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INHIBITION OF BONE FORMATION BY HIGH INTENSITY PULSED ELECTROMAGNETIC FIELD IN MC3T3-E1 CELLS

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Abstract—To investigate the effects of pulsed electromagnetic field (PEMF) with high electric field intensity on bone formation in murine osteoblast-like MC3T3-E1 cells, proliferation, alkaline phosphotase (ALP) activity, mineralized nodule formation, Collagen Type I (COL-I) and core-binding factor (Cbf)a1 mRNA expression, and bone morphogenetic protein (BMP)2/4 and mothers against decapentaplegic (Smad)1/5/8 protein expression were examined in cultured MC3T3-E1 cells after exposure to PEMF at the field intensities of 0 kV/m, 50 kV/m or 400 kV/m for 400 consecutive pulses daily for 7 consecutive days. After 50 kV/m of PEMF exposure, none of the above parameters of MC3T3-E1 cells changed significantly when compared to the control groups. However, the proliferation, ALP activity and mineralized nodule formation of MC3T3-E1 cells in 400 kV/m PEMF exposure groups decreased significantly although COL-I and Cbfa1 mRNA expression and BMP2/4 and Smad1/5/8 protein expression did not change. The PEMF we used at high electric field intensity suppressed proliferation, differentiation and

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mineralization of MC3T3-E1 cells in culture and appeared to be harmful for bone formation.

1. INTRODUCTION

Pulsed electromagnetic field (PEMF) are extensively applied to the treatment of orthopaedic conditions involving non-union fractures, fresh fractures and osteoporosis [1, 2]. Despite much evidence that PEMF can increase the osteoblast proliferation [3–7], differentiation [8–11] and extracellular matrix calcification [7, 8, 12, 13], there were still some opposite or negative reports [14–19]. So results of investigations dealing with this issue are still being debated. The difference between results may be due to the different frequency or intensity of PEMF used in different studies. The present study was carried out in response to the great diversity of the results reported in previous studies [20].

In previous reports the electric field intensities of PEMF were relatively low. For example, Ozawa et al. [4] stimulated murine osteoblast-like cells by a PEMF with an electric field intensity of 3.19 kV/m; McLeod and Collazo [14] exposed MC3T3-E1 cells to a PEMF with an electric field intensity of 0.9 mV/m; Hartig et al. [9] used a PEMF with an electric field of 6 kV/m; Chang et al. [17] applied a PEMF with an electric field intensity of 0.2 V/m to expose neonatal mouse calvarial bone cell cultures (Table 1). So far there were no reports available on the effects of electromagnetic field with high electric field intensity on bone formation of osteoblasts. In this study, the PEMF we used is with high electric field intensity which is up to 400 kV/m. In order to determine whether high electric field intensity PEMF affects bone formation and to analyze the underlying mechanism, we investigated the effects of PEMF on cell growth, alkaline phosphotase (ALP) activity, mineralized nodule formation, Collagen Type I (COL-I) and core-binding factor (Cbf)a1 mRNA expression, and bone morphogenetic protein (BMP)2/4 and mothers against decapentaplegic (Smad)1/5/8 protein expression in murine osteoblast-like MC3T3-E1 cells.

2. METHODS

2.1. Cell Culture

The murine osteoblast-like cell line MC3T3-E1 was obtained from the Department of Biochemistry and Molecular Biology of the Fourth Military Medical University, China. Cells were cultured in α-modified minimal essential medium (α-MEM; GIBCO, Grand Island, NY, USA)

Table 1. Detail parameters of some of the previous reports.

Cell type	Electric field intensity	Frequency	Magnetic flux density	Results	References
MC3T3-E1	3.19 kV/m	10 Hz		stimulation of proliferation	[4]
MC3T3-E1	0.9 mV/m	30 Hz	1.8 mT	inhibition of differentiation	[14]
osteoblast- like primary cells	6 kV/m	16 Hz		stimulation of proliferation, differentiation and matrix formation	[9]
neonatal mouse calvarial bone cell	0.2 V/m	15 Hz	0.1 mT	stimulation of proliferation, but no effects on differentiation and matrix formation	[17]

supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Hangzhou, Zhejiang, China), 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

In order to minimize the difference in cell growth between the control and treatment groups, the same batch of cells were used in each experiment. In addition, the same batch of serum was used in all experiments.

