Low-frequency pulsed electromagnetic field inhibits RANKL-induced osteoclastic differentiation in RAW264.7 cells by scavenging reactive oxygen species

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Abstract. Bone homeostasis is a dynamic balance maintained by bone formation and resorption. An increase in the number and activity of osteoclasts leads to excessive bone resorption, which in turn results in bone disease, including osteoporosis. Therefore, inhibiting the differentiation and activity of osteoclasts is important for maintaining bone mass. Several studies have revealed that the use of a low-frequency pulsed electromagnetic field (PEMF) is an effective method to treat osteoporosis. However, its exact mechanism remains to be fully clarified. Therefore, the present study was designed to examine the effects that PEMF exerts on receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis and intracellular reactive oxygen species (ROS) production in RAW264.7 cells. The viability of cells was determined using a Cell Counting Kit-8 assay, and gene and protein expression were investigated via reverse transcription-quantitative polymerase chain reaction and western blot analyses. Furthermore, microscopy was performed to detect the levels of intracellular ROS and tartrate-resistant acid phosphatase (TRAP). Following the culture of RAW264.7 cells with RANKL (50 ng/ml) for 4 days (3 h/day) under PEMF (75 Hz, 1 mt) exposure, it was observed that PEMF had an inhibitory effect on RANKL-induced osteoclastic differentiation. Multinucleated osteoclast formation, the activity of TRAP and the expression of osteoclastogenesis-associated genes, including cathepsin K, nuclear factor of activated T cells cytoplasmic 1 and TRAP, were significantly reduced by PEMF. Furthermore, PEMF effectively decreased the generation of intracellular ROS during osteoclastic differentiation. In addition, the results demonstrated that ROS are the key factor in osteoclast differentiation and formation. Reducing intracellular ROS with diphenylene-iodonium chloride significantly inhibited RANKL-induced osteoclast differentiation. Taken together, the results of the present study demonstrated that PEMF may inhibit RANKL-induced osteoclastogenesis by scavenging intracellular ROS. These results may provide the groundwork for future PEMF clinical applications in osteoclast-associated bone disease.

Introduction

It is well known that bone homeostasis is a dynamic balance maintained by bone formation and bone resorption (1,2). Osteoclasts are derived from hematopoietic precursors and function in bone resorption. The classic receptor activator of nuclear factor-κB (RANK)/RANK ligand (RANKL)/osteoprotegerin signaling pathway regulates the activity and differentiation of osteoclasts. RANKL is considered to be an important factor that regulates osteoclast differentiation and bone metabolism (3). Excessive bone resorption is closely associated with the formation of osteoclasts, which can lead to bone disease, including osteoporosis (4). It has been shown that osteoporosis significantly increases the risk of fracture, particularly hip fractures, in postmenopausal women (5). Therefore, regulating osteoclast differentiation and activity is important for preserving bone mass and reducing the incidence of fractures. At present, the treatment of osteoporosis predominantly includes estrogen replacement therapy and medical treatment, including the use of calcitriol and caltrate D; however, clinical evidence has demonstrated that these treatments are associated with serious side effects, including cancer (6,7). Therefore, identifying novel treatments is critical.

As a non-invasive and inexpensive method, low-frequency pulsed electromagnetic fields (PEMF) have shown efficacy for a wide range of diseases of the skeletomuscular system (8,9). In previous years, it has been reported that PEMF may

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inhibit osteoclast differentiation *in vitro* (10,11), however, the underlying mechanisms remain to be fully elucidated. PEMF exposure is likely to produce satisfying therapeutic effects for certain bone diseases, including osteoporosis, which is closely associated with osteoclast function (4,12,13). Therefore, *in vitro* studies examining the effects of PEMF on osteoclasts and the potential underlying mechanisms are essential to understand the efficacy of PEMF for treating osteoclast-associated diseases.

Reactive oxygen species (ROS) are produced intracellularly as byproducts during mitochondrial electron transport or through the action of certain enzymes, including NADPH oxidase and cyclooxygenase (14). ROS consist of radical and non-radical oxygen species, including the superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) (15). Changes in ROS levels are implicated in regulating cellular signal transduction. The excessive production of ROS results in oxidative stress, which in turn may cause apoptosis, ischemia and inflammation (16). However, low, non-toxic levels of ROS may act as secondary messengers in several receptor signaling pathways (17-19). Various studies have shown that ROS are implicated in bone metabolism and promote osteoclast differentiation and bone resorption (20-23). The increased production of ROS during osteoclast formation appears to activate the peroxisome proliferator-activated-receptor-γ coactivator 1β, which regulates mitochondrial biogenesis, thus facilitating osteoclast differentiation (24). A previous study illustrated the destructive effects of antioxidants on osteoclastogenesis in mouse models lacking the forkhead box O transcription factor, which drives transcription of the antioxidant catalase (25). To the best of our knowledge, the inhibitory effects of PEMF on RANKL-induced osteoclast differentiation have not been investigated. In the present study, the effects of PEMF on osteoclast differentiation were examined. It was demonstrated that PEMF served as a RANKL-mediated inhibitor of osteoclastogenesis. The mechanism underlying this inhibitory effect may be via the suppression of ROS generation, which is required for osteoclast differentiation. Therefore, the results of the present study demonstrated that PEMF may inhibit osteoclast differentiation by scavenging intracellular ROS.

