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Ishige okamurae extract suppresses osteoclastogenesis in RANKL-induced bone marrow-derived macrophages and ovariectomized mouse model

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ABSTRACT

Objective: To investigate the inhibitory effects of *Ishige okamurae* extract (IOE) against osteoclastogenesis *in vitro* and *in vivo*.

Methods: The effects of IOE on osteoclast differentiation were examined in receptor activator of nuclear factor- κ B ligand (RANKL)-stimulated bone marrow macrophages. Cell viability was examined using an MTT assay, while osteoclast formation and differentiation were determined by tartrate-resistant acid phosphatase (TRAP) staining kit. Protein expression levels of osteoclast-related factors, transcriptional factors, and signaling molecules involved in osteoclastogenesis were analyzed by Western blotting. The effect of IOE was examined in the ovariectomy (OVX) mouse model following oral administration, and osteoclast-related parameters were analyzed using 3D X-ray microscopy.

Results: IOE significantly reduced RANKL-induced osteoclast formation and differentiation without causing cytotoxic effects on bone marrow macrophages. It suppressed the formation of TRAP-positive multinuclear osteoclasts and downregulated the expression of osteoclast-related factors, such as TRAP (ACP5), matrix metalloproteinase-9, calcitonin receptor, and cathepsin K. Furthermore, IOE significantly inhibited the expression of key transcriptional factors, such as nuclear factor of activated T cells, cytoplasmic 1, and c-Fos. Mechanistically, IOE attenuated the activation of upstream extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and nuclear factor- κ B (NF- κ B) cascades. In OVX mice, administration of IOE significantly improved bone microstructural parameters such as trabecular thickness, number, volume, and separation.

Conclusions: The present findings demonstrate that IOE inhibits osteoclastogenesis *via* blockage of ERK, JNK, and NF- κ B signaling pathways *in vitro*. Furthermore, IOE administration reduces

bone loss in OVX mice. These results show that IOE has the potential to be a functional food for the prevention of osteoporosis.

KEYWORDS: *Ishige okamurae*; Bone marrow macrophages; Osteoclasts; Ovariectomy; 3D X-ray microscopy; Mice; JNK; ERK; NF- κ B

Summary

Question: Does *Ishige okamurae* extract (IOE) regulate osteoclast differentiation *in vitro* and *in vivo*?

Findings: IOE attenuated receptor activator of nuclear factor- κ B ligand (RANKL)-stimulated osteoclastogenesis *via* controlling osteoclast-related proteins, transcriptional factors, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and nuclear factor- κ B (NF- κ B) in bone marrow macrophages. Moreover, 3D X-ray microscopy results showed that IOE administration improved osteoclast-related parameters in mice.

Meaning: This study demonstrated that IOE prevents osteoporosis by suppressing excessive osteoclastogenesis, supporting its potential as a functional food for treatment of bone diseases.

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1. Introduction

Bone is an essential organ that performs various functions. It enables mobility, provides structural support to the body, protects internal organs, acts as a storage site for minerals, and produces blood[1–3]. Bone homeostasis refers to the tightly regulated balance of metabolic processes between bone-forming and bone-resorbing processes[3]. When this balance is disrupted, particularly due to dysregulation between the forming activity of osteoblasts and the bone-resorbing activity of osteoclasts, skeletal abnormalities such as fractures may occur, in addition to the development of metabolic disorders, including osteoporosis[3,4]. Accordingly, maintaining tightly regulated bone homeostasis is essential for the prevention and therapeutic management of these conditions.

Osteoclasts are generated from monocyte/macrophage precursors through a tightly regulated differentiation process orchestrated by receptor activator of nuclear factor- κ B ligand (RANKL) and osteoblast-derived macrophage colony-stimulating factor (M-CSF)[5,6]. M-CSF serves as a pivotal factor regulating the survival and proliferation of osteoclast precursors; it facilitates their differentiation into mature osteoclasts by inducing receptor activator of nuclear factor- κ B (RANK) expression in osteoclasts[6,7]. Engagement of RANK by osteoblast-derived RANKL promotes the recruitment of tumor necrosis factor receptor-associated factor 6 (TRAF6), which initiates osteoclastogenesis[8]. The recruitment of TRAF6 around RANKL triggers multiple downstream mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) cascades, driving osteoclast differentiation and maturation[9,10]. The activation of these signaling cascades subsequently induces activator protein-1 and facilitates the nuclear translocation of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), a key factor of osteoclast differentiation. These factors cooperatively control the levels of osteoclast-related factors, including those that encode tartrate-resistant acid phosphatase (TRAP), matrix metalloproteinase (MMP)-9, calcitonin receptor (CTR), and cathepsin K (CTK)[8–11].