2.2. PEMF Exposure

The PEMF (a pulsed electromagnetic wave at a repetition rate of 0.5 Hz, pulse-width 350 ns) was generated by a spark gap pulse generator. A tapered parallel plate Gigahertz Transverse Electromagnetic cell (GTEM cell) with a flared rectangular coaxial transmission line was used to expose the MC3T3-E1 cells. The PEMF generator and the GTEM-cell were both devised by the Department of Mechanical Engineering, Southeast University (Nanjing, Jiangsu, China). To study the effects of PEMF on bone formation, MC3T3-E1 cells in culture were exposed to PEMF at the electric field intensities of 0 kV/m, 50 kV/m or 400 kV/m for 400 consecutive pulses daily for 7 consecutive days. The magnetic flux densities were 0 mT, 0.17 mT and 1.33 mT respectively. The temperature measurements were done with thermometer immediately before and after PEMF exposure. The exposure produced a rise in the culture medium temperature less than 0.1°C.

2.3. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide (MTT) Assay

To evaluate osteoblast proliferation, the MC3T3-E1 cells were suspended in complete medium, plated into 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 1×10^4 cells/well ($n = 6$ for each time course in each group) and allowed to grow for 12 hours. The cells were then subjected to PEMF exposure as described above and the cell proliferation was measured by MTT assay every other day for 10 days. Medium was changed every 3 days. MTT (Amresco, Solon, OH, USA) was dissolved in 0.01 M Phosphate Buffered Saline (PBS; Invitrogen, Carlsbad, CA, USA) at a concentration of 5 g/l and sterilized by passage through a 0.22 μm filter (Millipore, Carrightwohill, Cork, Ireland). 20 μl of this stock solution was added to each well of culture plates, and the plates were incubated at 37°C for 4 hours. The medium was then removed and replaced with 150 μl of Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to dissolve the dark blue formazan crystals. The multi-well plates were shaken for 10 min at room temperature to ensure that all the crystals were dissolved, and the plates were read on a microplate reader at a wavelength of 570 nm (Model 680, Bio-Rad, Chicago, IL, USA).

2.4. ALP Assay

MC3T3-E1 cells were suspended in complete medium, plated into 24-well culture plates (Nunc, Roskilde, Denmark) at a density of 5×10^4 cells/well and allowed to grow to confluence ($n = 6$ in each group). The cells were then subjected to PEMF exposure as described above. Medium was changed every 3 days. After the final PEMF exposure, the cells were rinsed twice with ice-cold PBS, scraped into 0.2% Triton X-100 and incubated at 37°C for 30 min. The lysed cells were centrifuged at 8,000 g force for 10 min at 4°C. Aliquots of supernatants were subjected to ALP activity measurement and protein assay. In brief, ALP activity was measured using a quantitative colorimetric assay with para-nitrophenol phosphate as substrate (Jiancheng Biolotechnology Co., Nanjing, Jiangsu, China). All values were normalized against the total protein concentration determined with the use of bicinchoninic acid (BCA) Protein Assay Reagent (Biosynthesis Biotechnology Co., Beijing, China).

2.5. Mineralized Nodule Formation Assay

MC3T3-E1 cells were suspended in complete medium, plated into 24-well culture plates (Nunc, Roskilde, Denmark) at a density of 5×10^4 cells/well and allowed to grow to confluence ($n = 6$ in each group). The cells were then subjected to PEMF exposure as described above, followed by culturing in growth media supplemented with 50 µg/ml ascorbic acid and 10 mM glycerophosphate for 2 weeks to promote differentiation and initiate mineralization of matrix formed by the osteoblasts [21]. Medium was changed every 3 days. At the end of the culture, the cells were fixed with 70% ethanol and stained for calcium with alizarin red S (1%) to identify mineralized bone nodules. For quantitative analysis, plates were scanned and analyzed with Image J software (NIH, Bethesda, MD, USA). Individual nodule areas were calculated and expressed as a percentage of the total cell culture areas analyzed.

