1–2 T static magnetic field combined with Ferumoxytol prevent unloading-induced bone loss by regulating iron metabolism in osteoclastogenesis

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\textbf{ABSTRACT}

\textbf{Objective:} With the deepening of magnetic biomedical effects and electromagnetic technology, some medical instruments based on static magnetic field (SMF) have been used in orthopedic-related diseases treatment. Studies have shown SMF could combat osteoporosis by regulating the differentiation of mesenchymal stem cells (MSCs), osteoblast and osteoclast. With the development of nanotechnology, iron oxide nanoparticles (IONPs) have been reported to regulate the process of bone anabolism. As for SMF combined with IONPs, studies indicated osteogenic differentiation of MSCs were promoted by the combination of SMF and IONPs. However, there are few reports on the effects of SMF combined with IONPs on osteoclast. Herein, the purpose of this study was to investigate the effects of high static magnetic field (HiSMF) combined with IONPs on unloading-induced bone loss in vivo and osteoclastic formation in vitro, and elucidated the potential molecular mechanisms.

\textbf{Methods:} \textit{In vivo}, C57BL/6 J male mice were unloaded via tail suspension or housed normally. The hindlimb of mice were fixed and exposed to 1–2 T SMF for 1 h every day, 10 mg/kg of Ferumoxytol or saline were injected by tail vein once a week, last for 4 weeks. Bone microstructure, mechanical properties, and osteoclastogenesis were examined respectively. \textit{In vitro}, the RAW264.7 cells were used to assess the effects of 1–2 T SMF combined with IONPs in osteoclastogenesis. The iron content was detected by atomic absorption spectrometry and Prussian blue staining. DCFH-DA and MitoSOX™ fluorescence staining were used to assess oxidative stress levels. NF-κB and MAPK signaling pathways were examined by western blot assay.

\textbf{Results:} \textit{In vivo}, the results showed 1–2 T SMF and IONPs prevented the damage to bone microstructure and improved the mechanical properties, diminished the number of osteoclasts in unloaded mice, 1–2 T SMF combined with IONPs was found more effective. The iron content in the liver and spleen was reduced by the combination of 1–2 T SMF and IONPs, enhancing iron levels in the femur. \textit{In vitro}, osteoclast formation was inhibited by 1–2 T SMF and IONPs treatment, and 1–2 T SMF combined with IONPs had a more pronounced effect. Moreover, iron uptake of IONPs in osteoclast was reduced to 1–2 T SMF exposure. Oxidative stress levels were decreased in osteoclast differentiation under 1–2 T SMF combined with IONPs treatment. Molecularly, the expression of NF-κB and MAPK signaling pathways were inhibited under 1–2 T SMF combined with IONPs in osteoclastogenesis.

\textbf{Abbreviations:} BMC, Bone mineral content; BMD, Bone mineral density; BV/TV, Bone volume/tissue volume; Ct.Ar, Cortical area; Ct.Ar/T.Ar, Cortical area to total area; FTTH1, Ferritin heavy polypeptide 1; FPN, Ferroprotein; HiSMF, High static magnetic fields; HyMF, Hypomagnetic field; IONPs, Iron oxide nanoparticles; MAR, Mineral apposition rate; MDA, Malondialdehyde; MMP9, Matrix metalloproteinase-9; MRI, Magnetic resonance imaging; MSCs, Mesenchymal stem cells; NFATC1, Nuclear factor of activated T cells 1; N.Ob/BS, The number of osteoblast per bone surface; N.Oc/BS, The number of osteoclast per bone surface; O VX, Ovariectomized; RANKL, Receptor activator of nuclear factor κB ligand; ROI, Region of interest; ROS, Reactive oxygen species; SMF, Static magnetic field; SOD, Superoxide dismutase; SR-A I/II, Scavenger receptors type A I/II; T-AOC, Total Antioxidant Capacity; Th.N, Trabecular number; Tb.Sp, Trabecular separation; Tb.Th, Trabecular thickness; TIR1, Transferrin receptor 1; TMD, Tissue mineral density; Tt.Ar, Total cross-sectional area; TRAP5b, Tartrate-resistant acid phosphatase 5b.

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Osteoporosis is a common multifactor bone disease characterized by low bone mass, decreased bone density, impaired bone microstructure, increased bone fragility, and fracture [1]. Bone remodeling occurs throughout life to support the integrity of the skeleton. During bone remodeling, the old and damaged bone are removed by osteoclasts (bone resorption) and new bone is yielded by osteoblasts (bone formation) [2]. Osteoporosis can be divided into primary and secondary osteoporosis. Primary osteoporosis is caused by aging or decreased secretion of sex hormones, such as postmenopausal osteoporosis [3]. Conversely, secondary osteoporosis is usually caused by other health problems, such as disuse osteoporosis. Disuse osteoporosis results from systemic or local reductions in bone mineral content due to practice limitation, functional impairment, and weightlessness. The main pathogenesis is due to the reduction of stress stimulation on bones, enhancement of bone resorption and inhibition of bone formation, resulting in an imbalance in the regulation of bone remodeling, which eventually leads to bone loss, microstructure damage and increases the incidence of fragility fractures. Disuse osteoporosis is prevalent in the conditions of prolonged bed rest, immobilization, spinal cord injury, muscle atrophy, and weightlessness [4,5]. In previous research, most of them were about the analysis of osteoporosis in the ground population. Considering long-term space-flight-induced bone loss as a major obstacle for astronauts in space missions, the reduction of mechanical stimulation induced by spatial weightlessness is believed to be the major factor in causing spatial bone loss [6]. The hindlimb unloading (HLU) model is used in animal experiments to simulate the weightlessness of astronauts in spaceflight [7]. Nowadays, in addition to drugs, the treatment for osteoporosis is also included physical therapy.

