Diosmetin inhibits osteoclast formation and differentiation and prevents LPS-induced osteolysis in mice

Siyuan Shao1* | Fangsheng Fu1* | Ziyi Wang2 | Fangming Song1,2 | Chen Li1 | Zuo-xing Wu1 | Jiaxing Ding1 | Kai Li1 | Yu Xiao1 | Yiji Su1 | Xixi Lin1 | Guixin Yuan4 | Jinmin Zhao1 | Qian Liu1,3 | Jiake Xu1,2

1Research Centre for Regenerative Medicine, Guangxi Key Laboratory of Regenerative Medicine, Guangxi Medical University, Nanning, Guangxi, China
2School of Biomedical Sciences, the University of Western Australia, Perth, Western Australia, Australia
3Department of Trauma Orthopedic and Hand Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China
4Department of Orthopedics, The Second Affiliated Hospital, Shantou University Medical College, Shantou, Guangdong, China

Correspondence
Jiake Xu, School of Biomedical Sciences, The University of Western Australia, Perth, Western Australia, Australia. Email: jiake.xu@uwa.edu.au.
Qian Liu, Research Centre for Regenerative Medicine, Guangxi Medical University, Guangxi, China. Email: luoboqian@hotmail.com

Funding information
Australian Health and Medical Research Council, Grant/Award Numbers: APP1107828, APP1027932, APP1163933; National Natural Science Foundation of China, Grant/Award Number: 81501910; Natural Science Foundation of Guangxi Province, Grant/Award Numbers: 2015GXNSFCAA14001, 2015GXNSFDA139019

Abstract
Osteolytic bone diseases are closely linked to the over-activation of osteoclasts and enhancement of bone resorption. It has become a major health issue in orthopedic practice worldwide. Inhibition of osteoclasts is proposed to be the main treatment for osteolytic disorders. Diosmetin (DIO) is a natural flavonoid with properties of antioxidant, anti-infection, and antishock. The effect of DIO on osteoclast differentiation is poorly understood. In this study project, we found that DIO could inhibit osteoclast formation induced by receptor activator of nuclear factor kappa B ligand (RANKL) in a dose-dependent manner. The expression of the osteoclast differentiation marker genes, cathepsin K, nuclear factor of activated T-cells 1 (NFATc1), Acp5, Ctr, Atp6v0d2, and Mmp9 were also decreased by the treatment of DIO. In addition, DIO attenuated the formation of actin ring and the ability of bone resorption. Further, the western blotting showed that DIO inhibits the phosphorylation of the mitogen-activated protein kinases signaling pathway induced by RANKL, accompanied by the downregulation of NFATc1 and c-Fos expression. We also found that DIO could reduce the accumulation of reactive oxygen species (ROS) induced by RANKL. In vivo, the study revealed that DIO can significantly reduce LPS-induced osteolysis in mice. Collectively, our study shows that DIO can inhibit osteoclast formation and activation, and could serve as a potential therapeutic drug for osteolytic bone diseases.

KEYWORDS
diosmetin (DIO), MAPK, nuclear factor of activated T-cells 1 (NFATc1), osteoclasts, osteolysis

1 INTRODUCTION

Bone is a dynamic organ that makes up the vertebrate skeleton (Teitelbaum, 2000). The homeostasis of the bone depends on bone formation and bone resorption, which are regulated by osteoblasts and osteoclasts (Kular, Tickner, Chim, & Xu, 2012). The imbalance or uncoupling between these two types of cells can result in diseases such as osteoarthritis, bone sclerosis, and bone destruction or osteolysis. Osteoclasts are polymeric bone resorption cells derived from hematopoietic stem cells (Manolagas & Jilka, 1995). The formation of mature osteoclasts with the bone resorption activity depends on two necessary cytokines, receptor activator of nuclear factor-kappa B ligand (RANKL) and macrophage colony stimulation factor (M-CSF; Enomoto et al., 2003; Kim...
et al., 2016). M-CSF promotes osteoclasts precursor differentiation and fusion into multinucleated cells. RANKL plays an essential role in promoting the differentiation and maintenance of osteoclasts. Binding of RANKL to RANK receptor in osteoclasts precursors recruits tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and then activates mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) signal pathways (Ang et al., 2011; Deepak, Kasonga, Kruger, & Coetzee, 2015). Subsequently, several essential transcription factors required for osteoclast differentiation are induced, such as nuclear factor of activated T-cells 1 (NFATc1) and activator protein 1 (AP-1; Feng et al., 2009). Among them, NFATc1 is believed to be the main transcription factor regulating the downstream osteoclast marker genes such as cathepsin K (Ctsk), Mmp9, and Acp5, which are essential to osteoclast formation and resorption activity (Maruya et al., 2011).

Bacterial endotoxin or wear particles after artificial joint replacement may lead to inflammatory osteolysis (Lee et al., 2010). The immune cells are activated to regulate osteoclasts, which could enhance the osteoclastic resorption activity near and around the implant interface, causing local bone destruction (Hotokezaka et al., 2010; Islam et al., 2007). Moreover, the pathological osteolytic condition induced by lipopolysaccharide (LPS) was characterized by the production of inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and M-CSF (Hotokezaka et al., 2010). As a result, osteoclasts are produced in large quantity accompanied by abnormally functional activation in osteolytic conditions (Xu et al., 2009).

Therefore, reducing the formation or the activity of osteoclasts can inhibit bone resorption and prevent excessive loss of bone mass (Sato et al., 1991). For example, estrogen and bisphosphonates have been used in the clinical treatment of osteolyis. However, these treatments have some concerning side effects. Estrogen therapy is associated with the increased risks of breast cancer, stroke, and thromboembolism. Bisphosphonates can lead to osteonecrosis of the jaw (Otto, Sotlar, Ehrenfeld, & Pautke, 2011; Rodan & Martin, 2000). Consequently, it’s necessary to look for natural compounds with lower toxicity but effective against osteoclasts.