2.6. RNA Extraction, cDNA Synthesis and Polymerase Chain Reaction (PCR)

MC3T3-E1 cells were suspended in complete medium, plated into 6-well culture plates (Nunc, Roskilde, Denmark) at a density of 2×10^5 cells/well and allowed to grow to confluence. The cells were then subjected to PEMF exposure as described above. Medium was changed every 3 days. After the final PEMF exposure, the cells were rinsed twice with ice-cold PBS and scraped into TRIZOL (Invitrogen, Carlsbad, CA, USA). Total RNA of MC3T3-E1 cells was extracted according to the manufacturer's instructions. First-strand cDNA was synthesized using 5 µg of the total RNA, Oligo$(dT)_{16-18}$ (Sangon Biotech Co., Shanghai, China) and Molony murine leukemia virus reverse transcriptase (SuperScript II reverse transcriptase, Invitrogen, Carlsbad, CA, USA). Primers of the selected genes for PCR reactions were synthesized (Sunbiotech Co., Beijing, China) as follows [22, 23]:

(a) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (expected product size: 452bp)
forward, 5-ACCACAGTCCATGCCATCAC-3 and
reverse, 5-TCCACCACCCTGTTGCTGTA-3;

(b) COL-I (expected product size: 244bp)
forward, 5-TTTGTGGACCTCCGGCTC-3 and
reverse, 5-AAGCAGAGCACTCGCCCT-3;

(c) Cbfa1 (expected product size: 289bp)
forward, 5-CCGCACGACAACCGCACCAT-3 and
reverse, 5-CGCTCCGGCCCACAAATCTC-3.

The PCR reaction mixture (in a total volume of 25 μl) contained 2 μl of cDNA, 1 μl of each primer, 0.2 mM of dNTP, 1.5 mM of MgCl2, and 1 U Taq DNA polymerase (Tiangen Biotech Co., Beijing, China). PCR amplification was performed on a MyCycler PCR system (Bio-Rad, Chicago, IL, USA) using the cycling conditions as follows: denaturation at 94° for 4 min, amplification for 25 cycles (94° for 30 s; 55° for 30 s; and 72° for 30 s) and final extension at 72° for 5 min. The PCR products were analyzed on 1% agarose gel by electrophoresis. Optical densities of ethidium bromide-stained DNA bands were quantified using Bio-Rad image scanning software and the COL-I and Cbfa1 mRNA expression levels were normalized to the expression level of the housekeeping gene GAPDH. At least two independent experiments from cell culture to PCR were conducted, and each PCR was performed three times.

2.7. Western Blot

MC3T3-E1 cells were suspended in complete medium, plated into 6-well culture plates (Nunc, Roskilde, Denmark) at a density of 2×10^5 cells/well and allowed to grow to confluence. The cells were then subjected to PEMF exposure as described above. Medium was changed every 3 days. After the final PEMF exposure, the cells were rinsed twice with ice-cold PBS and scraped into lysis buffer [20 mM Tris-HCl, pH 8.0, 2% Nonident P-40, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM ethylene diamine tetraacetie acid (EDTA), 2 mM NaN_3, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After centrifugation at 12,600 g for 10 min at 4°C, the supernatants (40 μg protein/sample) were separated on sodium dodecyl sulfate (SDS)-10% polyacrylamide gel. After electrophoresis, the separated proteins were blotted onto a nitrocellulose membrane (Osmonics, Minnetonka, MN, USA) using a semi-dry electroblotter, and the membrane was blocked at 4°C overnight in 5% non-fat milk in tris buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Subsequently, the membrane was incubated at room temperature for 2 h with the primary antibodies (Rabbit polyclonal antibody directed against mouse BMP2/4 and goat polyclonal antibody directed against mouse Smad 1/5/8, Santa Cruz, CA, USA), and the bound antibody was detected with the horseradish peroxidase (HRP)-conjugated secondary antibody (HRP-conjugated goat anti-rabbit antibody and HRP-conjugated rabbit anti-goat antibody, Zhongshan Goldenbridge Biotechnology Co., Beijing, China). The desired proteins were visualized by use of the ECL Western blotting detection system (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The protein bands were quantified using Bio-Rad image

scanning software and the protein expression levels were normalized to the expression of a housekeeping protein, actin. At least two independent experiments from cell culture to Western Blot were conducted, and each Western Blot was performed three times.

2.8. Statistical Analysis

The data are expressed as mean ± standard deviation (SD). Data were analyzed using analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. The statistical analyses were performed using the SPSS 13.0 for Windows.