Static magnetic field (SMF) refers to the magnetic field with magnetic induction intensity and direction do not change with time, usually generated by permanent magnet or constant current. The SMF can be divided into hypomagnetic field (HyMF) (<5 μT), weak magnetic field (5 μT-1 mT), moderate SMF (1 mT-1 T) and high SMF (HiSMF) (>1 T) according to the magnetic induction intensity [8]. Our previous results found that osteoblast differentiation was inhibited [9] and osteoclast differentiation was promoted by HyMF in vitro [10], the unloading induced bone loss were aggravated and the recovery of unloading induced bone loss was also inhibited by HyMF in vivo [11–13]. Considerable evidence has demonstrated that a certain intensity of moderate SMF has positive effects on the skeletal system. Chen et al. [14] found that BMSCs were treated with 0.2–0.6 T moderate SMF for 3 days and 21 days respectively. The expression of ALP and the formation of mineralized nodules for osteogenic differentiation was promoted. Yamamoto et al. [15] found the formation of mineralized nodules was significantly promoted in primary rat osteoblasts treated with 160 mT SMF for 21 days. In addition, Kim et al. [16] showed that 15 mT SMF treated BMsMs for 6 days, and the osteoclast differentiation was inhibited. Our previous study found that 0.2–0.4 T SMF treatment for 4 weeks could promote the recovery of HLU-induced bone loss in mice [17]. In recent years, with the application of new technologies such as magnetic resonance imaging (MRI), the possibility of human exposure to HiSMF has gradually increased, which has promoted the investigation of the biological effects of HiSMF. Kotani H et al. [18] found that the differentiation of MC3T3-E1 osteoblast was accelerated via 8 T SMF in vitro. Our previous research found that osteoblast mineralization was promoted and the osteoclast differentiation was inhibited by 16 T SMF in vitro [9,19], and bone microstructure and mechanical properties were improved under 2–4 T SMF in vivo [20]. However, previous studies have exposed the whole body of the animal to SMF, and few studies have focused on localized exposure of the legs to SMF.

With the rapid development of nanotechnology, nanomaterials are used in biological detection, disease diagnosis and treatment [21]. Iron oxide nanoparticles (IONPs) have been used in the bone tissue engineering research field due to their positive effects in vivo. For example, IONPs are not only used as drug delivery systems, but also participate in bone metabolism to exert therapeutic effects [22]. Studies have shown that osteoclastic differentiation was promoted by hydroxyapatite-coated Fe3O4 IONPs [23]. Up to now, the FDA has approved a superparamagnetic IONPs Ferumoxytol (Feraheme™, AMAG Pharmaceuticals Inc.) for the treatment of iron deficiency anemia in patients with chronic kidney disease, also as an MRI contrast agent [24]. Liu L et al. [25] found that the ovariectomized (OVX) induced bone loss was ameliorated by blocking osteoclast formation following Ferumoxytol treatment. In addition, studies have shown that Fe3O4@PSC nanoparticles could promote osteogenic differentiation and inhibit osteoclast differentiation by scavenging reactive oxygen species (ROS), and improved the bone microstructure in OVX mice [26,27]. However, there are few studies on the effects of IONPs on unloading-induced bone loss.

IONPs have magnetic responsiveness and biocompatibility that other non-magnetic materials do not possess. Therefore, IONPs can be rapidly magnetized and targeted to a specific site to exert their medicinal effect under an external magnetic field. At present, IONPs is used in precisely targeted therapy of tumors. For example, SMF can target IONPs to tumor parts and inhibit tumor growth [28]. Our previous studies were also found HSMF could target iron preparations into tumor cells. In recent years, SMF targeting nanoparticles have gradually increased in bone tissue engineering research. Studies have shown that the osteogenic differentiation of osteoblast and mesenchymal stem cells (MSCs) were promote by the combination of SMF and magnetic nanoparticles, synergistically promoted osteogenesis in bone defects mice [29–31]. However, there are few reports on the effects of HSMF combined with IONPs on osteoclast. Herein, the aim of this study to investigate the effects of 1–2 T SMF combined with Ferumoxytol on unloading-induced bone loss in vivo and on osteoclast formation in vitro, and elucidated the underlying molecular mechanism.

2. Materials and methods

2.1. SMF exposure systems

In this experiment, a small magnet device of the Halbach array was used. The overall structure is a single hollow cylindrical magnet, and the cavity in the middle of the magnet is used for mice and cells exposure experiment. The diameter of the magnet inner chamber was 30 mm, and the magnetic induction intensity of the exposed area was 1–2 T. In animal experiment, the hindlimb of the mice was fixed and placed in the magnet inner chamber. In cells experiment, the cell culture dishes were placed in the magnet inner chamber (Fig. 1).
In this study, we used eight-week-old male C57BL/6 J mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were fed with standard food and water provided ad libitum, and kept at a controlled temperature, with a 12 h light/dark cycle. All experiment mice were randomly divided into eight groups: Ctrl, HLU, Ctrl + Fera, HLU + Fera, Ctrl + HiSMF, HLU + HiSMF, Ctrl + Fera + HiSMF, HLU + Fera + HiSMF (n = 8). During the unloading, the hindlimb of mice were immobilized and exposed to 1–2 T SMF for 1 h every day, and 10 mg/kg of Ferumoxytol or saline were injected via tail vein once a week, last for 4 weeks. On day 28 of the experiment, all mice were anesthetized and scanned for bone density, and the blood samples were collected by cardiac puncture. The femurs were used for micro-CT scanning or Histomorphometry analysis, and the tibias were collected for biomechanics analysis. All laboratory animals were approved by the Laboratory Animal Ethics and Welfare Committee of Northwestern Polytechnical University.

2.3. BMD and BMC evaluation

Dual-energy X-rays absorptiometry (DXA, InAlyzer; MEDIKORS, Seongnam, Korea) was performed to evaluate bone mineral content (BMC) and bone mineral density (BMD) of femur and tibia in vivo. On day 28 of the experiment, all mice were anesthetized and placed in an absorbance analyzer to scan the femur and tibia by DXA, and the results were analyzed by using digital imaging software.

