Cell medium was collected 1 hr after stimulation and assayed by ELISA. Values significantly different from sham treated groups are indicated (*) for p < 0.05. (n=10)