Materials and methods

Cell culture and PEMF exposure. 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 cultured in 24-well culture plate at 1x10^6 cells/ml, followed by culture in α-minimum essential media (α-MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco; The Fisher Scientific, Inc.) and 1% penicillin/1% streptomycin in the presence of 50 ng/ml RANKL (PeproTech, Inc., Rocky Hill, NJ, USA). The medium was placed at 37°C in a humidified atmosphere containing 5% CO_2 for 4 days and refreshed every 48 h.

For PEMF, the cells were placed in an incubator containing a 75 Hz sinusoidal PEMF with a density of 1 mT. The magnetic field used for the test was produced via a Helmholtz coil. The cells in the control group were placed in another incubator under the same conditions, but without a PEMF in the incubator.

Cell Counting Kit-8 (CCK-8) proliferation assay. Cell viability was quantified according to the manufacturer's protocol using the CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, the RAW264.7 cells were seeded in 96-well plates at a cell density of 1,000 cells/well and cell proliferation was determined at different time points (each group had a set of three sub-wells). The PEMF group was exposed to PEMF for 4 days (3 h/day), whereas control cells were cultured under the same conditions without PEMF. After 24, 48, 72 and 96 h, 10 µl CCK-8 solution was added to each well. Following incubation at 37°C for 4 h, the optical density (OD) of each well was determined using an enzyme mark instrument (Multiskan FC; Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm.

Tartrate-resistant acid phosphatase (TRAP) staining, cell counting and TRAP activity assay. Following exposure to PEMF, the cells were washed with PBS and fixed. A TRAP staining kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to identify the formation of mature osteoclasts according to the manufacturer's protocol. The cell culture plate was transferred to an optical microscope, and cells that were TRAP-positive with more than three nuclei were confirmed to be osteoclasts. Five random fields of view were selected, and the number of osteoclasts was counted. TRAP activity was measured at a wavelength of 540 nm with a microplate reader.

