

# Magnolol prevents ovariectomy-induced bone loss by suppressing osteoclastogenesis via inhibition of the nuclear factor- $\kappa$ B and mitogen-activated protein kinase pathways

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**Abstract.** Magnolol is the active component of the traditional Chinese medicine *Magnolia officinalis*, and has antioxidant, anti-inflammatory and anticancer activities, as well as an effect on bone metabolism *in vitro*. In the present study, it is reported that magnolol suppresses osteoclastogenesis *in vivo* and *in vitro*. Magnolol prevented ovariectomy-induced bone loss and osteoclastogenesis *in vivo*, and decreased the serum levels of C-terminal telopeptide of type 1 collagen, interleukin-6, tumor necrosis factor (TNF)- $\alpha$  and tartrate-resistant acid phosphatase 5B. *In vitro*, magnolol inhibited the osteoclastogenesis induced by the receptor activator for nuclear factor- $\kappa$ B ligand, and impaired the osteoclast function in bone marrow monocytes and RAW264.7 cells in a dose-dependent manner. Furthermore, magnolol suppressed the expression levels of the osteoclastogenesis markers cathepsin K, calcitonin receptor, matrix metalloproteinase 9, TNF receptor-associated factor 6 and tartrate-resistant acid phosphatase by inhibiting the nuclear factor- $\kappa$ B and mitogen-activated protein kinase pathways. Therefore, magnolol is a promising agent for the treatment of osteoporosis and associated disorders.

## Introduction

Bone homeostasis is dependent on the balance between bone formation and bone resorption, which are mediated by osteoblasts and osteoclasts (1). In patients with osteoporosis, principally postmenopausal women, the rate of osteoclastogenesis surpasses that of osteogenesis, resulting in decreased bone density and strength (2-4). Osteoporosis is associated with

an increased incidence of bone fractures, which are particularly dangerous in the elderly, and with a reduced quality of life (5,6). The prevalence of osteoporosis is increasing worldwide (7). In the United States, ~10 million individuals aged >50 years suffer from osteoporosis and 34 million suffer from osteopenia. In China, the prevalence of osteoporosis was 14.94% prior to 2008 and 27.96% between 2012 and 2015 (8).

Osteoclasts differentiate from the mononuclear phagocyte system and are involved in bone resorption. Inflammation leads to overactivation of osteoclasts, and thus suppression of inflammation inhibits osteoclastogenesis (3). Osteoclastogenesis has been reported to involve several signaling pathways (6,9,10). Receptor activator for nuclear factor- $\kappa$ B ligand (RANKL) is vital for the formation and function of osteoclasts (11,12), and binding of RANKL to its receptor RANK results in activation of the nuclear factor (NF)- $\kappa$ B, mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) signaling pathways. The activation of these pathways induces the expression of several osteoclast-specific genes, including cathepsin K, calcitonin receptor (CTR), matrix metalloproteinase 9 (MMP9), TNF receptor-associated factor 6 (TRAF6), and tartrate-resistant acid phosphatase (TRAP) (13-15).

Magnolol is the active component of the Chinese medicine *Magnolia officinalis* and is used clinically for its anti-inflammatory and antioxidant effects (16-19). Magnolol reportedly inhibits RANKL-induced osteoclastic differentiation of RAW264.7 macrophages (20). However, further research is required to assess the effect of magnolol on osteoporosis and the underlying mechanism(s). Therefore, the present study aimed to investigate the effect of magnolol on osteoclastogenesis *in vivo* and *in vitro*, as well as to examine the underlying mechanism at the molecular level.

## Materials and methods

**Reagents and cells.** Magnolol was provided by Shanghai Nodel Biotech Co., Ltd. (Shanghai, China) and reconstituted in phosphate-buffered saline (PBS), serving as the vehicle. RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS), penicillin, streptomycin and antibodies were

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purchased from BioTNT (Shanghai, China). The primary antibodies (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) used included anti-cathepsin K (1:500; cat. no. sc-6506), anti-CTR (1:200; cat. no. sc-8858), anti-MMP9 (1:400; cat. no. sc-21733), anti-TRAF6 (1:250; cat. no. sc-8409), anti-TRAP (1:350; cat. no. sc-100314), anti-p65 (1:350; cat. no. sc-398442), anti-phosphorylated (p)-p65 (1:500; cat. no. sc-136548), anti-p50 (1:250; cat. no. sc-166580), anti-p-p50 (1:400; cat. no. sc-271908), anti-inhibitor of  $\kappa$ B (I $\kappa$ B; 1:350; cat. no. sc-74451), anti-p-I $\kappa$ B (1:500; cat. no. sc-8404), anti-extracellular signal-regulated kinase (ERK; 1:200; cat. no. sc-135900), anti-p-ERK (1:400; cat. no. sc-81492), anti-c-Jun N-terminal kinase (JNK; 1:500; cat. no. sc-7345), anti-p-JNK (1:400; cat. no. sc-135642), anti-p38 (1:300; cat. no. sc-136210), anti-p-p38 (1:300; cat. no. sc-7973) and anti- $\beta$  actin (1:1,000; cat. no. sc-8432). Horseradish peroxidase-conjugated anti-mouse (cat. no. ZB2301) and anti-rabbit (cat. no. ZB2305) antibodies (1:5,000; OriGene Technologies, Inc., Beijing, China) were also used. Dulbecco's modified Eagle's medium was purchased from Corning Inc. (Corning, NY, USA).

**Animals and experimental design.** Experiments involving mice were approved by the Ethics Committee on Animal Experiments of the Central Hospital of Zibo (Shandong University, Zibo, China). A total of 36 C57BL/6 female mice (8-week-old; weight, 20-25 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The mice were housed under standard laboratory conditions (20-25°C; humidity, 40-70%; 16/8-h light/dark cycle), and provided with food and water *ad libitum*. The mice were randomly divided into the sham-operated, saline-treated ovariectomy (OVX), and magnolol-treated OVX groups (n=6 per group). OVX was performed using a standard protocol (21) Sham operation was performed following the same procedure as OVX without the resection of ovaries. At 24 h postoperatively, the OVX mice were treated with an intraperitoneal injection of 10 ml/kg magnolol or saline daily for 6 weeks. The mice were then euthanized by cervical dislocation, and femur and blood samples were obtained.

