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Radiofrequency field inhibits RANKL-induced osteoclast differentiation in RAW264.7 cells via modulating the NF-κB signaling pathway

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ABSTRACT

In this study, we investigated the inhibitory effects of radiofrequency exposure on RANKL-induced osteoclast differentiation in RAW264.7 cells, along with the underlying mechanisms. RAW264.7 cells were subjected to radiofrequency exposure at three distinct power densities: 50 μW/cm^2, 150 μW/cm^2, and 450 μW/cm^2. The results showed that, among the three dosage levels, exposure to 150 μW/cm^2 of radiofrequency radiation significantly reduced the proliferation capacity of RAW264.7 cells. RF exposure at three power densities resulted in significant increases in the level of osteoclast apoptosis and notable decreases in osteoclast differentiation. Notably, the most pronounced effects on apoptosis, differentiation in RAW 264.7 cells were observed at the 150 μW/cm^2 power density. These effects were accompanied by concurrent decreases in mRNA and protein levels of osteoclast-specific genes, including RANK, NFATc1, and TRACP. Furthermore, radiofrequency exposure at power density of 150 μW/cm^2 induced a significant decrease in cytoplasmic NF-κB protein levels while increasing its nuclear fraction, thereby counteracting the effects of RANKL-induced NF-κB activation. These data suggest that radiofrequency exerts inhibitory properties on RANKL-induced NF-κB transcriptional activity, subsequently indirectly suppressing the expression of downstream NF-κB target genes, such as NFATc1 and TRACP. In conclusion, our study demonstrates that radiofrequency radiation effectively inhibits osteoclast differentiation by modulating the NF-κB signaling pathway. These findings have important implications for potential therapeutic interventions in osteoporosis.

PLAIN LANGUAGE SUMMARY

- Osteoporosis is a common bone disease where bones become weak and brittle, often leading to fractures. It frequently occurs in older adults, especially postmenopausal women, due to low estrogen levels and inadequate calcium intake. This causes increased activity of bone cells called osteoclasts which break down bone tissue, resulting in severe bone loss. Currently, the primary treatment is long-term use of medications like bisphosphonates. However, these drugs can have side effects. The main adverse reactions include fever, vomiting, rash, diarrhea, dizziness, abdominal pain, musculoskeletal pain, headache, allergic-like reactions, indigestion, edema, and ocular symptoms.
- This study explored using radiofrequency (RF) radiation as a safe, non-invasive alternative therapy for osteoporosis. RF radiation is a type of energy used in communications like cell phones and WiFi. We tested whether exposure to 900MHz RF radiation could inhibit the formation and activity of osteoclasts to prevent excessive bone breakdown.
- We treated osteoclast precursor cells with RANKL, a protein that stimulates osteoclast formation. Cells were then exposed to RF radiation at various intensities. The results showed that medium-level RF radiation (150 μW/cm^2) significantly suppressed RANKL-induced osteoclast differentiation and bone resorption capacity. This effect was like the osteoclast inhibition seen with estrogen treatment.
- Further analysis revealed that RF radiation blocks the activation of NF-κB, a key signaling molecule that promotes osteoclast formation when RANKL is present. This in turn reduced production of downstream signals like NFATc1 and TRACP which are essential for osteoclast differentiation.
- In summary, this study demonstrates that medium-intensity RF radiation could potentially prevent excessive osteoclastic bone resorption in osteoporosis patients by interfering with NF-κB signaling cascade. The research highlights RF radiation's promise as a novel, non-invasive osteoporosis therapy.

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Radiofrequency field; osteoclast differentiation; RANKL; NF-κB; RAW 264.7 cells

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Introduction

Bone is a rigid and dynamic organ undergoing continuous remodeling orchestrated by a delicate balance between osteoblast-driven formation and osteoclast-mediated resorption. Disruptions in this equilibrium, particularly excessive bone resorption, could result in bone loss and the development of osteolytic diseases, such as postmenopausal osteoporosis (Boyle et al. 2003; Kim et al. 2012). Thus, the maintenance of both the normal quantity and function of osteoclasts plays a pivotal role in the orchestration of bone remodeling and the prevention of pathological bone degradation within the human body.

Osteoclasts, unique multinucleated macrophage-derived cells originating from hematopoietic stem cells, are exclusively responsible for the bone resorption process (Teitelbaum 2000). The Receptor Activator of Nuclear Factor-κB Ligand (RANKL) acts as the principal molecule regulating osteoclast differentiation. Additionally, the Receptor Activator of Nuclear Factor-κB (RANK), in conjunction with RANKL, has been identified as vital mediators in the process of osteoclast differentiation. The RANK/RANKL signaling pathway holds paramount importance in bone metabolism. The interaction between RANKL and RANK initiates differentiation of osteoclast precursor cells, consequently promoting the formation of mature multinucleated osteoclasts (Vega et al. 2007). Conditions such as osteoporosis are often characterized by an increase in both the number and resorption activity of osteoclasts. The excessive formation of mature multinucleated osteoclasts represents a pivotal event in the progression of osteoporosis.

At present, drug therapy is the principal approach to treating osteoporosis. However, prolonged utilization of chemical medications may result in adverse effects, including increased susceptibility to cardiovascular disease, esophageal cancer, and other ailments (McClung et al. 2013). In contrast, non-invasive physical therapy techniques such as low-frequency electromagnetic field, radiofrequency radiation, and ultrasound have shown promise in treating osteoporosis compared to traditional drug therapy. Radiofrequency radiation (RF) refers to non-ionizing radiation with a frequency range of 100 kHz to 300 GHz, characterized by strong penetration capabilities (Breckenkamp et al. 2003; Trock 2000). Several studies have indicated that RF could penetrate the bone marrow and affect the functionality of various hematopoietic cells (Cao et al. 2011; Gläser et al. 2016). Nuclear factor kappa-light-chain-enhancer activated B cells (NF-κB), a pivotal signaling molecule implicated in the process of osteoclast differentiation, can be activated by the RANK/RANKL system, thereby initiating its downstream signaling cascade and subsequently activating nuclear factor of activated T-cells cytoplasmic 1 (Nfatc1) (Boyce et al. 2015). Research has indicated that exposure to 900 MHz RF could impede the nuclear translocation of NF-κB in bone marrow stromal cells (Zong et al. 2019). Given that osteoclasts are derived from hematopoietic cell lines, it is plausible that RF could exert an influence on osteoclast formation. Despite the limited research into the impact of RF on osteoporosis, the precise effects and mechanisms of RF on this condition remain uncertain. Previous studies have confirmed that different doses of pulsed electromagnetic fields (PEMF) can reduce bone loss in osteoporotic rats (Androjna et al. 2021). Based on this, we hypothesize that RF, like PEMF, may exhibit energy-dependent effects on osteoclast differentiation. Therefore, this study aims to investigate the effects of RF on RANKL-induced osteoclast differentiation in RAW264.7 cells, as well as to elucidate the underlying mechanisms.

