Differential Intensity-Dependent Effects of Pulsed Electromagnetic Fields on RANKL-induced Osteoclast Formation, Apoptosis, and Bone Resorbing Ability in RAW264.7 Cells

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Pulsed electromagnetic fields (PEMF) have been proven to be effective for promoting bone mass and regulating bone turnover both experimentally and clinically. However, the exact mechanisms for the regulation of PEMF on osteoclastogenesis as well as optical exposure parameters of PEMF on inhibiting osteoclastic activities and functions remain unclear, representing significant limitations for extensive scientific application of PEMF in clinics. In this study, RAW264.7 cells incubated with RANKL were exposed to 15 Hz PEMF (2 h/day) at various intensities (0.5, 1, 2, and 3 mT) for 7 days. We demonstrate that bone resorbing capacity was significantly decreased by 0.5 mT PEMF mainly by inhibiting osteoclast formation and maturation, but enhanced at 3 mT by promoting osteoclast apoptosis. Moreover, gene expression of RANK, NFATc1, TRAP, CTSK, BAX, and BAX/BCL-2 was significantly decreased by 0.5 mT PEMF, but increased by 3 mT. Our findings reveal a significant intensity window for low-intensity PEMF in regulating bone resorption with diverse nature for modulating osteoclastogenesis and apoptosis. This study not only enriches our basic knowledge for the regulation of PEMF in osteoclastogenesis, but also may lead to more efficient and scientific clinical application of PEMF in regulating bone turnover and inhibiting osteopenia/osteoporosis. Bioelectromagnetics. © 2017 Wiley Periodicals, Inc.

Keywords: PEMF; osteoclastogenesis; bone resorption; osteoclast differentiation; apoptosis

INTRODUCTION

Osteoporosis increases the risk of fragility fractures in about 50% of postmenopausal women and 30% of older men, which results from dysfunctions in the osteoclastic bone breakdown and osteoblastic bone formation process [Reid, 2015; Weitzmann and Ofotokun, 2016]. Traditional pharmacological agents either promoting bone formation (e.g., parathyroid hormone, insulin-like growth factor, and growth hormone) or inhibiting bone resorption (e.g., calcitonin, estrogen, and bisphosphonate) may partially help prevent and reverse osteoporosis [Canalis, 2013; Charles and Aliprantis, 2014; Reid, 2015]. However, these pharmacological drugs have issues of high costs or undesirable side effects, which are non-negligible limitations for their clinical application [Burge et al., 2007].

Pulsed electromagnetic fields (PEMF) as a kind of safe, inexpensive, and noninvasive physical approach

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have shown therapeutic potential in various diseases of the skeletomuscular system [Jing et al., 2013; Hannemann et al., 2014]. Several clinical investigations have demonstrated that PEMF stimulation could enhance bone mineral density, accelerate bone fracture healing, and reduce the risk of fractures [Bassett et al., 1974; Tabrah et al., 1998; Liu et al., 2013]. Substantial studies by our group and others have also demonstrated that PEMF could increase in vivo bone mass with obvious enhancement of bone formation rate in osteoporotic animals and also enhance in vitro osteoblast activity and osteoblastic mineralization potential [Luben, 1991; Bodamyali et al., 1998; Jing et al., 2013; Jing et al., 2014; Zhai et al., 2016]. Although it has been documented that PEMF stimulation has the capacity of promoting osteoblastogenesis, we still relatively lack adequate understanding for the regulatory effects and related mechanisms of PEMF on osteoclastic activities and functions.