**Bone histomorphometric analysis.** Femur samples were decalcified for 2 weeks in an aqueous solution of 10% tetradsodium-EDTA at 4°C. Next, bone sections (4  $\mu$ m) were stained with hematoxylin and eosin (H&E) at room temperature for 5-10 min, followed by TRAP staining (cat. no. BB-4421-1; BestBio, Shanghai, China) to identify osteoclasts according to the manufacturer's protocol. Bone trabecula was observed in H&E-stained sections, and the osteoclasts were observed in TRAP-stained sections. Histomorphometric measurements were then performed under a microscope, and the trabecular bone area was calculated using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

**Microcomputed tomography analysis.** Bone samples were analyzed by microcomputed tomography (Skyscan; Bruker Corporation, Billerica, MA, USA) using the following parameters: 80 kV, 124  $\mu$ A and 8- $\mu$ m resolution. The built-in software was used to analyze various indices, including bone mineral density (BMD), bone surface area/bone volume

(BS/BV), BS/total volume (BS/TV), BV/TV and trabecular number (Tb.N).

**Serum biochemistry.** Following anesthesia, blood samples were collected from the fundus venous plexus. Subsequent to centrifugation (1,811 x g; 15 min; 4°C), the serum levels of C-terminal telopeptide of type 1 collagen (CTX-1; cat. no. MBS726456; MyBiosource, Inc., San Diego, USA), interleukin (IL)-6 (cat. no. MEC1008; Anogen-Yes Biotech Laboratories, Ltd., Mississauga, Canada), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ; cat. no. MEC1003; Anogen-Yes Biotech Laboratories, Ltd.) and TRAP 5b (TRAcP5B; cat. no. MBS776993; MyBiosource, Inc.) were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol.

**In vitro osteoclastogenesis assay.** Bone marrow monocytes (BMMCs) were isolated from the bone marrow of healthy C57BL/6 mice by flushing femurs and tibias with  $\alpha$ -MEM and culturing in  $\alpha$ -MEM with 10% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and macrophage colony-stimulating factor (M-CSF; 30 ng/ml) overnight. Non-adherent cells were collected and further cultured in the presence of M-CSF (30 ng/ml) for 3 days. Floating cells were discarded and adherent cells were used as bone marrow-derived macrophages. BMMCs and RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with glucose, 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The culture medium was changed once per day during the first 3 days. After removing nonadherent cells, colonies were cultured for 14 days, then passaged and subcultured. BMMCs treated with 0, 5, 10, 20, 40, 80 or 160  $\mu$ g/ml magnolol were subjected to an MTT assay.

Based on the MTT assay results, third-generation BMMCs (5x10<sup>3</sup>/well) and RAW264.7 cells (3x10<sup>3</sup>/well) were cultivated in 96-well plates and divided into a control group and four magnolol-treated (0, 5, 10 and 20  $\mu$ g/ml) groups. To induce osteoclast formation, 20 ng/ml M-CSF and 50 ng/ml RANKL were applied. After 5 days, staining for TRAP was performed using the TRAP Staining kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). TRAP-positive cells with three or more nuclei were regarded as osteoclasts. In addition, in order to visualize F-actin rings by fluorescent microscopy, cells treated with M-CSF/RANKL were cultured for 48 h with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 and incubated with rhodamine-conjugated phalloidin (cat. no. ab235138; Abcam, Cambridge, MA, USA) for 60 min at room temperature. Fluorescein isothiocyanate-conjugated F-actin antibodies (1:600; cat. no. ab205; Abcam) were used to detect F-actin. Assays were performed in at least triplicate, and mean values were calculated.

**Bone-pit formation assay.** RAW264.7 cells were divided into five groups, including the untreated control and four groups treated with magnolol of various concentration. Next, cells (3x10<sup>3</sup>/well) were seeded in 48-well plates containing a collagen gel matrix, and then treated with 20 ng/ml M-CSF and 50 ng/ml RANKL for 6 days. The resulting mature osteoclasts were digested with collagenase and seeded onto a biosynthetic bone surface (Osteo Assay Surface 24-well Multiple-Well