Diosmetin (DIO) is a natural compound extracted from lemon peel. It has been shown to exhibit an inhibitory effect on CYP1A1 and CYP1B1 (Doostdar, Burke, & Mayer, 2000), and strong anticancer and anti-inflammatory properties (Ciellino, Wang, & Yeh, 1998). In the skeletal system, DIO can induce the differentiation of human osteoblasts through the pathways of protein kinase C/p38 and extracellular signal-regulated kinase 1/2 (Hsu & Kuo, 2010). However, the role of DIO in osteolytic disorders and osteoclast differentiation has not been reported.

On the basis of the potential pharmacological effects of DIO, we explored the role of DIO in RANKL-induced osteoclast differentiation in vitro and in the LPS-induced osteolysis model in mice as well as the key molecular signaling mechanisms involved.

2 | MATERIALS AND METHODS

2.1 | Materials

DIO with a purity >97% was purchased from Mansite (Chengdu, China) and prepared at a stock concentration of 100 mM in dimethyl sulfoxide. Alpha minimum essential medium (α-MEM) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Sydney, Australia). Recombinant mouse M-CSF was obtained from R&D Systems (Minneapolis, MN). Recombinant GST-rRANKL was prepared as previously described (Xu et al., 2000). Antibodies against ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK, IκBα, c-Fos, and β-actin were obtained from Abcam (Cambridge, MA). The antibody specific for NFATc1 was obtained from Santa Cruz Biotechnology (Dallas, TX). The cell counting kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Kumamoto, Japan). The tartrate-resistant acid phosphatase (TRAcP) staining kit was purchased from Sigma-Aldrich (St. Louis, MO).

2.2 | Bone marrow-derived macrophages isolation and osteoclast culture

Six-week-old C57BL/6 J mice were killed and the bone marrow cells obtained by the exposure of medullary cavity of femur and tibia and cultured in α-medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml M-CSF (complete medium). To induce osteoclastogenesis, bone marrow-derived macrophages (BMMs) at a density of 6 × 10⁴ were seeded into 96-well plates with complete medium containing M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 7 days with indicated concentrations of DIO. The cells were incubated in a standard atmosphere, and culture medium renewed every 2 days until mature osteoclast formation. The cells were then fixed with 4% paraformaldehyde for TRAcP staining. TRAcP-positive multinucleated cells with more than three nuclei were counted as mature osteoclasts.

2.3 | Cell viability assay

The CCK-8 kit was used to detect the cytotoxicity of DIO on BMMs. BMMs at a density of 6 × 10⁵ were cultured in a 96-well plate with a complete α-MEM medium containing M-CSF (50 ng/ml) and different concentrations of DIO for 48 hr. Subsequently, the CCK-8 kit solution (10 μ/l/well) was added to each well and incubated for 2 hr without light. The absorbance at 450 nm was measured by a microplate reader (Thermo Fisher Scientific, MA).

2.4 | Bone resorption pit assay

To assess the effect of DIO on the osteoclast resorption activity, BMMs, at a density of 6 × 10⁵, were cultured in hydroxyapatite-coated plates (Corning, Inc., Corning, NY) with the presence of M-CSF (50 ng/ml) and RANKL (100 ng/ml) and treated with indicated
concentrations of DIO for 5–7 days to generate multinucleated osteoclasts. 10% sodium hypochlorite solution was then added to remove the cells to display the resorption area. Next, resorption areas were quantified by Standard light microscopy and the Image J software (NIH).

2.5 | Real-time quantitative polymerase chain reaction

To detect the expression of the marker genes in osteoclasts, BMMs at a density of $1 \times 10^5$ were seeded in a six-well plate with complete α-medium containing of M-CSF (50 ng/ml) and RANKL (100 ng/ml) and treated with indicated concentrations of DIO for 5 days. When osteoclasts appeared in the positive group, cells were lysed by TRizol buffer at 4°C according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from 1 µg of total RNA using the reverse transcriptase kit (Takara Biotechnology, Otsu, Japan). And glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as a housekeeping gene. The real-time quantitative polymerase chain reaction (RT-PCR) was performed using the following procedures: 94°C for 5 min, followed by 35 cycles (94°C for 40 s, 60°C for 40 s, and 72°C for 40 s) and a final extension step of 5 min at 72°C. The relative expression levels of osteoclast marker genes were analyzed using the 2$^{-\Delta\Delta Ct}$ method. The mouse-specific primer sequence (5’-3’) for the experiment is shown as follows: TRAcP (Acp5; forward: ACCAGCTTGAAGMGCACC; reverse: CCAGAGCTCCACATTAGTGGG), Ctsk (forward: GGCACAGCTAAGAACAAAAC; reverse: GTGCTTGGCTTCCTTGG), MMP-9 (Mmp9; forward: AGTCTGTGTCGGCGGCAC; reverse: TACATGAGCGCTTCGGAC) NFATc1 (Nfatc1; forward: GGGTGCTCAGACAGAT; reverse: GGAAGTCAAGTCTGGTG), V-ATPase-d2 (Atp6vd2; forward: GTGACAGCTTGGAGACCTTAA; reverse: GAGAAATGTGCTCAGGGCGC), CTR (Calc; forward: TGTTGAATTGTTGCGCCA; reverse: TCTGGTGGGCTTCATC) GAPDH (Gadph; forward: AACTTGGCATTGTGGAAGG; reverse: ACACATTGGCAGACCCCA).