In the past few years, several investigators have also explored the potential of PEMF in regulating osteoclastic activities, and inconsistent findings have been documented. Chang et al. [2006] reported that 7.5 Hz, 3.0 μV/cm PEMF exposure has the ability of speeding up apoptosis of osteoclasts. He et al. [2015] found that the formation of osteoclast-like cells was potently prevented with PEMF exposure at 3.8 mT for 3 days. However, Barnaba et al. [2012] found no obvious impact of PEMF on osteoclast TRAP activity at 0.4 mT, 50 Hz. Moreover, our previous in vivo investigations have also revealed no significant effect of PEMF at 20 mT, 15 Hz on osteoclastogenesis and bone resorption in osteoporotic rats [Jing et al., 2013, 2014]. According to these inconsistent findings, we believe that significant parameter-dependent effects for the regulation of PEMF on osteoclastogenesis may exist. However, we still lack essential basic knowledge regarding optimal exposure parameters of PEMF on inhibiting osteoclastic activities and functions. Moreover, another important question regarding the exact mechanism about how osteoclasts transduce external PEMF stimulation remains unanswered thus far. These issues represent significant limitations for the extensive and scientific application of PEMF in clinics.

In the present study, osteoclastic activities under PEMF stimulation with various intensities (0.5, 1, 2, and 3 mT) were evaluated by using the murine monocytic/macrophagic RAW264.7 cell line. We aimed to (i) examine whether the effects of PEMF on osteoclastic activities were intensity-dependent; (ii) investigate whether the osteoclastic activities were inhibited or promoted under PEMF exposure with different intensities; (iii) elucidate the exact paths about how PEMF regulated osteoclastic activities (by inhibiting osteoclast formation or promoting osteoclast apoptosis) together with relevant molecular mechanisms. We hypothesize in the present study that the regulatory effects of PEMF exposure on RANKL-induced osteoclast formation, apoptosis, and bone resorbing ability in RAW264.7 cells are intensity-dependent.

**MATERIALS AND METHODS**

**Cell Culture and Osteoclast Differentiation**

The RAW264.7 cells were purchased from the Cell Bank of the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). For the osteoclastogenesis experiment, the cells were seeded in six-well culture plate (Corning, Corning, NY) at $1 \times 10^6$ cells/ml and cultured in Dulbecco’s Modified Eagle medium (DMEM, Hyclone, Logan, UT) with high glucose (1%), supplemented with 10% (v/v) fetal bovine serum (GE Healthcare, Pittsburgh, PA) at 37 °C in a humidified atmosphere of 95% air and 5% CO2. After overnight incubation, the RAW264.7 cells could differentiate into osteoclast-like cells in the presence of 50 ng/ml RANKL (Peprotech, Rocky Hill, NJ) during the following 7 days. The medium was refreshed every 48 h.
PEMF Stimulation

Cells were exposed to PEMF stimulation with different intensities by using a PEMF exposure device (GHY-III, FMMU, Xi’an, China; China Patent no. ZL02224739.4). The device was composed of pulsed signal generator and a Helmholz coils assembly with two-coil array (Fig. 1). The Helmholz coils were placed within an incubator during the period of PEMF exposure. The PEMF waveform generated and utilized in this experiment was comprised of a pulsed burst (burst width, 5 ms; pulse width, 0.2 ms; pulse wait, 0.02 ms; burst wait, 60 ms; pulse rise, 0.3 μs; pulse fall, 2.0 μs) repeated at 15 Hz. This waveform has proven to be effective on inhibiting in vivo bone loss in osteoporotic animals and enhancing in vitro osteoblastic activities in our previous studies [Jing et al., 2014; Wang et al., 2014; Zhai et al., 2016]. A 2-Ω resistor was placed in series with the coils, and the voltage drop across the resistor was observed with an oscilloscope. Based on the obtained current value, the peak intensity of the magnetic fields could be calculated. In addition, the control cultures were placed in other inactivated Helmholz coils with the same culture conditions. A Gaussmeter (Model 455 DSP, Lake Shore Cryotronics, Westerville, OH) was used to confirm the accuracy of the magnetic field measurement. The measured background electromagnetic field was 50 ± 2 μT. In order to determine the induced electric field within the coils, a custom-designed electrical potential detecting circular coil (5 cm coil diameter, 1 mm coil diameter, 20 turns) was placed in the mid-center of the Helmholz coils with the coil parallel to the Helmholz coils. The current detecting coil was connected with the oscilloscope, and the induced peak electrical field was determined to be approximately 0.5, 1, 2, and 3 mV/cm for 0.5, 1, 2, and 3 mT magnetic field intensity, respectively. The peak magnetic field exhibited <0.01 mT fluctuation during daily 2 h PEMF exposure. An accelerometer (VIB-5, Shanghai Xingsheng Detecting Instrument, Shanghai, China) was employed to determine potential mechanical vibrations induced by the electromagnetic stimulation on the exposed cell culture plate. We did not detect mechanical vibratory signals throughout the stimulation period via the measurement of accelerometer. The cell culture plate in the PEMF group was placed in the center of the coils to maximize the uniformity of the magnetic field along the axial plane of the coils. Cells in the Control group were placed similarly, but in the sham PEMF stimulation where the coils were inactivated. A thermometer was placed in the center of the coils for both Control and PEMF groups to determine heating effects throughout the experiment, respectively. We found <0.2 °C deviation between the two groups throughout the experiment.