Bisphosphonates are widely used as antiresorptive agents that inhibit the activity of mature and precursor osteoclasts by targeting RANKL[12,13]. However, these drugs typically cause severe rebound effects such as jaw osteonecrosis, hematological and lymphatic toxicity, hypocalcemia, nephrotoxicity, acute flu-like syndrome, and gastrointestinal problems[14,15]. Various studies investigating the biological activity of natural products have been conducted to find alternative therapeutics for osteoporosis with few side effects[16].

Seaweeds, as abundant and sustainable marine resources, have been an important traditional dietary resource in China, Japan, and Korea[17,18]. They exhibit various biological properties that can treat diseases and promote health[18]. *Ishige okamurae*, belonging to Ishigeaceae (Phaeophyceae), is an edible brown seaweed found in intertidal zones of coastal areas in China, Japan, and Korea[19]. It

was recently found to exhibit anti-inflammatory, antibacterial, anti-atopic-dermatitis, and anti-obesity activities[20–23]. Our previous study has demonstrated that *Ishige okamurae* extract (IOE) inhibits osteoclastogenesis in RANKL-induced RAW 264.7 cells and dexamethasone-induced bone loss in zebrafish larvae[24]. Hence, we hypothesized that IOE may effectively inhibit osteoclastogenesis in mice subjected to ovariectomy (OVX) and exhibiting bone loss. Therefore, this study aimed to investigate the effect of IOE on bone marrow macrophages (BMMs) and on bone loss induced by OVX in mice.

2. Methods

2.1. Cell culture

BMMs were derived using a modification of the method reported by Lee *et al.*[25]. They were harvested from the femur and tibia of an 8-week-old ICR male mouse and cultured on α -minimum essential medium (α -MEM; Gibco BRL, USA) containing 1% antibiotic-antimycotic (Gibco BRL). The obtained cells were initially plated in a 100 mm culture dish with α -MEM containing 10% fetal bovine serum (FBS; Welgene, Korea) and 1% antibiotic-antimycotic for 24 h. Non-adherent cells were subsequently transferred to a 100 mm petri dish with α -MEM containing 10% FBS, 1% antibiotic-antimycotic, and 30 ng/mL of M-CSF for 3 d to promote adherence. Attached BMMs were used for subsequent experiments.

2.2. Cell viability

BMMs were plated in a 96-well plate (2×10^4 cells/well) for 24 h. BMMs were treated with various doses of IOE (12.5, 25, 50, and 100 μ g/mL) in presence of M-CSF (30 ng/mL) for 3 d. Cell viability was determined *via* MTT assay, as previously described by Cho *et al.*[24]. Absorbance was quantified at 540 nm with a SpectraMax M2/M2e Multimode Microplate Reader (Molecular Devices, USA).

2.3. TRAP staining

BMMs were plated in a 96-well plate (2×10^4 cells/well) for 24 h. BMMs were then exposed to IOE at various concentrations (25, 50, and 100 μ g/mL) in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 3 d. TRAP activity was determined using a commercially available TRAP staining kit (Cosmo Bio, Japan) following the manufacturer's instructions. Stained cells were observed under a light microscope. The area and nuclear number of TRAP-positive multinucleated giant cells were quantified using the ImageJ program (NIH, USA).