2.6 | Western blot analysis

BMMs, at a density of $5 \times 10^5$, were cultured in six-well plates with complete medium containing M-CSF (50 ng/ml). Until the BMMs almost covered each well, the cells were starved for 1 hr and then treated with or without 10 µM DIO for 3 hr. BMMs were then treated with 100 ng/ml RANKL for 0, 5, 10, 20, 30, or 60 min. Radioimmunoprecipitation assay lysis buffer was added to the cells for 30 min at 4°C. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes and blocked in 5% Albumin from bovine serum (BSA) for 90 min. The membranes were washed with phosphate-buffered saline (PBS) and incubated for more than 12 hr with specific primary antibodies. After that, membranes were washed with Tris Buffered Saline Tween for three times and incubated with the corresponding second antibody at normal temperature for 2 hr. Finally, protein bands are displayed through the Odyssey imaging system (LI-COR, NE) and measured by Image J software.

2.7 | Actin ring formation assays and 4′,6-diamidino-2-phenylindole staining

BMMs at a density of $6 \times 10^2$ were cultured in 96-well plates with complete α-MEM medium containing M-CSF (50 ng/ml) and RANKL (100 ng/ml) and treated with 5 µM or 10 µM DIO for 5 days. When osteoclast formation was observed, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were blocked by 3% BSA in PBS for 30 min. Next, cells were incubated with 0.2% phalloidin in PBS at room temperature for 60 min. 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to observe the nucleus. Multiple images of the cells were taken using a fluorescent microscope.

2.8 | Intracellular ROS detection

The reactive oxygen species (ROS) levels in the cells were determined by the ROS assay kit (Beijing time, Shanghai, China) with 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA). BMMs were seeded at a density of $6 \times 10^3$ cell/well in the 96-well plates with M-CSF (50 ng/ml) and RANKL (100 ng/ml) and treated with the indicated concentration of DIO for 3 days. Then, the complete medium was replaced with a pure medium; cells were incubated with 10 µM DCFH-DA in the cell incubator for 40 min and washed with PBS four times, the images were obtained by fluorescence microscopy.

2.9 | LPS-induced osteolysis in vivo

All mice involved in this study were obtained from the Animal Experiment Center of Guangxi Medical University and experimental procedures following the guidelines of the Animal Care Committee of Guangxi Medical University. A total of 24 male eight-week-old C57/BL6J mice were randomly assigned to four groups, including the following groups: Sham group (PBS; control), vehicle group (LPS; 5 mg/kg body weight), LPS with low dose DIO (5 mg/kg body weight) and LPS with high dose DIO (10 mg/kg body weight). All treatments were implemented after the mice were anesthetized with chloral hydrate, and the subcutaneous injection was made at the sagittal midline of the calvaria. Then, injection of PBS or DIO was performed as prophylactic therapy on the day before LPS injection. The Sham and vehicle groups were administered with PBS alone in an interval of every two days. The DIO groups were treated with different concentrations of DIO every second day. After 7 days of treatment, all the mice were euthanized. Mouse calvarias were collected and subjected to histocyte fixative for micro-CT and histological analysis. During the whole experiment, all four groups of mice survived and the DIO treatment group mice showed no abnormal activity. The bone volume/tissue volume ratio (BV/TV), number of porosity, and percentage of total porosity were analyzed by micro-CT (Skyscan 1176; Skyscan,
Aartselaar, Belgium). After micro-CT analyses, the calvaria samples were decalcified for 2 weeks in 10% EDTA. Then, paraffin-embedded histopathological sections were used for TRACP staining, hematoxylin and eosin (H&E) staining, and immunohistochemical staining as previously described (Song et al., 2017; Wei et al., 2018). The TRACP-positive osteoclasts were determined by ImageJ software.

### 2.10 Statistical analysis

All experiments were performed three times or more independently, and all experimental data were presented as the mean ± standard deviation (SD). The statistical analyses between the two groups were conducted by using Student’s t tests and SPSS 22.0 software (SPSS Inc.). And the p < 0.05 were considered statistically significant.

### 3 RESULTS

#### 3.1 DIO inhibited osteoclastogenesis in vitro

To investigate whether DIO can effectively inhibit osteoclast differentiation induced by RANKL, BMMs were cultured in the presence of M-CSF and RANKL, then treated with designated concentrations of DIO. After 7 days, mature osteoclasts were found in the positive control group. The experimental data showed that DIO could inhibit RANKL-induced osteoclast differentiation in a dose-dependent manner (Figure 1a–b). Compared to the control group, the total quantity of TRACP-positive multinucleated cells had a decreasing trend with an increase in the concentration of DIO from 0 μM to 10 μM (Figure 1c). To further explore the time course effect of DIO on the osteoclastic formation, DIO was added in several designed time periods (1–3 days, 3–5 days, 5–6 days, and 1–6 days; Figure 1d). The results suggested that DIO significantly reduces RANKL-induced osteoclast differentiation at three different stages (1–3 days, 3–5 days, and 1–6 days; Figure 1e,f), and most potently in the mid-stage. To eliminate the toxicity of DIO to BMM cells, which could influence the differentiation of osteoclasts, we did the cell proliferation test (CCK-8). Our data confirmed that DIO with concentrations lower than 10 μM has no cytotoxic effect on BMMs (Figure 1g). Taken together, DIO could inhibit RANKL-induced osteoclast differentiation in a concentration-dependent manner within the non-cytotoxic concentration range.