Determination of intracellular ROS. The level of intracellular ROS generated by H_2O_2 was measured using a ROS assay kit (Beyotime Institute of Biotechnology, Haimen, China). H_2O_2 was used as a ROS inducer. DCFH-DA interacts with ROS in viable cells to generate 2',7'-dichlorofluorescein (DCF). DCF is highly fluorescent at 530 nm. The cells were washed with PBS three times and cultured for 30 min at 37°C in the dark following the addition of DCFH-DA at a final concentration of 10 µM/ml. The relative expression of ROS was evaluated using a fluorescent microscope, and the fluorescence intensity of DCF was determined using a fluorometric plate reader. To further investigate the involvement of ROS in the promotion of osteoclast precursor differentiation, diphenylene-iodonium chloride (DPI; Sigma-Aldrich; Merck KGaA), a widely used NADH oxidase 1 inhibitor that completely scavenges generated ROS, was added to the cultured system (RANKL).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. TRIzol® reagent (Thermo Fisher Scientific, Inc.) was used to extract intracellular total RNA, and a Prime Script RT kit (Takara Bio, Inc., Otsu, Japan) was used to synthesize single-stranded cDNA. qPCR was performed using an ABI 7500 real-time PCR system (Thermo Fisher Scientific, Inc.). qPCR was conducted using cDNA (1 µl) with SYBR Green-1 (20 µl; Takara Bio, Inc.) according to the manufacturer's protocol under the following conditions: Activation at 95°C for 10 min, then 40 cycles of amplification (95°C for 10 sec, 60°C for 24 sec and 72°C for 20 sec) and a final extension at 72°C for 1 min. All the reactions were performed in triplicate and target gene expression
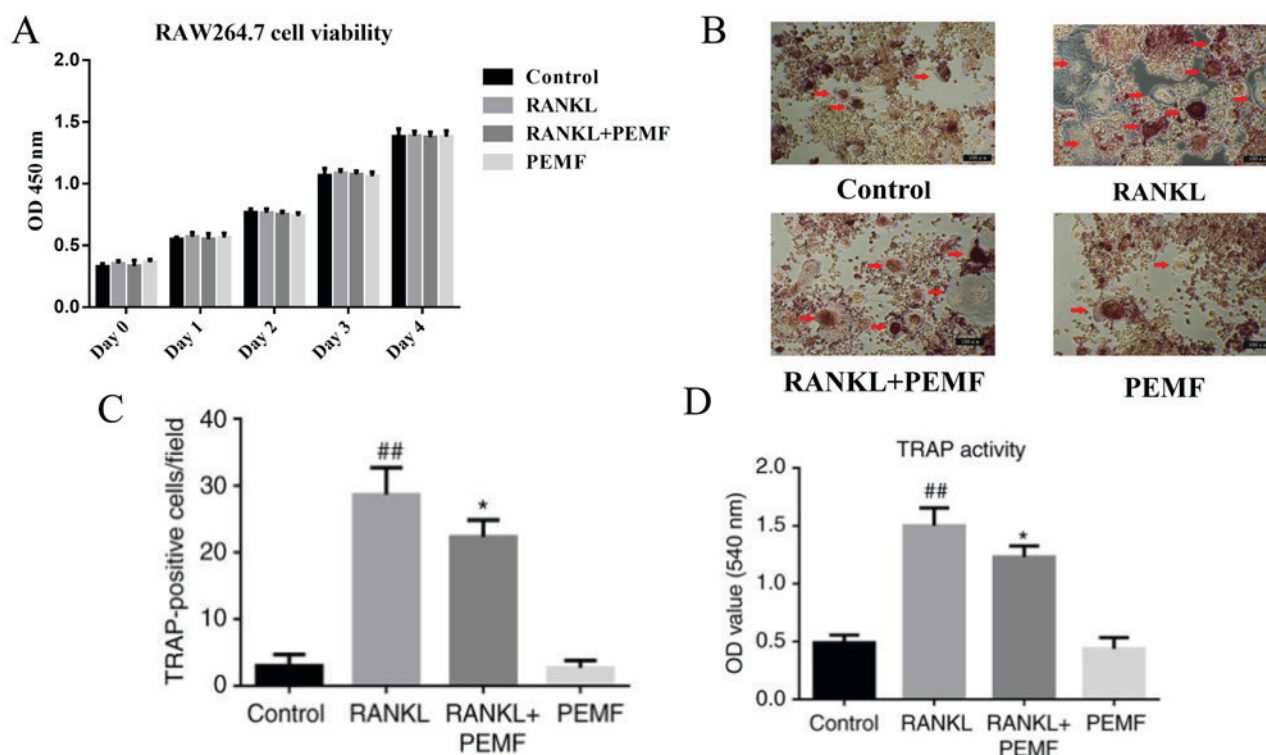
Figure 1. PEMF inhibits osteoclastic differentiation, but not the viability of RANKL-induced RAW264.7 cells. Cells were treated with or without RANKL (50 ng/l) under PEMF stimulation for 4 days. (A) Cell viability was analyzed using the Cell Counting Kit-8 assay. (B) After 4 days in culture, cells were fixed and stained using the TRAP kit. Osteoclasts were visualized under an optical microscope. Scale bar=100 μm. Red arrows indicate multinucleated osteoclasts. (C) TRAP-positive multinucleated osteoclasts containing more than three nuclei were counted in five random fields. (D) TRAP activity was measured at λ=540 nm. Values are expressed as the mean ± standard deviation. (n=3). ##$P<0.01$ compared with the control group; *$P<0.05$ compared with RANKL alone. PEMF, pulsed electromagnetic field; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase; OD, optical density.

was normalized to the reference gene β-actin. The $2^{-\Delta\Delta Cq}$ method (26) was applied to calculate the relative expression level of the target genes. The PCR products were subjected to melting curve analysis and a standard curve to confirm the correct amplification. The primers used for PCR were as follows: Cathepsin K (CTSK), forward 5'-AGA ACG GAG GCA TTG ACT CT-3', reverse 5'-GAT GGA CAC AGA GAT GGG TC-3'; TRAP, forward 5'-CGA TCA CAA TCT GCA GTA CC-3', reverse 5'-ACC CAG TGA GTC TTC AGT CC-3'; nuclear factor of activated T cells cytoplasmic 1 (NFATc1), forward 5'-CGC AAG TAC AGT CTC AAT GG-3', reverse 5'-CAG GTA TCT TCG GTC ACA CT-3'; and β-actin, forward 5'-AGG CCA ACC GTG AAA AGA TG-3' and reverse 5'-TGG CGT GAG CGA GAC CAT AG-3'.