Materials and methods

Materials

RAW264.7 mouse macrophage cell line was purchased from Procell Company (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin and streptomycin were obtained from Hyclone (Cytiva, USA). Recombinant mouse soluble RANK ligand (RANKL) was obtained from Peprotech (Peprotech, USA). The Tartrate-resistant acid phosphatase (TRACP) & Alkaline Phosphatase (ALP) Double Stain Assay Kit was procured from Takara (Takara, Japan), while the Reverse Transcriptase Kit was acquired from Thermo Fisher Scientific (Thermo, USA). Scientific antibodies against RANK, NF-κB, NFATc1, TRACP, Histone-3 and GAPDH were purchased from Abcam (Abcam, USA). The Cell Counting Kit (CCK-8), Annexin V-FITC cell apoptosis analysis kit, BCA Protein Assay Kit, HRP-conjugated goat-anti-rabbit IgG secondary antibody and BAY11–7082 (a NF-κB inhibitor) were all purchased from Beyotime (Beyotime, China). The SYBR Green PCR master mix was purchased from Invitrogen (Invitrogen, USA), and the Nuclear Extraction Kit was obtained from Invent Biotechnologies (USA).

Radiofrequency fields

The selection of power intensities at 50, 150, and 450 μW/cm^2 for our RF exposure was based on China's national standards, with 400 μW/cm^2 as the safety limit at 900 MHz. The 50 μW/cm^2 level ensures safety, the 150 μW/cm^2 provides a moderate increase for comparison, and the 450 μW/cm^2 tests potential risk areas, offering a comprehensive assessment of RF effects on osteoclast differentiation. The radiofrequency radiation exposure system was described in detail earlier (Cao et al. 2010). The system utilized a continuous-wave 900 MHz RF signal, which was generated, amplified, and directed into a GTEM chamber (Giga-hertz Transverse Electro-magnetic chamber) via an antenna sourced from Southeast University, Nanjing, Jiangsu, China. The radiofrequency field within the GTEM chamber was meticulously assessed using a field strength meter (PMM, Cisano sul Neva, Italy) to precisely determine the positions that provided power intensities of 50, 150, and 450 μW/cm^2, as required for the experimental setup.

Cell culture and cell treatment

The RAW264.7 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS and antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin) at 37°C in a humidified atmosphere of 5% CO_2. For the osteoclast differentiation experiment, the cells were cultured in media containing 50 ng/mL of RANKL for 5 days, with the culture media being refreshed every other day. To evaluate the roles of NF-κB in mediating the effect of RF exposure, the cells were treated with NF-κB inhibitor BAY11–7082 (10 μM). To assess the effect of RF in osteoclastogenesis, 17β-Estrodiol (E2) was used as a positive control. All cells were divided into following groups with different treatments: Sham (kept in GTEM without RF transmission), RANKL (treated with 50 ng/mL of RANKL for 5 days), LRF+RANKL (subjected to RF exposure at power density of 50 μW/cm^2,4 h/d for 5d, in the presence of 50 ng/mL of RANKL), MRF+RANKL (subjected to RF exposure at power density of 150 μW/cm^2,4 h/d for 5d, in the presence of 50 ng/mL of RANKL), HRF+RANKL (subjected to RF exposure at power density of 450 μW/cm^2, 4 h/d for 5d, in the presence of 50 ng/mL of RANKL), E2+RANKL (treated with 10^{-7} mol/L of 17β-Estrodiol for 5 days in the presence of 50 ng/mL of RANKL), MRF (subjected to RF exposure at power density of 150 μW/cm^2,4 h/d for 5d), and RANKL +BAY11-7082 (treated with 10 μM of NF-κB inhibitor BAY11–7082 in the presence of 50 ng/mL of RANKL for 5 days). *Cell viability assay*

Cell viability was assessed using a Cell Counting Kit-8 assay, following the manufacturer's instruction. Briefly, the cells were seeded in 96-well plates at a density of 1000 cells per well, with three replicates for each group. After specific treatments, the culture medium was removed, then 100 μL of fresh medium containing 1/10 (v/v) CCK-8 reagent was added to each well. The plates were then incubated at 37°C for 4 hours. The optical density (OD) of each well was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Apoptosis assay

Cell apoptosis was assessed using Cell Apoptosis Assay Kit according to the manufacturer's instruction. Briefly, RAW264.7 cells were seeded in 6-well plates at a density of 2 × 10^4 cell per well, with three replicates for each group. After specific treatments, cells were collected and washed twice with FBS. The cells were then resuspended in 195 μL of 1× binding buffer and incubated with 5 μL Annexin Ⅴ-FITC and 10 μL of propidium iodide (PI) staining solution on ice for 10 min in the dark. The samples were subjected to flow cytometry analysis within 1 h.

Tartrate-resistant acid phosphatase (TRACP) staining assay

For the TRACP staining assay, cells were seeded in 24-well plates at a density of 8 × 10^3 cell per well, with three replicates for each group. After specific treatments, cells were fixed and subjected to TRACP staining, following the manufacturer's instructions, and then TRACP-positive multinucleated cells were counted under an inverted microscope (Olympus, Japan).

Real-time PCR analysis

Total RNA was extracted using Trizol method according to the manufacturer's instruction. Then 1 μg of RNA was reverse-transcribed to generate first-strand cDNA, utilizing a Reverse Transcriptase Kit as per the manufacturer's recommendations. Gene-specific amplification was carried out in an ABI 2720HT real-time PCR system.

The reaction system contained 1 μl of cDNA, 10 μl of SYBR Green PCR master mix and 200 nM of the appropriate oligonucleotide primers. All measurements were

performed in triplicate, following a standardized thermal cycling protocol: initial denaturation at 95°C for 6 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 min. To determine the relative expression levels of mRNA, a normalization process was applied using the 2-ΔΔCt method with GAPDH as the reference gene. The primers employed in this analysis were as follows: *Rank* (forward: CATCTTCGGCGTTTACTACAGG, reverse: TCCACTT-AGACTACTGCAAGCA); *Nfatc1* (forward: GGAGAGTCCGAGAATCGAGAT, reverse: TTGCAGCTAGGAAGTACGTCT); *TRACP* (forward: GCAACATCCCCTG-GTATGTG, reverse: GCAAAC GGTAGTAAGGGCTG); *GAPDH* (forward: AGGTC-GGTGTGAACGGATTTG, reverse: TGTAGACCA TGTAGTTGAGGTCA).