#### 3.2 DIO inhibited osteoclast marker gene expression in vitro

To estimate the effect of DIO on osteoclastic formation and resorption related gene, including Ctsk, Nfatc1, Acp5, Atp6v0d2, Ctr, and Mmp9. BMMs were treated with RANKL with DIO at concentrations of 5 μM and 10 μM. And the results of RT-PCR showed that the expression of osteoclast marker gene is down-regulated in a dose-dependent manner relative to the control group (Figure 2a–f). These data suggest that DIO inhibited the expression of the signature gene in osteoclasts, thus affected the formation and function of osteoclasts.

#### 3.3 DIO impairs osteoclast bone resorption in vitro

To further evaluate the effect of DIO on the osteoclastic resorption activity, hydroxyapatite plates were used to measure the bone resorption ability of osteoclasts. BMMs were seeded in 96-well hydroxyapatite plates until mature osteoclasts were formed. Obvious erosion of hydroxyapatite plates could be observed in the positive group. As expected, the resorption of hydroxyapatite was significantly inhibited by 5 μM and 10 μM of DIO (Figure 2g,h). We also explored whether DIO influenced the formation of the F-actin ring, which is an important hallmark for the function of osteoclasts. F-actin ring was observed by fluorescence microscope. In the group treated with RANKL only, we were able to observe a large number of intact actin rings. However, the formation of the actin ring was significantly inhibited by 5 μM and 10 μM DIO (Figure 3a). This is consistent with the observed decline in bone resorption. Based on these findings, DIO was able to inhibit the resorption activity of osteoclast accompanied by the impairment of F-actin ring formation in vitro.

#### 3.4 DIO inhibited RANKL-induced ROS level in vitro

To determine whether DIO plays an antioxidant role in osteoclasts, BMMs were seeded in 96-well plates with M-CSF and RANKL, and treated with PBS or DIO for 72 hr. The level of ROS was determined in the cells. Obviously, intracellular ROS levels were increased by the RANKL treatment. Notably, the active oxygen products were reduced in BMM cells after treatment with 5 μM and 10 μM DIO (Figure 3b). The signal intensity and quantity of ROS positive cells after the DIO treatment showed a decreasing trend in a concentration-dependent manner (Figure 3c).

#### 3.5 DIO repressed the MAPK signaling pathway but almost no effect on NF-κB pathways during RANKL-induced osteoclastogenesis in vitro

The MAPK and NF-κB signaling pathways in RANKL-induced osteoclastogenesis were also investigated. The results of western blot analysis showed that the phosphorylation of ERK1/2 and JNK1/2 was markedly inhibited by DIO at 10 and 20 min compared with the control group (Figure 4a–c), however there was only little effect on the phosphorylation of the p38 signaling pathway (Figure 4d). The degradation of the inhibitor of nuclear factor kappa-B α (IκBα) was not inhibited by DIO compared with the control group (Figure 4e). Collectively, we found that DIO effectively suppresses phosphorylation of the MAPK signaling pathway induced by RANKL. But has no effect on the NF-κB signaling pathway.
FIGURE 1  DIO inhibited RANKL-induced osteoclastogenesis in vitro. BMMs were cultured in the presence of M-CSF and RANKL. (a) A diagram showing the DIO chemical formula. (b) BMMs were treated with various concentrations of DIO for 7 days and then subjected to TRAcP staining (magnification = 100×; scale bar = 100 µM). (c,f) The numbers of TRAcP-positive multinucleated cells (≥3 nuclei) were measured. (d) Treatment periods of DIO are listed. (e) The time course effect of DIO (10 µM) during the 7 days process of osteoclast formation. (g) BMMs were cultured for 48 hr with 50 ng/ml M-CSF at the indicated concentrations of DIO. Cell viability was determined by the CCK-8 assay. (**p < 0.01 ***p < 0.001 compared with control). BMMs: bone marrow-derived macrophages; CCK-8: cell counting kit-8; DIO: diosmetin; M-CSF: macrophage colony stimulation factor; RANKL: receptor activator of nuclear factor κ-B ligand; TRAcP: tartrate-resistant acid phosphatase [Color figure can be viewed at wileyonlinelibrary.com]
DIO reduced RANKL-induced gene expression in osteoclast and bone resorption activity in vitro. BMMs were incubated for 5 days with M-CSF and RANKL and treated with the indicated concentrations of DIO. The relative mRNA expression of (a) NFATc1, (b) Acp5, (c) Atp6v0d2, (d) Ctr, (e) Ctsk, and (f) Mmp9 was analyzed by RT-PCR. The gene expression was normalized to GAPDH. (g) BMMs were cultured in hydroxypatite-coated plates and treated with the indicated concentrations of DIO until mature osteoclasts were formed. Osteoclast was removed, and images were obtained by light microscope (magnification = 40×; scale bar = 200 µm). (h) The total areas of resorption pits were measured using ImageJ. (*p < 0.05  **p < 0.01 ***p < 0.001 relative to control). BMM: bone marrow-derived macrophage; Ctsk: cathepsin K; DIO: diosmetin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; M-CSF: macrophage colony stimulation factor; RANKL: receptor activator of nuclear factor-κB ligand; RT-PCR: real-time polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 3  DIO suppressed formation of actin ring and ROS level induced by RANKL.
(a) DIO disrupts actin ring formation in osteoclasts. Cultured cells were stained with rhodamine-phalloidin (F-actin) and DAPI, then examined by immunofluorescence microscopy.
(b) BMMs were cultured in the absence or presence of RANKL and treated with or without DIO for 3 days. The images were taken by using a fluorescence microscope after incubation with DCFH-DA for 30 min (magnification = 100×; scale bar = 100 µM).
(c) Detection of the number of ROS positive cells. (***p < 0.001 relative to control). BMM: bone marrow-derived macrophages; DAPI: 4',6-diamidino-2-phenylindole; DCFH-DA: 2, 7-dichlorodihydrofluorescein diacetate; DIO: diosmetin; RANKL: receptor activator of nuclear factor κB ligand; ROS: reactive oxygen species [Color figure can be viewed at wileyonlinelibrary.com]
3.6 | DIO suppressed RANKL-induced NFATc1 expression

