Original Article
Effects of PEMFs on Osx, Ocn, TRAP, and CTSK gene expression in postmenopausal osteoporosis model mice

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Abstract: *Objective:* Ovariectomized mice were used to simulate the symptoms of postmenopausal women with osteoporosis, and observe the effects of PEMF treatment on expression of Osx, Ocn, TRAP, and CTSK in ovariectomized mice. *Methods:* Thirty-week-old wild-type C57BL/6 mice were randomly divided into three groups (n=10, each group): sham operation group, ovariectomy (OVX) group, and PEMF group. Mice in the sham group underwent sham ovariectomy, while mice in the remaining two groups were ovariectomized. On postoperative day two, mice in the PEMF treatment group received PEMF treatment at a frequency of 8 Hz and an intensity of 3.8 mT for one hour daily for four weeks. At the same time, mice in the remaining two groups were placed in the PEMF treatment area under power-down state daily, similar to that in the PEMF group. After four weeks, all relevant indicators were tested. *Results:* (1) Compared with mice in the sham group, the number of trabecular bones significantly decreased, the thickness of the trabecular bone became thinner, the number of osteoclasts significantly increased, the gene expression of Osx and Ocn significantly decreased, and the gene expression of TRAP and CTSK significantly increased in the OVX group (P<0.01). (2) Compared with the blank controls without operation, the number of osteoblasts increased in the PEMF group. (3) Compared with the OVX group, the number of osteoclasts significantly decreased, the expression of Osx and Ocn significantly increased, and the gene expression of TRAP and CTSK significantly decreased in the PEMF group (P<0.01). *Conclusion:* PEMF treatment can significantly promote bone formation, which may be realized through inhibition of osteoclast formation, achieving bone morphological protection. PEMFs can significantly upregulate Osx and Ocn osteogenesis-related genes, which affect bone formation, and downregulate TRAP and CTSK osteoclast-related genes, which affect bone resorption. PEMFs may be used to treat postmenopausal osteoporosis by regulating Osx, Ocn, TRAP, and CTSK gene expression.

Keywords: PEMFs, postmenopausal osteoporosis, Ocn, Osx, TRAP, CTSK

Introduction

Postmenopausal osteoporosis (PMOP), also known as type I primary osteoporosis, is the most common form of osteoporosis. In postmenopausal women, this systemic metabolic bone disease is induced by ovarian function decline and estrogen deficiency and characterized by bone mass reduction in per unit volume, bone tissue microstructure degradation, and decreased strength [1]. At present, the absolute number of osteoporosis cases in China has been ranked first in the world [2]. In China's population of over 40 year olds, osteoporosis occurs in approximately 140 million people, accounting for 24.62% of the total population,

and its incidence is higher particularly in postmenopausal women [3]. Osteoporosis can easily lead to the occurrence of fractures, increasing mortality. Hence, the prevention and treatment of this disease has become an urgent concern and public health problem for society [4, 5]. Pulsed electromagnetic field (PEMF) treatment is a new physical therapeutic approach for the treatment of PMOP, which can enhance expression of bone local growth factors and reduce postmenopausal bone loss [6]. However, its specific mechanism of action remains unclear. In the present study, the effects of PEMF on osteoporosis in the treatment of postmenopausal osteoporosis were investigated.

Materials and methods

All animal experiments were approved by the Institutional Animal Care and Use Committee at West China Hospital, Sichuan University. C57BL/6 wild-type (WT) 12-week-old male mice was purchased from Jackson Laboratory (Bar Harbor, ME, USA), maintained in a temperature-controlled facility with a 12-hour light/dark cycle, and given *ad libitum* standard chow and water. Mice were randomized into three groups (*n*=10, each group): WT sham operation group, WT bilateral ovariectomy group (referred as OVX group), WT ovariectomy, and PEMF treatment group (referred as PEMF group). Mice in the WT sham operation group were resected above the ovaries, and mice in the remaining two groups were ovariectomized. After the completion of the 4-week feeding, all mice were sacrificed under general anesthesia. The soft tissue attached to the right femur was dissected under general anesthesia and immersed in a 4% paraformaldehyde fixative for histological section and staining. The soft tissue attached to the left femur was peeled, and soaked in PBS sterile buffer for osteoblast culture. The soft tissue attached to the bilateral tibia was peeled, the metaphysis was removed, and the marrow in the bone cavity was washed down with PBS buffer and frozen at -80°C in a refrigerator for the quantitative detection of fluorescence. For genotyping, genomic DNA was extracted from the tail tips using a Genomic DNA kit (Tiangen, DP304-03, China). Standard polymerase chain reaction (PCR) was performed using the PCR Master Mix (Takara, Japan).