Western blot analysis

Western blot was performed to investigated the expression levels of cytoplasmic and nuclear proteins. The extraction of these proteins was carried out using Minute™ Cytoplastic and Nuclear Extraction Kit according to the manufacturer's instructions. The protein concentrations were determined using the BCA Protein Assay Kit. Equal amounts of cytoplasmic and nuclear proteins were loaded onto 10% sodium dodecyl sulfate-polyacrylamide for gel electrophoresis. Then, proteins were transferred onto a polyvinylidene difluoride membranes. To minimize nonspecific binding, membranes were blocked with 5% non-fat milk for 90 minutes at room temperature. The membranes were then incubated with primary antibodies overnight at 4°C. Afterward, the membranes were washed three times with Tris-buffered saline containing 1‰ Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C for 1 h. Following three additional washes, bands were detected using an enhanced chemiluminescence system (G: BOX, USA). Band density was analyzed using ImageJ software and standardized to the GAPDH signal. Densitometry analysis was performed, and results were normalized using GAPDH and Histone-3 (housekeeping genes) to correct for variations in protein loading. Results are presented as mean ± standard deviation from three independent experiments.

Statistical analysis

All the experiments were independently repeated three times and the data were expressed as the mean±S.D. One-way ANOVA and Dunnett Multiple Comparison Test from SPSS22.0 software were used for statistical evaluation and $p < 0.05$ was considered to be statistically significant.

Results

Radiofrequency exposure suppressed osteoclast cell viability

The effect of three different power densities of RF on the cell viability of RAW264.7 cells in the presence or absence of RANKL was investigated using CCK-8 assay. Our results showed that the RANKL group, LRF+RANKL group, and HRF+RANKL group did not exhibit significant inhibition of cell viability while both the MRF +RANKL group and the E2+RANKL group showed a significant decrease in cell viability compared to the sham group ($p < 0.05$). The results are shown in Figure 1. These findings suggest that neither low-dose (LRF) nor high-dose (HRF) radiofrequency radiation significantly affected cell viability. However, medium dose RF radiation was found to effectively inhibit cell viability in the presence of RANKL, exhibiting a comparable inhibitory effect to E2, which serves as a positive control for an osteoclast proliferation inhibitor.

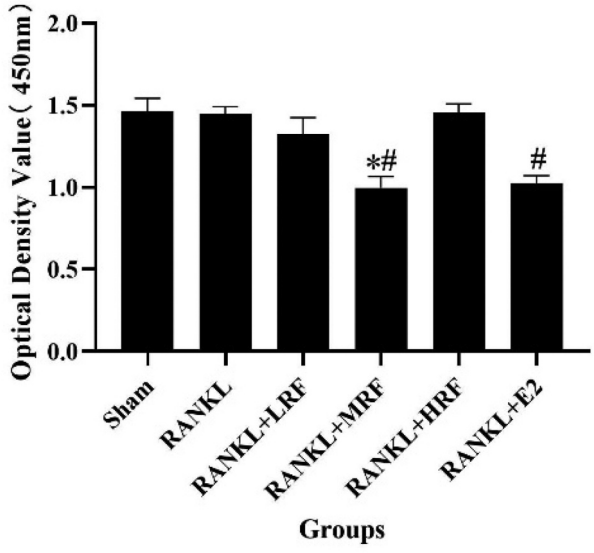


Figure 1. Effects of radiofrequency exposure on osteoclast cell viability. Sham: the sham group where the cells were placed in the exposure chamber but not subjected to any treatment; RANKL: an agent known necessary for osteoclast formation; LRF, MRF, and HRF represent low, medium, and high doses of RF radiation, respectively; E2: estrogen, a positive control for inhibition of osteoclast proliferation. The data presented in the figures are expressed as the mean ± SD. *Significantly different from Sham-exposed cells ($p < 0.05$). #Significantly different from cells treated with RANKL alone ($p < 0.05$).

RF exposure promoted RANKL-independent osteoclastic apoptosis in RAW264.7 cells

The effect of three different power densities of RF on the apoptosis of RAW264.7 cells in the presence of RANKL was investigated using an apoptosis assay. Our results showed that the apoptosis levels in the RANKL group were significantly higher than those in the Sham group ($p < 0.05$). Furthermore, treatment with three different doses of RF, as well as E2, resulted in significantly higher apoptosis levels compared to the RANKL group (Figure 2). These findings suggest that RF radiation at power density of 50, 150, and 450 μW/cm^2 could promote apoptosis during the process of RANKL-induced differentiation of RAW264.7 cells into

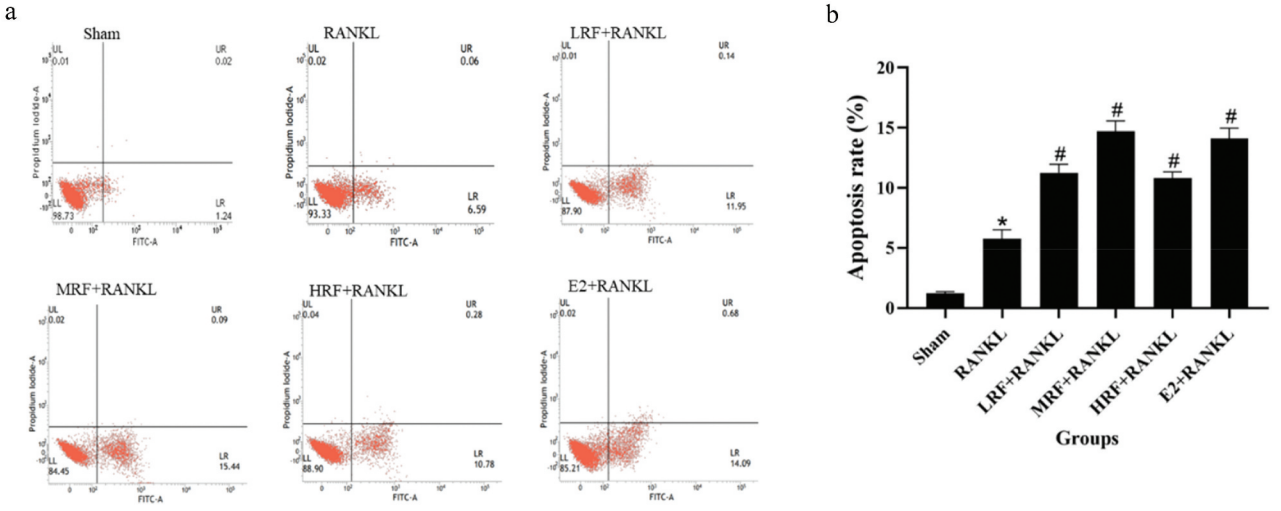


Figure 2. RF promoted rankl-independent apoptosis in RAW264.7 cells. (a) Cell was stained with annexin V/7-AAD and analyzed by flow cytometry following RF treatment in the presence of RANKL and E2. (b) The ratio of cell apoptosis was evaluated for each group. Sham: the control group where the cells were placed in the exposure chamber but not subjected to any treatment; RANKL: an agent known necessary for osteoclast formation; LRF, MRF, and HRF represent low, medium, and high doses of RF radiation, respectively; E2: estrogen, a positive control for inhibition of osteoclast proliferation. The data presented in the figures are expressed as the mean ± SD. *Significantly different from Sham cells ($P < 0.05$). #Significantly different from cells treated with RANKL alone ($P < 0.05$).

