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

Siyuan Shao[1*], Fangsheng Fu[1*], Ziyi Wang[2], Fangming Song[1,2], Chen Li[1], Zuo-xing Wu[1], Jiaxing Ding[1], Kai Li[1], Yu Xiao[1], Yiji Su[1], Xixi Lin[1], Guixin Yuan[4], Jinmin Zhao[1], Qian Liu[1, 3#] and Jiake Xu[1. 2 #]

[1] Research Centre for Regenerative Medicine, Guangxi Key Laboratory of Regenerative Medicine, Guangxi Medical University, Nanning, Guangxi, China

[2] School of Biomedical Sciences, the University of Western Australia, Perth, Western Australia, Australia

[3] Department of Trauma Orthopedic and Hand Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China

[4] Department of Orthopedics, The Second Affiliated Hospital, Shantou University Medical College, Shantou, Guangdong, China

[*] These authors contributed equally to this work.

[#] **Corresponding to:**

Jiake Xu,

School of Biomedical Sciences, The University of Western Australia, Perth, Western Australia, Australia. E-mail: jiake.xu@uwa.edu.au.

Qian Liu,

Research Centre for Regenerative Medicine, Guangxi Medical University, Guangxi, China. E-mail: luoboqian@hotmail.com

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 anti-shock. The effect of DIO on osteoclast differentiation is poorly understood. In this research project, we found that DIO could inhibit osteoclastic formation induced by RANKL in a dose-dependent manner. The expression of the osteoclast differentiation marker genes, *Ctsk, NFATc1, Acp5, Ctr, Atp6v0d2, 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, Western blotting showed that DIO inhibits the phosphorylation of MAPK signaling pathway induced by RANKL, accompanied with the downregulation of NFATc1 and c-Fos expressions. We also found that DIO could reduce the accumulation of reactive oxygen species (ROS) induced by RANKL. *In vivo* 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.

Keyword: Diosmetin; osteoclasts; osteolysis; MAPK; NFATc1

Introduction:

Bone is a dynamic organ that makes up the vertebrate skeleton (Teitelbaum, 2000). The homeostasis of bone depends on the bone formation and bone resorption, which are regulated by osteoblasts and osteoclasts (Kular et al., 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 polynuclear bone resorption cells derived from hematopoietic stem cells (Manolagas and Jilka, 1995). The formation of mature osteoclasts with 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. And RANKL plays an essential role in promoting the differentiation and maintenance of osteoclasts. Binding of RANKL to RANK receptor in osteoclasts precursors recruits 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 et al., 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 *Ctsk, Mmp9,* and *Acp5*, which are essential to osteoclastic 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 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 is able to inhibit bone resorption and to prevent excessive loss of bone mass (Sato et al., 1991). For example, estrogen and bisphosphonates have been used in clinical treatment for osteolysis. However, these treatments have some concerning side effects. Estrogen therapy is associated with increased risks of breast cancer, stroke and thromboembolism. Bisphosphonates can lead to osteonecrosis of the jaw (Otto et al., 2011; Rodan and 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 et al., 2000), and strong anti-cancer and anti-inflammatory properties (Ciolino et al., 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 and Kuo, 2010). However, the role of DIO in osteolytic disorders and osteoclast differentiation has not been reported.

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

Materials and methods

Materials

DIO with a purity > 97% was purchased from Mansite (Chengdu, China) and prepared at a stock concentration of 100 mM in dimethylsulfoxide (DMSO). Alpha minimum essential medium (α-MEM) and fetal bovine serum (FBS) were obtained from ThermoFisher (Sydney, Australia). Recombinant mouse M-CSF was obtained from R&D Systems (Minneapolis, MN, USA). 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, USA). Antibody specific for NFATc1 was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The cell viability

kit (CCK-8) was obtained from Dojindo Molecular Technologies (Kumamoto, Japan). Tartrate-resistant acid phosphatase (TRAcP) staining kit was purchased from Sigma-Aldrich (St. Louis, MO, USA).

BMM isolation and osteoclast culture

6-week-old C57BL/6J mice were sacrificed and bone marrow cells obtained by exposure of medullary cavity of femur and tibia and cultured in α-medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin and 50 ng/ml M-CSF (complete medium). To induce osteoclastogenesis, bone marrow-derived macrophages (BMMs) at a density of 6×103 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. Cells were incubated in 37°C standard incubators with 5% CO2 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 3 nuclei were counted as mature osteoclasts.

Cell viability Assay

CCK-8 Kit was employed to detect the cytotoxicity of DIO on BMMs. BMMs at a density of 6×10^3 were cultured in 96-well plate with complete α-MEM medium containing M-CSF (50 ng/ml) and different concentrations of DIO for 48 h. Subsequently, CCK-8 Kit solution (10μl/well) was added to each well and incubated for 2 h without light. The absorbance at 450 nm was measured by a microplate reader (Thermo, USA).