Tartrate-Resistant Acid Phosphatase (TRAP) Staining and Cell Counting

After 7-day PEMF stimulation, the RAW264.7 cells were washed and then fixed in 4% paraformaldehyde (PFA) for 10 min, and the osteoclast-like cells were then stained by using a commercially available TRAP staining kit (Sigma–Aldrich, St. Louis, MO) per manufacturer’s instructions. The cell culture plate was then transferred to a light microscope (Olympus, Tokyo, Japan), and cells were identified as osteoclast-like cells if they were both TRAP-positive and exhibited more than three nuclei. Twenty random fields of view were selected under the light microscope in each well, and the osteoclast-like cells were counted in each group. The experiment was repeated three times.

F-Actin Cytoskeleton Staining

The RAW264.7 cells were cultured in confocal petri dishes with RANKL (50 ng/ml) and stimulated with different intensities of PEMF for 7 days and 10 days, respectively. Then, cells were fixed in 4% PFA for 30 min and permeabilized with 0.1% Triton X-100 for 10 min. After being washed with PBS, the cells were stained for F-actin with FITC-conjugated phalloidin (Sigma–Aldrich) for 40 min at 37 °C. The samples were then stained for cell nucleus with DAPI (Bioworld Technology, St. Louis Park, MN) for 10 min at 37 °C. The samples were imaged using a confocal microscope (FluoView 1000, Olympus). All the images were captured without changing the camera settings. The experiment was repeated three times.

Osteoclast Apoptosis Analysis

The RAW264.7 cells with 1 × 10^6 cells/ml were seeded in confocal petri dish for apoptosis staining and cultured overnight to resume the exponential growth. Cells were then cultured in complete medium containing 50 ng/ml RANKL and exposed to 2 h/day PEMF stimulation for 7 days and 10 days. The apoptosis of osteoclasts was examined by using an annexin V-FITC apoptosis detection kit (Beyotime, Jiangsu, China). After being washed with PBS, cells were incubated with annexin V binding buffer and incubated with annexin V-FITC in the dark for 10 min. The morphological alterations of osteoclast apoptosis were then observed under a confocal microscope (FluoView 1000, Olympus). The numbers of live
osteoclasts and apoptotic osteoclasts in either early or late stage were counted and analyzed for quantitative results among 20 randomly selected osteoclast cells under the confocal microscope in each group. The experiment was repeated three times.

**Resorption Capability Determination**

The sterilized fresh bovine femoral bone disk with 5 mm diameter was placed into the six-well culture plate. Then, the RAW264.7 cells were seeded into each well. After overnight incubation, the cells were incubated in the presence of RANKL (50 ng/ml) and treated by PEMF stimulation for 7 days. Then, the cells were removed from the bone disks by using an ultrasonic clearing machine (Kejie, Shenzhen, China). The bone chips of each group were fixed in 2.5% glutaraldehyde in phosphate buffer for 24 h. The specimens were then dehydrated in graded series of aqueous ethanol solutions of 50%, 70%, 90%, and 100% ethanol for 1 h. After being air-dried, samples were mounted on aluminum SEM stubs with silver paint and sputter-coated with gold using a coater. The specimens were examined using a scanning electron microscope (Hitachi JSM-4800, Tokyo, Japan). The areas of 20 pits formed by osteoclasts were measured using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) in each group. The experiment was repeated three times.