3. RESULTS

To detect the effects of PEMF on proliferation of MC3T3-E1 cells, the growth curve of the cells in culture was observed by MTT assay after PEMF exposure. The cells grew steadily with time and a three-fold increase in optical density (OD) 570 was seen in each group during the first 6 days after the cells were exposed to PEMF. At day 8 and day 10, OD570 in 400 kV/m group (1.08 ± 0.027 and 1.12 ± 0.032, respectively) was significantly lower than OD570 in 0 kV/m group (1.13 ± 0.022 and 1.24 ± 0.015, respectively). However, there was no significant difference in OD570 between 50 kV/m group (1.15 ± 0.015 and 1.22 ± 0.027 at day 8 and day 10, respectively) and 0 kV/m group (Fig. 1).

The ALP activity in MC3T3-E1 cells was analyzed to examine

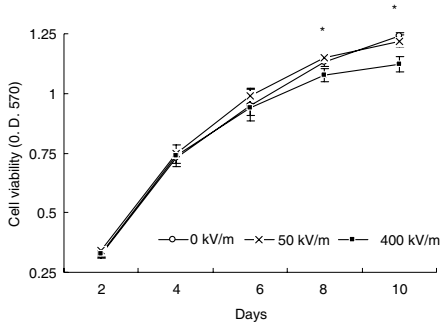

Figure 1. Effects of PEMF on the growth of MC3T3-E1 cells. Cells were exposed to PEMF for 400 consecutive pulses daily for 7 consecutive days. Cells were harvested at day 2, 4, 6, 8, 10 after the first PEMF exposure. Values are the mean ± SD of six independent cultures. $^*P < 0.05$ vs. corresponding 0 kV/m group.

whether PEMF exposure can influence osteoblastic differentiation. After 7 days of PEMF exposure, the ALP activity per unit protein in MC3T3-E1 cells was detected. Compared with 0 kV/m group, the ALP activity in 400 kV/m group decreased significantly (37.63 ± 3.244 vs. 41.67 ± 3.050). However, the ALP activity in 50 kV/m group (39.85 ± 1.461) was not significantly different from that in the 0 kV/m group (Fig. 2(a)).

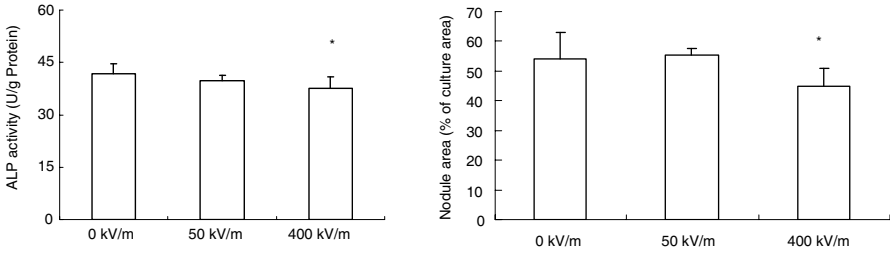

Figure 2. Effects of PEMF on (a) ALP activity and (b) mineralized bone nodule formation of MC3T3-E1 cells. Cells were exposed to PEMF for 400 consecutive pulses daily for 7 consecutive days. Values are the mean ± SD of six independent cultures. $^*P < 0.05$ vs. 0 kV/m group.

To investigate the effects of PEMF on mineralized nodule formation, cultures of MC3T3-E1 cells were subjected to PEMF exposure after reaching confluence. At the end of the cultures, the cells were stained by alizarin red S. As shown in Fig. 2(b), the percentage of mineralized nodule area in 400 kV/m group (44.8 ± 6.11) was significantly smaller than that in 0 kV/m group (54.2 ± 8.70). However, there was no obvious difference in this parameter between 50 kV/m group (55.5 ± 1.99) and 0 kV/m group.

PEMF exposure had no effect on COL-I and Cbfa1 mRNA expression in MC3T3-E1 cells. After 7 days of PEMF exposure, COL-I and Cbfa1 mRNA were expressed by MC3T3-E1 cells in each group, but the relative intensity of both COL-I and Cbfa1 as normalized to GAPDH did not differ statistically among the three groups although the relative levels of both COL-I and Cbfa1 mRNA in 400 kV/m group appeared lower than those in 0 kV/m group (Fig. 3).