Next, we evaluated the expression levels of RANKL-induced transcription factors NFATc1 and c-Fos, which are critical to the terminal differentiation of osteoclasts. By western blotting, we found that the protein expression of NFATc1 was suppressed on Day 1, 3, and 5 (Figure 4f,g) whereas the expression of c-Fos was mainly inhibited on Day 1 (Figure 4h) relative to the control group. These
results revealed that DIO inhibits the protein expression of key transcription factors NFATc1 and c-Fos.

3.7 DIO protected against LPS-induced osteolysis

Finally, to measure the therapeutic effect of DIO in vivo, we used an osteolytic model of C57/BL6J mice. LPS was injected subcutaneously near the suture of the mouse skull to induce osteolysis at the site of the calvaria. Micro-CT scanning and 3D image reconstruction were used to analyze the extent of bone erosion. LPS injection (LPS group) showed significant bone erosion on the calvaria compared to the sham group (PBS injection). Meanwhile, the DIO treated group (5 mg/kg group and 10 mg/kg group) displayed an inhibitory effect on inflammatory osteolysis induced by LPS (Figure 5a). This finding was confirmed by a further quantitative analysis of bone parameters, which showed that the DIO’s treatment effectively protects against the loss of bone volume/tissue volume and decreased the bone porosity (Figure 5b-d). Further, histological analysis and assessment confirmed that DIO reduces LPS-induced bone loss, inflammatory bone erosion, and a number of osteoclasts (Figure 5e-g). Finally, immunohistochemical staining showed that the expression of CTSK was downregulated and the expression of runt-related transcription factor 2 (RUNX2) was enhanced (Figure 6). Collectively, our findings suggested that DIO could effectively suppress LPS-induced osteolysis in mice.

4 DISCUSSION

Bone resorption and bone remodeling occur continuously to maintain healthy bone homeostasis (Kular et al., 2012; Zhu et al., 2018). The abnormal increase of osteoclasts and bone resorption can lead to osteoporosis and aseptic loosening of the prosthesis after joint replacement (Citak, Cross, Gehrke, Dersch, & Kendoff, 2015; Harris, 2001). Therefore, effective measures to prevent and treat osteolytic diseases were to target the inhibition of osteoclastic formation and function (Boyle, Simonet, & Lacey, 2003; Kular et al., 2012; Xu et al., 2009). In our research, DIO was demonstrated to have the pharmacological characteristics of inhibiting RANKL-induced osteoclastogenesis and ROS level as well as LPS-induced bone loss in mice. Given the previously proven anti-inflammatory and anti-infection activity of DIO, it is suggested that DIO might serve as a candidate for the treatment of inflammatory osteolysis. Therefore, it is of significance to elucidate the potential molecular and cellular mechanisms by which DIO inhibits RANKL-induced osteoclast formation.

The differentiation of osteoclasts is mainly regulated by the classical signal pathways of RANKL/RANK (Takayanagi, 2009), in which TRAF6 was recruited to initiate downstream signal pathways, such as NF-κB, MAPKs, and to activate and amplify intracellular calcium signal to dephosphorylation of calcineurin (Boyle et al., 2003; Takayanagi, 2007). The subsequent activation of NFATc1 is essential for the production and maintenance of osteoclasts (Negishi-Koga & Takayanagi, 2009). According to the relevant research, the MAPK signaling pathway is composed of ERK, p38, and JNK signaling pathway (Shimo et al., 2007). The activity of MAPK is thought to be mainly regulated by the phosphorylation sites in the amino acid sequence of the activated domain (Liu, Shepherd, & Nelin, 2007). ERK regulate the expression of c-Fos and promote the precursor transformation to mature osteoclasts (Miyazaki et al., 2000). In our study, the phosphorylation of ERK and JNK were suppressed by DIO, but the mechanism of the inhibition of phosphorylation of the MAPK pathway by DIO needs further investigation. After the suppression of MAPK phosphorylation by DIO, the expression of NFATc1 and c-Fos was downregulated (Lee, Lee, Sung, & Yoo, 2014). These transcription factors mediate the end stage of osteoclast differentiation and the expression of Ctsk, Atp6vod2, Acp5, and other genes (Crotti et al., 2008; Matsumoto et al., 2004). Based on the results shown by PCR, we found that the expression of Ctsk, Atp6vod2, and Acp5 was inhibited after the treatment with DIO, which is consistent with the inhibitory effect of DIO on the NFATc1 protein expression (Song et al., 2016). In addition, DIO also suppresses the expression of c-Fos, which participates in the formation of AP-1 complexes and promotes the differentiation and function of osteoclasts (Matsuo et al., 2000). In simple terms, DIO decreases the expression of NFATc1 by inhibiting the phosphorylation of the MAPK pathway, which we believe is the main molecular mechanism of DIO inhibiting the differentiation of osteoclasts induced by RANKL.

Apart from the RANKL-induced MAPK signaling pathway, NF-κB signaling pathway is also crucial in osteoclast differentiation (Baud’Huin et al., 2007). NF-κB exists in the cytoplasm in the form of homologous dimer and binds to IκBα protein in the resting state (Chen, Wu, & Ghosh, 2003). When RANKL interacts with RANK, the recruitment of TRAF6 makes the complex of NF-κB and IκBα disintegrate and NF-κB is translocated to the nucleus to perform its function (Ea, Sun, Inoue, & Chen, 2004). However, there was no significant degradation of IκBα with the treatment of DIO, so the activation of NF-κB is largely not affected.