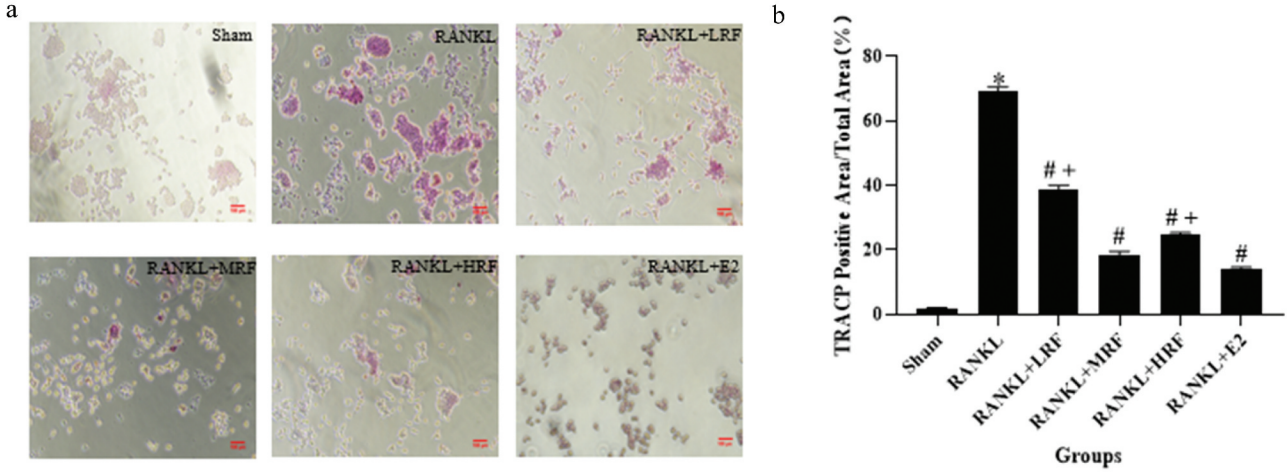


Figure 3. RF suppressed the rankl-dependent osteoclast differentiation in RAW264.7 cell. (a) Representative images of TRACP staining of RAW264.7 cells subjected to specific treatment (scale bar = 100 μm). (b) The ratio of TRACP-positive area to total area was evaluated for each group. Sham: the control group where the cells were placed in the exposure chamber but not subjected to any treatment; RANKL: an agent known necessary for osteoclast formation; LRF, MRF, and HRF represent low, medium, and high doses of RF radiation, respectively; E2: estrogen, a positive control for inhibition of osteoclast proliferation. The data presented in the figures are expressed as the mean ± SD. *Significantly different from Sham cells ($P < 0.05$). #Significantly different from cells treated with RANKL alone ($P < 0.05$). +Significantly different from cells treated with RANKL+MRF alone ($P < 0.05$).

osteoclasts. Among the three RF dose groups, the highest level of apoptosis was observed at the 150 μW/cm^2.

RF exposure suppressed the RANKL induced osteoclast differentiation in RAW264.7 cell

To examine the effects of RF exposure on the RANKL-induced osteoclast differentiation, RAW264.7 cells were subjected to RF at power densities of 50, 150, 450 μW/cm^2, 4 h/d for 5d, in the presence of 50 ng/mL of RANKL. Figure 3a shows that RANKL significantly induced TRCAP$^+$-osteoclast differentiation evidenced by the most substantial cell fusion area and a darker coloration compared with sham group. In contrast, the osteoclast differentiation exhibited varying degrees of decline in the three RF dose groups and the estrogen group, in comparison to the RANKL group. Particularly remarkable was the substantial decrease observed in the MRF+RANKL group among the three RF groups. Consistent with these results, the ratio of TRACP$^+$ area to total area was decreased in response to RF and estrogen exposure when compared to RANK exposure (Figure 3b). This data collectively underscores the profound impact of radiofrequency treatment on osteoclast differentiation, with implications for potential therapeutic interventions.

RF exposure suppressed the expression of osteoclastic-specific genes related to osteoclastic differentiation

The effect of RF exposure on RANKL-induced osteoclastic genes expression were examined using quantitative