Western blotting. The treated RAW264.7 cells ($5x10^4$ cells per well) were lysed using cOmplete™ Lysis-M and Phos-STOP (Roche Diagnostics, Indianapolis, IN, USA). The concentration was measured using a Bicinchoninic Acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), Proteins (40 μg/lane) were separated via 10% SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, and were blocked for 1.5 h at room temperature with 3% non-fat dry milk in TBS-Tween-20 (150 mM NaCl, pH 7.4, 25 mM Tris-HCl and 0.2% Tween-20). The membranes were incubated with specific primary antibodies overnight at 4˚C. The primary rabbit polyclonal antibodies used were as follows: Anti-β-actin (1:1,000; cat. no. ab8226, Abcam, Cambridge, MA, USA); anti-CTSK (1:2,000; cat. no. 11239-1-AP, ProteinTech Group,

Inc., Chicago, IL, USA); anti-NFATc1 (1:3,000; cat. no. 4389, Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-TRAP (1:2,000; cat. no. 10325-1-AP, ProteinTech Group, Inc.). Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (1:1,000; cat. no. 7074, Cell Signaling Technology, Inc.). Immunoreactive bands on the PVDF membranes were detected by enhanced chemiluminescence (Tanon Science and Technology Co., Ltd., Shanghai, China).

Statistical analysis. Data are presented as the mean ± standard deviation. Each experiment was repeated at least three times. Statistical analyses were performed using one-way analysis of variance with subsequent post hoc multiple comparisons with Dunnett's test using SPSS version 20 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PEMF inhibits the osteoclastic differentiation, but not the viability of RANKL-induced RAW264.7 cells. Cell viability was assessed using a CCK-8 assay following exposure of the RAW264.7 cells to PEMF. The RAW264.7 cells were treated with or without RANKL (50 ng/l) for 4 days under PEMF (75 Hz, 1 mt, 3 h/day). The results revealed no significant difference in cell viability between groups (Fig. 1A).
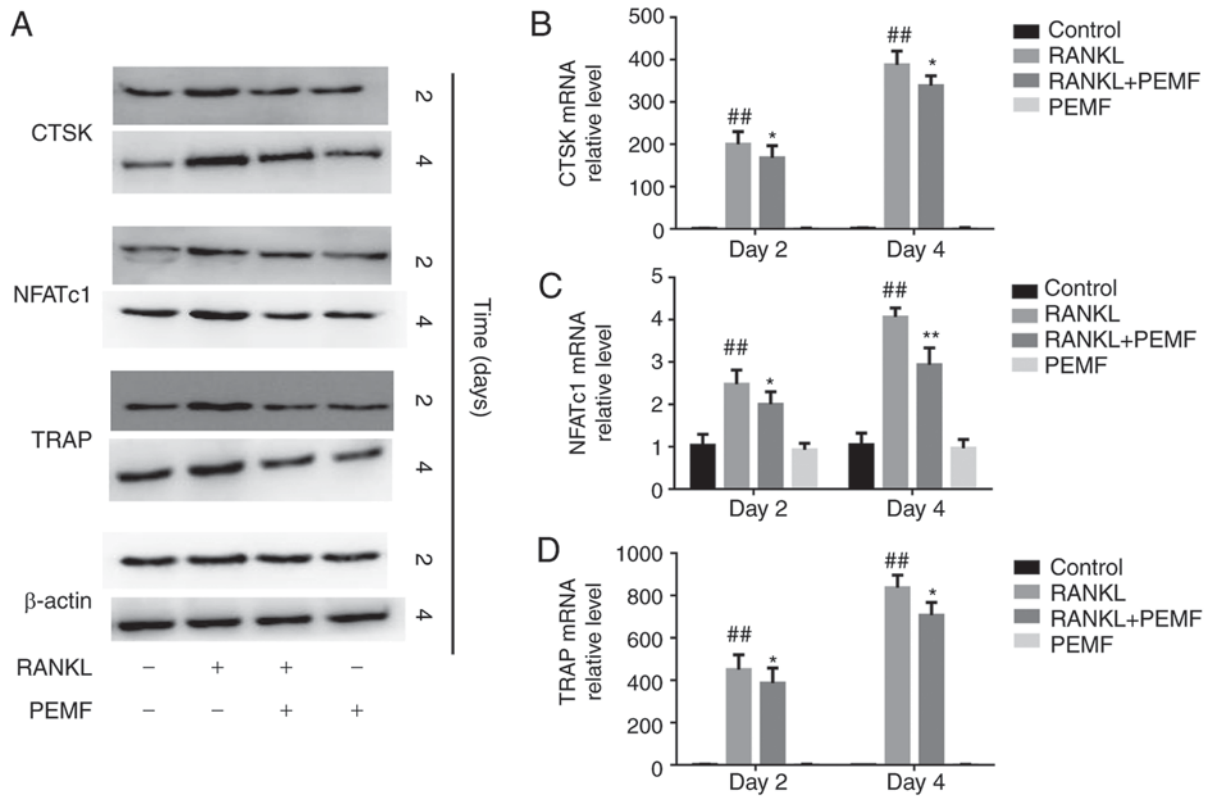
Figure 2. PEMF inhibits the expression of osteoclastogenesis-associated genes. RAW264.7 cells were cultured with RANKL (50 ng/ml) for 2 or 4 days with PEMF exposure. (A) Expression of osteoclastogenesis-related genes (CTSK, NFATc1 and TRAP) was determined by western blotting. Expression levels of osteoclastogenesis-(B) CTSK, (C) NFATc1 and (D) TRAP were examined by reverse transcription-quantitative polymerase chain reaction analysis. Values are expressed as the mean ± standard deviation (n=3). The relative expression level of the target gene was normalized to that of β-actin. $^{##}$P<0.01 compared with the control group; *P<0.05 and **P<0.01 compared with RANKL alone. PEMF, pulsed electromagnetic field; RANKL, receptor activator of nuclear factor-κB ligand; CTSK, cathepsin K; NFATc1, nuclear factor of activated T cells cytoplasmic 1; TRAP, tartrate-resistant acid phosphatase.