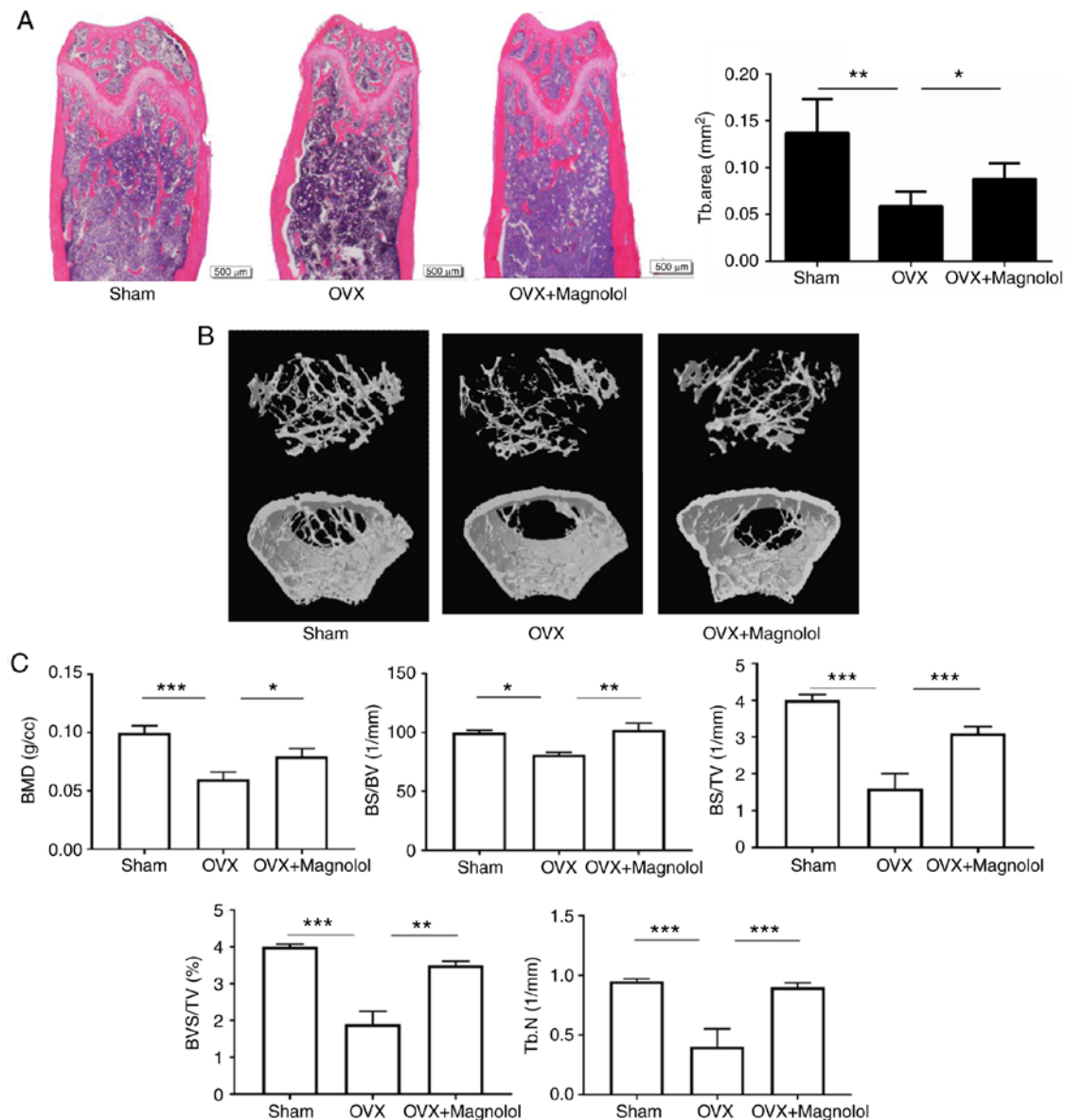


Figure 1. Magnolol prevents OVX-induced bone loss. (A) Representative femurs and trabecular areas stained with hematoxylin and eosin at 6 weeks postoperatively (magnification,  $\times 40$ ; bar=500  $\mu\text{m}$ ; n=6). (B) Representative three-dimensional structures of femurs examined by microcomputed tomography. (C) BMD, BS/BV, BS/TV, BV/TV and Tb.N values. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . OVX, ovariectomy; BMD, bone mineral density; BS, bone surface area; BV, bone volume; TV, total volume; Tb.N, trabecular number.

Plates; Corning, Inc., Corning, NY, USA) and incubated for 7 days at room temperature. The medium was changed on day 3, and the plates were washed with PBS and air-dried for 3 h on day 7. The bone resorption area was assessed by light microscopy (BX53; Olympus Corporation, Tokyo, Japan) and Image-Pro Plus software.

**Western blotting.** In order to assess the effects of magnolol on the NF- $\kappa$ B and MAPK signaling pathways, RAW264.7 cells ( $3 \times 10^3$ /well) were seeded into 6-well plates and treated with RANKL without or with magnolol (0 or 20  $\mu\text{g}/\text{ml}$ ). Following incubation for 0, 15, 30, 45 or 60 min, the phosphorylation levels of p38, p50, p65, I $\kappa$ B, ERK and JNK were evaluated by western blotting. In addition, in order to assess the protein expression levels of osteoclastogenesis markers, RAW264.7 cells ( $3 \times 10^3$ /well) were cultured for 7 days in the presence of RANKL without or with 0, 5, 10, or 20  $\mu\text{g}/\text{ml}$  magnolol.

Subsequently, the expression levels of cathepsin K, CTR, MMP9, TRAF6, and TRAP were assessed by western blotting using antibodies obtained from Abcam (Cambridge, MA, USA).

The total protein was extracted from the cells using M-PER mammalian protein extraction reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts of protein (10  $\mu\text{g}/\text{lane}$ ), estimated by a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) were loaded onto 11% SDS-PAGE gels and transferred onto nitrocellulose membranes. Blocking was performed with blocking solution (cat. no. P0023B; Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 1 h. Membranes were then incubated with the primary (4°C; overnight) and secondary antibodies (room temperature; 1 h) detailed above (p38, p50, p65, I $\kappa$ B, ERK, JNK, cathepsin K, CTR, MMP9, TRAF6 and TRAP). Bands were detected by chemilumines-

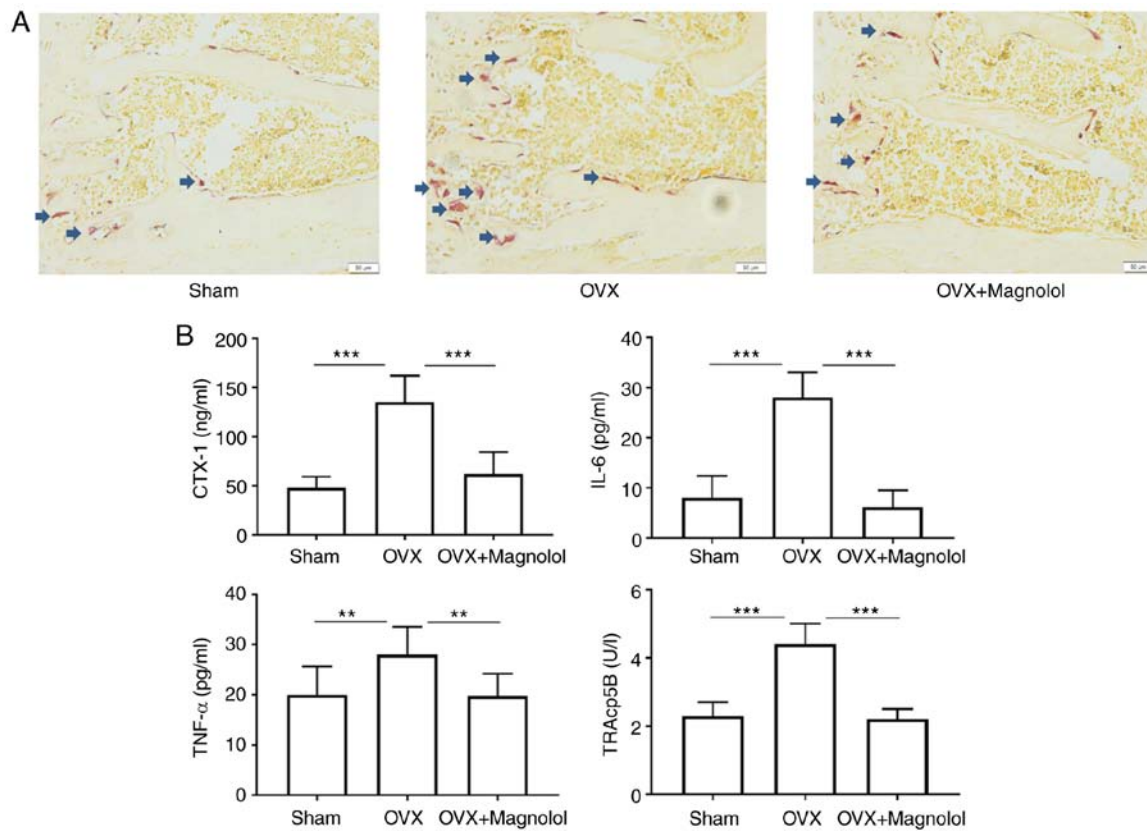


Figure 2. Magnolol inhibits osteoclastogenesis *in vivo*. (A) Representative TRAP-stained femur tissues from the sham, OVX model and magnolol-treated OVX groups (magnification, x40; bar=50  $\mu$ m; n=6). Arrows indicate the TRAP+ cells. (B) Serum CTX-1, IL-6, TNF- $\alpha$  and TRAcP5B levels, detected by enzyme-linked immunosorbent assay. \*\*P<0.01 and \*\*\*P<0.001. OVX, ovariectomy; TRAP, tartrate-resistant acid phosphatase; CTX-1, C-terminal telopeptide of type 1 collagen; IL-6, interleukin-6; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TRAcP5B, tartrate-resistant acid phosphatase 5b.