ROS is the product of living cells under stress, including superoxide radical, hydrogen peroxide, hydroxyl radical, and so on (Simon, Hajyehia, & Levischaffer, 2000). RANKL can significantly increase the ROS level in osteoclasts (Song et al., 2018; Yip et al., 2010). According to the relevant studies, ROS production may be due to the transfer of excess unpaired electrons in the oxidative respiratory chain to O2 (Kovac et al., 2015). ROS production in BMMs not only activates the MAPK signaling pathways but also increases the Ca2+ activity and activates the transcription factor NFATc1 (Ha et al., 2004; Koh et al., 2010). Our experimental data showed that DIO effectively decreased the RANKL-induced ROS signals in osteoclasts. The reduction of intracellular ROS level may also be one of the reasons for inhibiting RANKL-induced osteoclast differentiation.

Based on the in vitro experimental data, an LPS animal model was used for the evaluation of the role of DIO in vivo (Song et al., 2017). LPS, as a classical gram-negative bacterial endotoxin (Bi et al., 2010), not only induces the recruitment of monocytes and macrophages but
also stimulates the production of many inflammatory factors. These cytokines can promote osteoclast formation, induce bone resorption, and lead to osteolysis or bone loss (Bi et al., 2010; Islam et al., 2007; Nair et al., 1996). In our study, DIO can rescue the bone destruction mediated by LPS, accompanied with abatement of inflammatory invasion and the reduction of the number of osteoclasts. In addition, immunohistochemical staining was performed to investigate the expression of CTSK associated with osteoclasts and RUNX2.

**FIGURE 5** DIO protected LPS-induced bone resorption in vivo. (a) Representative 3D reconstructed µCT images of the inside and outside of calvarias. (b,c,d) Percentage bone volume to tissue volume (BV/TV%), number of porosities, and the percentage of total porosity of whole calvaria for each sample were measured. (e) Representative images of decalcified bone stained with H&E and TRAcP from sham mice, LPS-injected mice, and LPS-injected mice treated with 5 mg/kg or 10 mg/kg DIO. (f) Number of TRAcP-positive cells. (g) Quantitative analyses of osteoclast surface/bone surface. Scale bars represent 200 µM in 40× and 100 µM in 100×. All data are expressed as mean ± SD. (*p < 0.05, **p < 0.01, ***p < 0.001 relative to the LPS group). DIO: diosmetin; H&E: hematoxylin and eosin; LPS: lipopolysaccharide; SD: standard deviation TRAcP: tartrate-resistant acid phosphatase [Color figure can be viewed at wileyonlinelibrary.com]
DIO: diosmetin; LPS: lipopolysaccharide; RUNX2: runt-related transcription factor 2

In summary, our study demonstrated for the first time that DIO has protective effect on LPS-induced osteolysis in mice. These results indicate that DIO might have potential clinical application value for osteoclast-related diseases.

ACKNOWLEDGMENTS

The authors acknowledged all the members of our laboratory for their assistance. Qian Liu, Fangming Song, and Jiake Xu designed the study, Siyuan Shao, Fangsheng Fu, Xixi Lin, Yiji Su, Ziyi Wang, Zuo-xing Wu, Jiaxing Ding, Kai Li, Yu Xiao, and Guixin Yuan performed the experiments, analyzed the data, and prepared the figures. Siyuan Shao wrote the main manuscript. Jinmin Zhao, Jiake Xu, and Qian Liu revised the manuscript and supervised the project. All authors reviewed the manuscript. This study was supported in part by National Natural Science Foundation of China (81501910), the Natural Science Foundation of Guangxi Province (2015GXNSFC414001, 2015GXNSFDA139019). This study was also supported in part by grants from the Australian Health and Medical Research Council (NHMRC, APP1107828, APP1027932, APP1163933).

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

Ang, E. S. M., Yang, X., Chen, H., Liu, Q., Zheng, M. H., & Xu, J. (2011). Naringin abrogates osteoclastogenesis and bone resorption via the inhibition of RANKL-induced NF-κB and ERK activation. FEBS Letters, 585(17), 2755–2762.

Baud, H., M., Duplomb, L., Velasco, C. R., Fortun, Y., Heymann, D., & Padrines, M. (2007). Key roles of the OPG–RANK–RANKL system in bone oncology. Expert Review of Anticancer Therapy, 7(2), 221–232.

Bi, Y., Seabold, J. M., Kaar, S. G., Ragab, A. A., Goldberg, V. M., Anderson, J. M., & Greenfield, E. M. (2010). Adherent endotoxin on orthopedic wear particles stimulates cytokine production and osteoclast differentiation. Journal of Bone & Mineral Research, 16(11), 2082–2091.

Boyle, W. J., Simonet, W. S., & Lacey, D. L. (2003). Osteoclast differentiation and activation. Nature, 423(6937), 337–342.

Chen, Y., Wu, J., & Ghosh, G. (2003). xB-Ras binds to the unique insert within the ankyrin repeat domain of IκBζ and regulates cytoplasmic retention of IκBζ-NF-κB complexes. Journal of Biological Chemistry, 278(25), 23101–23106.

Ciolo, H. P., Wang, T. T., & Yeh, G. C. (1998). Diosmin and diosmetin are agonists of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. Cancer Research, 58(13), 2754–2760.