2.4. Western blotting

BMMs were plated in a 6-well plate (2×10^4 cells/well) for 24 h. BMMs were exposed to various doses of IOE (25, 50, and 100 $\mu\text{g/mL}$) in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL) for specific durations depending on the target: osteoclast differentiation-related markers (3 d), transcriptional factors (2 d), NF- κB (5 min), and MAPK (20 min). Protein levels were determined as described previously by Cho *et al.*[24]. The membranes were exposed overnight at 4 °C with primary antibodies against TRAP (ACP5) (1:1 000; Invitrogen, Cat. No. BS-6434R, USA), MMP-9 (1:1 000; Abcam, Cat. No. ab38898, USA), CTK (1:1 000; Abcam, Cat. No. ab19027), CTR (1:1 000; Invitrogen, Cat. No. 720296), NFATc1 (1:1 000; BD Pharmingen™, Cat. No. 556602, USA), c-Fos (1:1 000; Cell Signaling Technology, Cat. No. 2250S, USA), phospho-I κB (1:1 000; Invitrogen, MA5-15224), phospho-p65 (1:1 000; Cell Signaling Technology, Cat. No. 3033S), phospho-extracellular signal-regulated kinase (p-ERK; 1:1 000; Cell signaling Technology, 9101S), ERK (1:1 000; Cell signaling Technology, 4695S), phospho-c-Jun N-terminal kinase (p-JNK; 1:1 000; Cell Signaling Technology, Cat. No. 9251S, USA), JNK (1:1 000; Cell signaling Tehcnology, 9252S, USA) and β -actin (1:1 000; Santa Cruz Biotechnology, Cat. No. SC-47778, USA). Following the washing step, the membranes were exposed to HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (1:3 000; Cell Signaling Technology) for 2 h at room temperature. Protein signals were detected with a Fusion FX Chemiluminescence system (Vilber Lourmat, France).

2.5. Immunofluorescence (IF) staining

BMMs were plated in a confocal slide chamber (2×10^4 cells/well) for 24 h. BMMs were exposed to IOE (100 $\mu\text{g/mL}$) in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 20 min. p65 staining was carried out as previously reported by Cho *et al.*[24]. Blocked and permeabilized cells were incubated with a primary antibody against p65 (1:100; Cell Signaling Technology). The cells were then treated with Alexa Fluor 488-labeled goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (1:800; Thermo Fisher Scientific) for 1.5 h. Following washing steps, nuclear staining was performed using Hoechst 33342 (40 $\mu\text{g/mL}$; Sigma-Aldrich) at room temperature for 20 min. Subsequently, the cells were washed, mounted, and imaged using a Zeiss LSM 700 confocal laser microscope (Carl Zeiss, Germany).

2.6. Animals and experimental design

Female C57BL/6 mice (6 weeks old) were obtained from DooYeo Biotech (Korea) and subjected to OVX on the 8th week. The mice were maintained under strict environmental conditions, including a temperature of (23 ± 2) °C, humidity of (55 ± 5)%, and a 12 h light/dark cycle, with *ad libitum* access to food and water.

To evaluate the protective effect of IOE against the OVX model, mice were bilaterally ovariectomized and randomly divided into four groups with at least five mice in each group: sham (1); OVX (2); OVX with (3) 50 and (4) 200 mg/kg of IOE[26–28]. Following surgery, animals were daily orally administered either distilled water or IOE at the indicated doses for 8 weeks. The body weight of each mouse was measured once every week.

2.7. Micro-computed tomography (Micro-CT) analysis

Femurs fixed in 4% paraformaldehyde solution were scanned using an Xradia 620 Versa micro-CT imaging system (Zeiss, Dublin, CA, USA) in the Korea Basic Science Institute (KBSI, KJ318) at 10 μm resolution under the following settings: 60 kV, 108 μA , 6.5 W, and 801 projections. The structural parameters for trabecular bone—trabecular volume (Tb.V), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp), bone mineral density (BMD)—were measured using Analyze software (version 12.0; AnalyzeDirect, Overland Park, KS, USA), and described in detail as previously reported[29].

2.8. Ethical statement

All animal experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee at Korea Basic Science Institute (KBSI, Republic of Korea) and were approved under protocol number KBSI_IACUC-23-24 on 16 May 2023.

2.9. Statistical analysis

In vitro data were evaluated using one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test and were presented as mean $\pm$ standard deviation (SD). *In vivo* data are expressed as mean $\pm$ standard error of the mean (SEM), and statistical significance was determined using ANOVA followed by Duncan's test. All statistical analyses were conducted using GraphPad PRISM software (version 8.0; GraphPad Software, USA).

