Bone Defect Healing Enhanced by Pulsed Electromagnetic Fields Stimulation: *in Vitro* Bone Organ Culture Model

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Abstract

Pulsed Electromagnetic Field (PEMF) has many medical applications. Previous animal and clinical studies have clearly shown a positive effect of PEMF on the rate of osseous repair. The present *in vitro* study was designed to elucidate the specific response of bony tissue to PEMF treatment. Bilateral femora were obtained from 40 mature male Wistar rats, and a bone defect was created at the center of each distal metaphysis. The femora were maintained for 1, 2, or 3 weeks *in vitro* organ culture and received 8 hours of PEMF stimulation or sham-exposure. Healing of the osseous defect was evaluated by histomorphological examination. The prostaglandin E_2 (PGE_2) and alkaline phosphatase (ALP) concentrations in culture medium were harvested and analyzed by enzyme-linked immunosorbent assay reader and spectrophometer. The results showed that PEMF stimulation can accelerate defect healing. All the experimental femoral defects treated with PEMF stimulation healed faster than the untreated control defects, and the ALP concentration of supernatants was significantly elevated on 1- and 2-week periods. When an osseous defect was created at the femoral metaphysis, the synthesis and release of PGE_2 was elevated and then decreased gradually. With PEMF stimulation, the PGE_2 level in the culture medium of the experimental group was increased at the end of week 2 and 3 compared to the sham group. This highly controlled and well-studied model of PEMF stimulation of bone healing *in vitro* can be used to further examine the biological mechanisms involved.

Keywords: Pulsed Electromagnetic Field, Bone Defect, Stimulation, Healing Rate, Histomorphology, Prostaglandin E_2, *In vitro* Organ Culture

Introduction

The application of low frequency (3-3000 Hz) pulsed electromagnetic field (PEMF) stimulation to heal fractures in animal model and clinical trials has been shown to increase the mechanical strength of callus, including the strength to failure and the stiffness, and also to reduce the time required to achieve union [1-5]. An important issue related to these studies is whether the active agent is the magnetic flux density itself or the induced time-varying electric field arises in any system exposed to a time-varyinging magnetic field [6]. The observations from previous studies might imply that cells in the fracture callus can sense and respond to the electrical energy transferred by the PEMF stimulation. The host tissue responses to these PEMF models are generally assessed by morphological and histological examinations to evaluate their effect. It is difficult to exam the in-vivo reaction of a specific tissue to the treatment modality because numerous cell populations and chemical factors are involved. In order to determine the sequences of events and the parameters influencing the interactive process, a model of organ culture in the presence of PEMF stimulation is of great importance.

In 1974, Bassett et al. introduced the technique of PEMF stimulation. The therapeutic result of this technique has been comparable to the other types of electrical stimulation with a healing rate of 72 – 87% [7-11]. In 1994, we compared the therapeutic effects between the frequency of 72 Hz (proposed by Bassett in 1977 with an impulse width of 0.38 ms) and 7.5 Hz (proposed by our group with an impulse width of 0.3 ms) [12], and it showed that the fracture healing rate were 100% with our pulse parameter and 90% achieved by Bassett's parameter, but there was no statistical significance. This might implicate more potential in developing new electric devices of very low frequency PEMF for clinical use [12]. We also tested our pulse parameter by an *in vitro* organ culture model of suckling Wistar rat femur growth in 1996 [13], and it

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demonstrated that the length of rat femur with exposure was increased significantly more than the untreated group. We examined the frequency of 7.5 Hz were benefit for our mechanism studies of osseous defects.

Electrical perturbations serve as extracellular signals to a variety of cells, including osteoblasts and osteoclasts. Several authors have found increased cellular proliferation [14-16] and production of prostaglandin E_2 after electrical stimulation of bone cells by various means. Our lab also reported that PEMF may be useful in the prevention of osteoporosis resulting from ovariectomy and that PGE_2 might relate to these preventive effects in vivo and *in vitro*[17, 18]. In this study, we use an *in vitro* bone defect organ culture model to investigate the effect of low frequency, time-varying PEMF stimulation. The goals of this study were (a) to determine whether PEMF stimulation increase the growth of osseous defects, (b) to elucidate the relationships between prostaglandin E_2 release and bone defect healing. With the use of a model that employs a drill hole in the metaphysis of distal femur, we demonstrated the acceleration of the normal defect repair process by PEMF stimulation.