PEMF exposure also had no effect on BMP2/4 and Smad1/5/8 protein expression in MC3T3-E1 cells. As shown in Fig. 4, the relative level of both BMP2/4 and Smad1/5/8 as normalized to actin in MC3T3-E1 cells was not significantly different between PEMF exposed groups (both 400 kV/m and 50 kV/m) and 0 kV/m group after 7 days of PEMF exposure.

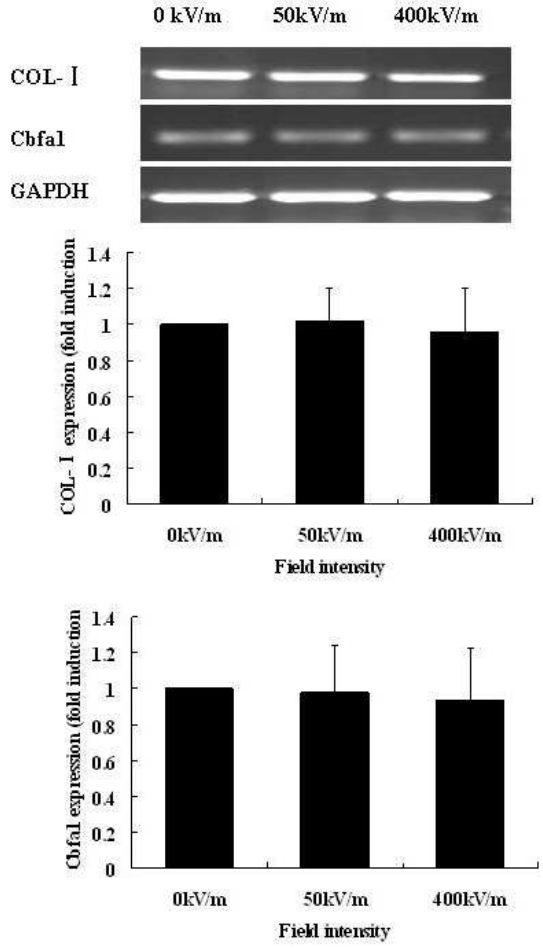

Figure 3. Effects of PEMF on COL-I and Cbfa1 mRNA expression in MC3T3-E1 cells. Cells were exposed to PEMF for 400 consecutive pulses daily for 7 consecutive days. Cells were harvested immediately after the final PEMF exposure. Total RNA was isolated and subjected to semi-quantitative RT-PCR analysis. The COL-I and Cbfa1 mRNA expression was normalized by an internal control gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). COL-I and Cbfa1 mRNA expression were quantitated by densitometry and plotted as fold induction. Results were obtained from two independent experiments and expressed as mean $\pm$ SD ($n = 3$ in each experiment).

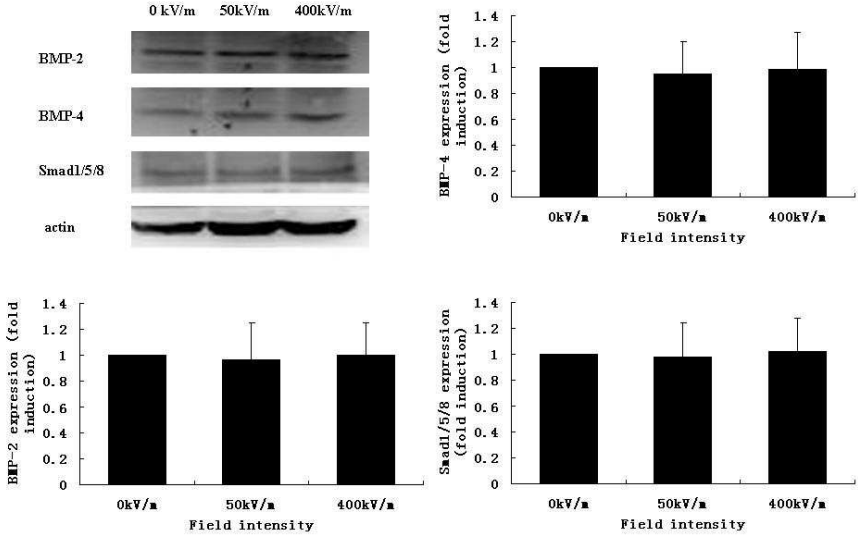

Figure 4. Effects of PEMF on BMP2/4 and Smad1/5/8 protein expression in MC3T3-E1 cells. After cells were exposed to PEMF for 400 consecutive pulses daily for 7 consecutive days, equal aliquots of cell lysates were prepared, and BMP-2, BMP-4 and Smad1/5/8 protein levels were determined by Western blotting. Actin was used as an internal control. BMP2/4 and Smad1/5/8 protein expression were quantitated by densitometry and plotted as fold induction. Results were obtained from two independent experiments and expressed as mean ± SD ($n = 3$ in each experiment).