3. Results

3.1. IOE inhibits osteoclast differentiation in RANKL-stimulated BMMs

IOE showed no cytotoxic effects at a concentration up to 100 $\mu\text{g/mL}$; hence, this concentration was used for subsequent experiments (Figure 1). RANKL treatment elevated the number of TRAP-positive cells and promoted osteoclast differentiation. Treatment with IOE, however, significantly attenuated this RANKL-induced osteoclastogenesis in BMMs ($P < 0.001$) (Figure 2).

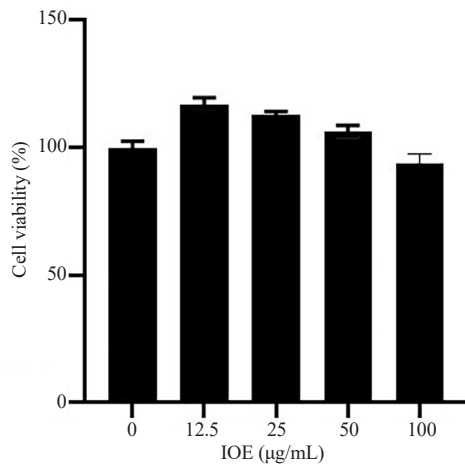


Figure 1. Influence of *Ishige okamurai* extract (IOE) on cell viability of bone marrow macrophages (BMMs). Results are presented as mean $\pm$ SD of at least three independent experiments.

3.2. IOE inhibits the levels of osteoclast differentiation-related mediators and transcriptional factors in RANKL-stimulated BMMs

RANKL treatment significantly upregulated the osteoclast differentiation-associated markers, including ACP5, MMP-9, CTK, and CTR ($P < 0.001$). In contrast, IOE treatment significantly attenuated the levels of these markers ($P < 0.001$) (Figure 3). Additionally, RANKL exposure significantly elevated the expression of NFATc1 and c-Fos, whereas IOE significantly suppressed their expression in a dose-dependent manner in RANKL-induced BMMs ($P < 0.001$) (Figure 4).

3.3. IOE inhibits NF- κ B and MAPK signaling pathways in RANKL-stimulated BMMs

RANKL treatment induced the phosphorylation of I κ B and p65; however, IOE significantly suppressed NF- κ B activation in

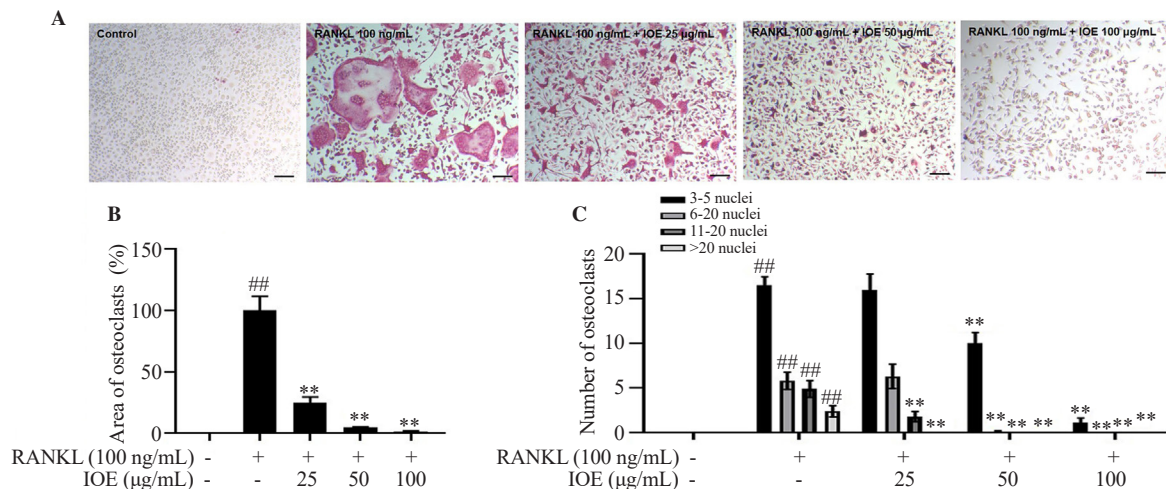


Figure 2. Inhibitory effect of IOE on tartrate-resistant acid phosphatase (TRAP) activity in receptor activator of nuclear factor κ B ligand (RANKL)-stimulated osteoclastogenesis in BMMs. (A) Representative micrographs of TRAP-positive multinucleated osteoclasts (scale bar: 100 μ m). (B) Area of osteoclasts. (C) Number of osteoclasts. Results are presented as mean $\pm$ SD of at least three independent experiments. $^{##}P < 0.001$ vs. normal control. $^{**}P < 0.001$ vs. RANKL-treated group.