In the present study, the inhibitory effect of PEMF on osteoclast differentiation was investigated. RANKL (50 ng/ml) induced the differentiation of RAW264.7 murine macrophage cells into osteoclasts. Osteoclasts were visualized under an optical microscope (Fig. 1B). However, PEMF significantly reduced the number of TRAP-positive multinucleated osteoclasts (Fig. 1C). Compared with the control group, RANKL treatment increased the activity of TRAP by 150%, whereas PEMF reduced the activity of TRAP stimulated by RANKL (Fig. 1D). No significant difference was observed between the control group and PEMF group.

PEMF inhibits the expression of osteoclastogenesis-associated genes. The expression of several osteoclastogenesis-associated genes, including CTSK, NFATc1 and TRAP, were determined by western blotting (Fig. 2A) and RT-qPCR analysis (Fig. 2B-D) on days 2 and 4. The results showed that PEMF inhibited the RANKL-induced increase in the expression of CTSK, NFATc1 and TRAP, which was consistent with the above results.

Antioxidant capacity and intracellular ROS levels are reduced by PEMF. To confirm whether PEMF can scavenge intracellular ROS, the accumulation of intracellular ROS was evaluated by fluorescence microscopy. The RAW264.7 cells were exposed to PEMF for 3 h following treatment with 1 mM H_2O_2 for 30 min. A previously reported study demonstrated

that during RANKL-induced differentiation of RAW264.7 cells into osteoclasts, maximum intracellular ROS levels were reached at day 2 (27). Therefore, the inhibitory effect of PEMF on ROS production was assessed following 2 days of induction with RANKL (50 ng/ml). The ROS-positive cells were visualized as bright green spots, and H_2O_2 treatment increased the level of intracellular ROS, compared with that in the control group. In addition, treatment with PEMF inhibited the H_2O_2-induced increase in intracellular ROS (Fig. 3A). All cells were collected and analyzed on a fluorometric plate reader to measure the OD values of DCF. The fluorescence intensity of DCF was reduced in the PEMF + H_2O_2 group compared with that in the H_2O_2-treated group (Fig. 3B). In the RAW264.7 cells treated with RANKL and exposed to PEMF for 2 days (3 h/day), it was determined that RANKL treatment significantly increased ROS, and PEMF prevented this increase (Fig. 3C). Similar results were observed for the OD values of DCF (Fig. 3D). These results suggested that PEMF had antioxidant capacity and reduced intracellular ROS.