cence (cat. no. E003-1007; Sea Biotech, Shanghai, China) and imaged with X-ray films.  $\beta$ -actin was used as an endogenous reference for normalization. ImageJ software (v1.46; National Institutes of Health, Bethesda, MD, USA) was used to analyze densitometry.

**Immunofluorescence staining.** The effect of magnolol on the nuclear translocation of p65 was evaluated by immunofluorescence staining. Briefly, BMSCs treated with RANKL in the absence or presence of 20  $\mu$ g/ml magnolol were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were washed with 0.1% BSA-PBS and incubated with an anti-p65 monoclonal antibody overnight at 4°C, followed by a biotinylated goat anti-mouse IgG and fluorescein-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature and Hoechst (Vector Laboratories) was used for counterstaining for 2 days. The localization of p65 was visualized by immunofluorescence analysis (x400 magnification) and the percentage of nuclei fluorescence intensity of p65 in 3 random fields were measured in each group with ImageJ software.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. Student's t-test was used for comparisons of two groups, while one-way analysis of variance was performed to compare three or more groups. A value of P<0.05 was considered to denote a difference that was statistically significant.

## Results

**Magnolol prevents OVX-induced bone loss.** According to H&E staining, magnolol prevented the loss of trabecular bone in OVX mice (Fig. 1A). This was further confirmed by microcomputed tomography, and the three-dimensional structure of the bone is shown in Fig. 1B. When comparing Sham and OVX groups, it was observed that the BMD (P<0.001), BS/BV (P<0.05), BS/TV (P<0.001), BV/TV (P<0.001) and Tb.N (P<0.001) values were decreased in OVX mice. When comparing OVX and OVX+Magnolol groups, the BMD (P<0.05), BS/BV (P<0.01), BS/TV (P<0.001), BV/TV (P<0.01) and Tb.N (P<0.001) values were increased in OVX+Magnolol mice, indicating that magnolol treatment reversed OVX-induced bone loss (Fig. 1C).

**Magnolol inhibits osteoclastogenesis *in vivo*.** To determine whether magnolol suppressed OVX-induced bone loss by inhibiting the formation of osteoclasts, TRAP staining was performed, and the serum levels of markers of osteoclastogenesis and inflammation were assessed. It was observed that there were more TRAP-positive cells in OVX mice when compared with the sham group. However, magnolol reduced the number TRAP-positive cells in OVX+Magnolol mice compared with the OVX group (Fig. 2A). As determined by ELISA, the serum CTX-1 (P<0.001), IL-6 (P<0.001), TNF- $\alpha$  (P<0.01) and TRAcP5B (P<0.001) levels were significantly increased in OVX mice compared with those in the sham