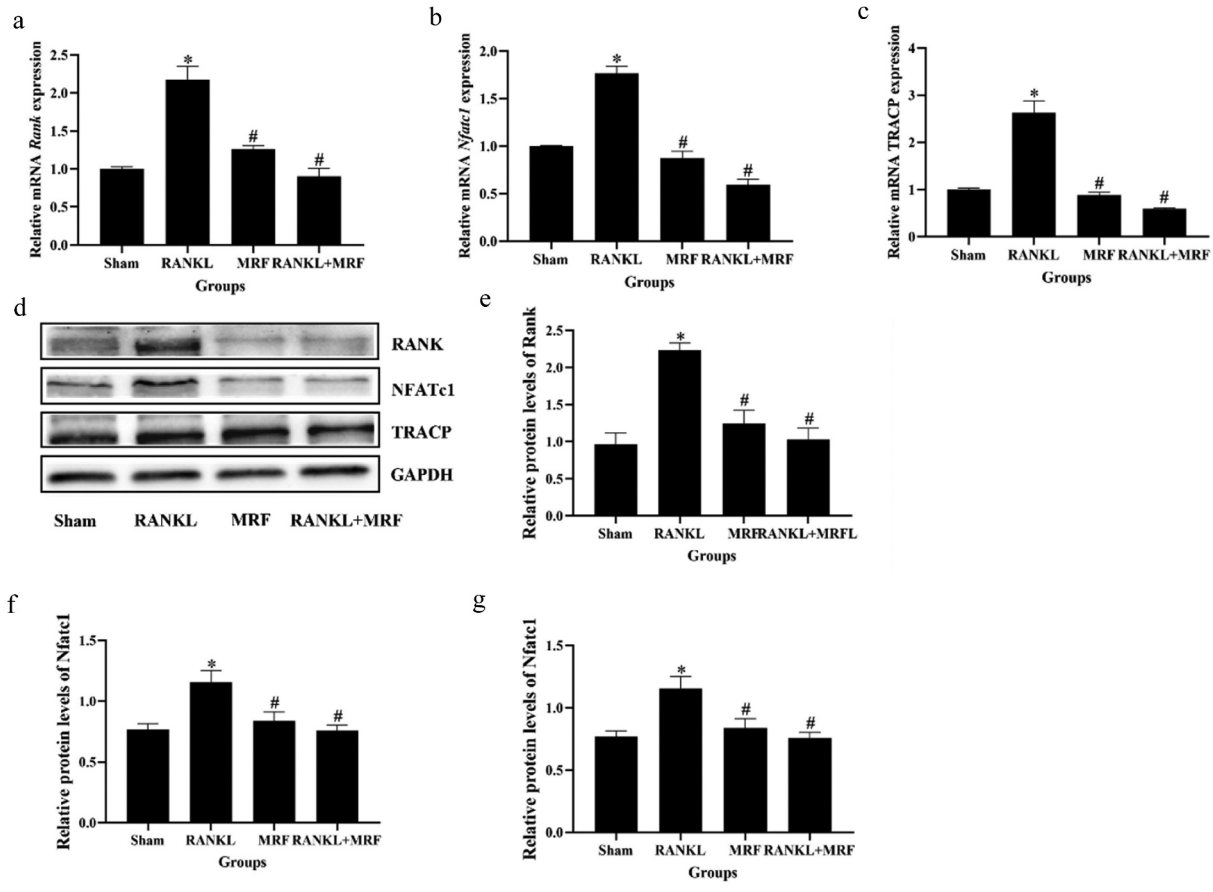


Figure 4. RF inhibited the expression of genes related to osteoclastic differentiation. Sham:the sham group where the cells were placed in the exposure chamber but not subjected to any treatment; RANKL: an agent known necessary for osteoclast formation; MRF represents medium dose of RF radiation; RANKL+MRF represents the group exposed to medium dose of RF radiation in the presence of RANKL. (A-C) RT-PCR analysis of *Rank*, *TRACP and Nfatc1* mRNA levels in RAW 264.7 cells exposed to RF while culturing with or without RANKL. (D) Western blot analysis of RANK, NFATc1 and TRACP protein in RAW 264.7 cells exposed to RF while culturing with or without RANKL. (E-G) The bar chart showed data normalized to house-keeping gene (GAPDH) and the gray values of RANK, NFATc1 and TRACP proteins were semi-quantitatively analyzed in each group. The data presented in the figures are expressed as the mean ± SD. *Significantly different from Sham-exposed cells ($P < 0.05$). #Significantly different from cells treated with RANKL alone ($P < 0.05$).

real-time PCR. Our results revealed a significant downregulation of osteoclastic marker genes, such as NFATc1, Rank, and TRACP, in response to RF exposure, as illustrated in Figure 4(a-c). Furthermore, western blot analysis confirmed a marked decrease in the protein levels of NFATc1, RANK, and TRACP in the RF-treated groups compared to the RANKL-only groups (Figure 4(d-g)). These findings provided further evidence supporting the hypothesis that RF exerts a suppressive effect on osteoclastic differentiation at the molecular level.

RF exposure inhibited rankl-induced activation of NF-κB signal pathway

To elucidate the underlying mechanism responsible for the inhibitory effect of RF, we further investigated whether RF impedes the activation of NF-κB induced by RANKL. The relative levels of NF-κB protein in both the cytoplasm and nucleus were assessed utilizing western blot analysis 4 h post RF exposure (Figure 5).

Notably, the NF-κB protein levels in the cytoplasm were significantly reduced in the presence of RANKL, concurrent with a corresponding increase in the nuclear fraction compared to the sham group. In contrast, the exposure of RF and the NF-κB inhibitor, BAY11–7082, effectively mitigated the activation effect of RANKL on nuclear translocation of NF-κB protein. These findings provided compelling evidence that RF possessed significant inhibitory properties against the RANKL-induced activation of NF-κB.

Furthermore, we conducted studies to assess the potential of RF in attenuating the activation of NFATc1 during osteoclastogenesis. Given the crucial role of NF-κB activation in the initial induction of NFATc1, we examined whether RF attenuate the activation of NFATc1 via the inhibition of NF-κB activation. Our results demonstrated that treatment with RANKL resulted in increased NFATc1 protein levels. However, the administration of RF and BAY11–7082 effectively suppressed the NFATc1 protein levels. Additionally, we observed a concomitant inhibition of TRACP protein levels following treatment with RF and BAY11–7082. These results not only demonstrate the inhibitory effect of RF on NF-κB but also showcase its potential to regulate downstream effectors like NFATc1 and TRACP, key players in osteoclast differentiation and