Materials and Methods

Bone Defect Model

Forty healthy skeletal mature male Wistar rats, weighing 200-250 g, were used in this study. The rats were initially killed by an overdose of intraperitoneal pentobarbital. Under aseptic technique, the bilateral hindlimbs of the rat were disarticulated at the hip and knee joints. Soft tissues were dissected from the femora, with the periosteum carefully preserved. The dissected femora were soaked in and triply washed with prewarmed (37°C) phosphate-buffered saline (PBS) (Dulbecco′s PBS without calcium chloride or magnesium chloride; Atlanta Biologicals, USA) solution. A 1.77 ± 0.07 mm^2 bone defect was created at the center of the distal femoral metaphysis using a stainless-steel wire as a drill. The femora were maintained in BGJb organ culture medium (Fitton-Jackson medium, Life Technologies) supplemented with 20% fetal calf serum, penicillin G sodium 100 units/ml and streptomycin 100 mg/ml, β-glycerophosphate 0.216 g/100 ml (Sigma, St. Louis, MO, USA), and L-ascorbic acid 0.005 g/100 ml (Sigma, St. Louis, MO, USA), and were incubated at 37 ℃ in air supplemented with 5% carbon dioxide [19]. The femora were maintained for 1, 2, 3, or 4 weeks *in vitro* organ culture and received 8 hours of PEMF stimulation or sham-exposure for 1, 2 or 3 weeks. The medium in each well was changed at the 3rd, 7th, 10th, 14th, 17th, 21st, and 24th day before daily exposure, during the experiment periods.

PEMF Stimulation on Bone Defect

Active- and sham-treatment were indistinguishable from the outside both for their shape and for their weight using wound solenoid coils to generate uniform time-varying electromagnetic fields. The 13.5-cm-long by 7-cm-diameter coils were each wound with two parallel windings of 22 AWG magnet wire, resulting in a total winding resistance of 7.45 ohms. The stimulation magnetic field waveform was generated

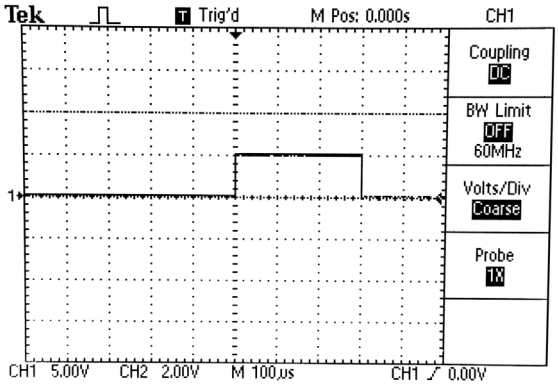

Figure 1. Diagram showing the outline of 7.5 Hz single pulse stimulation waveform coil driving potential with period (T_1) 133.3 msec, pulse width (T_2) 0.3 msec and amplitude (A) 5 volt.

by a single-chip pulse generator (PIC/16C5X series, Microchip Technology Inc., AZ, USA) (Fig. 1). Active stimulators supplied the coil intermittently with a single pulse of electrical current at a frequency of 7.5 Hz, with an impulse width of 0.3 ms, generating induced electric fields of 6 mV/cm, which measured by search coil (50 turns of No. 30 AWG magnet wire wound on a 2.5-mm radius bobbin). The peak strength of the magnetic field in the active coils was 3 Gauss measured by Gauss Meter (MG-5DAR, WALKER Scientific Inc., USA). Sham-treatment stimulators were manufactured so that the current flow in the coil was zero and no induced electric field could be recorded by means of a coil probe connected to an oscilloscope (LBO-522, Leaders, Taipei, Taiwan). The magnetic field was uniform to within 10% over the length of the coil, and uniform to within 3% over the 3.5-cm width of the organ culture wells, which were placed in a central position within the coils during the experiments. The magnetic field is directed parallel to the plane of the culture wells, and parallel to the axis of the femora. Concurrent sham exposures were accomplished by connecting the same solenoid, but without any input current. All solenoids were placed on a single shelf of a organ culture incubator to ensure similar environmental conditions. Extraneous fields in the incubator included the geomagnetic field, measured by Gauss Meter (MG-5DAR, WALKER Scientific Inc., USA), at 49-μT, 13 degrees from the vertical, and a 14-μT flux at 60 Hz due to the circulation fan. The incubator was maintained at 37℃ with a 5% CO_2 in air environment at 100% humidity. The solenoid coils were surrounded by water pipe (with inner diameter of 0.4 cm) connected to an externally regulated thermostatic water bath in order to maintain the temperature within the coil in an isothermal state, ensuring that the maximum variation of the temperature was 37℃ within ± 0.1°C.