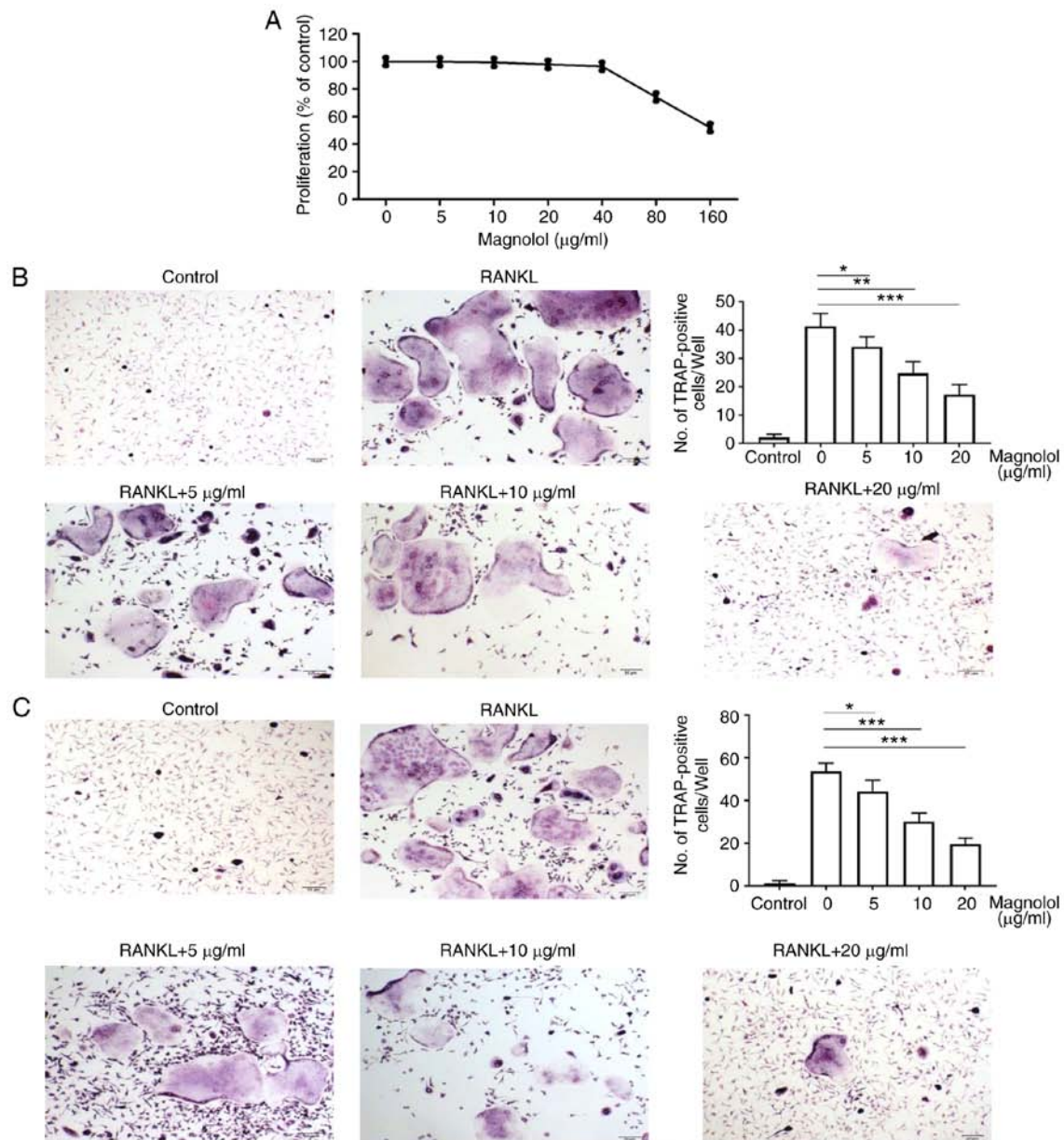


Figure 3. Magnolol inhibits osteoclastogenesis *in vitro*. (A) Cell proliferation examined by an MTT assay in bone marrow monocytes. The number of TRAP-positive (B) bone marrow monocytes and (C) RAW264.7 cells was assessed in the different groups (magnification, x100; bar=50 μm). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator for nuclear factor-κB ligand.

group. However, magnolol treatment markedly decreased the serum levels of CTX-1 (P<0.001), IL-6 (P<0.001), TNF-α (P<0.01) and TRAcP5B (P<0.001) in the OVX+Magnolol group compared with the OVX group (Fig. 2B), suggesting inhibition of osteoclastogenesis and inflammation.

**Magnolol inhibits osteoclastogenesis *in vitro*.** Based on the results of the MTT assays, magnolol concentrations of 0, 5, 10 and 20 μg/ml were selected for use in subsequent experiments, since the cell proliferation was not markedly reduced following treatment at these doses (Fig. 3A). Compared with the positive control (RANKL alone), magnolol decreased the number of TRAP-positive BMMCs (Fig. 3B) and RAW264.7 cells (Fig. 3C) in a concentration-dependent manner. Therefore, magnolol appeared to suppress RANKL-induced osteoclastogenesis in BMMCs and RAW264.7 cells.

**Magnolol inhibits osteoclast function *in vitro*.** To evaluate the effect of magnolol on osteoclast function *in vitro*, F-actin ring formation and bone-pit formation were assayed. Magnolol inhibited F-actin ring formation in M-CSF-stimulated and RANKL-stimulated RAW264.7 cells in a concentration-dependent manner (Fig. 4). In addition, magnolol significantly decreased the area of bone resorption pits (Fig. 5). Taken together, these results suggested that magnolol inhibited RANKL-induced osteoclast activity.

**Magnolol suppresses the expression of osteoclastogenesis markers.** To evaluate the mechanism underlying the effect of magnolol, the protein expression levels of cathepsin K, CTR, MMP9, TRAF6 and TRAP were assessed. It was observed that magnolol significantly suppressed the RANKL-induced expression of cathepsin K, CTR, MMP9, TRAF6 and TRAP

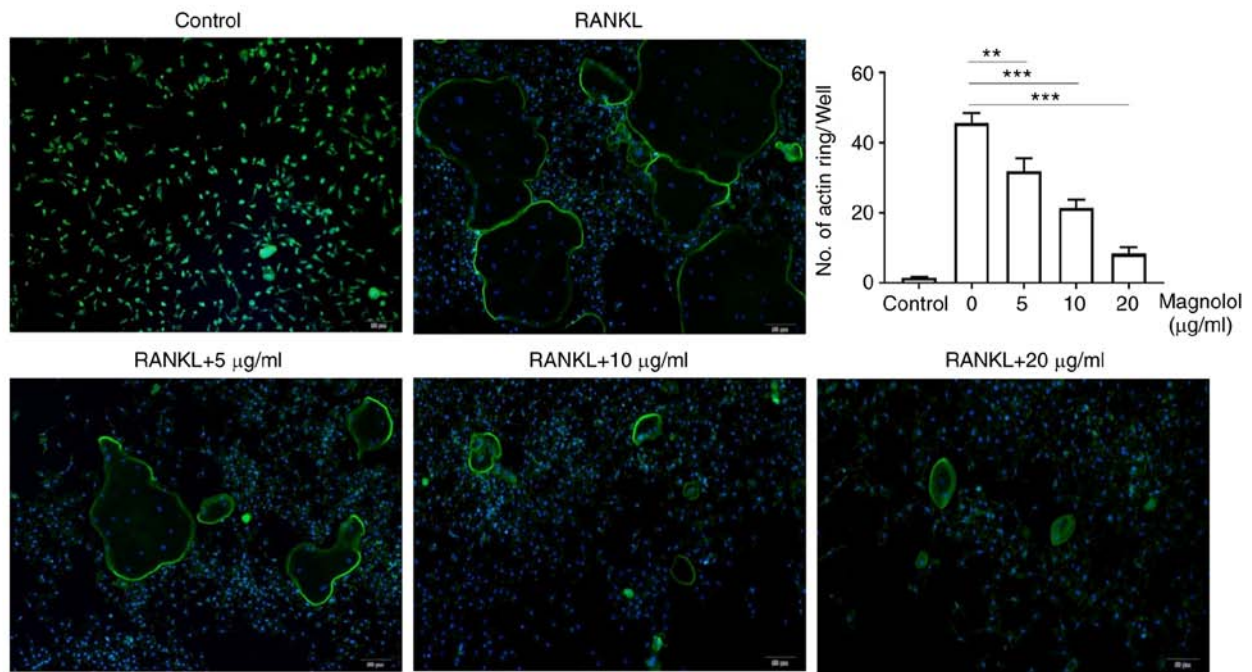


Figure 4. Magnolol inhibits F-actin ring formation, as determined by rhodamine-conjugated phalloidin staining (magnification,  $\times 100$ ; bar= $50 \mu\text{m}$ ).  $^{**}P < 0.01$  and  $^{***}P < 0.001$ . RANKL, receptor activator for nuclear factor- $\kappa\text{B}$  ligand.