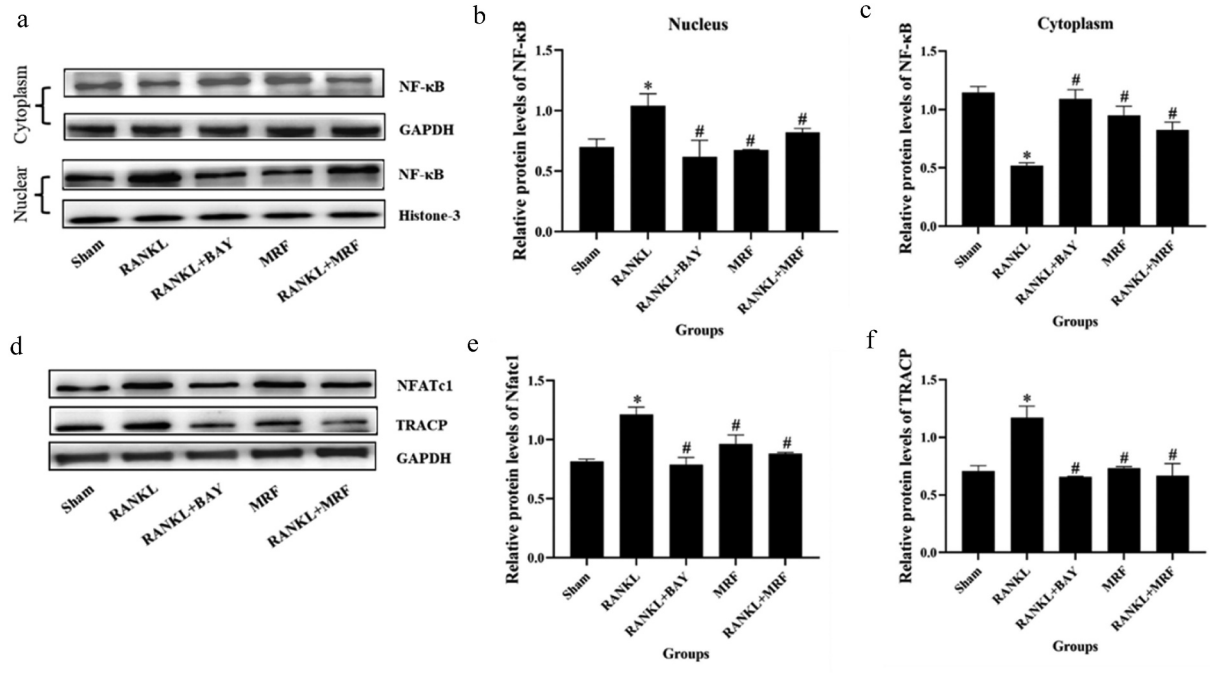


Figure 5. RF suppresses rankl-induced activation of nf-κB signal pathway. Sham:sham group where the cells were placed in the exposure chamber but not subjected to any treatment; RANKL: an agent known necessary for osteoclast formation; RANKL+BAY represents the group exposed to BAY11-7082, a NF-κB inhibitor in the presence of RANKL; MRF: medium dose of RF radiation; RANKL +MRF represents the group exposed to medium dose of RF radiation in the presence of RANKL. (A) Western blot analysis of NF-κB in cytoplasm and nucleus in RAW264.7 cells cultured in the presence of RANKL were exposed to specific treatment. (B-C) The bar graph shows data normalized to house-keeping gene (Histone-3) and the gray values of NF-κB proteins in nucleus and cytoplasm were semi-quantitatively analyzed in each group. (D) Western blot analysis of NFATc1 and TRACP in RAW264.7 cells cultured in the presence of RANKL were exposed to specific. (E-F) The bar chart showed data normalized to house-keeping gene (GAPDH) and the gray values of NFATc1 and TRACP proteins were semi-quantitatively analyzed in each group. The data presented in the figures are expressed as the mean ± SD. *Significantly different from Sham cells ($P < 0.05$). #Significantly different from cells treated with RANKL alone ($P < 0.05$).

bone resorption. These findings strongly supported the notion that RF possessed the ability to attenuate the RANKL-dependent osteoclast differentiation via NF-κB-NFATc1 signaling pathway.

Discussion

RF radiation is a form of electromagnetic radiation with a frequency range between 3 kHz and 300 GHz, which is known to interact with biological systems in various ways.

Studies have shown that high-dose RF radiation exposure can have deleterious effects on cellular and organismal health, including increased oxidative stress and DNA damage (Alkis et al. 2019; Delen et al. 2021). However, intriguingly, low-to-moderate levels of RF radiation exposure have been demonstrated to exert beneficial effects on cellular functions and overall health (He et al. 2017). These findings highlight the complex and context-dependent nature of RF radiation interactions with biological systems.

Osteoporosis is commonly observed in older adults, particularly postmenopausal women. Abrupt declines in estrogen and inadequate uptake of dietary calcium can lead to substantial activation of osteoclasts and excessive bone resorption, ultimately resulting in significant bone loss and the eventual development of osteoporosis (Compston et al. 2019). Given the detrimental effects of osteoporosis, a common bone disease characterized by reduced bone mass and increased risk of fractures, novel therapeutic strategies are urgently needed. Pulsed electromagnetic fields (PEMF) have been reported to impede the osteoclast formation induced by RANKL (Zhang et al. 2017). Our previous research demonstrated that the application of low-dose radiofrequency radiation could elicit adaptive responses in bone marrow stromal cells, thereby ameliorating the detrimental cellular consequences and mortality resulting from subsequent exposure to γ-radiation (Ji et al. 2016). In light of this context, our study investigated the inhibition effect of RF on osteoclastogenesis and the underlying mechanism.

In the context of RANKL-induced differentiation, RAW264.7 cells primarily exhibit differentiation rather than proliferation. Therefore, primarily focuses on assessing the progression of RANKL-induced differentiation rather than osteoclast proliferation. The results indicated that among the three RF exposure dosages, only RF exposure at 150 μW/cm^2 significantly inhibited cellular viability. However, it is essential to note that this inhibition of cellular viability was not necessarily attributed to cytotoxicity. Additionally, the results of apoptosis assay and TRACP staining presented in this study clearly demonstrated that three doses of RF could induce apoptosis of RAW264.7 cells in the differentiation process and caused a noteworthy inhibitory effect of RF on RANKL-induced osteoclast differentiation. Our study observed an increase in osteoclast apoptosis in response to RANKL, a finding that contrasts with some previous research suggesting that RANKL inhibits apoptosis. This discrepancy might be attributed to the complex interplay of various factors within the bone microenvironment. For instance, RANKL's interaction with receptors like leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4) could activate pathways that inhibit NF-κB, thereby promoting osteoclast apoptosis (Duan et al. 2020). Additionally, RANKL-induced osteoclast differentiation may lead to the release of transforming growth factor-β (TGF-β), which can induce apoptosis in mature osteoclasts (Bahia et al. 2020). Regardless, further research is needed to clarify the precise role of RANKL in osteoclast apoptosis and to elucidate the complex interplay of factors that govern this process. Therefore, these findings suggest that the reduction in viable cells observed in CCK-8 assay following RF exposure is attributed to RF's inhibition of RANKL-induced osteoclast differentiation rather than any cytotoxic effects. These findings align with the research conducted by Chang et al (Chang et al. 2005; Wang et al. 2017), which also demonstrated the efficacy of low-dose electromagnetic fields in preventing osteoclastogenesis in RAW264.7 cells.

In this study, we observed the inhibitory effects of different radiofrequency (RF) power intensities (50 μW/cm^2, 150 μW/cm^2, and 450 μW/cm^2) on osteoclast differentiation. Surprisingly, only the 150 μW/cm^2 RF group exhibited an inhibitory effect comparable to the positive control group treated with E2. This observation suggests that the most effective inhibition was achieved by 150 μW/cm^2 900 MHz RF. Meanwhile, this also suggested that the effect of RF on osteoclastogenesis did not follow a linear dose-response relationship, instead, it displayed a unique "window effect" where specific RF intensities yield more significant biological effects than lower or higher intensities. The precise mechanism underlying this phenomenon remains uncertain, nevertheless, it is plausible that it is associated with the resonance between RF at specific power intensity and distinct molecular activities (Cao and Tong 2007). The "window effect", a distinctive characteristic of RF, offers significant advantages in the realm of medical applications. By pinpointing the optimal power intensity that yields the most substantial beneficial impact, one can potentially avoid the adverse consequences arising from high-dose interventions, while striving for optimized therapeutic outcomes.