2.4. Micro-CT analysis

The femur was measured by using a Micro-computed tomography scanner (SkyScan-1176CT, Bruker; USA). Scanning was set under 80 KV, 305 mA, 525 ms, and a resolution of 8.96 μm/pixel. Three-dimensional reconstruction images and data-analysis were detected by using NRecon and CTAn software respectively. The region of interest (ROI) was selected and analyzed by CTAn software, including trabecular bone and cortical bone analysis area. The main parameters of trabecular included
bone volume fraction (BV/TV), trabecular separation (Th.Sp), trabecular number (Th.N) and trabecular thickness (Th.Th). Cortical parameters included cortical area (Cl.Ar), cortical area fraction (Cl.Ar/TL.Ar), tissue mineral density (TMD) and total cross-sectional area (TL.Ar).

2.5. Mechanical properties

The mechanical properties of tibia were evaluated by three-point bending test using a universal material testing machine (Instron 5943; Instron, MA). In brief, the tibia was fixed on the two supports of the testing machine, and the bending load rate was 1 mm/min until the tibia fracture. Then, the inner and outer diameters in the fracture were detected by using digital microscope KH-8700 (HIROX, Japan). The stiffness and the ultimate load were calculated from the load–displacement curve, and the ultimate stress and elastic modulus were calculated through the stress–strain curve.

2.6. Histological and histomorphometry analysis

The femur was decalcified in 10% EDTA (replaced with fresh solution every 3 days) for 3 weeks. Then, the samples were embedded in paraffin and sectioned at 5 μm thick through using a semiautomated rotary microtome (Leica Biosystems RM2245, Nussloch, Germany). The sections were stained by H&E and TRAP kit to examine the number of osteoblast (N.Ob/BS) and osteoclast (N.Oc/BS). All mice were intraperitoneally injected with calcine at 10 and 3 days before euthanasia. The femurs were fixed in 4% paraformaldehyde for 2 days, embedded in methylmethacrylate and sectioned at 50 μm. Fluorescence microscope and ImageJ software were used to analyze the histomorphometric parameter.

2.7. Biochemical assay

The blood samples of mice were collected and centrifuged with 3000 rpm for 10 min at 4 °C, separated the serum and stored at −80 °C. The contents of serum tartrate-resistant acid phosphatase 5 b (TRAP5b) were detected by ELISA kit ((Jianglai Biotech, Shanghai, China).

2.8. Cell culture

The pre-osteoclast RAW264.7 cells were used in this study and cultivated in α-minimum essential medium (αMEM, Gibco, USA), supplemented with 2 mM t-glutamine, 10% Fetal Bovine Serum (FBS, Gibco) and 1% penicillin–streptomycin (Beyotime) at 37 °C with 5% CO2 humid atmosphere. The medium was added with 50 ng/ml RANKL (PeproTech, Rocky Hill) and changed every 2 days to induce osteoclast differentiation.

2.9. Osteoclast differentiation assay

RAW264.7 cells were seeded in 18 mm dishes with 2 × 10^4 cells per well. After overnight adherence, the medium was added with 50 ng/ml RANKL and different concentrations of Ferumoxytol. Then, the dishes were placed in geomagnetic field (GMF) as a control and 1–2 T SMF for 4 days, and the medium was changed every 48 h. Osteoclast formation was detected by TRAP staining Leukocyte Acid Phosphatase Kit (Sigma–Aldrich). F-actin Filament formation was examined by rhodamine-labeled phallolidin staining overnight at 4 °C.

Osteoclast bone resorption function was assessed by seeding RAW264.7 cells in 96-well Corning Osteo Assay Plate (Coring) with approximately 3000 cells per well. As similar with the previous exposure method, after 10 days of induction, the medium was removed and the cells were bleached in 10% sodium hypochlorite for 5 min at room temperature, and the wells were washed and dried. The resorption pits were observed by using light microscopy (Nikon) and analyzed by ImageJ software.

2.10. Iron content measurements

The levels of iron in the liver, spleen, femur, and serum were evaluated by atomic absorption spectrometry (AAS; Analytik Jena, AG Germany). Bone tissue samples were dried at 180 °C and dry weight were measured. Then, they were placed in a resistance furnace at 600 °C for ashing. Finally, dissolved with 65% nitric acid and iron contents of tissue were detected by atomic absorption. Moreover, iron content in osteoclast was detected by atomic absorption spectrometry and Prussian blue staining. Briefly, Raw264.7 cells were differentiated into osteoclasts by RANKL as similar with the previous exposure method, then washed with PBS three times, lysed with 65% nitric acid at 70 °C for 2 h, and diluted with triple distilled water. Finally, the iron content was determined by AAS, the detection results were normalized to total cell protein concentration, and the total cellular protein was detected by the BCA protein assay kit. In addition, for Prussian blue staining, the medium was removed, and washed with PBS, fixed with 4% paraformaldehyde for 20 min, incubated with the prepared Prussian blue staining at 37 °C for 30 min and counterstained for 1 min, washed with water and dried, observed with microscopy and photographed.

2.11. Measurement of oxidative stress levels

Intracellular ROS content was determined by using Reactive Oxygen Species Assay kit (Beyotime Biotechnology, China). In brief, the medium was removed and incubated with 10 μM DCFH-DA solution in the dark for 30 min at 37 °C, washed with PBS three times. The levels of intracellular ROS were measured by observing fluorescence intensity. Osteoclast mitochondria superoxide was visualized by MitoSOX™ Red indicator (M36008, Invitrogen, Australia). Briefly, cells were incubated with 5 μM MitoSOX™ Red solution for 40 min at 37 °C and washed with PBS. Mitochondrial ROS production was observed by fluorescence microscopy.