4. DISCUSSION

Although many studies supported the role of PEMF in promoting the osteoblastic proliferation [3–7], some reported contradictory outcomes. Lohmann et al. [15, 16] found that PEMF, which consisted of 4.5 ms bursts of 20 pulses repeating at 15 Hz and was applied for 8 hours per day for 1 to 4 days, did not affect ROS 17/2.8 cell number but caused a reduction in MG63 cell proliferation. Similarly, Hannay et al. [19] found that PEMF consisting of 5 ms pulse bursts of 20 pulses repeating at 15 Hz and applied for 24 h inhibited proliferation of SaOS-2 cells. The contradictory results were likely attributed to the different PEMF and different experimental conditions employed. The PEMF used in our study is a unique PEMF that can reach the highest electric field intensity of 400 kV/m. We found that after

7 days of PEMF exposure, the proliferation of MC3T3-E1 cells did not change in 50 kV/m group but decreased significantly in 400 kV/m group, indicating that PEMF used at high electric field intensity did suppress osteoblastic proliferation.

ALP is a typical marker of the differentiation of osteoblast whereas mineralized bone nodule formation is a feature of osteoblast mineralization in vitro. We found that both ALP activity and the percentage of mineralized nodule area decreased significantly after 400 kV/m of PEMF exposure but remained unchanged after 50 kV/m of PEMF exposure, suggesting that PEMF used at high electric field intensity suppresed the differentiation and mineralization of osteoblast. Our results are consistent with those reported by others using PEMF exposure. McLeod and Collazo [14] reported an inhibition of differentiation of MC3T3-E1 cells after PEMF exposure (30 Hz, 1.8 mT, 0.9 mV/m, for 4 to 64 h). Chang et al. [17] noted that the mineralization nodules formation did not change, but the ALP activity of the osteoblasts decreased significantly after PEMF stimulation (15 Hz, 0.1 mT, 0.2 V/m, for 14 days). Cohly et al. [18] reported the mRNA expression of ALP in osteoblast were down regulated after PEMF stimulation (0.618 mT), indicating PEMF may be detrimental to bone formation.

The inhibitive effects of proliferation, differentiation and mineralization of MC3T3-E1 cells indicate that PEMF used at high electric field intensity (400 kV/m) was possibly a harmful factor for bone formation in vitro. Interestingly, COL-I mRNA expression in MC3T3-E1 cells did not change significantly after 400 kV/m PEMF exposure although bone formation was suppressed. COL-I is the main component of bone and is important for calcification leading to bone formation. Unchanging of COL-I mRNA implies that the bone formation inhibition by PEMF at high electric field intensity might be due to the alteration of post-transcription of COL-I or some other calcification regulative factors such as ALP.

BMP2/4 has been proposed to act as an important factor in osteoblast differentiation. A fundamental function of BMP2/4 is to induce the differentiation of mesenchymal cells toward cells of the osteoblastic lineage to promote osteoblastic maturation and function [24]. Smad1/5/8 are receptor regulated Smads which can be activated by BMP2/4 [25, 26]. Smads can interact with Cbfa1/ runt-related transcription factor (Runx)-2 [27]. The effect on osteoblastic differentiation requires interactions of Smads with Cbfa1/ Runx-2 [28]. The importance of interactions between Cbfa1/Runx-2 and Smad is emphasized further by recent studies demonstrating that the presence of Cbfa1/Runx-2 is required for the targeting of BMP-2

dependent Smads to subnuclear sites. In the absence of Cbfa1/Runx-2, Smad1 and Smad5 are not translocated to the nucleus after BMP activation. Cbfa1/Runx-2 allows for the recruitment of Smads to sites of active transcription, and this effect is coupled with the regulation of gene expression [29]. We found that BMP2/4, Smad1/5/8 protein expression and Cbfa1 mRNA expression in MC3T3-E1 cells did not change after the PEMF exposure that inhibited the bone formation. The inhibitory effects of PEMF on bone formation might therefore be mediated by signal transduction pathways other than the BMP-Smad pathway.