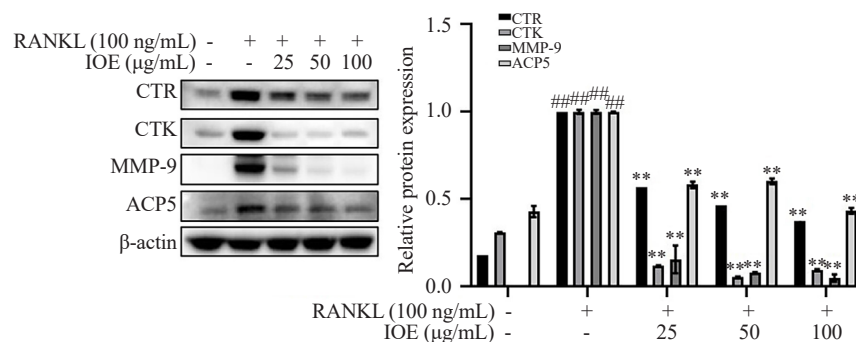


Figure 3. Inhibitory effect of IOE on osteoclast-related mediators in RANKL-stimulated osteoclastogenesis in BMMs. Results are presented as mean $\pm$ SD of at least three independent experiments. CTR: calcitonin receptor, CTK: cathepsin K, MMP-9: matrix metalloproteinase-9; ACP5: acid phosphatase 5. $^{##}P < 0.001$ vs. normal control. $^{**}P < 0.001$ vs. RANKL-treated group.

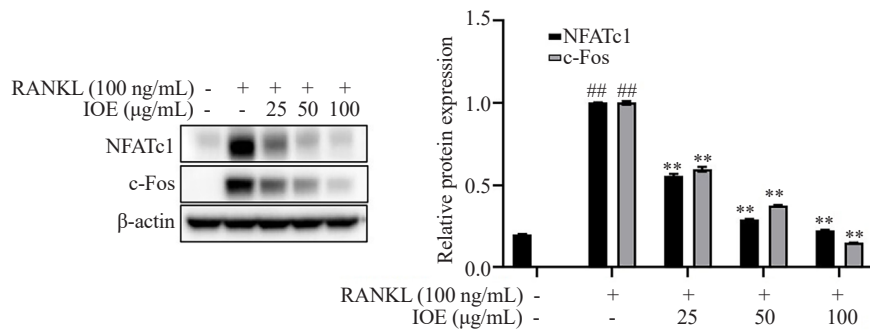


Figure 4. Inhibitory effect of IOE on osteoclastogenesis-related transcriptional factors in RANKL-stimulated BMMs. Results are presented as mean $\pm$ SD of at least three independent experiments. NFATc1: nuclear factor of activated T cells, cytoplasmic 1. $^{##}P<0.001$ vs. normal control. $^{**}P<0.001$ vs. RANKL-treated group.