Bone histological analysis

The left femur was dissected from the soft tissue, fixed in 4% paraformaldehyde solution for two days, washed with PBS for three times, immersed into 20% ethylenediaminetetraacetic acid (EDTA) buffer solution, and decalcified at 37°C. The decalcifying fluid was changed every 3-4 days until complete decalcification of the femurs was achieved. The femurs were then dehydrated and embedded in paraffin wax. Longitudinal sections from the middle of the femur (5 ml) were stained with tartrate-resistant acid phosphatase (TRAP; Nanjing Jiancheng, D023, China) and hematoxylin and eosin (H&E) for histological analysis. TRAP-positive multinucleated cells (three or more nuclei as osteoclasts, indicated by red arrows in the figure) were observed along the bone edge, and counted at six different visual fields (magnification, ×40) using a light microscope (Olympus, PA, USA) to evaluate osteoclast formation.
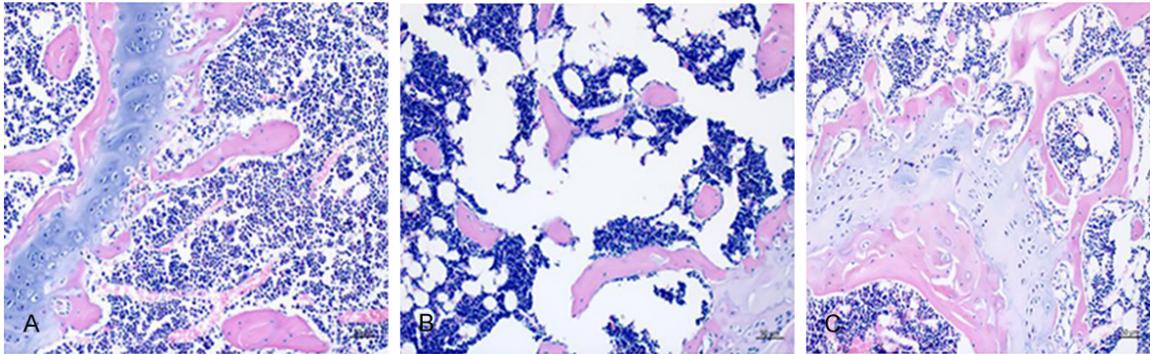
TRAP staining

According to TRAP staining kit instructions, cells were removed from the fixed slide, a few drops of the working fluid were dropped, incubated in a wet box at 37°C for 60 minutes, rinsed with water, counterstained, fixed in 1% alcohol-hydrochloric acid and 0.5% ammonia for 1-5 seconds, air dried, and sealed with neutral resin. Under light microscopic observation, TRAP (+) multi-nuclear cells (≥3) were observed as osteoclasts. Three slides were prepared for each treatment and observed at 40× microscopy using a light microscope. Six fields were randomly selected for osteoclast counting, the average was taken, and the results were presented in number of cells/slide. Osteoblast isolation and culture was performed as described [7].

Real-time quantitative PCR for bone- and adipose-related gene expression

Total RNA was extracted from cultured bone marrow stromal cells (BMSCs) or the distal metaphysis of right femurs, according to manufacturer's protocol (Trizol, Invitrogen, California, USA). RNA quality was evaluated by the A260/A280 ratio. RNA samples with an A260/A280 ratio between 1.8 and 2.0 were selected for reverse-transcription and real-time quantitative PCR. The cDNA was synthesized using 20 μl of the reverse transcription reaction solution (Takara, Japan). Real-time quantitative PCR was performed using an iQ SYBR green Supermix kit (Bio-Rad, USA) in a iCycler PCR machine (Bio-Rad), according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS 5.0 software. One-way analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) post-hoc test was used to compare significant differenc-