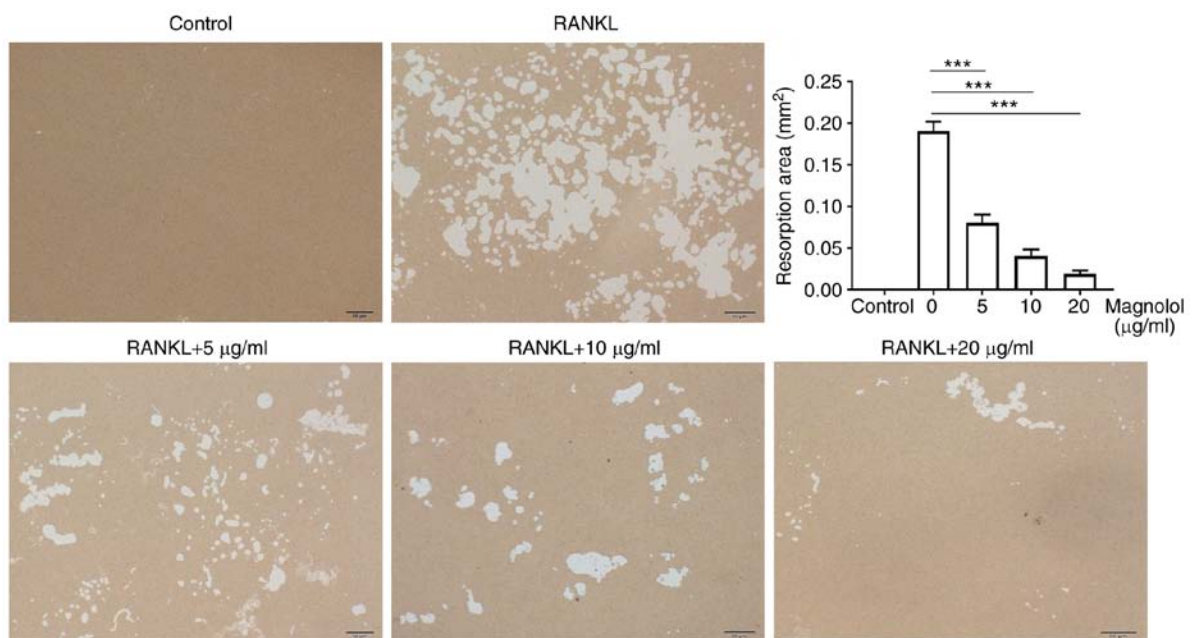


Figure 5. Magnolol decreases the area of resorption pits formed in RANKL-treated RAW264.7 cells (magnification,  $\times 100$ ; bar= $50 \mu\text{m}$ ).  $^{***}P < 0.001$ . RANKL, receptor activator for nuclear factor- $\kappa\text{B}$  ligand.

(Fig. 6). Therefore, magnolol suppressed the expression levels of osteoclastogenesis markers.

*Magnolol suppresses the NF- $\kappa\text{B}$  and MAPK pathways.* To assess the effect of magnolol on the NF- $\kappa\text{B}$  pathway, immunofluorescence staining of p65 was performed. The results revealed that the RANKL-induced translocation of p65 to the nucleus was inhibited by magnolol (Fig. 7A). Furthermore, the phosphorylation of the key proteins of the NF- $\kappa\text{B}$  pathway, namely I $\kappa\text{B}$ , p65 and p50, were assessed by western blotting.

Magnolol inhibited the phosphorylation of I $\kappa\text{B}$ , p65 and p50 (Fig. 7B) at different time points. These results indicate that magnolol suppressed the NF- $\kappa\text{B}$  signaling pathway.

The MAPK pathway is also important in osteoclastogenesis (13,15); thus, the phosphorylation of the key proteins, namely ERK, p38 and JNK were further examined by western blotting. It was observed that magnolol suppressed the RANKL-induced phosphorylation of ERK, p38 and JNK at different time points (Fig. 8). Therefore, magnolol may also suppress the activation of the MAPK signaling pathway.

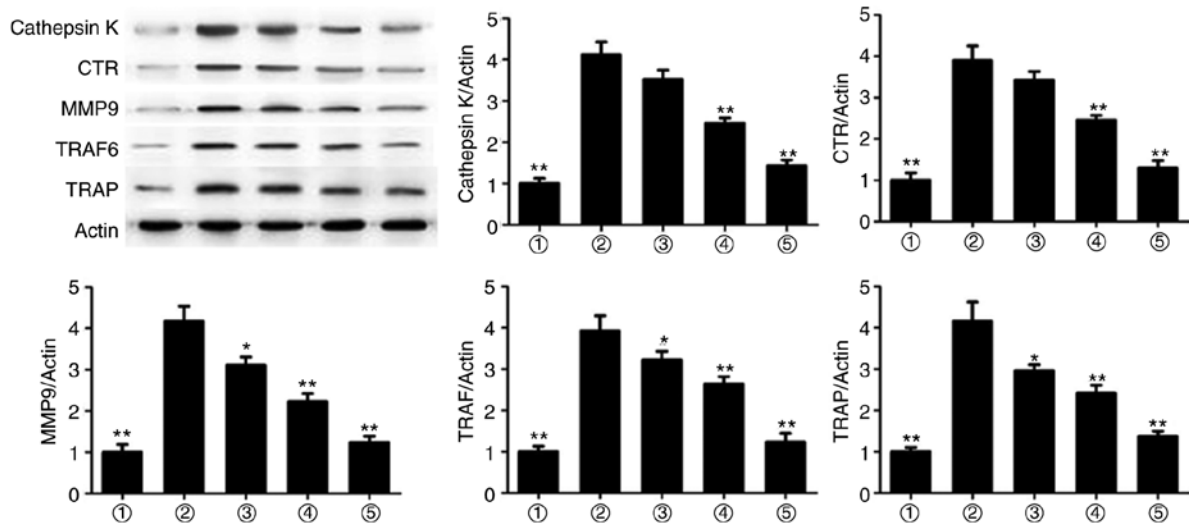


Figure 6. Magnolol suppresses the expression levels of the osteoclastogenesis markers cathepsin K, CTR, MMP9, TRAF6 and TRAP in RAW264.7 cells. \* $P < 0.05$  and \*\* $P < 0.01$ , vs. RANKL-only group. 1, Control; 2, RANKL; 3, RANKL+5  $\mu\text{g/ml}$ ; 4, RANKL+10  $\mu\text{g/ml}$ ; 5, RANKL+20  $\mu\text{g/ml}$ . RANKL, receptor activator for nuclear factor- $\kappa\text{B}$  ligand; CTR, calcitonin receptor; MMP9, matrix metalloproteinase 9; TRAF6, TNF receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase.