In conclusion, PEMF of higher electric field intensity suppressed proliferation, differentiation and mineralization of MC3T3-E1 cells in culture. These results suggest that the PEMF we used might be harmful for bone formation. However, the related mechanism still needs further study.

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REFERENCES

1. Bassett, C. A., "Beneficial effects of electromagnetic fields," *J. Cell. Biochem.*, Vol. 51, 387–393, 1993.
2. Walker, N., C. Denegar, and J. Preische, "Low-intensity pulsed ultrasound and pulsed electromagnetic field in the treatment of tibial fractures: A systematic review," *J. Athl. Train.*, Vol. 42, 530–535, 2007.
3. Liboff, A. R., T. Williams, Jr., D. M. Strong, and R. Wistar, Jr., "Timevarying magnetic fields: Effect on the DNA synthesis," *Science*, Vol. 223, 818–819, 1984.
4. Ozawa, H., E. Abe, Y. Shibasaki, T. Fukuhara, and T. Suda, "Electric fields stimulate DNA synthesis of mouse osteoblast-like cells (MC3T3-E1) by a mechanism involving calcium ions," *J. Cell. Physiol.*, Vol. 138, 477–483, 1989.
5. Diniz, P., K. Shomura, K. Soejima, and G. Ito, "Effects of pulsed electromagnetic field (PEMF) stimulation on bone tissue like formation are dependent on the maturation stages of the osteoblasts," *Bioelectromagnetics*, Vol. 23, 398–405, 2002.

6. De Mattei, M., N. Gagliano, C. Moscheni, C. Dellavia, C. Calastrini, A. Pellati, M. Gioia, A. Caruso, and G. Stabellini, "Changes in polyamines, c-myc and c-fos gene expression in osteoblast-like cells exposed to pulsed electromagnetic fields," *Bioelectromagnetics*, Vol. 26, 207–214, 2005.
7. Icaro Cornaglia, A., M. Casasco, F. Riva, A. Farina, L. Fassina, L. Visai, and A. Casasco, "Stimulation of osteoblast growth by an electromagnetic field in a model of bone-like construct," *Eur. J. Histochem.*, Vol. 50, 199–204, 2006.
8. Takano-Yamamoto, T., M. Kawakami, and M. Sakuda, "Effect of a pulsing electromagnetic field on demineralized bone-matrix-induced bone formation in a bony defect in the premaxilla of rats," *J. Dent. Res.*, Vol. 71, 1920–1925, 1992.
9. Hartig, M., U. Joos, and H. P. Wiesmann, "Capacitively coupled electric fields accelerate proliferation of osteoblast-like primary cells and increase bone extracellular matrix formation in vitro," *Eur. Biophys. J.*, Vol. 29, 499–506, 2000.
10. Landry, P. S., K. K. Sadasivan, A. A. Marino, and J. A. Albright, "Electromagnetic fields can affect osteogenesis by increasing the rate of differentiation," *Clin. Orthop. Relat. Res.*, 262–270, 1997.
11. Yamamoto, Y., Y. Ohsaki, T. Goto, A. Nakasima, and T. Iijima, "Effects of static magnetic fields on bone formation in rat osteoblast cultures," *J. Dent. Res.*, Vol. 82, 962–966, 2003.
12. Aaron, R. K. and D. M. Ciombor, "Acceleration of experimental endochondral ossification by biophysical stimulation of the progenitor cell pool," *J. Orthop. Surg.*, Vol. 14, 582–589, 1996.
13. Aaron, R. K., D. M. Ciombor, and G. Jolly, "Stimulation of experimental endochondral ossification by low-energy pulsing electromagnetic fields," *J. Bone Miner. Res.*, Vol. 4, 227–233, 1989.
14. McLeod, K. J. and L. Collazo, "Suppression of a differentiation response in MC-3T3-E1 osteoblast-like cells by sustained, low-level, 30 Hz magnetic-field exposure," *Radiat. Res.*, Vol. 153, 706–714, 2000.
15. Lohmann, C. H., Z. Schwartz, Y. Liu, H. Guerkov, D. D. Dean, B. Simon, and B. D. Boyan, "Pulsed electromagnetic field stimulation of MG63 osteoblast-like cells affects differentiation and local factor production," *J. Orthop. Res.*, Vol. 18, 637–646, 2000.
16. Lohmann, C. H., Z. Schwartz, Y. Liu, Z. Li, B. J. Simon, V. L. Sylvia, D. D. Dean, L. F. Bonewald, H. J. Donahue, and B. D. Boyan, "Pulsed electromagnetic fields affect phenotype and connexin 43 protein expression in MLO-Y4 osteocyte-like cells and

ROS 17/2.8 osteoblast-like cells," *J. Orthop. Res.*, Vol. 21, 326–334, 2003.