Figure 1. Group of mice analyzed for pathology by H&E staining (20×). Group A: SHAM group. Group B: ovx group. Group C: wild-type bilateral ovarian castration + PEMFs group.
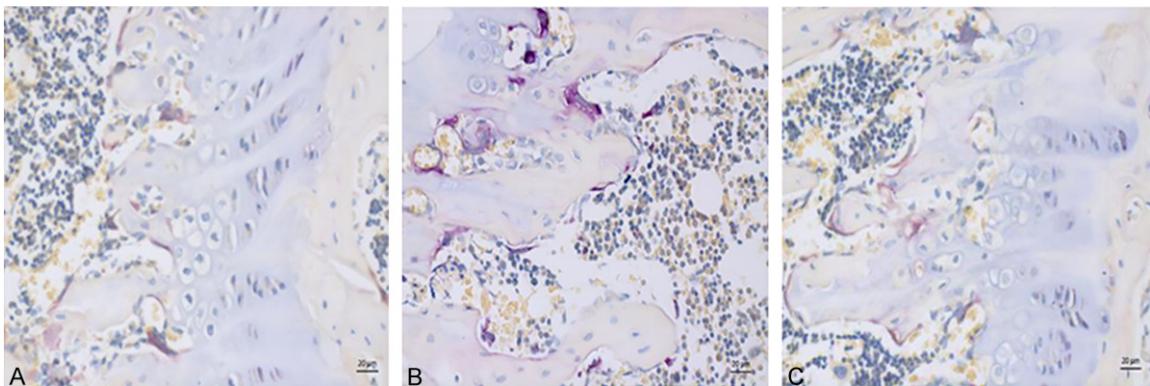
Figure 2. Group of mice analyzed for pathology by tissue TRAP staining (40×). Group A: SHAM group. Group B: ovx group. Group C: wild-type bilateral ovarian castration + PEMFs group.
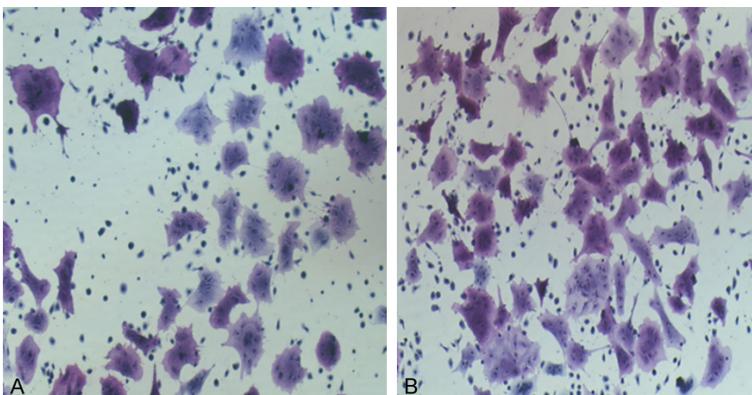
Figure 3. Group of mice with their osteoblasts cultured. Group A: vehicle control. Group B: PEMFs treatment group.

es between groups. Two-way analysis was used to determine interactions between genotype and diet treatment. All data are expressed as mean ± standard error of the mean (SEM).

Differences were considered to be significant at $P<0.05$.

Results

Effects of PEMF treatment on bone tissue morphology

Figures 1 and **2** demonstrate that the number of trabecular bones is significantly lower and the thickness of the trabeculae is thinner in WT mice than in mice in the sham group, suggesting that ovarian castration has a destructive effect on bone tissue morphology. The number of osteoclasts in WT mice was significantly higher than that in mice in the sham group ($P<0.01$). Compared with the OVX group, the number of osteoclasts in WT mice
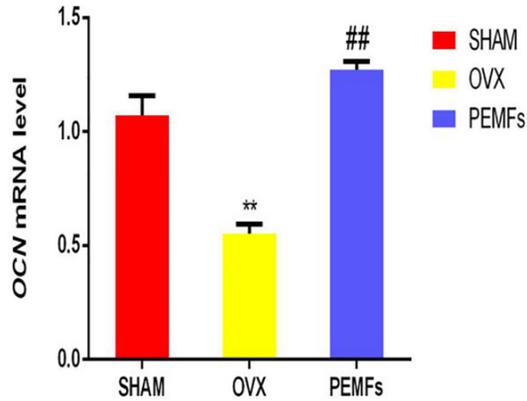
Figure 4. Ocn mRNA expression levels in each group.