Reducing intracellular ROS levels inhibits RANKL-induced osteoclast differentiation. The results showed that DPI administration inhibited the effect of RANKL on osteoclast differentiation and reduced the number of TRAP-positive multinuclear cells (Fig. 4A and B). The decreased activity of TRAP (Fig. 4C) was consistent with the observed alterations in the TRAP staining. DPI treatment also
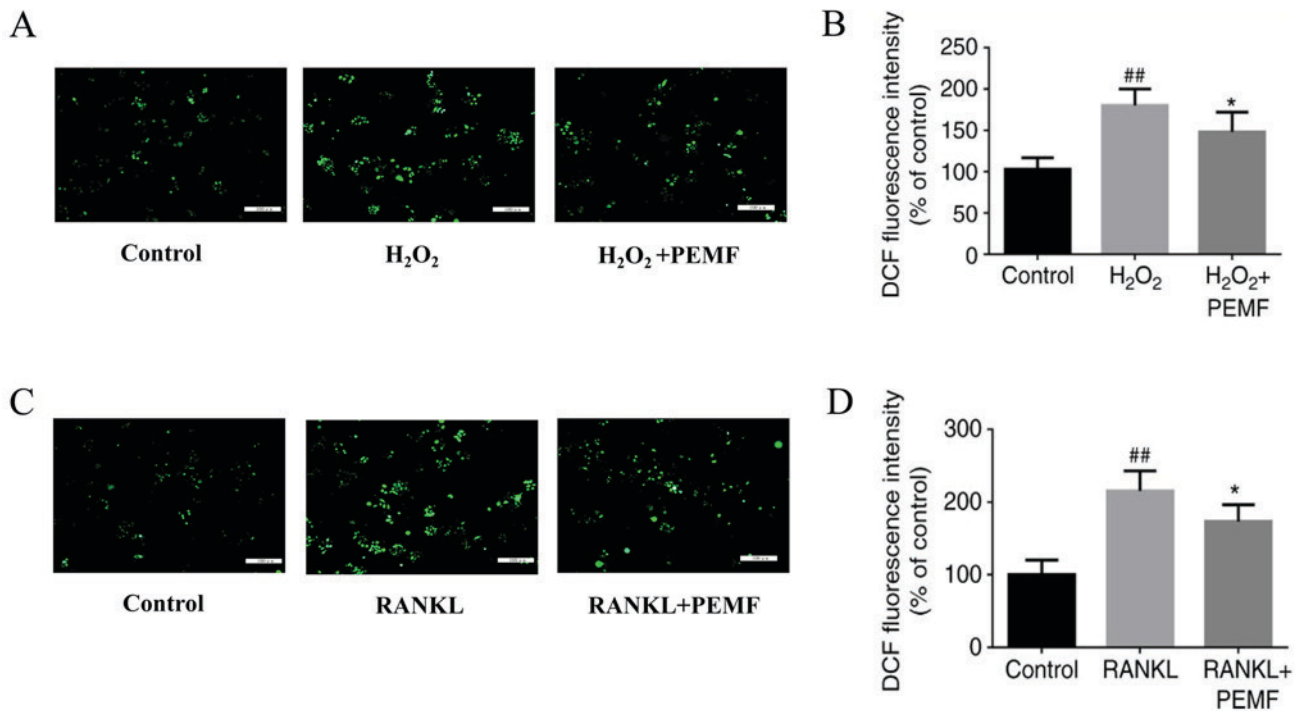
Figure 3. PEMF reduces the intracellular level of induced ROS. RAW264.7 cells were treated with H_2O_2 (1 mM) for 30 min or incubated with RANKL for 2 days in the absence and presence of PEMF. (A) Accumulation of intracellular ROS in H_2O_2-treated groups was assessed by fluorescence microscopy (scale bar=100 μm) and (B) OD values of DCF in these groups were measured using a fluorescent reader at λ=530 nm. (C) Accumulation of intracellular ROS in the RANKL-treated groups was assessed by fluorescence microscopy (scale bar=100 μm) and (D) OD values of DCF in these groups were measured using a fluorescent reader at λ=530 nm. All values are expressed as the mean ± standard deviation (n=3). ##$P<0.01$ compared with the control group; *$P<0.05$ compared with H_2O_2 or RANKL alone. PEMF, pulsed electromagnetic field; RANKL, receptor activator of nuclear factor-κB ligand; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; DCF, 2',7'-dichlorofluorescein; OD, optical density.

downregulated the expression of osteoclastogenesis-associated genes (Fig. 4D). These data revealed ROS to be the key factor in RANKL-induced osteoclast formation, and scavenging intracellular ROS effectively inhibited osteoclast formation.

Discussion

Osteoclasts are unique in their ability to resorb mineralized bone and have critical functions in bone remodeling and physical skeletal morphogenesis (28). In recent decades, it has been confirmed that electromagnetic fields, including PEMF, have potential in curing skeletal muscle system diseases and/or injury, including fracture healing, particularly in tendon-to-bone healing and joint and bone injury (29-31). Abnormalities in osteoclast number and activity are associated with osteoporosis. In the present study, it was evident that 75 Hz, 1 mt PEMF exhibited a distinctly inhibitory effect on RANKL-induced osteoclast differentiation. Therefore, it is of clinical significance.