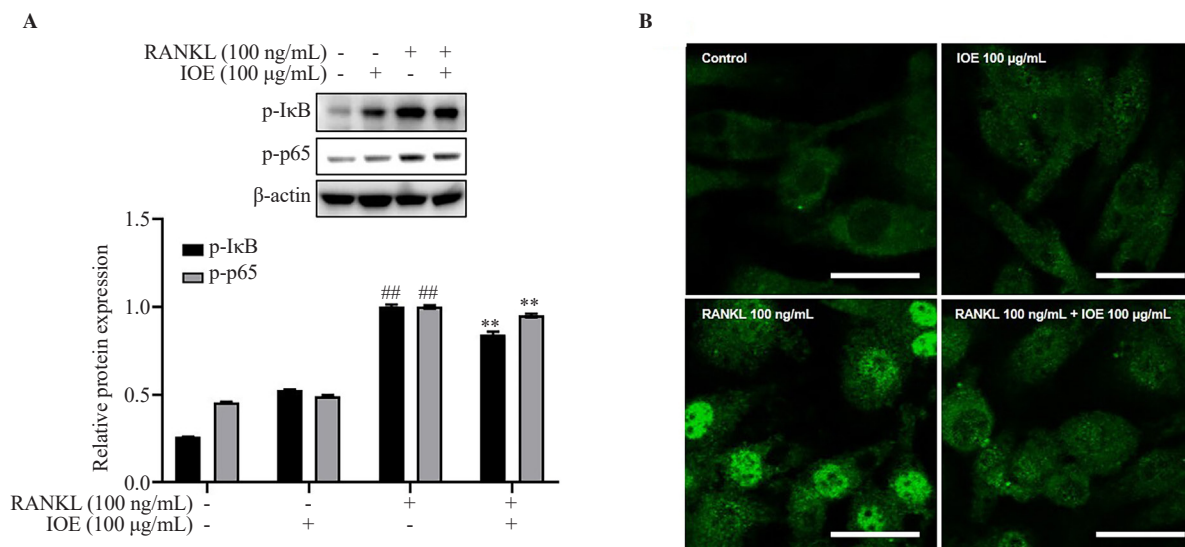


Figure 5. Inhibitory effect of IOE on the phosphorylation of NF- κ B in RANKL-stimulated BMMs. (A) Protein expression levels of p-I κ B and p-p65. (B) Representative images of nuclear translocation of p65 (scale bar: 10 μ m). Results are presented as mean $\pm$ SD of at least three independent experiments. $^{##}P<0.001$ vs. normal control. $^{**}P<0.001$ vs. RANKL-treated group.

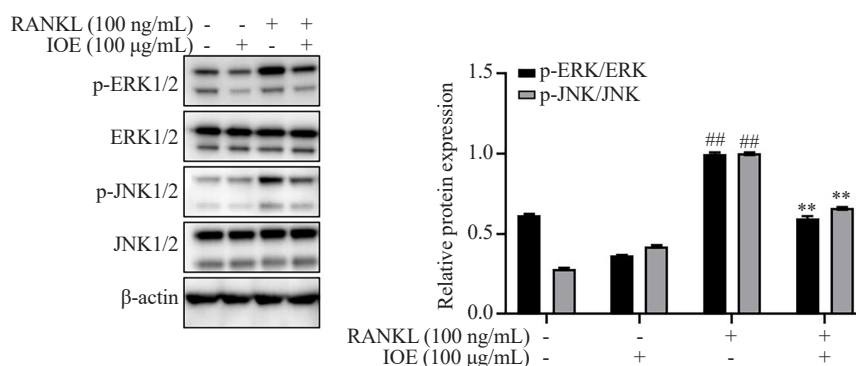


Figure 6. Inhibitory effect of IOE on the phosphorylation of mitogen-activated protein kinase in RANKL-stimulated BMMs. Results are presented as mean $\pm$ SD of at least three independent experiments. $^{##}P<0.001$ vs. normal control. $^{**}P<0.001$ vs. RANKL-treated group.

BMMs ($P<0.001$) (Figure 5A). IOE also significantly blocked RANKL-induced nuclear translocation of p65 ($P<0.001$) (Figure 5B). Moreover, treatment with IOE significantly reduced the phosphorylation of MAPKs, including ERK and JNK, in RANKL-stimulated BMMs ($P<0.001$) (Figure 6).

3.4. IOE inhibits bone loss and osteoclast differentiation in the OVX mouse model

IOE did not affect the body weight in comparison with the sham group (Figure 7A). Tb.Th, Tb.N, and Tb.V were significantly reduced in the OVX group compared with those in the sham group ($P<0.001$). However, the IOE-administered groups differed from the OVX group in these parameters. Both Tb.Th and Tb.N were

significantly increased in the 50 and 200 mg/kg IOE-administered groups ($P<0.05$), whereas Tb.V did not show a significant difference in the 50 and 200 mg/kg IOE-administered groups. The Tb.Sp values for the OVX and sham groups also differed considerably ($P<0.001$). Treatment with 50 mg/kg IOE induced greater changes in this parameter compared with the OVX group ($P<0.05$). There was no significant difference in BMD between the OVX group and the IOE-treated groups (Figure 7B-G).