Citak, M., Cross, M. B., Gehrke, T., Dersch, K., & Kendoff, D. (2015). Modes of failure and revision of failed lateral unicompartmental knee arthroplasties. The Knee, 22(4), 338–340.

Crottì, T. N., Sharma, S. M., Fleming, J. D., Flannery, M. R., Ostrowski, M. C., Goldring, S. R., & McHugh, K. P. (2008). P1.1 and NFATc1 mediate osteoclast induction of the mouse β3 integrin promoter. Journal of Cellular Physiology, 215(3), 636–644.

Deepak, V., Kasona, A., Kruger, M. C., & Coetzee, M. (2015). Inhibitory effects of eugenol on RANKL-induced osteoclast formation via attenuation of NF-κB and MAPK pathways. Connective Tissue Research, 56(3), 195–203.

Doostdar, H., Burke, M. D., & Mayer, R. T. (2000). Bioflavonoids: Selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1. Toxicology, 144(1), 31–38.

Ea, C. K., Sun, L., Inoue, J. I., & Chen, Z. J. (2004). TIFA activates IκB kinase (IKK) by promoting oligomerization and ubiquitination of TRAF6. Proceedings of the National Academy of Sciences of the United States of America, 101(43), 15318–15323.
Enomoto, H., Shiojiri, S., Hoshi, K., Furuichi, T., Fukuyama, R., Yoshiida, C. A., ... Komori, T. (2003). Induction of osteoclast differentiation by Runx2 through receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin regulation and partial rescue of osteoclastogenesis in Runx2-/- mice by RANKL transgene. *Journal of Biological Chemistry*, 278(26), 23971–23977.

Feng, H., Cheng, T., Steer, J. H., Joyce, D. A., Pavlos, N. J., Leong, C., ... Xu, J. (2009). Myocyte enhancer factor 2 and microphthalmia-associated transcription factor cooperate with NFATc1 to transactivate the V-ATPase d2 promoter during RANKL-induced osteoclastogenesis. *Journal of Biological Chemistry*, 284(21), 14667–14676.

Ha, H., Bok kwak, H., Woong lee, S., Mi jin, H., Kim, H. M., Kim, H. H., & Hee lee, Z. (2004). Reactive oxygen species mediate RANK signaling in osteoclasts. *Experimental Cell Research*, 301(2), 119–127.

Harris, W. H. (2001). Wear and periprosthetic osteolysis: The problem. *Clinical Orthopaedics*, 393(393), 66–70.

Hottokezaka, H., Sakai, E., Ohara, N., Hottokezaka, Y., Gonzales, C., Matsuo, K., ... Nakayama, K. (2010). Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF-α, lipopolysaccharide, or peptidoglycan. *Journal of Cellular Biochemistry*, 101(1), 122–134.

Hsu, Y. L., & Kuo, P. L. (2010). Diosmetin induces human osteoblastic differentiation through the protein kinase C/p38 and extracellular signal-regulated kinase 1/2 pathway. *Journal of Bone & Mineral Research*, 23(6), 949–960.

Islam, S., Hassan, F., Tumurkhuu, G., Dagvadorj, J., Koide, N., Naiki, Y., ... Yokochi, T. (2007). Bacterial lipopolysaccharide induces osteoclast formation in RAW 264.7 macrophage cells. *Biochemical & Biophysical Research Communications*, 360(2), 346–351.

Kim, H. J., Ohk, B., Yoon, H. J., Kang, W. Y., Seong, S. J., Kim, S. Y., & Yoon, Y. R. (2016). Docosahexaenoic acid signaling attenuates the proliferation and differentiation of bone marrow-derived osteoclast precursors and promotes apoptosis in mature osteoclasts. *Cellular Signalling*, 29, 226–232.

Koh, J. M., Lee, Y. S., Kim, Y. S., Kim, D. J., Kim, H. H., Park, J. Y., ... Kim, G. S. (2010). Homocysteine enhances bone resorption by stimulation of osteoclast formation and activity through increased intracellular ROS generation. *Journal of Bone & Mineral Research*, 21(7), 1003–1011.

Kovac, S., Angelova, P. R., Holmström, K. M., Zhang, Y., Dinkovakostova, A. T., & Abramov, A. Y. (2015). Nrf2 regulates ROS production by mitochondria and NADPH oxidase. *BBA - General Subjects*, 1850(4), 794–801.

Kular, J., Tickner, J., Chim, S. M., & Xu, J. (2012). An overview of the regulation of bone remodelling at the cellular level. *Clinical Biochemistry*, 45(12), 863–873.

Lee, M. S., Ikenoue, T., Trindade, M. C., Wong, N., Goodman, S. B., Schurman, D. J., & Smith, R. L. (2010). Protective effects of intermittent hydrostatic pressure on osteoarthritic chondrocytes activated by bacterial endotoxin in vitro. *Journal of Orthopaedic Research*, 28(1), 117–122.

Liu, Y., Shepherd, E. G., & Nelin, L. D. (2007). MAPK phosphatases—regulating the immune response. *Nature Reviews Immunology*, 7(3), 202–212.

Manolagas, S. C., & Jilka, R. L. (1995). Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *New England Journal of Medicine*, 49(10), 305–311.

Maruya, M., Suzuki, K., Fujimoto, H., Miyajima, M., Kanagawa, O., Wakayama, T., & Fagarasan, S. (2011). Vitamin A-dependent transcriptional activation of the nuclear factor of activated T cells 1 (NFATc1) is critical for the development and survival of B1 cells. *Proceedings of the National Academy of Sciences of the United States of America*, 108(2), 722–727.