The levels of oxidative stress biomarkers including superoxide dismutase (SOD), malondialdehyde (MDA) and total antioxidant capacity (T-AOC) were examined by Total SOD Assay Kit (Beyotime, China), lipid peroxidation MDA kit (Beyotime Biotechnology, China) and Total Antioxidant Capacity Assay Kit with a Rapid ABTS method (Beyotime Biotechnology, China) respectively. In brief, the medium was removed and washed with PBS, the cells were lysed, and centrifuged at 12,000 × g for 10 min to obtain the supernatant at 4 °C. Levels of oxidative stress marker were performed using the previously described kits, and the detection results were normalized to total cell protein concentration. The total protein concentration was detected by BCA protein assay (Beyotime Biotechnology, China). All procedures were following the manufacturer’s instructions.

2.12. Measurement of ATP

ATP is mainly produced in mitochondria, which is the most direct source of energy for cells, and energy is consumed during osteoclast differentiation. ATP levels were determined by using an ATP Assay Kit (Beyotime Biotechnology, China) according to the manufacturer’s instructions. The medium was removed and the cells were lysed with lysis buffer to obtain the supernatant at 12,000 × g for 5 min at 4 °C. The total protein content was measured by BCA protein assay as previously described. The detection results were normalized to total cell protein concentration.

2.13. Quantitative real-time PCR analysis

Total RNA from cells was extracted by using HiPure Total RNA Mini Kit (Magen, Guangzhou, China) and reverse-transcribed was performed using HiScript II Q RT SuperMix (Vazyme, Nanjing, China) following the manufacturer’s instructions. The expression of the target gene was normalized to the GAPDH mRNA levels. The primer sequences are shown:
Fig. 2. 1–2 T SMF combined with Ferumoxytol prevent bone loss in HLU mice. BMC and BMD of the femur(A) and tibia (B) were measured by DXA; Three-dimensional imaging of trabecular bone (C) and cortical bone (D) by micro-CT scanning (E) Trabecular bone parameters, including BV/TV, Tb.N, Tb.Sp, Tb.Th (F) Cortical bone parameters, including Ct.Ar, Ct.Ar/Tt.Ar, TMD, Tt.Ar (G) Bone mechanical property were detected by three-point bending test, including stiffness, ultimate load, ultimate stress, elastic modulus; n = 6–8. All data shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
2.14. Western blot analysis

The tissue samples and cells were lysed with RIPA lysis buffer on ice and total cell lysates were centrifuged to obtain the supernatant at 12,000×g for 10 min at 4 °C. Then, total protein was separated with SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane.

SR-AI: Forward primer: CAACATCACCAACGACCTCA, Reverse primer: CAGTAAGCCCTCTGTCTCCC;

GAPDH: Forward primer: TGACACCACAAGCTTATTAG, Reverse primer: GGATGCAGGGATGATGTTC.
blocked with 5% skim milk. Next, the membranes were incubated with specific primary antibody, including osteoclast differentiation related proteins: matrix metalloproteinase-9 (MMP9; Abcam, ab38898, a major protease responsible for the degradation of various extracellular matrices), nuclear factor of activated T cells 1 (NFATC1; Cell Signaling Technology, 8032, key transcription factors during osteoclast differentiation), nuclear transcription factor kappa-B (NF-κB; Affinity, AF5006, regulate osteoclast differentiation by initiating or regulating gene transcription), IκB-α (Affinity, AF5002, a family member of IκB that mainly regulates the activation and transcription of NF-κB); Iron metabolism related proteins: transferrin receptor1 (TFR1; Cell Signaling Technology, 13,113, iron uptake protein), H-ferritin1 (FTH1; Cell Signaling Technology, 3998, iron storage protein), ferroportin 1 (FPN1; Abcam, ab78066, iron exporter protein); MAPK signaling pathway: JNK (Cell Signaling Technology, 9252), P-JNK (Cell Signaling Technology, 9251 S), ERK (Abclonal, a16686), P-ERK (Abclonal, ap0472), p38 (Cell

Fig. 4. 1–2 T SMF combined with Ferumoxytol inhibit osteoclastogenesis in vitro (A–B) Osteoclast formation was detected by TRAP staining (C) F-actin filament formation was examined by rhodamine-labeled phallloidin staining (D) Bone resorption ability of osteoclast was evaluated by pit formation assay; n = 3, scale bar = 50 μm. All data shown as mean ± SD. *P < 0.05 vs Ctrl, #P < 0.05 vs 0 in Ctrl, &P < 0.05 vs 0 in 1–2 T SMF.
Fig. 5. Effects of 1–2 T SMF combined with Ferumoxytol on iron content of tissue. Iron deposits in liver (A, Bar = 50 μm), spleen (B, Bar = 100 μm) and femur (E, Bar = 20 μm) were detected by Prussian blue staining; total iron content in liver (D), spleen (E), femur (F) and serum (G) were detected by atomic absorption spectrometry; n = 6–8. All data shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
2.15. Statistical analysis

GraphPad Prism 8.2.1 software (GraphPad Software, USA) was used for the statistical analysis. The data were displayed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was utilized to compare the differences between groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. 1–2 T static magnetic field combined with Ferumoxytol prevent bone loss in unloaded mice

To investigate the effects of local 1–2 T SMF combined with IONPs on bone loss in HLU mice. The BMC and BMD of the femur and the tibia were analyzed, and the DXA data showed that the BMC and BMD were statistically significantly reduced in the HLU group. After 4 weeks of 1–2 T SMF and IONPs treatment, BMC and BMD of the femur, and BMC of the tibia was
Fig. 7. 1–2 T SMF combined with Ferumoxytol reduce oxidative stress levels during osteoclastogenesis in vitro (A) Cellular ROS levels were assessed by ROS Assay kit (B) Mitochondrial superoxide levels were visualized by MitoSOX™ Red indicator fluorescence staining (C–E) Oxidative stress biomarkers (SOD, MDA, and T-AOC) were examined in osteoclastogenesis; (F) ATP content was detected (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
obviously increased compared with the HLU group, and 1–2 T SMF combined with IONPs had a joint promotion effect (Fig. 2A), while there was no visible difference in the BMD of tibia (Fig. 2B).