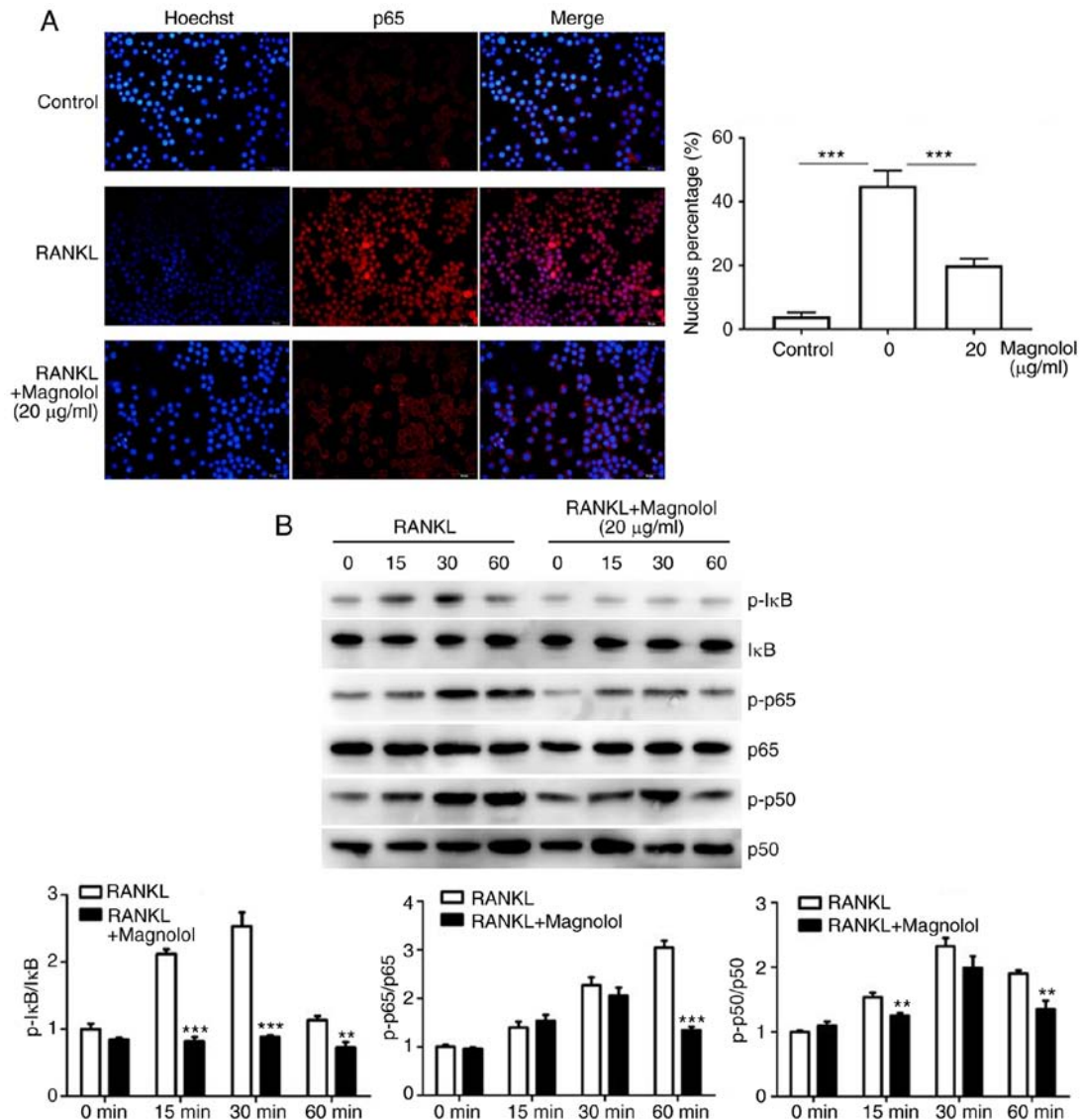


Figure 7. Magnolol suppresses the NF- $\kappa\text{B}$  pathway. (A) Magnolol inhibits RANKL-induced p65 translocation from the cytoplasm to the nucleus (magnification,  $\times 100$ ; bar=20  $\mu\text{m}$ ). (B) Magnolol inhibits the phosphorylation of I $\kappa\text{B}$ , p65 and p50, which are involved in the activation of the NF- $\kappa\text{B}$  pathway. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , vs. corresponding RANKL-only group. RANKL, receptor activator for nuclear factor- $\kappa\text{B}$  ligand; NF- $\kappa\text{B}$ , nuclear factor- $\kappa\text{B}$ ; I $\kappa\text{B}$ , inhibitor of  $\kappa\text{B}$ .