**Fig. 2. Effects of 15 Hz PEMF exposure with various intensities (0.5, 1, 2, and 3 mT) on RANKL-induced osteoclast differentiation on RAW264.7 cells. Cells were exposed to PEMF stimulation (2 h/day) with various intensities in the presence of RANKL (50 ng/ml) for 7 days, and then fixed and stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells were counted as osteoclasts in 20 random sights in each group. (A–E) Representative images showing osteoclast formation after cells exposed to PEMF stimulation with various intensities (original magnification, 100×). (F) Statistical comparison of osteoclast number per version between Control group and PEMF groups with various intensities (0.5, 1, 2, and 3 mT). Values are all expressed as mean ± S.D. (n = 20). *Significant difference from Control group with P < 0.05.**
**Real-Time PCR**

Total RNA was extracted from the cultured cells using TRIzol RNA isolation reagents (Thermo Fisher Scientific, Waltham, MA) and quantified with spectrophotometry (Bio-Rad, Hercules, CA). The cDNA was synthesized from 2 μg of RNA using FastQuant RT kit (Tiangen Biotech, Beijing, China). Real-time PCR was performed in a real-time PCR detection system (Bio-Rad) by using a Maxima SYBR Green qPCR kit (Thermo Fisher Scientific). The primers used in this study are shown in Table 1. After an initial denaturation at 95°C for 10 min, the amplification reaction was conducted for 40 cycles with annealing at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 55°C for 15 s. Gene expression levels were normalized to β-Actin using 2^−ΔΔCT analysis. The experiment was repeated three times.

**Statistical Analysis**

All data are presented as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was performed for evaluating differences among the five groups. Bonferroni’s post hoc analysis was used to determine the significance between each two groups. Differences were significant at P < 0.05. All statistical analyses were performed by using SPSS 13.0 software (SPSS, Chicago, IL).

**RESULTS**

**TRAP Staining**

The effects of 15 Hz PEMF exposure with various intensities (0, 0.5, 1, 2, and 3 mT) on osteoclastogenesis via TRAP staining on RAW264.7 cells are shown in Figure 2A–E. Our results demonstrate that osteoclastogenesis was significantly regulated by PEMF stimulation. We found that PEMF exposure at 0.5 and 1 mT suppressed osteoclastogenesis, whereas PEMF stimulation with 3 mT largely promoted the formation of osteoclasts. Statistical comparisons further show that the average number of formed osteoclasts per version was significantly decreased by PEMF exposure with the 0.5 mT group (P < 0.05, −55.6%) and 1 mT group (P < 0.05, −48.9%) as compared with the Control group. We also observe that PEMF stimulation with 3 mT significantly increased the number of osteoclasts as compared with the Control group (P < 0.05, +33.3%).

![Fig. 3. Effects of 15 Hz PEMF exposure with various intensities (0.5, 1, 2, and 3 mT) on osteoclast cytoskeletal organization. RAW264.7 cells were exposed to different PEMF (2 h/day) at different intensities in the presence of 50 ng/ml RANKL for 7 or 10 days. Cells were then fixed and stained with FITC-conjugated phalloidin (green) and DAPI (blue), and then imaged under confocal microscope. Scale bars in A and B represent 10 μm.](image-url)
Osteoclast Cytoskeletal Organization

The effects of 15 Hz PEMF burst with various intensities (0, 0.5, 1, 2, and 3 mT) for 7 days and 10 days on osteoclast cytoskeletal organization during osteoclastogenesis via F-actin staining are shown in Figure 3. As shown in Figure 3A, smooth cellular outlines with no filopodia were observed in the Control, 2 mT, and 3 mT PEMF groups. However, the 0.5 mT PEMF group exhibited many dendritic pseudopodia at the cell edge. Moreover, we also found several thin filopodia around the cellular periphery in the 1 mT group. The osteoclasts exhibited reticular structures of microfilaments when they had smooth edges, whereas osteoclasts with dendritic filopodia exhibited chaotic cellular cytoskeletal structure. It is notable that a podosome belt was formed in the 3 mT PEMF stimulation group. After PEMF exposure for 10 days (Fig. 3B), the morphology of most osteoclasts in the 0.5 and 1 mT PEMF groups exhibited typical mature osteoclast organizations with condensed nuclei and nuclear margin at the chromatin, which were