Representative three-dimensional imaging of trabecular bone and cortical bone was illustrated (Fig. 2C and D). The micro-CT results exhibited significantly decreased in BV/TV, Tb.N, Ct. Ar, Ct. Ar/T.Ar, TMD and obviously increased in Tb. Sp and Tb. Ar in HLU group. While, the deterioration of bone microstructure in HLU mice was ameliorate under 1–2 T SMF and IONPs treatment, and the combined effect of 1–2 T SMF and IONPs on bone microstructure were more effective (Fig. 2E and F). However, there was no obvious difference in Tb.Th.

The three-point bending experiment showed the stiffness, ultimate load, ultimate stress of tibia was significantly decreased in HLU group (Fig. 2G). Compared with the HLU group, mechanical properties were restored to both 1–2 T SMF and IONPs treatment, 1–2 T SMF and IONPs were found to exert stronger protective effects. Whereas, there was no difference in elastic modulus. Together, these results indicate that local 1–2 T SMF and IONPs could prevent the reduction of bone mass in HLU mice, and the combination of 1–2 T SMF and IONPs could better resistance to HLU-induced bone loss in mice.

3.2. Effects of 1–2 T static magnetic field combined with Ferumoxytol on bone remodeling in unloaded mice

The osteoblastogenesis and osteoclastogenesis were detected by H&E and TRAP staining in femur (Fig. 3A and B). Compared with ctrl group, the N. Ob/BS exhibited significantly decreased and the N. Oc/BS was distinctly increased in HLU mice. Whereas, 1–2 T SMF and IONPs treatment for 4 weeks, the osteoblast numbers were increased and osteoclast levels were diminished, and 1–2 T SMF combined with IONPs had a significant effect (Fig. 3C). In addition, serum TRAP5b levels were obviously reduced in 1–2 T SMF and IONPs group compared with the HLU group (Fig. 3D). Western blot assay demonstrated that the expression levels of osteoblastogenesis-related proteins including NFATC1, MMP9 were increased in HLU group. However, the expression of NFATC1, MMP9 were decreased both 1–2 T SMF and IONPs treatment, and 1–2 T SMF combined with IONPs had a joint inhibition effect (Fig. 3E and F). Moreover, the bone formation in vivo was examined based on calcine double-labeling, the mineral apposition rate (MAR) was obviously reduced in HLU group. And the MAR was increased with 1–2 T SMF and IONPs treatment, but no significant difference between 1 and 2 T SMF and IONPs group compared with the 1–2 T SMF combined with IONPs group. In conclusion, these results indicated that the local 1–2 T SMF combined with IONPs could prevent the imbalance of bone remodeling by inhibiting osteoclastogenesis in HLU mice.

3.3. 1–2 T static magnetic field combined with Ferumoxytol inhibit osteoclastogenesis in vitro

To further elucidate effects of 1–2 T SMF and Ferumoxytol on osteoclast, the differentiation capacity of osteoclast was evaluated in vitro. It was found that osteoclast differentiation was significantly inhibited by 1–2 T SMF and IONPs, including osteoclast formation (Fig. 4A and B), F-actin ring formation (Fig. 4C) and bone resorption ability in a dose dependent manner (Fig. 4D and E), and 1–2 T SMF combined with IONPs had a more obvious inhibitory effect. Therefore, we focused on the mechanism of 1–2 T SMF and IONPs to inhibit osteoclastogenesis.

3.4. Effects of 1–2 T static magnetic field combined with Ferumoxytol on iron content in vivo and in vitro

Studies have shown a close relationship between iron and bone homeostasis. In this study, iron deposition was observed in the liver, spleen, and femur in HLU group. Meanwhile, iron content in the liver and spleen were also increased in IONPs group (Fig. 5A and B). Whereas, the combination of 1–2 T SMF and IONPs was found to reduce iron content in the liver and spleen compared with the IONPs group, and the reduced iron may be targeted to bone tissue (Fig. 5D and E). Next, iron content in bone tissue was demonstrated higher in 1–2 T SMF combined with IONPs group than IONPs group by atomic absorption spectrometry in HLU mice, the results indicated that 1–2 T SMF could effectively target IONPs to bone tissue (Fig. 5C, F). On the other hand, Ferumoxytol is clinical drug used to treat anemia. The hemoglobin and red blood cell in peripheral blood were also detected, the results showed hemoglobin and red blood cell counts were promoted under 1–2 T SMF combined with IONPs group compared to the HLU group (Supplemental Fig. 1). Moreover, serum iron levels were also examined. Consistent with previous studies, iron content was significantly increased in the HLU mice. After 1–2 T SMF exposure for 4 weeks, serum iron content was reduced compared with the HLU group, and there was no significant difference in the IONPs group (Fig. 5G).

To examine whether the effects of 1–2 T SMF and IONPs on osteoclast differentiation in vitro were associated with iron metabolism. Iron content was assessed during RANKL induced osteoclastogenesis by atomic absorption spectrometry and Prussian blue staining. The results showed iron uptake of IONPs by osteoclast was reduced under 1–2 T SMF exposure (Fig. 6A and B). Furthermore, the expression levels of iron metabolism-related proteins were assessed, including iron uptake protein transferrin receptor 1 (TR1), iron storage protein H-ferritin (FTH1) and iron exporter protein FPN. The date showed the expression of FTH1 was increased after IONPs treatment, but decreased with 1–2 T SMF exposure. There were no differences in protein expression of TR1 and FPN (Fig. 6C and D). Previous studies have shown IONPs can enter cells through scavenger receptors type A/II (SR-A/II). Then, the mRNA expression of SR-AI was detected in this study, and the results proved the expression of SR-AI mRNA was increased with IONPs treatment, but decreased with 1–2 T SMF exposure (Fig. 6E). Therefore, it was confirmed that IONPs entered osteoclast in the form of SR-A1.