17. Chang, W. H., L. T. Chen, J. S. Sun, and F. H. Lin, "Effect of pulse-burst electromagnetic field stimulation on osteoblast cell activities," *Bioelectromagnetics*, Vol. 25, 457–465, 2004.
18. Cohly, H. H., G. E. Abraham, K. Ndebele, J. J. Jenkins, J. Thompson, and M. F. Angel, "Effects of static electromagnetic fields on characteristics of MG-63 osteoblasts grown in culture," *Biomed. Sci. Instrum.*, Vol. 39, 454–459, 2003.
19. Hannay, G., D. Leavesley, and M. Pearcy, "Timing of pulsed electromagnetic field stimulation does not affect the promotion of bone cell development," *Bioelectromagnetics*, Vol. 26, 670–676, 2005.
20. Jorge-Mora, T., M. Alvarez-Folgueiras, J. Leiro, F. J. Jorge-Barreiro, F. J. Ares-Pena, and E. Lopez-Martin, "Exposure to 2.45 GHz microwave radiation provokes cerebral changes in induction of hsp-90 a/b heat shock protein in rat," *Progress In Electromagnetics Research*, Vol. 100, 351–379, 2010.
21. Bodamyali, T., B. Bhatt, F. J. Hughes, V. R. Winrow, J. M. Kanczler, B. Simon, J. Abbott, D. R. Blake, and C. R. Stevens, "Pulsed electromagnetic fields simultaneously induce osteogenesis and upregulate transcription of bone morphogenetic proteins 2 and 4 in rat osteoblasts in vitro," *Biochem. Biophys. Res. Commun.*, Vol. 250, 458–461, 1998.
22. Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R. T. Bronson, Y.-H. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki, and T. Kishimoto, "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts," *Cell.*, Vol. 89, 755–764, 1997.
23. Mikihiko, M., T. Nakamoto, K. Hino, K. Tsuji, Z. J. Shen, K. Nakashima, A. Nifuji, H. Yamamoto, H. Hirai, and M. Noda, "The nucleocytoplasmic shuttling protein CIZ reduces adult bone mass by inhibiting bone morphogenetic protein-induced bone formation," *J. Exp. Med.*, Vol. 6, 961–970, 2005.
24. Yamaguchi, A., T. Ishizuya, N. Kintou, Y. Wada, T. Katagiri, J. M. Wozney, V. Rosen, and S. Yoshiki, "Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3–G2/PA6," *Biochem. Biophys. Res. Commun.*, Vol. 220, 366–371, 1996.
25. Kawabata, M., T. Imamura, and K. Miyazono, "Signal transduction by bone morphogenetic proteins," *Cytokine Growth*

Factor Rev., Vol. 9, 49–61, 1998.

26. Miyazono, K., "Signal transduction by bone morphogenetic protein receptors: Functional roles of Smad proteins," *Bone.*, Vol. 25, 91–93, 1999.
27. Lee, K. S., H. J. Kim, Q. L. Li, X. Z. Chi, C. Ueta, T. Komori, J. M. Wozney, E. G. Kim, J. Y. Choi, H. M. Ryoo, and S. C. Bae, "Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12," *Mol. Cell. Biol.*, Vol. 20, 8783–8792, 2000.
28. Ernesto, C., N. E. Aris, and G. Elisabetta, "Bone morphogenetic proteins, their antagonists, and the skeleton," *Endocrine Reviews*, Vol. 2, 218–235, 2003.
29. Zaidi, S. K., A. J. Sullivan, A. J. van Wijnen, J. L. Stein, G. S. Stein, and J. B. Lian, "Integration of Runx and Smad regulatory signals at transcriptionally active subnuclear sites," *Proc. Natl. Acad. Sci. USA*, Vol. 99, 8048–8053, 2002.