Fig. 4. Effects of 15 Hz PEMF exposure with various intensities (0.5, 1, 2, and 3 mT) on osteoclast apoptosis. RAW264.7 cells were cultured in confocal petri dishes and exposed to 2 h/day PEMF for 7 days and 10 days, and stained with annexin V-FITC (green) and propidium iodide (red) for evaluating early apoptosis and late apoptosis, respectively, after PEMF exposure for (A) 7 days and (B) 10 days. Scale bars represent 10 μm. (C) Statistical comparisons of overall apoptosis rate, early apoptosis rate, and late apoptosis rate between Control group and PEMF groups with various intensities (0.5, 1, 2, and 3 mT). *Significant difference from Control group with $P < 0.05$. 

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regarded as important characteristics of early apoptosis via DAPI staining. However, osteoclasts in the 2 and 3 mT PEMF groups could not maintain their cell shape, and the cell nuclei and cell cytoskeleton fell into cell fragmentation, which was a typical phenomenon of late-stage apoptosis.

Osteoclast Apoptosis

PEMF stimulation exhibited significant regulatory effects on osteoclast apoptosis as shown in Figure 4. Most cells exposed to PEMF with 0.5 and 1 mT were non-apoptotic or in early stage apoptosis at day 7, whereas most osteoclasts in the 2 and 3 mT PEMF groups were in late-stage apoptosis as shown in Figure 4A. According to the quantitative results, we found that 3 mT PEMF group induced higher ratio of late apoptosis ($P < 0.05$) and relatively lower ratio of early apoptosis in osteoclasts as compared with the Control group. However, PEMF stimulation at 0.5 mT showed relatively lower ratio of late apoptosis and higher ratio of early apoptosis as compared with the Control group ($P < 0.05$).

Bone Resorption Capability

To quantify the resorption ability of the derived osteoclasts, we determined the number and area of pits by seeding osteoclasts on the bone slices (Fig. 5). In comparison with the Control group, the 2 and 3 mT PEMF groups showed relatively higher pit area, whereas the 0.5 and 1 mT PEMF groups exhibited lower pit area (Fig. 5A–E). Statistical comparison demonstrates that PEMF stimulation with 0.5 mT induced significantly lower average pit area than the Control group (Fig. 5F, $P < 0.05$). However, PEMF exposure with 2 and 3 mT significantly increased average pit areas as compared with the Control group ($P < 0.05$).

Osteoclastogenesis-Associated Gene Expression

Real-time PCR examination results for total mRNA expression of RANK, cathepsin K (CTSK), matrix metalloproteinase-9 (MMP-9), BAX, BCL-2, NFATc1, and TRAP are demonstrated in Figure 6. mRNA gene expression levels of RANK,
TRAP, MMP-9, CTSK, BAX, and NFATc1 in the 0.5 mT group were significantly lower than the Control group ($P < 0.05$), and BCL-2 gene expression levels in the 0.5 mT PEMF group were significantly higher than those in the Control group ($P < 0.05$).

Significant increases were observed in the 2 mT PEMF group in RANK, TRAP, and NFATc1 mRNA expression ($P < 0.05$), but not in BAX/BCL-2 ratio and BCL-2, CTSK, and MMP-9 mRNA expression. Moreover, the 3 mT PEMF group exhibited significantly higher RANK, TRAP, BAX, CTSK, MMP-9, and NFATc1 mRNA expression than the Control group ($P < 0.05$).