3.5. 1–2 T static magnetic field combined with Ferumoxytol reduce oxidative stress levels during osteoclastogenesis in vitro

Studies have shown that iron is a trace element necessary for osteoclast differentiation. Excess iron will catalyze the production of ROS through the Fenton reaction, and excess ROS is scavenged via antioxidants in the cells, such as SOD. In order to explore the underlying mechanism of SMF and IONPs, the levels of ROS during osteoclast differentiation were assessed. Fluorescence micrographs showed that cellular ROS levels were decreased under the 1–2 T SMF and IONPs treatment, and the combination of 1–2 T SMF and IONPs had a more pronounced inhibitory effect (Fig. 7A). Mitochondria are the main sites of endogenous ROS production. Next, the levels of mitochondrial ROS were also determined. The results showed the content of mitochondrial ROS was reduced in the presence of 1–2 T SMF and IONPs, and 1–2 T SMF combined with IONPs was found more effective (Fig. 7B). As expected, the content of SOD and T-AOC were increased, and MDA content were decreased in the presence of 1–2 T SMF and 0.06 mg/ml Ferumoxytol (Fig. 7C–E). Since ATP is a high energy phosphate compound and mainly produced in mitochondria through cellular respiration, which is the most direct source of energy in cells. Consistent with our previous research, the ATP levels were enhanced under the 1–2 T SMF exposure and 0.06 mg/ml Ferumoxytol treatment in this study, and 1–2 T SMF combined with IONPs had a synergistic promotion effect (Fig. 7F). Taken together, the results suggested the 1–2 T SMF combined with IONPs inhibited RANKL induced osteoclast differentiation by reducing oxidative stress levels.

3.6. 1–2 T static magnetic field combined with Ferumoxytol inhibit activation of NF-κB and MAPK signaling pathway during osteoclastogenesis in vitro

Previous studies have shown osteoclast nuclear transportation is activated by NF-κB and MAPK (JNK, ERK, p38) signaling pathways when
RANKL bind to RANK. Therefore, in order to assess whether the inhibitory effects in the presence of 1–2 T SMF and IONPs on osteoclastogenesis were mediated by breaking NF-κB and MAPK signaling pathways. In this research, the results showed the expression of NFATC1, NF-κB and MMP9 were decreased, and degradation of IκB-α were suppressed after stimulating with RANKL in Raw264.7 cells in the presence of IONPs and 1–2 T SMF (Fig. 8A and B). Moreover, MAPK signaling pathway was also inhibited by blocking the JNK, ERK and p38 phosphorylation. The combination of 1–2 T SMF and IONPs had a synergistic inhibition effect (Fig. 8C and D).

4. Discussion

As a non-invasive physical condition, SMF are widely used in orthopedic-related diseases, and SMF with a certain strength show a certain positive effect. In addition, IONPs have also been used in the field of bone tissue engineering. Studies have shown IONPs could promote the osteogenic differentiation of MSCs [32], and SMF combined with IONPs can synergistically promote the differentiation of osteoblasts [31]. However, effects of SMF and IONPs on osteoclasts have not yet been elucidated. Therefore, this research aimed to investigate effects of 1–2 T SMF and Ferumoxytol on osteoclast formation in vitro and on HLU-induced bone loss in vivo.

Our previous research showed 2–4 T SMF improved the bone microstructure and mechanical properties in mice [20]. Studies have shown that Ferumoxytol could obviously improve the bone mass in OVX mice. This study showed the BMC, BMD, and microstructure in the femur were obvious increased in HLU mice with 1–2 T SMF and IONPs.
treatment. There was no significant change in BMD of the tibia, but the mechanical properties in tibial were improved under the 1–2 T SMF and IONPs treatment in mice. This result may be due to more trabecular bone in the femur than in the tibia. Therefore, BMD changes in the femur are more pronounced than in the tibia. Furthermore, the results showed the N. Oc/BS was distinctly increased in HLU mice, but 1–2 T SMF and IONPs treatment caused lower osteoclast levels than HLU mice. Meanwhile, RANKL-induced differentiation of RAW264.7 into osteoclast was inhibited in the presence of 1–2 T SMF and IONPs, including osteoclast formation and bone resorption capacity in vitro, and 1–2 T SMF combined with IONPs had a stronger inhibitory effect. Thus, the 1–2 T SMF complexed with IONPs prevented unloading-induced bone loss by inhibiting osteoclastogenesis.