Histomorphological Analysis of Defect Healing

For histomorphologic examination, the femora were fixed for 2-3 days in 10% neutral-buffered formalin and 2 days in Bouin′s solution; they were then decalcified in 10% acetic acid, 0.85% NaCl, and 10% formaline solution [20]. Paraffin-embedded specimens were sectioned longitudinally and stained with hematoxyline and eosin. The histological

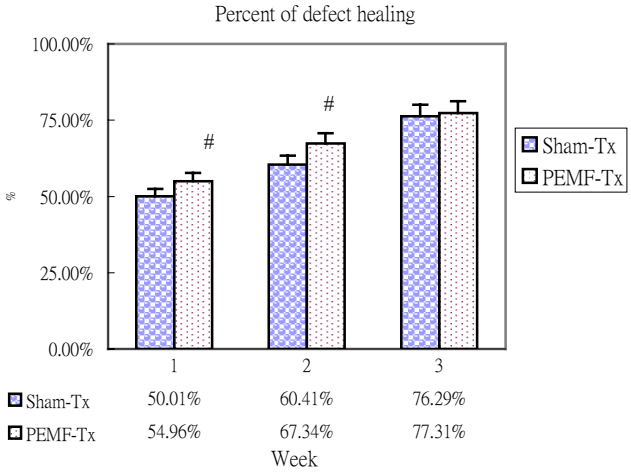

Figure 2. Mean percentage of bone defect healing at various exposing time intervals with pulsed electromagnetic field stimulation. Mean percentage of bone defect healing = [1－(hole area after exposing / original defect area)]. Femora-defect healing increased to a statistically significantly degree after 1 week ($p = 0.00006$) and 2 weeks ($p = 0.00002$) of PEMF stimulation, but percentage of defect healing did not show a significant difference at the third week between the simulation and the dummy group. # mean $p<0.05$ with t-test.

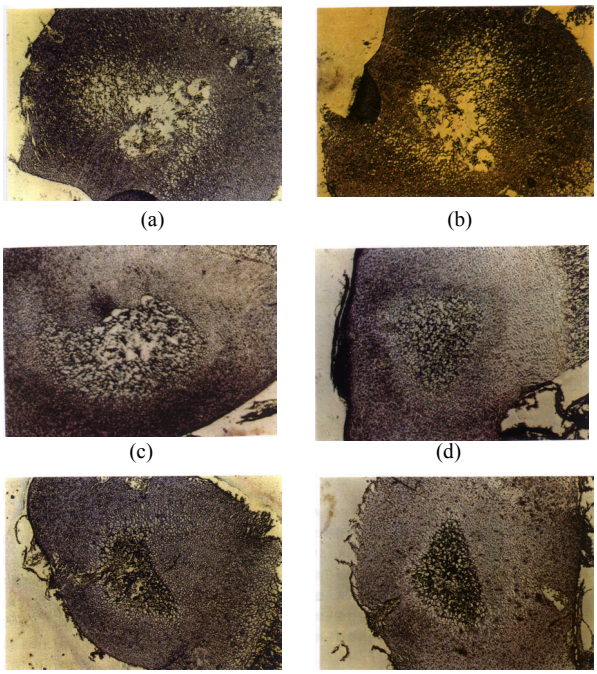

Figure 3. Histological appearance of a femoral defect following PEMF stimulation (×50): (a) dummy-exposing specimen for 1 week; (b) study specimen stimulated for 1 week; (c) dummy-exposing specimen for 2 weeks; (d) study specimen stimulated for 2 weeks; (e) dummy-exposing specimen for 3 weeks; (f) study specimen stimulated for 3 weeks.

progression of healing was photographed by inverted microscopy (IMT-II; Olympus, Japan) and analyzed with an MCID image analyzing system (MCID Software Series; Image Research, Ontario, Canada). The mean percentage of defect-area healing was determined by dividing the observed defect area after experiment with original defect area (i.e., 1.77 ± 0.07 mm^2). The mean percentage of trabecular regeneration of the bone defect was determined by dividing the observed area occupied by trabecular bone after experiment by the original defect area.

Analysis of Prostaglandin E_2 (PGE_2) in Culture Medium

The media removed from all the specimens, stimulated and **sham-exposure**, were sampled periodically at 1, 2, or 3 weeks, divided into aliquot samples of 1000 μL, and then deep-frozen at -80℃ for further analysis. The concentration of PGE_2 in the culture medium was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, either 50 μL of standard PGE_2 (Cayman Chemical Company, Ann Arbor, MI) or an experimental sample was added per well. All samples were incubated for 18 hours at room temperature on the benchtop. Following this, 200 μl of Ellman′s Reagent was added to each well and incubated for 1.5 hour at room temperature. The reaction was stopped and read by a MicroELISA reader (Emax Science Corp., Sunnyvale, CA) at 405 nm. The synthesis of PGE_2 was determined as the concentration of PGE_2 in the medium at various test periods.