**DISCUSSION**

Osteoclasts play a critical role in maintaining calcium balance and regulating bone quantity and bone quality [Matsuo and Irie, 2008]. Abnormality of osteoclast activities is associated with various congenital and metabolic diseases (e.g., osteoporosis, osteopetrosis, periodontal disease, etc.). Thus, it has great clinical significance. Low-intensity PEMF exposure, as a safe and non-invasive biophysical method, has been proven to be effective for regulating bone turnover and promoting bone mass [Jing et al., 2013; Hong et al., 2014]. However, the exact mechanisms...
for the regulation of PEMF on osteoclastogenesis as well as optical exposure parameters of PEMF on inhibiting osteoclastic activities and functions remain unknown. In this study, we evaluated the effects of 15 Hz PEMF with various intensities (0.5, 1, 2, and 3 mT) on osteoclast activities and functions. We also quantified gene expression levels which were related with osteoclastic formation, resorption capability, and apoptosis under PEMF exposure with different intensities. Our results reveal novel findings that bone resorbing capacity was significantly decreased by 0.5 mT PEMF mainly by inhibiting osteoclast formation and maturation, but enhanced at 3 mT by promoting osteoclast apoptosis. Moreover, our results also demonstrate that osteoclastogenesis-associated gene expression was also suppressed by 0.5 mT PEMF, but enhanced by 3 mT PEMF. Considering the confirmed effects of PEMF on regulating osteoclastogenesis [Chang et al., 2004; Chang et al., 2005; Zhang et al., 2017], no positive control (chemicals or physical factors which are already known to regulate osteoclast activity and function) was used in our present study, which may be a limitation of our study. Our present findings reveal an important intensity window for low-intensity PEMF in regulating bone resorption with diverse nature for modulating osteoclastogenesis and apoptosis, and can enrich our basic knowledge for understanding the mechanism of PEMF-mediated inhibition of osteoclastic activities.

The differentiation, maturation, and fusion of osteoclasts as well as osteoclast-mediated bone resorption function are directly related to the integrity of the actin cytoskeleton [Jurdic et al., 2006]. Two major types of actin structures can be formed in osteoclasts depending on the substrate they spread: sealing zone on surfaces containing apatite crystals for bone resorption, and podosomes on surfaces without apatite crystals [Saltel et al., 2008]. Podosomes are organized in clusters at the beginning of differentiation, followed by evolvement into dynamic rings and final stabilization at the cell edge to form a podosome belt [Song et al., 2014; Ti et al., 2015]. The cell periphery of immature osteoclasts shows dendritic filopodial extensions, while mature osteoclasts exhibit smooth plasma membranes [Wang et al., 2015]. Mature osteoclasts can form a resorptive microenvironment consisting of a refuded border and an actin ring on the bone-cell interface. Lysosomal proteases, H^+ and Cl^-, are secreted into the resorption lacuna so that bone can be digested effectively [Teitelbaum, 2011; Boyce et al., 2012]. Our F-actin staining results indicate that osteoclast formation can be delayed under relatively low-intensity PEMF stimulation (0.5 and 1 mT). It has been shown that dendritic pseudopods of immature osteoclasts were transformed into smooth-edged podosome belt in mature osteoclasts during cell fusion process on days 4–6 [Song et al., 2014]. We thus observed that most cells still exhibited dendritic filopodia in relatively low-intensity (0.5 and 1 mT) PEMF groups on day 7, indicating that these osteoclasts were still in the immature stage. In contrast, F-actin cytoskeleton in relatively high-intensity (2 and 3 mT) PEMF groups and the Control group showed typically mature osteoclast cellular morphology with smooth cell edge and reticular intracellular structure [Ti et al., 2015]. To investigate the longer-term change of these immature osteoclasts, the experiment was expanded to 10 days and osteoclastic cytoskeleton structure changes were analyzed. Our results show that most osteoclasts in the 0.5 mT PEMF group exhibited typical mature osteoclastic cytoskeleton organization. Thus, these results indicate that the immature osteoclasts at day 7 could grow into mature OC cells, and this process was delayed but not interrupted.