Iron is an essential trace element for organisms and has a variety of biological functions, including hemoglobin formation synthesis, oxidative stress process involvement, cell growth. Studies have shown that iron accumulation could accelerate bone loss in OVX mice by promoting osteoclast differentiation [33]. Our previous study found that iron stores in the liver and spleen were obviously increased in mice with HLU-induced bone loss [11], and Yu et al. [26] found that FAC treatment aggravated O VX-induced bone loss in mice. However, in this study, we found that IONPs treatment could prevent HLU-induced bone loss. Studies have shown that Ferumoxytol differs from typical iron supplements, its carbohydrate coating is tightly bound to the iron core, limiting the release of large amounts of iron ions in a short period of time when entering the body, thereby avoiding tissue damage [34]. After intravenous injection, IONPs are captured by the reticuloendothelial system (RES) and accumulate in the liver, spleen, and bone marrow [35]. To further illustrate the relationship between 1 and 2 T SMF and IONPs inhibiting unloading-induced bone loss and iron metabolism in osteoclastogenesis. First, the iron levels in the liver, spleen, and femur were measured. The results showed iron content in the liver, spleen, and femur was increased after IONPs treatment, and 1–2 T SMF combined with IONPs decreased iron content in liver and spleen, while enhancing iron levels in the femur, the results indicated that local 1–2 T SMF has a good bone targeting ability. In addition, the effects of 1–2 T SMF and IONPs on the iron content in RANKL-induced osteoclastogenesis were further examined in vitro. The results showed that 1–2 T SMF reduced iron uptake of IONPs in osteoclast. Meanwhile, studies have demonstrated that IONPs could be recognized by macrophages through scavenger receptors [36]. This study found that SMF decreased osteoclast SR-A1 expression and decreased uptake of IOPNs by osteoclasts. This phenomenon may be due to stronger inhibitory ability of SMF on osteoclastogenesis, and the combined effect of SMF and IONP masked the increase of osteoclastogenesis caused by the decreased intake of IONPs, and showed a certain joint effect. In addition, at the animal level, local SMF could target IONPs to bone tissue sites, resulting in increased IONP concentrations in the tissue, which further attenuates SMF reduction ability of uptake of IONPs by osteoclasts, thereby enhancing the inhibitory effect on osteoclasts. In the local treatment of clinical osteoporosis, such as the treatment of lumbar vertebral osteoporosis, it has a good application prospect and transformation significance. In addition, Ferumoxytol is clinical drugs...
used to treat iron-deficiency anemia. Thus, the hemoglobin and red blood cell counts were assessed in mice. The results showed that 1–2 T SMF combined with IONPs increased hemoglobin and red blood cell counts in mice compared to the HLU group, suggesting the IONPs were targeted in bone marrow may contribute to hemoglobin production.

ROS is an important factor in cell signal transduction, and the iron can catalyze the production of ROS through the Fenton reaction. Mitochondria are the main sites of endogenous ROS production; excessive ROS could increase cellular oxidative stress. Studies have shown that ROS plays an important role in osteoclast differentiation, and increasing ROS content could promote osteoclast differentiation [37]. Our previous research found that the production of cellular ROS in osteoclast was decreased under 16 T SMF exposure [19]. Yu et al. [26] found that Fe3O4@PSC IONPs could inhibit osteoclastogenesis by scavenging ROS, but the levels of mitochondria ROS and oxidative stress in osteoclast have not been elucidated. In this study, the results showed that the ROS content of osteoclast and the levels of mitochondrial superoxide were reduced with 1–2 T SMF and IONPs treatment, and the combination of 1–2 T SMF and IONPs had an obvious effect. In addition, the oxidative stress biomarkers including SOD, T-AOC and MDA levels were examined. The results showed that SOD and T-AOC content were increased and MDA levels were diminished in the presence of 1–2 T SMF and 0.06 mg/ml Ferumoxylotol, and 1–2 T SMF combined with Ferumoxylotol had a joint effect. Since ATP is the most direct source of energy in cells and mainly produced in mitochondria via cellular respiration. Studies have shown that osteoclast need to consume a large amount of ATP during differentiation, so only have lower ATP levels [38]. Consistent with previous research, the present study showed the production of ATP was raised with 1–2 T SMF and 0.06 mg/ml Ferumoxylotol presence, and the 1–2 T SMF combined with IONPs were found more obvious. Therefore, 1–2 T SMF combined with IONPs could decrease the oxidative stress levels in osteoclastogenesis. NF-κB signaling and MAPK (including JNK, ERK and p38) signaling are typical signaling pathways in RANKL-induced osteoclastogenesis [39]. It was found that NF-κB signaling were reduced, and the JNK, ERK and p38 phosphorylation were inhibited by 1–2 T SMF and IONPs treatment in this study. Taken together, these results demonstrated 1–2 T SMF and IONP inhibited osteoclast differentiation by blocking NF-κB and MAPK signaling pathways.

5. Conclusion

In summary, this study indicated 1–2 T SMF combined with Ferumoxylotol prevented unloading-induced bone loss by regulating iron metabolism in osteoclastogenesis (Fig. 9). These results will provide a theoretical foundation for prevention of osteoporosis with SMF and IONPs, and it has a basis in translational therapy for orthopedic-related diseases in the future.

Author contributions

Study design: PS and GZ. Study conduct: GZ, CZ and YW. Data collection: GZ, ZZ, YW and JC. Data analysis: GZ, JY and CZ. Drafting manuscript: GZ and PS. Approving final version of manuscript: all authors. GZ and PS takes responsibility for the integrity of the data analysis.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2022.10.007.