Analysis of Alkaline phosphatase (ALP) in Culture Medium

The activity of alkaline phosphatase released from the femora into the medium was harvested periodically and measured at 1, 2, or 3 weeks with a commercially available assay kit (procedure no. ALP-10; Sigma, Louis, MO, USA). Briefly, an aliquot (20 μl) from the media was mixed with 1 ml ALP reagent. The absorbance at 405 nm caused by p-nitrophenol production was measured by spectrophometer and followed for 5 minutes at 30°C. The change in rate of absorbance was directly proportional to the ALP activity.

Statistical Analysis

The differences between various tested conditions were evaluated by ANOVA test. The level of statistical significance was defined as $p < 0.05$.

Results

Mean Percentage of Bone Defect Healing

PEMF stimulation can increase the speed of defect healing. After 1 week of PEMF stimulation, the mean defect healing in treated femora (54.96 ± 0.46) was greater than that of the sham-exposed femora (50.01 ± 0.55) statistically ($p < 0.005$). After 2 weeks of PEMF stimulation, the mean defect healing (67.34 ± 0.51) was statistically greater than that of the sham-exposed femora (60.41 ± 0.69), too ($p < 0.005$). In the group treated for 3 weeks with PEMF stimulation, the mean defect healing of treated femora (77.31 ± 0.94) was greater than that of the sham-exposed group (76.29 ± 1.06), but was not statistically significant ($p = 0.245$) (Fig. 2). In the group incubated for 4 weeks with bone defect healing naturally, the mean defect healing of femora was 87.34 ± 1.03.

Mean Percentage of Trabecular Regeneration

PEMF stimulation increased the rate of trabecular regeneration (Fig. 3). In the group treated with PEMF stimulation, the mean defect healing of treated femora (47.38 ±

Table 1. Prostaglandin E_2 Concentration at Various Time Intervals with Pulsed Electromagnetic Field Stimulation (n = 10)

Weeks of Stimulation	**Sham-Treated** (pg/ml) (SD)	**PEMF-Stimulated** (pg/ml) (SD)	P Value
1	12768 (113.1)	12945 (124.2)	0.1577
2	12333 (247.3)	14474 (421.0)	0.0033
3	9479 (206.6)	12111 (112.3)	0.0010

Table 2. Alkaline Phosphatase Activity at Various Time Intervals with Pulsed Electromagnetic Field Stimulation (n = 10)

Weeks of Stimulation	**Sham-Treated** (U/L) (SD)	**PEMF-Stimulated** (U/L) (SD)	P Value
1	122.61 (3.73)	141.20 (1.85)	0.000984
2	106.71 (5.02)	205.95 (8.07)	0.000002
3	96.97 (4.72)	111.71 (3.95)	0.020700

0.34) was greater than that of the sham-exposed group (43.12 ± 0.33), and reached a statistically significant level after 1 week of stimulation ($p < 0.005$) (Fig. 3a, 3b). After 2 weeks of PEMF stimulation, the enhancing effect on the trabecular bone regeneration (58.31 ± 0.45) was even more obvious comparing with sham exposure group (51.23 ± 0.39) ($p < 0.005$) (Fig. 3c, 3d). There was no significant difference between stimulated (65.44 ± 1.03) and sham-exposed (65.03 ± 1.16) groups in mean percentage of trabecular regulation after 3 weeks of PEMF stimulation (Fig. 3e, 3f).

PGE_2 in Culture Medium

When normal femoral bone without a defect was cultured *in vitro*, the PGE_2 level reached 12945 ± 124.18 pg/ml (n=10) in the 1st week and then increased to 14474 ± 421.02 pg/ml in the 2nd week, and 12111 ± 112.27 pg/ml in the 3rd week. When a bone defect was created at the femoral metaphysis, the PGE_2 level increased to 12768 ±113.11 pg/ml in the first week, then decreased to 9479 ±206.58 pg/ml after 3 weeks of culture (Table 1). The fleshly osseous defects induced the secretion of PGE_2. The concentration of PGE_2 was maintain with a higher value compared to the shammed group (Table 1) during the final two weeks.

ALP in Culture Medium

When a bone defect was created at the femoral metaphysis, the ALP activity decreased gradually from 122.61 U/L after 1 week of culture to 96.97 U/L after 3 weeks of culture. No matter what period was stimulated, the PEMF stimulation significantly increased the secretion of ALP ($p < 0.05$) compared to the sham-exposed group. It was found that the ALP activity nearly doubled when the osteoblasts were stimulated for 2 weeks ($p < 0.0005$). The differences in ALP activity level between stimulated and sham-exposed group elevated in the first to the second week of stimulation, and then decreased rapidly to the third week of stimulation (Table 2).