Figure 5. Osx mRNA expression levels in each group. Compared with SHAM group, *P<0.05, **P<0.01. Compared with OVX group, #P<0.05, ##P<0.01.
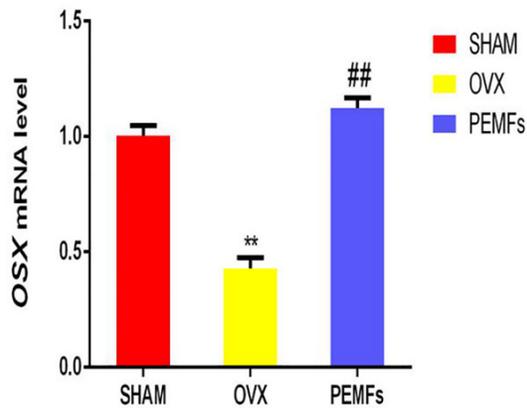

Figure 6. CTSK mRNA expression levels in each group.

Figure 7. TRAP mRNA expression levels in each group. Note: Compared with SHAM group, *P<0.05, **P<0.01. Compared with OVX group, #P<0.05, ##P<0.01.

treated with PEMF significantly decreased (P<0.01).

Effects of PEMF treatment on osteoblasts

Figure 3 shows that PEMF treatment significantly enhanced the ability of bone marrow mesenchymal stem cells to differentiate into osteoblasts, when compared to WT-controls (mice in the group did not undergo any surgery). The number of osteoblasts increased.

Effects of PEMF treatment on osteogenesis-related genes

In **Figures 4** and **5**, compared with mice in the sham group, the gene expression of osterix (Osx) and osteocalcin (Ocn) was significantly lower in mice in the OVX group (P<0.01). Expression of mouse-related genes was significantly higher in the PEMF group than in the sham group, but the difference was not statisti-
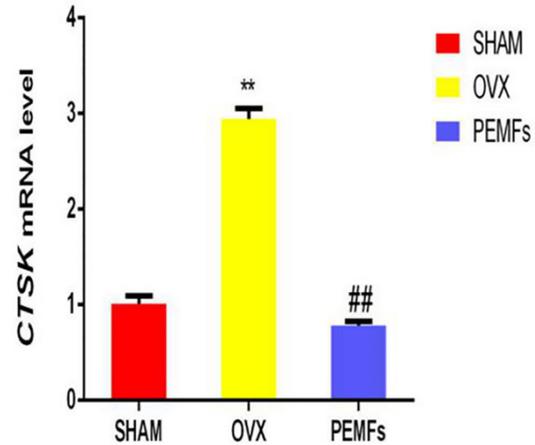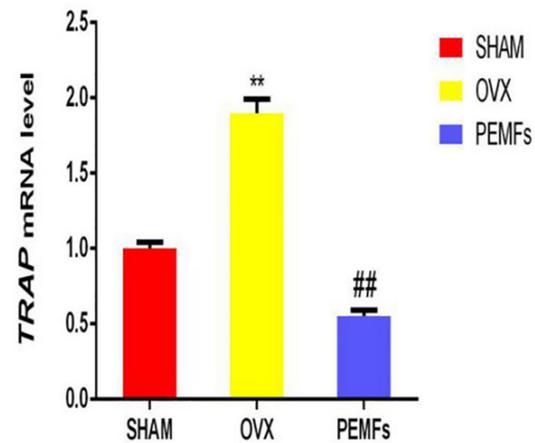
cally significant (P>0.05). Compared with the OVX group, expression of Osx and Ocn in mice was significantly higher in the PEMF group (P<0.01).

Effects of PEMF treatment on osteoclastic genes

In **Figures 6** and **7**, compared with the sham group, the gene expression of TRAP and cathepsin K (CTSK) was significantly higher in mice in the OVX group (P<0.01). However, gene expression of TRAP and CTSK was significantly lower in the PEMF group than in the sham group, but the difference was not statistically significant (P>0.05). Compared with the OVX group, expression of TRAP and CTSK was significantly higher in mice in the PEMF group (P<0.01).

Discussion

With an aging society, osteoporosis and its complications have brought about increasing concerns in the community. The present clinical treatment for PMOP is mostly through Western medicine, in which estrogen replacement therapy is often given in clinic, which has more adverse reactions and toxic side effects [8-10]. PEMF treatment is a new physical therapeutic approach for the treatment of PMOP. The study conducted by Jansen, Jing et al. [11-16] revealed that PEMFs can inhibit bone resorption and have no definite side effects, in addition to improving bone mass and promoting bone formation. A large number of basic studies have confirmed that PEMFs can stimulate osteoblast proliferation and differentiation [7, 17]. Furthermore, the study conducted by Chang et al. [18] revealed that PEMFs have an effect on osteoclast apoptosis, and that this effect is correlated with the time of action. Although PEMFs have been shown to be effective for PMOP, the specific mechanisms of action remain unclear. Hence, we attempted to reveal the mechanism of PEMFs by treating PMOP at the molecular level.