To further validate the impact of RF on RANKL-induced the osteoclasts differentiation, we evaluated the expression of key markers associated with osteoclast differentiation, namely RANK and TRACP. RANK is primarily expressed in osteoclast precursor cells and mature osteoclasts within the skeletal system, and serves as the exclusive target for RANKL (Boyce and Xing 2007). Upon binding to RANKL, RANK directly stimulates the process of osteoclast differentiation, resulting in an increase in its differentiation capacity. TRACP is also a key marker of osteoclast differentiation and plays an important role in bone resorption (Nowak et al. 2008). In this study, it was observed that exposure to 900 MHz RF at 150 μW/cm^2 resulted in a significant decrease in both *Rank* and *TRACP* mRNA levels, as well as protein levels in RANKL-treated RAW264.7 cells. Our findings are consistent with Lei's research, which found that electromagnetic fields of high-frequency (3000–50,000 Hz) and mixed-frequency (1–50,000 Hz) impeded osteoclast differentiation (Lei et al. 2018). These findings suggested that RF could inhibit the expression of differentiation markers in RANKL-induced osteoclasts, leading to a reduction in their differentiation capacity and consequents impairment of their bone resorption function.

NF-κB signal pathway plays a crucial role in osteoclast differentiation within the RANK/RANKL signal system. When RANKL and RANK are absent, increased expression of NF-κB leads to the differentiation of bone marrow stromal cells into osteoclasts [288]. NF-κB knockout mice exhibit bone sclerosis due to their inability to generate osteoclasts (Franzoso et al. 1997). Previous study demonstrated that aconitine could inhibit the differentiation of RAW264.7 cells induced by RANKL through the inhibition of NF-κB signaling pathway (Zeng et al. 2016). NF-κB typically forms a complex with its inhibitor IκBα, rendering it inactive within the cytoplasm. However, upon encountering inflammatory factors, radiation, or other stimulating signals, IκBα undergoes phosphorylation by IκB kinase (IKK), leading to the translocation of NF-κB from the cytoplasm to the nucleus. Once in the nucleus, NF-κB binds to a promoter region, thereby augmenting the expression of numerous genes (Novack 2011; Pordanjani and Hosseinimehr 2016; Sun et al. 2020). Therefore, the suppression of NF-κB activation has the potential to impede the process of osteoclast formation and differentiation (Novack 2011). Our results demonstrated that RF exposure at 150 μW/cm^2 power density effectively suppressed NF-κB expression and impeded its translocation from the cytoplasm to the nucleus. These results suggested that RF has the ability to hinder RANKL-induced osteoclastogenesis by obstructing the nuclear translocation of NF-κB. During osteoclast differentiation, RF inhibits RANKL-induced differentiation by downregulating NF-κB expression. Accordingly, we further conducted an additional analysis on the expression of genes associated with osteoclastic differentiation, which are regulated by NF-κB.

NFATc1, functioning as a downstream signal molecule of NF-κB, plays a pivotal role as a transcription factor in the process of osteoclast differentiation induced by RANKL. It is reported that embryonic stem cells lacking *Nfatc1* failed to undergo osteoclast differentiation (Hiroshi et al. 2002). Additionally, research has documented that PEMF has the potential to reduce *NFATc1* mRNA expression (Kim et al. 2012). In this study, we found that exposure to 150 μW/cm^2 of 900 MHz RF decreased *NFATc1* mRNA expression induced by RANKL treatment at the similar level exerted by BAY11–7082, a NF-κB inhibitor. Meanwhile, it was observed that the exposure of both 150μW/cm^2 900 MHz RF and BAY11–7082 effectively hindered the decrease in TRACP protein expression induced by RANKL. Therefore, the results suggested that the application of 150 μW/cm^2 900 MHz RF could impede the differentiation of osteoclasts by suppressing the translocation of NF-κB to the nucleus, inhibiting the expression of NF-κB-regulated transcription factor NFATc1, and subsequently reducing the expression of genes associated with osteoclast differentiation.

In conclusion, our results indicated that exposure to 900 MHz RF at a power density of 150 μW/cm^2 inhibited RANKL-induced differentiation of RAW264.7 cells into osteoclasts by inhibiting NF-κB nuclear translocation. The results suggest that RF may be a potentially non-invasive and effective therapeutic approach for addressing osteoporosis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors' contributions

Caihua Ding and Haiying Wang: cell experiment and writing. Chunyu Yang ang Yang Hang: cell experiment. Shunxing Zhu: revision. Yi Cao: design and revision.

References

Alkis, M. E., H. M. Bilgin, and V. Akpolat. 2019. Effect of 900-, 1800-, and 2100-MHz radiofrequency radiation on DNA and oxidative stress in brain. *Electromagn. Biol. Med.* 38:32–47. doi:10.1080/15368378.2019.1567526.

Androjna, C., C. S. Yee, and C. R. White. 2021. A comparison of alendronate to varying magnitude PEMF in mitigating bone loss and altering bone remodeling in skeletally mature osteoporotic rats. *Bone*. 143:115761. doi:10.1016/j.bone.2020.115761.

Bahia, M. T., M. B. Hecke, and E. G. F. Mercuri. 2020. A bone remodeling model governed by cellular micromechanics and physiologically based pharmacokinetics. *J. Mech. Behav. Biomed. Mater.* 104:103657. doi:10.1016/j.jmbbm.2020.103657.

Boyce, B. F., and L. P. Xing. 2007. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res. Theraphy.* 1:S1. 9 Suppl. doi:10.1186/ar2165.

Boyce, B. F., Y. Xiu, and J. B. Li. 2015. Nf-κB-Mediated regulation of Osteoclastogenesis. *Endocrinol. Metab.* 30:35. doi:10.3803/EnM.2015.30.1.35.

Boyle, W. J., W. S. Simonet, and D. L. Lacey. 2003. Osteoclast differentiation and activation. *Nature*. 423:337–42. doi:10.1038/nature01658.

Breckenkamp, J., G. Berg, and M. Blettner. 2003. Biological effects on human health due to radiofrequency/microwave exposure: A synopsis of cohort studies. *Biophysik.* 42:141–54. doi:10.1007/s00411-003-0203-x.

Cao, Y., and J. Tong. 2007. Advances in research on biological effects of electromagnetic radiation. *Sch. Radiat. Medice. Public. Health.* 24:222–26.

Cao, Y., Q. Xu, and Z. D. Jin. 2010. Effects of 900-MHz microwave radiation on gamma-ray-induced damage to mouse hematopoietic system. *J. Toxicol. Environ. Health, Part A*. 73:507–13. doi:10.1080/15287390903523451.