Since the life spans of osteoclasts were three to fourfold shorter than osteoblasts, osteoclast apoptosis was rarely observed in histological sections [Jobke et al., 2014]. Osteoclasts displayed rapid cell death in the absence of trophic factors (e.g., RANKL and M-CSF), which were caused by cellular apoptosis (programmed cell death) [Akiyama et al., 2008]. Consistent with the results of cytoskeletal structure analyses, our apoptosis examination results also demonstrate that PEMF exposure with relatively low intensity (0.5 mT) significantly inhibited osteoclast apoptosis, whereas the extent of apoptosis was aggravated under PEMF exposure with 3 mT. At day 7, most osteoclasts exposed to relatively low-intensity PEMF stimulation (0.5 mT) underwent early stage apoptosis. In contrast, most osteoclasts fell into cell fragmentation (a typical phenomenon of late-stage apoptosis). In conclusion, our results reveal intensity-dependent regulatory effects of PEMF on osteoclastic apoptosis and osteoclastic lifespan under PEMF stimulation.

Although we observed an obvious regulatory effect of PEMF exposure with different intensities on osteoclast differentiation and apoptosis, several previous studies have substantiated that osteoclast number and activity were not linearly correlated with bone resorption rate because of the involvement of non-resorbing osteoclasts in stimulating bone formation [Karsdal et al., 2007]. Thus, we quantified the bone resorptive function by seeding RANKL-induced osteoclasts on bone slices and measuring the average area of pits they formed on bone slices. Our results revealed that the osteoclast-induced bone resorption
rates were attenuated in low-intensity (0.5 mT) PEMF group, whereas 3 mT PEMF exposure largely promoted osteoclast-related bone resorption abilities. The bone resorption ability of osteoclasts was closely related to the number, apoptosis, maturation, and microstructure of osteoclasts. It has been proven that the structure of actin filaments in osteoclasts contributed to the attachment of bone surface, and thus influenced the bone resorption function [Furlan et al., 2007]. It can be assumed that the cytoskeleton structure of some osteoclasts formed on the bone slice may be less mature at day 7 in 0.5 mT PEMF group as well, so that bone resorption capability was receded when compared with other groups.

RANKL and RANK are crucial for regulating osteoclast differentiation [Rubin et al., 1996], and NFATc1 is a master regulator of osteoclast differentiation that is activated by RANKL; it regulates a number of osteoclast-specific gene expressions such as cathepsin K, osteoclast-associated receptor (OSCAR), and TRAP [Kim and Kim, 2014]. To evaluate the bone-resorption capability of derived osteoclasts, we detected the gene expression levels of TRAP, CTSK, and MMP-9, which were bone-degrading enzymes secreted by activated osteoclasts [Teitelbaum, 2011]. We also determined the gene expression of BCL-2 and BAX, which were apoptosis-related genes: BCL-2 inhibited apoptosis and BAX promoted cell death [Adams and Cory, 2007]. In this study, we found that gene expression levels of RANK, NFATc1, TRAP, CTSK, BAX, and BAX/BCL-2 ratio were significantly decreased after exposed to 0.5 mT PEMF; nonetheless these genes were significantly increased when exposed to higher-intensity PEMF stimulation (3 mT). The expression level of BCL-2 was significantly increased when exposed to 0.5 mT PEMF. These findings suggest that osteoclast differentiation, resorption ability, and apoptosis were largely inhibited by 0.5 mT PEMF, but promoted by 3 mT PEMF.

In conclusion, the present study clearly demonstrates that 15 Hz PEMF exposure with different intensities (0, 0.5, 1, 2, and 3 mT) exhibited significantly distinct regulatory effects on osteoclastogenesis, and resorption capability of RAW264.7 cell line induced osteoclast-like cells. We demonstrate that bone resorbing capacity was significantly decreased by 0.5 mT PEMF mainly by inhibiting osteoclast formation and maturation, but enhanced at 3 mT by promoting osteoclast apoptosis. Moreover, gene expression of RANK, NFATc1, TRAP, CTSK, BAX, and BAX/BCL-2 was significantly decreased by 0.5 mT PEMF, but increased by 3 mT. Our findings reveal a significant intensity window for low-intensity PEMF in regulating bone resorption with diverse nature for modulating osteoclastogenesis and apoptosis. These findings not only provide important experimental evidence for choosing the optimal PEMF exposure parameters, but also enrich our basic knowledge for understanding the underlying mechanism concerning PEMF regulate bone turnover and promote bone mass.

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