In the present study, WT C57BL/6 mice were used to replicate PMOP models. Mice have been widely used as experimental animals for the replication of PMOP models. However, in recent years, studies have suggested that mouse sexual maturity, sexual cycle, and bone mass changes after ovariectomy of mice were similar to those of rats [19].

Resection of the mice ovary is a classical method of replicating osteoporosis [20], which is very similar to PMOP. PEMF treatment was provided on day two after removal of the ovaries in WT mice. Osteoporosis is a long-term developmental process. After estrogen removal, estrogen levels rapidly decreased in the stage of bone loss. The next day after PEMF treatment was selected to explore its early intervention on osteoblasts and osteoclasts in the disease, and determine whether it could delay the process of PMOP. Compared with the sham group, the number of trabeculae bones significantly decreased, the thickness of the trabeculae was thinner, the bone marrow cavity was enlarged, the distance became wider, and the area of the trabecular bone decreased in mice in the OVX group. Compared with the sham group, the number of osteoclasts significantly increased in mice in the OVX group ($P<0.01$), suggesting

that the PMOP model was successfully replicated. There was no statistical conclusion on modeling time, but a vast majority of researchers consider that for mice modeled using the castration method, a four-week period of time is sufficient for successful modeling [21-25]. This is consistent with the modeling time in the present study. Furthermore, MOST et al. [26] observed that in adult mice castrated its number of osteoclasts in the bone marrow increased at seven days after castration. Compared with the OVX group, the number of osteoclasts significantly decreased in mice treated with PEMF ($P<0.01$). From the results of the osteoblast culture, it was found that PEMFs can significantly enhance the ability of bone marrow mesenchymal stem cells to differentiate into osteoblasts, when compared with WT blank controls. This indicates that PEMFs have a significant effect on bone formation, which is consistent with previous experimental results [7, 11-18].

Osx and Ocn are genes that reflex osteoblast activity. Osx is an osteoblast-specific transcription factor discovered by Nakashima [27], which belongs to the Sp/XKLF family and is a key transcription factor expressed in the development of bone tissues [28-34]. Ocn is secreted by osteoblasts, which is a specific biochemical indicator for the reaction of bones, which can respond to osteoblast activity. Compared with the sham group, expression of Osx and Ocn was significantly lower in mice in the OVX group ($P<0.01$). The expression of related genes was significantly higher in mice in the PEMF group than in the sham group ($P>0.05$). Compared with the OVX group, expression of Osx and Ocn was significantly upregulated in the PEMF group ($P<0.01$). This suggests that PEMFs may promote osteoblast formation by upregulating Osx and Ocn gene expression.

TRAP and CTSK are relevant factors that respond to osteoclast activity. TRAP is an isoenzyme of acid phosphatase secreted by osteoclasts, which is a marker of osteoclasts. TRAP staining can reflect the number and activity of osteoclasts [35-37]. CTSK is one of the most abundant osteolytic cells and the strongest osteolytic activity of cysteine protease. Compared with the sham group, gene expression of TRAP and CTSK was significantly higher in mice in the OVX group ($P<0.01$). TRAP and CTSK gene expression was downregulated in the PEMF group ($P>0.05$). Compared with the OVX group, TRAP and CTSK gene expression was significantly downregulated in mice in the PEMF

group (P<0.01). This suggests that PEMFs could downregulate the expression of osteoblast-related genes Osx and Ocn, and downregulate the expression of TRAP and CTSK.

In summary, PEMF stimulation is given in the stage with the presence of bone loss but without the full development of PMOP. Due to the early intervention time, this can effectively delay the progression of PMOP. Therefore, PEMFs can be used for postmenopausal women to prevent PMOP. Indeed, there are limitations in the experiment. Future studies with a control group should be conducted to further explore the effect at different treatment time windows. Early intervention can provide a new approach for clinical treatment. PEMFs might be used for postmenopausal women to prevent osteoporosis.

Acknowledgements

Disclosure of conflict of interest

None.

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