4. Discussion

Osteoporosis is a common metabolic bone disorder defined by reduced bone mass and structural deterioration of bone

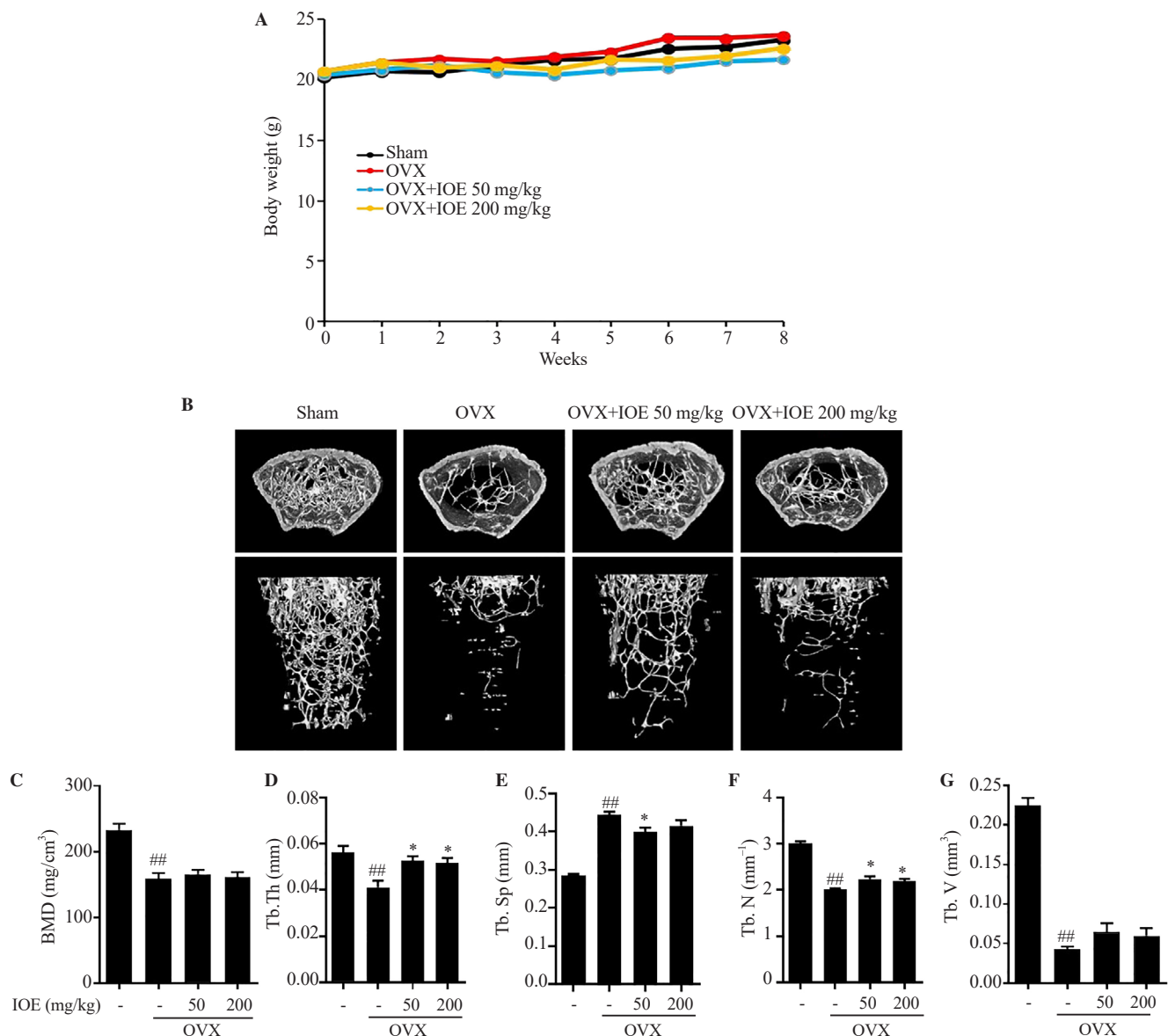


Figure 7. Protective effect of IOE against bone loss induced by ovariectomy (OVX) in mice. A: Body weight; B: Representative 3D images of trabecular bone in mouse femurs obtained through X-ray microscopy analysis; C: bone mineral density (BMD); D: trabecular thickness (Tb.Th); E: trabecular separation (Tb.Sp); F: trabecular number (Tb.N); G: trabecular volume (Tb.V). Results are presented as mean $\pm$ SEM ($n = 5$). ^{##} $P<0.001$ vs. normal control. ^{*} $P<0.05$ vs. RANKL-treated group.

microarchitecture, consequently increasing skeletal fragility and susceptibility to fracture[25]. Excessive bone resorption by over-activated mature osteoclasts causes bone loss, resulting in osteoporosis[30]. In this study, we used BMMs isolated from tibias and femurs of mice to investigate the effect of IOE on RANKL-induced osteoclast differentiation in primary cells.