Discussion

Electromagnetic stimulation and/or electrical stimulation is known to promote osteogenesis, and it has been investigated in several experimental and clinical models [1-2, 4, 7, 21-24]. It has been demonstrated that PEMF stimulation accelerate fracture healing [2] and promote the maturation of bone trabeculae [24]. It also has been stressed that the positive effect on bone growth and repair seems to be related to specific PEMF signal configuration [4,24-25]. Earlier studies on the host tissue responses to these electromagnetic stimulation models generally made assessments by morphological and histological examinations. We designed an *in vitro* study to elucidate the specific responses of bony organ to PEMF treatment. The object of this study is to elucidate whether PEMF treatment accelerates the repair of femoral defects in an *in vitro* organ culture model.

Our results demonstrate that PEMF stimulation can accelerate bone healing. All experimental femora with defects treated by PEMF stimulation resulted in shorter healing times. Trabecular regeneration was also accelerated by PEMF stimulation (Fig. 3). The acceleration effect reached a statistically significant level after only 1 week of PEMF stimulation (Fig. 2). The increased synthesis and release of PGE_2 may act as an autocrine or paracrine factor. Also, the administration of PGE_2 *in vitro* and in vivo models led to increased bone remodeling and turnover [26-31], attributable to increased bone resorption [32-35] or bone formation [36-39] or both. Nagata suggested that the administration of PGE_2 in the concentration of 10^{-7} M has an osteogenesis effect by the activation of the osteoblasts and by the synthesis of collagen [39]. It was also been shown that PGE_2 in the concentration of 10^{-5} to 10^{-8} M can increase the concentration of Ca^{2+} and synthesis of collagen protein [40]. In the current study, the synthesis and release of PGE_2 by normal intact femora decreased gradually during 4 weeks of culturing and it seemed that the value of PGE_2 level maintain about 500 to 600 pg/ml after 2 weeks incubation. When a bone defect was created at the femoral metaphysis, the synthesis and release of PGE_2 was elevated apparently and then decreased. It probably means that drill a hole to the femur (which is like a large harmful mechanical stimulation) would cause high PGE_2 production, and then PGE_2 level might decrease gradually accompanying with the healing period of bone defect. Finally, PGE_2 level might go down to the stable value when the healing process is finished. The results showed that healing changed at the end of the first week, but PGE_2 production increased at the end of week 2 and 3. It probably caused by harmful mechanical treatment of bone defect drilling at day zero of the experiment. That osseous defect might cause large production of PGE_2 initially, and the PGE_2 secretion accompanied by the PEMF stimulation would not so obviously in the first week of exposure. But the effects of PEMF exposure on PGE_2 level elevating in culture medium seemed to be distinguishable (17%, and 22% increasing) after 2 and 3 weeks stimulations comparing with sham-exposure groups (Table 1). According to these results, it might be hypothesized that the acceleration of

bone defect healing by PEMF stimulation could be at least partly due to increases in the synthesis and secretion of PGE_2. To determine how PEMF stimulation modulates osteoblast function, we measured ALP activity as a marker of osteoblast differentiation and we found that PEMF stimulation enhanced ALP activity in these cells. ALP is present in the osteoblast and in the matrix vesicle membrane and is a very good indicator of bone formation and matrix mineralization [41-43]. The ALP activity decreased gradually from 1 to 3 weeks after a bone defect created at the femoral metaphysis in the sham-exposed group, but the ALP activity level elevated significantly at the second week compared to the sham-exposed group (Table 2). After the third week of stimulation, the ALP activity level decreased, but it was still greater than the sham-exposed group. After 2 weeks of PEMF stimulation, the enhancing effect on the trabecular bone regeneration was even more obvious, and there was also no significant difference between stimulated and sham-exposed groups in mean percentage of trabecular regeneration after 3 weeks of PEMF stimulation. This could indicate that PEMF treatment stimulated bone defect healing by increasing the ALP activity level, especially during the 1 to 2 week stimulation period.

The specific mechanism by which PEMF stimulation accelerates the normal metaphyseal defect-repair process is still unknown, and the present study did not address this question. The results of this study support current findings in the literature suggesting that PEMF treatment may have a stimulatory effect on the bone-healing processes, although the appropriate PEMF dose required to achieve maximal stimulation remains to be determined for both animal and human subjects. We believe that the observation of PEMF stimulation of bone healing 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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