Previous studies have examined the action of PEMF in osteoporosis, attributing its mechanism of action to osteoblast precursor proliferation (32) and the inhibition of osteoclast activity (33), in addition to the enhancement of osteoblastic mineralization potential (9). Treatment of bone marrow cells derived from ovariectomized rats with PEMF led to significant suppression of osteoclast formation and osteoclast-associated cytokine expression (34). Furthermore, certain properties of deteriorated bone, including its stiffness, the maximum load

and the yield load, are inhibited following PEMF exposure, indicating that more bone mass was retained and there was greater mechanical bone strength to resist fractures (35). Wang *et al* (36) also reported that RAW264.7 cells incubated with RANKL and exposed to 15 Hz PEMF (2 h/day) at 3 mt for 7 days had decreased bone resorbing capacity, due to the promotion of osteoclast apoptosis. These results suggested that PEMF exerts biological effects on osteoclasts and/or osteoblasts. To better understand how PEMF affects osteoclasts, the present study also examined the effects of PEMF on osteoclast differentiation. Although it was demonstrated that PEMF inhibited RANKL-induced osteoclast differentiation by scavenging intracellular ROS, other possible mechanisms cannot be excluded. Another important regulating factor in osteoclasts is calcium. During RANKL-induced osteoclastogenesis, Ca^{2+} upregulates the downstream signal NFATc1, which is considered the master regulator of osteoclastogenesis (37). Previous studies have reported that PEMF affects the influx of Ca^{2+} in several cell types (38,39). Therefore, Ca^{2+} may be another indication that PEMF affects osteoclast differentiation.

In the classic RANKL signaling pathway, the production of ROS is mediated by NADPH oxidase I in response to RANKL (40). In the present study, it was found that RANKL significantly increased intracellular ROS, consistent with previous reports. ROS are byproducts of cell metabolism and are also important signaling molecules. The ability of ROS to serve as signaling molecules in pathways has been well established, particularly in osteoclast differentiation, where ROS are
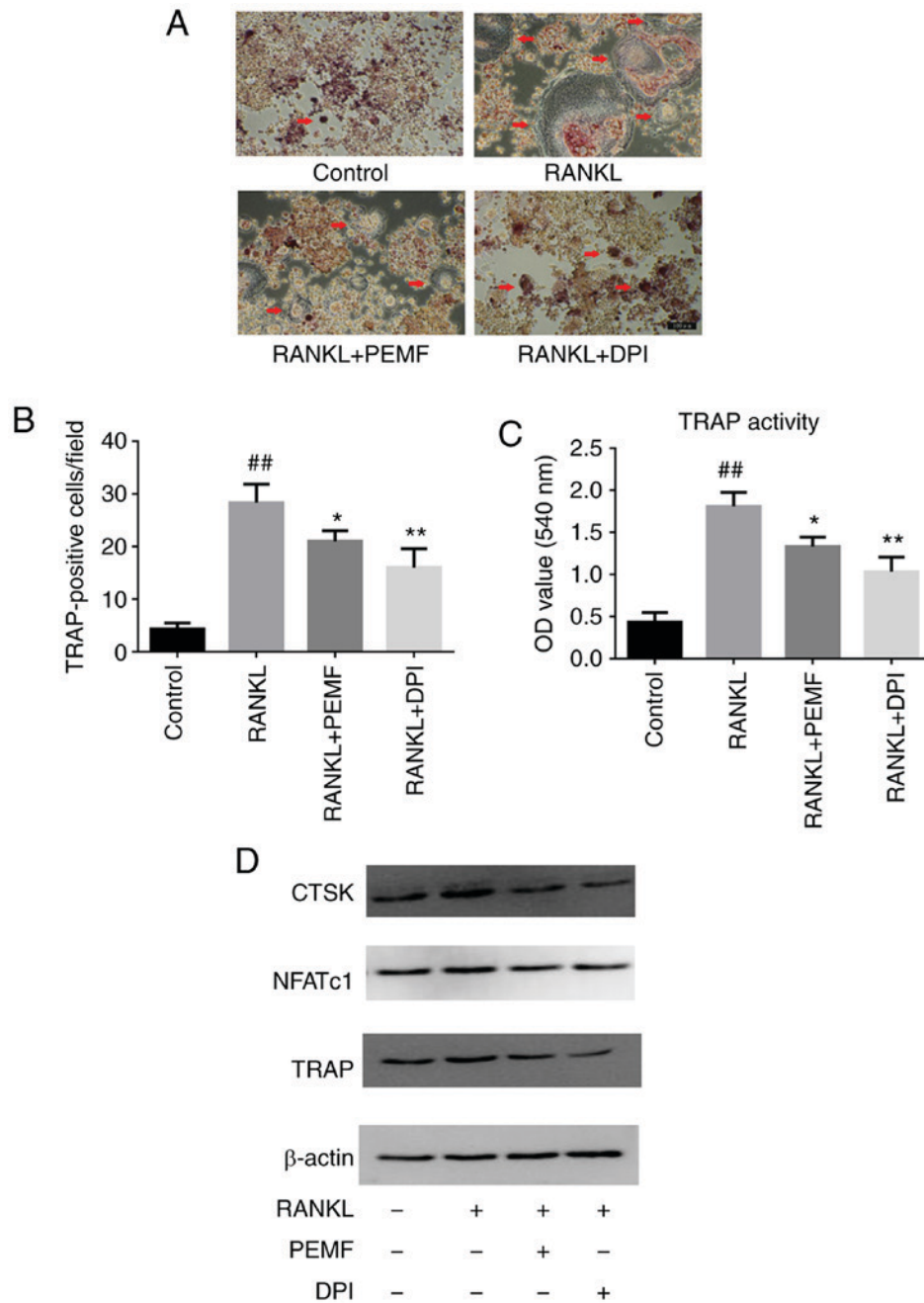
Figure 4. Reducing the level of intracellular ROS inhibits RANKL-induced osteoclast differentiation. RAW264.7 cells were treated with DPI (10 μM) together with RANKL (50 ng/ml) for 4 days. (A) TRAP staining of osteoclasts treated with PEMF (3 h/day) or DPI together with RANKL for 4 days. Scale bar=100 μm. (B) TRAP-positive multinucleated osteoclasts containing more than three nuclei were counted. (C) TRAP activity was measured at λ=540 nm in each group. All values are expressed as the mean ± standard deviation (n=3). The relative expression level of the target gene was normalized to β-actin. ##P<0.01 compared to the control group; *P<0.05, **P<0.01 compared to RANKL alone. (D) Expression levels of osteoclastogenesis-associated proteins was analyzed. PEMF, pulsed electromagnetic field; RANKL, receptor activator of nuclear factor-κB ligand; DPI, diphenylene-iodonium chloride; TRAP, tartrate-resistant acid phosphatase; OD, optical density.