References

[1] Who. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. World Health Organ Tech Rep Set 1994;843:1–129.
[2] Salhotra A, Shah HN, Levi B, Longaker MT. Mechanisms of bone development and repair. Nat Rev Mol Cell Biol 2020;21(11):965–711.
[3] Kim H, Kong H, Sudhin S, Van Hul W. A look behind the scene: the risk and pathogenesis of primary osteoporosis. Nat Rev Rheumatol 2015;11(8):462–74.
[4] Alexandre C, Vico L. Pathophysiology of bone loss in diseuse osteoporosis. Joint Bone Spine 2011;78(6):572–6.
[5] Baert G, Goldschätz GM, Hoppock GA, Friedman MA, Suja LJ, Donahue HJ. Similarities between size and age-induced bone loss. J Bone Miner Res 2002;37(8):1417–34.
[6] Vico L, Hargens A. Skeletal changes during and after spaceflight. Nat Rev Rheumatol 2018;14(4):229–45.
[7] Morrey-Holtz ER, Globus RK. Hindlimb unloading of growing rats: a model for predicting skeletal changes during space flight. Bone 1998;22(5 Suppl):83S–88.
[8] Mo W, Liu Y, He R. Hypomagnetic field, an ignorable environmental factor in space. Sci China Life Sci 2014;57(7):726–8.
[9] Yang J, Zhang J, Ding C, Dong D, Shang P. Regulation of osteoclast differentiation by static magnetic field. Electromagn Biol Med 2017;36(1):8–19.
[10] Yang J, Meng X, Dong D, Xue Y, Chen X, Wang S, et al. Iron overload involved in the enhancement of unloading-induced bone loss by hypomagnetic field. Bone 2018;114:235–45.
[11] Bia X, Xie L, Zheng Q, Yang PF, Zhang WJ, Ding C, et al. Hypomagnetic field aggravates bone loss induced by hindlimb unloading in rat femurs. PLoS One 2014;9(8):e105604.
[12] Xue Y, Yang J, Luo J, Ren L, Shen Y, Dong D, et al. Disorder of iron metabolism inhibits the recovery of unloading-induced bone loss in hypomagnetic field. J Bone Miner Res 2020;35(6):1163–73.
[13] Chen G, Zhou Y, Tao B, Liu Q, Shang W, Li Y, et al. Moderate SMFs attenuate bone loss in mice by promoting directional osteogenic differentiation of BMSCs. Stem Cell Res Ther 2020;11(1):487.
[14] Yamamoto Y, Ohnuki Y, Goto T, Nakasima A, Iijima T. Effects of static magnetic field on bone formation in rat osteoblasts. J Dent Res 2003;82(12):962–6.
[15] Kim EC, Park J, Koh G, Park SJ, Koh N, Kwon JK, et al. Effects of moderate intensity static magnetic fields on osteoclast differentiation in mouse bone marrow cells. Bioelectromagnetics 2018;39(5):394–404.
[16] Yang J, Zhou S, Lv H, Wei M, Fang Y, Shang P. Static magnetic field of 0.2–0.4 T promotes the recovery of hindlimb unloading-induced bone loss in mice. Int J Radiat Biol 2021;97(5):746–54.
[17] Kotani H, Kawaguchi H, Shimoakia T, Iwamaik M, Ueno S, Ozawa H, et al. Strong static magnetic field stimulates bone formation to a definitive orientation in vitro and in vivo. J Bone Miner Res 2002;17(10):1814–21.
[18] Dong D, Yang J, Zhang G, Huyan T, Shang P. 16 T high static magnetic field inhibits receptor activator of nuclear factor kappa-B ligand-induced osteoclast differentiation by regulating iron metabolism in Raw264.7 cells. J Tissue Eng Regen Med 2019;13(12):2811–90.
[19] Yang J, Wang S, Zhang G, Fang Y, Fang Z, Shang P, et al. Static magnetic field (2–4 T) improves bone microstructure and mechanical properties by coordinating osteoblast/osteoclast differentiation in mice. Bioelectromagnetics 2021;42(3):200–11.
[20] Pelaz B, Alexiou C, Alvarez-Puebla RA, Alves F, Andrews AM, Ashraf S, et al. Diverse applications of nanomedicine. ACS Nano 2017;11(13):2313–81.
[21] Tautzingerer A, Kotton A, Ignatus A. Nanoparticles and their potential for application in bone. Int J Nanomed 2012;7:4545–57.
[22] Tran N, Webster TJ. Increased osteoblast functions in the presence of hydroxyapatite-coated iron oxide nanoparticles. Acta Biomater 2011;7(3):1298–306.
[23] Coyne DW. Ferumoxytol for treatment of iron deficiency anemia in patients with chronic kidney disease. Expet Opin Pharmacother 2009;10(15):2563–8.
[24] Liu L, Jin B, Duan J, Yang L, Liu Z, Zhu W, et al. Bioactive iron oxide nanoparticles suppress osteoclastogenesis and ovariectomy-induced bone loss through regulating the TRAF6-p62-CYLD signaling complex. Acta Biomater 2020;103:281–92.
[25] Yu P, Zheng L, Wang P, Chai S, Zhang Y, Shi T, et al. Development of a novel polysaccharide-based iron oxide nanoparticle to prevent iron accumulation-related osteoporosis by scavenging reactive oxygen species. Int J Biol Macromol 2020;165(Pt B):1634–45.
[26] Zheng L, Zhang Z, Li Y, Shi T, Fu K, Yan W, et al. Bone targeting antioxidative nano-iron oxide for treating postmenopausal osteoporosis. Bioact Mater 2022;14:250–61.
[27] Wang B, Wu W, Lu H, Wang Z, Xin H. Enhanced anti-tumor of pep-1 modified superparamagnetic iron oxide/PTX loaded polymer nanoparticles. Front Pharmacol 2018;9:1556.
[28] Marycz K, Sobierajkia P, Roeckeen M, Kornicka-Gabarowska K, Kepka M, Iczdrazk R, et al. Iron oxides nanoparticles (IONPs) exposed to magnetic field promote expression
of osteogenic markers in osteoblasts through integrin alpha-3 (INTa-3) activation, inhibits osteoclasts activity and exerts anti-inflammatory action. J Nanobiotechnol 2020;18(1):33.

[30] Wu D, Kang L, Tian J, Wu Y, Liu J, Li Z, et al. Exosomes derived from bone mesenchymal stem cells with the stimulation of Fe3O4 nanoparticles and static magnetic field enhance wound healing through upregulated miR-21-5p. Int J Nanomed 2020;15:7979–93.

[31] Yun HM, Ahn SJ, Park KR, Kim MJ, Kim JJ, Jin GZ, et al. Magnetic nanocomposite scaffolds combined with static magnetic field in the stimulation of osteoblastic differentiation and bone formation. Biomaterials 2016;85:68–89.

[32] Xiao HT, Wang L, Yu B. Superparamagnetic iron oxide promotes osteogenic differentiation of rat adipose-derived stem cells. Int J Clin Exp Med 2015;8(1):698–705.

[33] Xiao W, Bin C, Jingyae S, Yu J, Hui Z, Peng Z, et al. Iron-induced oxidative stress stimulates osteoclast differentiation via NF-kappaB signaling pathway in mouse model. Metabolism 2018;83:167–76.