Matsumoto, M., Kogawa, M., Wada, S., Takayanagi, H., Tsujimoto, M., Katayama, S., ... Nogi, Y. (2004). Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. *Journal of Biological Chemistry*, 279(44), 45969–45979.

Matsuo, K., Owens, J. M., Tonko, M., Elliott, C., Chambers, T. J., & Wagner, E. F. (2000). Fos1 is a transcriptional target of c-Fos during osteoclast differentiation. *Nature Genetics*, 24(2), 184–187.

Miyazaki, T., Katagiri, H., Kanegae, Y., Takayanagi, H., Sawada, Y., Yamamoto, A., ... Tanaka, S. (2000). Reciprocal role of ERK and NF-κb pathways in survival and activation of osteoclasts. *Journal of Cell Biology*, 148(2), 333–342.

Nair, S. P., Meghji, S., Wilson, M., Reddi, K., White, P., & Henderson, B. (1996). Bacterially induced bone destruction: Mechanisms and misconceptions. *Infection & Immunity*, 64(7), 2371–2380.

Negishi-Koga, T., & Takayanagi, H. (2009). Ca 2+ -NFATc1 signaling is an essential axis of osteoclast differentiation. *Immunological Reviews*, 231(1), 241–256.

Otto, S., Sotlar, K., Ehrenfeld, M., & Paukte, C. (2011). Osteonecrosis of the jaw as a possible rare side effect of annual bisphosphonate administration for osteoporosis: A case report. *Journal of Medical Case Reports*, 5(1), 477.

Rodan, G. A., & Martin, T. J. (2000). Therapeutic approaches to bone diseases. *Science*, 289(5484), 1508–1514.

Sato, M., Grasser, W., Endo, N., Akins, R., Simmons, H., Thompson, D. D., Rodan, G. A. (1991). Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *Journal of Clinical Investigation*, 88(6), 2095–2105.

Shimo, T., Matsumura, S., Ibaragi, S., Isowa, S., Kishimoto, K., Mese, H., ... Sasaki, A. (2007). Specific inhibitor of MEK-mediated cross-talk between ERK and p38 MAPK during differentiation of human osteosarcoma cells. *Journal of Cell Communication & Signaling*, 1(2), 103–111.

Simon, H. U., Hajyehia, A., & Levishaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Aptosis*, 5(5), 415–418.

Song, D., Cao, Z., Liu, Z., Tickner, J., Qiu, H., Wang, C., ... Xu, J. (2018). *Cistanche deserticola* polysaccharide attenuates osteoclastogenesis and bone resorption via inhibiting RANKL signaling and reactive oxygen species production. *Journal of Cellular Physiology*, 233, 9674–9684.

Song, F., Zhou, L., Zhao, J., Liu, Q., Yang, M., Tan, R., ... Xu, J. (2016). Eriodictyol inhibits RANKL-induced osteoclast formation and function via inhibition of NFATc1 activity. *Journal of Cellular Physiology*, 231(9), 1983–1993.

Song, F., Wei, C., Zhou, L., Qin, A., Yang, M., Tickner, J., ... Xu, J. (2017). Luteoloside prevents lipopolysaccharide-induced osteolysis and suppresses RANKL-induced osteoclastogenesis through attenuating RANKL signaling cascades. *Journal of Cellular Physiology*, 233(2), 1723–1735.

Takayanagi, H. (2007). Osteoimmunology: Shared mechanisms and cross-talk between the immune and bone systems. *Nature Reviews Immunology*, 7, 292–304.

Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science*, 289(5484), 1504–1508.

Wei, C. M., Su, Y. J., Qin, X., Ding, J. X., Liu, Q., Song, F. M., ... Zhao, J. M. (2018). Monocrotaline suppresses RANKL-induced osteoclastogenesis in vitro and prevents LPS-induced bone loss in vivo. *Cellular Physiology & Biochemistry International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, 48(2), 644–656.

Lee, W.S., Lee, E.G., Sung, M.S., & Yoo, W.H. (2014). Kaempferol inhibits IL-1β-stimulated, RANKL-mediated osteoclastogenesis via downregulation of MAPKs, c-Fos, and NFATc1. *Inflammation*, 37(4), 1221–1230.

Xu, J., Tan, J. W., Huang, L., Cooper, D., Gao, X. H., Dan, L., ... Cooper, D. (2000). Cloning, sequencing, and functional characterization of the rat homologue of receptor activator of NF-κB ligand & dagger. *Journal of Bone and Mineral Research*, 15(11), 2178–86.
Xu, J., Wu, H. F., Ang, E. S., Yip, K., Wołoszyn, M., Zheng, M. H., & Tan, R. X. (2009). NF-κB modulators in osteolytic bone diseases. *Cytokine & Growth Factor Reviews, 20*(1), 7–17.

Yip, K. H., Zheng, M. H., Steer, J. H., Giardina, T. M., Han, R., Lo, S. Z., ... XM., J. (2010). Thapsigargin modulates osteoclastogenesis through the regulation of RANKL-induced signaling pathways and reactive oxygen species production. *Journal of Bone & Mineral Research, 20*(8), 1462–1471.

Zhu, S., Yao, F., Qiu, H., Zhang, G., Xu, H., & Xu, J. (2018). Coupling factors and exosomal packaging microRNAs involved in the regulation of bone remodelling. *Biological Reviews of the Cambridge Philosophical Society, 93*(1), 469–480.

---

**How to cite this article:** Shao S, Fu F, Wang Z, et al. Diosmetin inhibits osteoclast formation and differentiation and prevents LPS-induced osteolysis in mice. *J Cell Physiol.* 2019;234:12701–12713. [https://doi.org/10.1002/jcp.27887](https://doi.org/10.1002/jcp.27887)