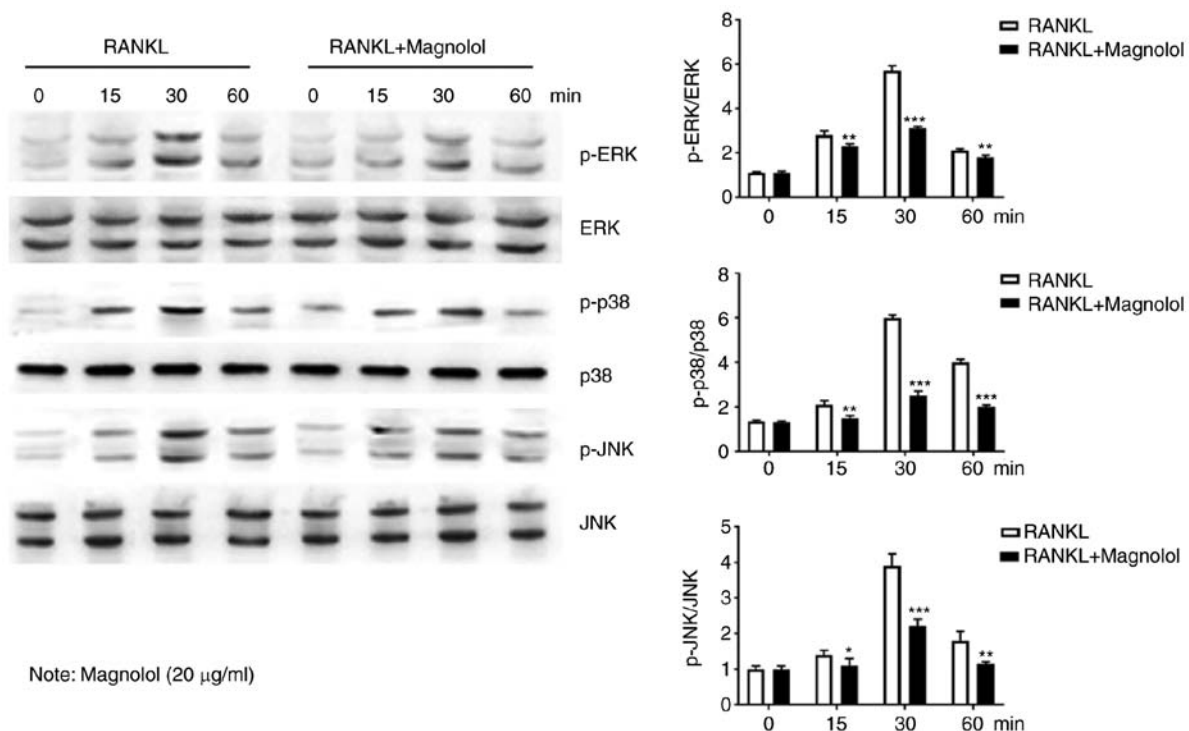


Figure 8. Magnolol inhibits the phosphorylation of ERK, p38 and JNK, which are involved in activation of the MAPK pathway. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , vs. corresponding RANKL-only group. RANKL, receptor activator for nuclear factor- $\kappa$ B ligand; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

## Discussion

A large number of middle-aged and elderly individuals worldwide suffer from osteoporosis (2,22). The pathogenesis of osteoporosis involves an imbalance between bone formation and resorption (23), which reflects an imbalance between osteoblasts and osteoclasts (24). Patients with osteoporosis are more vulnerable to fractures, which may lead to hypostatic pneumonia, bedsores, deep-vein thrombosis, pulmonary embolism, and urinary tract infection due to long-term immobilization (2,6,23). Calcium supplements and vitamin D are used to reverse bone loss (25); however, the clinical outcomes are unsatisfactory (6,23,25). For this reason, effort has been focused on developing drugs that are effective against osteoporosis (13,15,26).

Estrogen suppresses inflammation and inhibits osteoclast formation and function in bone tissue; therefore, postmenopausal women are more likely to develop osteoporosis (27,28). In the present study, the femurs of OVX mice were subjected to H&E staining and microcomputed tomography, as bone loss typically begins in regions with abundant cancellous bone (24). The data indicated that OVX-induced bone loss was significantly attenuated by magnolol treatment. To identify the underlying mechanism, TRAP-positive cells were counted and the concentration of TRAcP5B was determined, which reflects the degree of osteoclastogenesis (15); the results suggested that magnolol inhibits osteoclastogenesis. Therefore, magnolol assists the restoration of the osteoclast-osteoblast balance by suppressing the formation of osteoclasts. Magnolol alleviated OVX-induced osteoporosis by restraining osteoclastogenesis in a concentration-dependent manner. However, magnolol at greater than threshold concentrations suppressed cell proliferation. Furthermore, inflammation is

an important factor in the pathogenesis of osteoporosis (3). In the current study, the elevated serum IL-6 and TNF- $\alpha$  levels in OVX mice were reversed by magnolol treatment. Thus, inflammation serves an important role in osteoclastogenesis and bone resorption, and consequently anti-inflammatory agents may be useful against osteoporosis.

Traditional Chinese medicine provides an abundant source for drug development. A number of active components of Chinese herbs, such as matrine and epoxyecosatrienoic acids, can prevent bone loss (26,29,30). Magnolol is extracted from *Magnolia officinalis*, and has been reported to exhibit antioxidant, anti-inflammatory and anticancer effects (16-21). In addition, magnolol promotes the growth of osteoblasts and inhibits the differentiation of osteoclasts *in vitro* (27). Previous research has demonstrated that magnolol was able to ameliorate ligature-induced periodontitis in rats, which is an osteoclast-associated disease (31).

In the present study, magnolol suppressed osteoclastogenesis *in vivo* and *in vitro* by blocking the NF- $\kappa$ B and MAPK signaling pathways. *In vitro*, TRAP staining revealed that magnolol inhibited RANKL-induced osteoclastogenesis by BMMCs and RAW264.7 cells. Osteoclast function, in terms of F-actin ring formation and bone-pit formation, was also inhibited by magnolol. Furthermore, the expression levels of the osteoclastogenesis markers cathepsin K, CTR, MMP9, TRAF6 and TRAP were suppressed by magnolol.

RANKL serves a vital role in osteoclast maturation (32). RANKL binds to RANK on immature osteoclasts, resulting in the activation of the NF- $\kappa$ B and MAPK pathways (11,12,20). The NF- $\kappa$ B pathway is closely associated with osteoclastogenesis and inflammation, and agents targeting this pathway are used to treat osteoporosis and inflammatory conditions (13,28).



Following the induction of RANKL, p65 translocates to the nucleus to activate gene transcription, and this translocation was suppressed by magnolol in the present study. Furthermore, magnolol decreased the phosphorylation of I $\kappa$ B, p65 and p50 in a time-dependent manner, which subsequently suppressed gene transcription. It was also observed that magnolol suppressed the MAPK pathway, as indicated by inhibition of the phosphorylation of ERK, p38 and JNK.

As mentioned earlier, magnolol was demonstrated to inhibit osteoclastogenesis *in vivo* and *in vitro*; however, it remains unclear whether magnolol affects osteoblast formation. A previous study reported that magnolol promoted the function of MC3T3-E1 osteoblasts (27), but further research is required on this subject. Furthermore, the present study demonstrated that magnolol inhibited osteoclast maturation by suppressing the NF- $\kappa$ B and MAPK pathways. However, whether magnolol affects the AKT pathway or other signaling transduction pathways remains unclear. Identification of the genes targeted by magnolol that mediate its inhibition of osteoclast formation would provide further insight on the pathways involved. Finally, in the current study, magnolol inhibited bone loss in a model involving estrogen; however, the effects of magnolol on other osteoclastogenesis-associated diseases, including arthritis and bone tumors, warrant further investigation.

In conclusion, the current data indicated that magnolol suppressed osteoclastogenesis *in vivo* and *in vitro* by blocking the NF- $\kappa$ B and MAPK pathways, suggesting its potential use in the treatment of osteoporosis and associated disorders.

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#### Availability of data and materials

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

#### Authors' contributions

The study was conceived and designed by WYF and TL. WYF and LJQ conducted most of the experiments with assistance from QH and PQZ. The paper was written by WYF, with contributions from TL. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

The experiments on mice were approved by the Ethics Committee on Animal Experiments of the Central Hospital of Zibo (Zibo, China).

#### Patient consent for publication

No patients were involved in this study.

#### Competing interests

The authors declare that they have no competing interests and no financial associations to disclose.

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