Previous studies have employed BMMs isolated from mice to demonstrate the effect of candidate resources on osteoclastogenesis[31,32]. Based on these reports, we used BMMs treated with M-CSF and RANKL. TRAP, CTR, CTK, and MMP-9 are specific markers of osteoclast differentiation and maturation and are associated with bone resorption[24]. In this study, TRAP staining demonstrated that IOE significantly inhibits osteoclast differentiation and reduces the number of TRAP-positive multinucleated giant cells.

The differentiation of osteoclast precursors to mature osteoclasts is orchestrated by M-CSF and RANKL[33]. M-CSF mediates the differentiation of monocytes/macrophages and prolongs the survival and proliferation of osteoclast precursor cells[33,34]. RANKL is a key factor that enables the expression of osteoclast phenotypes, including fusion, expansion of lifespan, and differentiation, by stimulating the pool of M-CSF-expanded precursor cells[33]. Osteoclastogenesis is initiated by interaction of osteoblast-secreted RANKL and its receptor RANK, located on the surface of osteoclast precursor cells. This interaction triggers the recruitment of the direct downstream molecule TRAF6 to activate osteoclast differentiation, which subsequently activates the MAPK and NF- κ B signaling cascades[30,35].

M-CSF-mediated MAPK activation plays a critical role in osteoclast precursor proliferation and survival, while RANKL-induced MAPK mainly contributed to osteoclast differentiation[36]. The ERK signaling cascade is primarily implicated in the survival, proliferation, apoptosis, and formation of osteoclasts[36]. It is also involved in regulating osteoclast differentiation and migration, which result in bone resorption[36]. The JNK signaling cascade regulates the apoptosis, formation, and differentiation of osteoclasts[36,37]. NF- κ B activation by RANKL is critical to accelerating osteoclast differentiation[8]. I κ B degradation induces the activation of p50/p65 heterodimers, facilitating their nuclear translocation[8,38]. In this study, IOE was shown to suppress ERK, JNK, and NF- κ B signaling cascades in BMMs stimulated with M-CSF and RANKL. Activation of ERK, JNK, and NF- κ B accelerates activator protein-1 (consisting of c-Fos and c-Jun) activation and the nuclear translocation of NFATc1; thus, this process eventually promotes the upregulation of osteoclast-associated markers, including TRAP, CTR, CTK, and MMP-9[39,40]. IOE treatment significantly suppressed c-Fos activation and nuclear translocation of NFATc1, and further suppressed RANKL-induced osteoclast-related markers in BMMs. These findings demonstrate that IOE effectively inhibits osteoclastogenesis *in vitro*.

The OVX mouse model has been widely used to test candidate

drugs for osteoporosis treatment. 3D X-ray CT scanning analyses have confirmed that sham and OVX models exhibit significant differences[41–43]. In this study, X-ray microscopy demonstrated that IOE administration significantly altered trabecular parameters, including Tb.Th, Tb.N, and Tb.Sp, compared with those of the OVX group.

In conclusion, our study highlights the anti-osteoclastogenesis effect of IOE *in vitro* and *in vivo*. IOE interferes with the RANKL-induced ERK, JNK, and NF- κ B signaling pathways, thereby inhibiting osteoclastogenesis-related mediators and transcriptional factors in RANKL-induced BMMs. Moreover, OVX-induced bone loss was prevented upon the administration of IOE. Therefore, IOE exhibits great potential for the development of functional foods that can prevent osteoporosis.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

SHC developed the methodology, conducted the investigation, validated the results, performed the formal analysis, and wrote the original draft of the manuscript. HYJ developed the methodology,

conducted the investigation, and validated the results. SK developed the methodology. YJJ developed the methodology and conducted the investigation. JIP developed the methodology. KNK conceived the study, supervised the project, reviewed and edited the manuscript, administered the project, and acquired the funding.

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