involved in the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling pathways (19). Additionally, the ROS-mediated direct modification of inositol 1,4,5-trisphosphate receptor thiol groups promotes Ca^{2+} release from the endoplasmic reticulum (41). As mentioned previously, Ca^{2+} is involved in osteoclast differentiation. Through NF-κB, MAPK and Ca^{2+} signaling, ROS are involved in the differentiation and formation of osteoclasts. DPI scavenges RANKL-induced intracellular ROS, effectively preventing the differentiation of osteoclast precursors. Furthermore, RANKL may upregulate

the expression of superoxide dismutase 2 (SOD2) and sirtuin 3 (Sirt3), which exert feedback on osteoclastogenesis by inhibiting RANKL signaling via the downregulation of ROS (42). This mechanism physiologically prevents osteoclast overgrowth. Sirt3-deficient mice exhibit severe bone loss caused by elevated osteoclastogenesis (43). By contrast, evidence has revealed that several diseases are associated with ROS, including osteoporosis (15,44). Certain antioxidants, including alliin and lycopene, inhibit osteoclast formation and bone resorption via the inhibition of ROS generation (45,46).

Exposure to PEMF leads to the upregulation of proteins associated with proper protein folding (47-50). A previous study revealed that PEMF effectively prevents the pro-oxidant effects of H_2O_2 in SK-N-BE(2) human neuroblastoma cells by increasing Mn-dependent superoxide dismutase (MnSOD)-based antioxidant protection (51). Furthermore, Falone et al (52) found that PEMF markedly decreased ROS in human osteoclasts by inducing the expression of antioxidant enzymes, including glutathione peroxidase 3, SOD2, catalase and glutathione-s-reductase. However, whether the same mechanism exists in osteoclasts remains to be elucidated and requires further investigation.

There are certain limitations to the present study. First, the exact mechanism of PEMF scavenging of intracellular ROS in osteoclasts remains to be fully elucidated. As research in this area advances, there is no doubt that this issue can be addressed. Second, although RAW264.7 cells are widely used to induce the formation of osteoclasts in response to RANKL, they are not identical to osteoclasts derived from the body. For example, when comparing in vivo and in vitro conditions, there are discrepancies in vitamin D compound synthesis during osteoclastic bone resorption (53). Third, the present study did not determine the optimal PEMF parameters, including exposure time, strength and frequency, and did not perform bone resorption analysis or NOX expression assays. Therefore, further investigation is required.

In conclusion, the findings of the present study demonstrated that PEMF inhibited RANKL-induced osteoclast differentiation by scavenging intracellular ROS. These findings provide theoretical support for using PEMF in the treatment of osteoporosis and also indicates that ROS may be a potential therapeutic target in the treatment of osteoporosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JT conceived and designed the study. YP, HL, QY, YY, HX, YL and ZH performed the experiments. YP drafted the manuscript. HL, QY, YY, HX, YL and ZH reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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