Cao, Y., Q. Xu, and Z. D. Jin. 2011. Induction of adaptive response: Pre-exposure of mice to 900 MHz radiofrequency fields reduces hematopoietic damage caused by subsequent exposure to ionising radiation. *Int. J. Radiat. Biol.* 87:720–28. doi:10.3109/09553002.2010.550981.

Chang, K., S. Huang, and S. Huang. 2005. Pulsed electromagnetic fields stimulation affects osteoclast formation by modulation of osteoprotegerin, RANK ligand and macrophage colony-stimulating factor. *J. Orthopaedic Res.:* 23:1308–14. doi:10.1016/j.orthres.2005.03.012.

Compston, J. E., M. Mr, and W. D. Leslie. 2019. Osteoporosis. *Lancet*. 393:364–76. doi:10.1016/S0140-6736(18)32112-3.

Delen, K., B. Sırav, and S. Oruç. 2021. Effects of 2600 MHz radiofrequency radiation in brain tissue of male Wistar rats and neuroprotective effects of Melatonin. *Bioelectromagnetics* 42:159–72. doi:10.1002/bem.22318.

Duan, L., Y. Liang, and X. Xu. 2020. Noncoding RNAs in subchondral bone osteoclast function and their therapeutic potential for osteoarthritis. *Arthritis. Res. Ther.* 22:279. doi:10.1186/s13075-020-02374-x.

Franzoso, G., L. Carlson, and L. Xing. 1997. Requirement for nf-kappaB in osteoclast and B-cell development. *Genes Devel[J].* 11:3482–96. doi:10.1101/gad.11.24.3482.

Gläser, K., M. Rohland, and T. K. Ostmann. 2016. Effect of radiofrequency radiation on human hematopoietic stem Cells. *Radiat. Res.* 186:455–65. doi:10.1667/RR14405.1.

He, Q. N., L. Zong, and Y. L. Sun. 2017. Adaptive response in mouse bone marrow stromal cells exposed to 900 MHz radiofrequency fields: Impact of poly (adp-ribose) polymerase (PARP). *Mutat. Res./Genetic Toxicol. Environ. Mutagen.* 2017:19–25. doi:10.1016/j.mrgentox.2017.05.007.

Hiroshi, T., K. Sunhwa, and K. Takako. 2002. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell.* 3:889–901. doi:10.1016/S1534-5807(02)00369-6.

Ji, Y. X., Q. N. He, and Y. Cao. 2016. Adaptive response in mouse bone-marrow stromal cells exposed to 900-MHz radiofrequency fields: Gamma-radiation-induced DNA strand breaks and repair. *J. Toxicol. Environ. Health. Part A*. 79:419–26. doi:10.1080/15287394.2016.1176618.

Kim, H. S., K. S. Suh, and S. K. Lee. 2012. The inhibitory effect and the molecular mechanism of glabridin on rankl-induced osteoclastogenesis in RAW264.7 cells. *Int. J. Mol. Med.* 29:169–77. doi:10.3892/ijmm.2011.822.

Lei, T., F. J. Li, and Z. W. Liang. 2018. *In vitro and vivo studies on the effects of electromagnetic fields with different frequency spectrum bands on ovariectomized osteoporosis in mice [J]*. Air Force Medical University.

McClung, M., S. T. Harris, P. D. Miller, D. C. Bauer, K. S. Davison, L. Dian, D. A. Hanley, D. L. Kendler, C. K. Yuen, and E. M. Lewiecki. 2013. Bisphosphonate therapy for osteoporosis: Benefits, risks, and drug holiday. *Am. J. Med.* 126:13–20. doi:10.1016/j.amjmed.2012.06.023.

Novack, D. V. 2011. Role of nf-κB in the skeleton. *Cell. Res.* 21:169–82. doi:10.1038/cr.2010.159.

Nowak, Z., M. Konieczna, and M. Saracyn. 2008. Tartrate resistant acid phosphatase–TRACP-5b as a modern bone resorption marker. *Polski Merkuriusz Lekarski: Organ Polskiego Towarzystwa Lekarskiego[J]* 142:351–54.

Pordanjani, S. M., and S. J. Hosseinimehr. 2016. The role of nf-kB inhibitors in cell response to radiation. *Curr. Med. Chem.* 23:3951–63. doi:10.2174/0929867323666160824162718.

Sun, X. W., Z. A. Xie, and B. Hu. 2020. Teitelbaum SL. The Nrf2 activator RTA-408 attenuates osteoclastogenesis by inhibiting STING dependent nf-κb signaling. *Redox. Biol.* 28:101309. doi:10.1016/j.redox.2019.101309.

Teitelbaum, S. L. 2000. Bone resorption by osteoclasts. *Science* 289:1504–08. doi:10.1126/science.289.5484.1504.

Trock, D. H. 2000. Electromagnetic fields and magnets. Investigational treatment for musculoskeletal disorders. *Rheumatic. Disease. Clinics. North. Am.* 26:51–62. viii.10.1016/S0889-857X(05)70119-8.

Vega, D., N. M. Maalouf, and K. Sakhaee. 2007. The role of receptor activator of nuclear factor-kappaB (RANK)/RANK ligand/osteoprotegerin: Clinical implications. *[J]. J. Clin. Endocrinol. Metab.* 92:4514–21. doi:10.1210/jc.2007-0646.

Wang, P., J. Liu, and Y. F. Yang. 2017. Differential intensity-dependent effects of pulsed electromagnetic fields on rankl-induced osteoclast formation, apoptosis, and bone resorbing ability in RAW264.7 cells. *Bioelectromagnetics* 38:602–12. doi:10.1002/bem.22070.

Zeng, X. Z., L. G. He, and S. Wang. 2016. Aconine inhibits rankl-induced osteoclast differentiation in RAW264.7 cells by suppressing nf-κB and NFATc1 activation and DC-STAMP expression. *Acta. Pharmacologica. Sin.* 37:255–63. doi:10.1038/aps.2015.85.

Zhang, J., H. X. Xu, and Z. Y. Han. 2017. Pulsed electromagnetic field inhibits rankl-dependent osteoclastic differentiation in RAW264.7 cells through the Ca2þ-calcineurinNfatc1 signaling pathway. *Biochem. Biophys. Res. Commun.* 2017:289–95. doi:10.1016/j.bbrc.2016.11.056.

Zong, L., Z. Gao, and W. Xie. 2019. Role of nf-κB activation in mouse bone marrow stromal cells exposed to 900-MHz radiofrequency fields (RF). *J. Toxicol. Environ. Health. Part A.* 82:46–51. doi:10.1080/15287394.2018.1553372.