Bone resorption pit assay

In order to assess the effect of DIO on osteoclast resorption activity, BMMs at a density of 6×10^3 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 ImageJ software (NIH)

Real-time quantitative PCR

To detect the expression of marker genes in osteoclasts, BMMs at a density of 1×10^5 were seeded in a 6-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). cDNA was synthesized from 1 μg of total RNA using reverse transcriptase Kit (TaKaRa Biotechnology, Otsu, Japan). And GAPDH was amplified as a housekeeping gene. The PCR was performed using the following procedures: 94 °C 5 mins, followed by 35 cycles (94 °C 40 sec, 60 °C 40 sec and 72 °C 40 sec) and a final extension step of 5 mins at 72°C. 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 are shown as follows: TRAcP (*Acp5*) (forward: ACACAGTGATGTGTGTGGCAACTC; reverse: CCAGAGGCTTCCACATTATGATGG), cathepsin K (*Ctsk*) (forward: GGCCAACTCAAGAAGAAAAC; reverse: GTGCTTGCTTCCCTTCTGG), MMP-9 (Mmp9) (forward: AGTTTGGTGTCGCGGGCAC; reverse: TACATGAGCGCTTCCGCAC) NFATc1 (*Nfatc1*) (forward: GGGTCAGTGTGACCGAAGAT; reverse: GGAAGTCAGAAGTGGGTGGA), V-ATPase-d2(*Atp6v0d2*) (Forward: GTGAGACCTTGGAAGACCTGAA; Reverse: GAGAAATGTGCT CAGGGGCT), CTR (*Calcr*) (forward: TGGTTGAGGTTGTGCCCA; reverse: CTCGTGGGTTTGCCTCATC) GAPDH (*Gapdh*) (forward: AACTTTGGCATTGTGGAAGG; reverse: ACACATTGGGGGTAGGAACA).

Western blot

BMMs at a density of 5×10^5 were cultured in 6-well plates with complete medium containing M-CSF (50 ng/mL). Until the BMMs almost covered each well, the cells were starved for 1 h and then treated with or without 10 μM DIO for 3 h. BMMs were then treated with 100 ng/ml RANKL for 0, 5, 10, 20, 30, or 60 mins. Radio Immunoprecipitation Assay (RIPA) lysis buffer was added to cells for 30 mins at 4°C. The proteins were analyzed by sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose (NC) membranes and blocked in 5% BSA for 90 mins. The membranes were washed with phosphate buffer saline (PBS) and incubated more than 12 h with specific primary antibodies. After that, membranes were washed with Tris Buffered Saline Tween (TBST) for 3 times and incubated with the corresponding second antibody at normal temperature for 2 h. Finally, protein bands are displayed through Odyssey imaging system (LI-COR) and measured by Image J software.

Actin Ring Formation Assays and DAPI Staining

BMMs at a density of 6×10^3 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, **c**ells were fixed with 4% paraformaldehyde for 15 mins at room temperature and permeabilized with 0.1% triton X-100 in PBS for 5 mins. The cells were blocked by 3% BSA in PBS for 30 mins. Next, cells were incubated with 0.2% phalloidin in PBS at room temperature for 60 mins. 4',6-diamidino-2-phenylindole (DAPI) staining was performed to observe the nucleus. Multiple image of the cells was taken using a fluorescent microscope.

Intracellular ROS detection

The reactive oxygen species (ROS) levels in the cells were determined by reactive oxygen species assay kit (Beijing time, Shanghai, China) with 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA). BMMs were seeded at a density of 6×10^3 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 replace the complete medium with pure medium, cells were incubated with 10 μM DCFH-DA in the cell incubator for 40 mins and washed with PBS for 4 times, the images were obtained by fluorescence microscope.

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 followed to the guidelines of the Animal

Care Committee of Guangxi Medical University. 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 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 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). Following micro-CT analyses, the calvarias sample were decalcified for 2 weeks in 10% EDTA. Then paraffin embedded histopathological sections were used for TRAcP staining, H&E staining and immunohistochemical staining as previously described (Song et al., 2017; Wei et al., 2018). The TRAcP-positive osteoclasts were determined by Image J software.

Statistical analysis

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

Results

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 (Fig. 1A-B). Compared to the control group, the total quantity TRAcP positive multinucleated cells had a decreasing trend with an increase of the concentration of DIO from 0 μM to 10 μM (Fig. 1C). To further explore the time course effect of DIO on osteoclastic formation, DIO was added in several designed time periods (1-3 days, 3-5 days, 5-6 days,1-6 days) (Fig. 1D). The results suggested that DIO significantly reduces RANKL-induced osteoclast differentiation at three different stage (1-3 days, 3-5 days and 1-6 days) (Fig. 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 (CCK8). Our data confirmed that DIO with concentrations lower than 10 μM has no cytotoxic effect on BMMs (Fig. 1G). Taken together, DIO could inhibit RANKL-induced osteoclast differentiation in a concentration-dependent manner within the non-cytotoxic concentration range.

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 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 control group (Fig. 2A-F). These data suggest that DIO inhibited the expression of signature gene in osteoclasts, thus affected the formation and function of osteoclasts.

DIO impairs OC bone resorption *in vitro*

To further evaluate the effect of DIO on 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 are 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 (Fig. 2G, H). We also explore whether DIO influenced the formation of 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 actin ring was significantly inhibited by 5 μM and 10 μM DIO (Fig. 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 with the impairment of F-actin ring formation *in vitro*.

DIO Inhibited RANKL induced ROS level *in vitro*

In order 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 h, the level of ROS determined in cells. Obviously, intracellular ROS levels were increased by RANKL treatment. Notably, the active oxygen products were reduced in BMM cells after treated with 5 μM and 10 μM DIO (Fig. 3B). The signal intensity and quantity of ROS positive cells after DIO treatment showed a decreasing trend in a concentration-dependent manner (Fig. 3C).

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

MAPK and NF-κB signaling pathways in RANKL-induced osteoclastogenesis were also investigated. The results of western blot showed that the phosphorylation of ERK1/2 and JNK1/2 was markedly inhibited by DIO at 10 and 20 mins compared with the control group (Fig. 4A, B, C), however there was only little effect on the phosphorylation of p38 signaling pathway (Fig. 4D). The degradation of inhibitor of nuclear factor kappa-B α (IκBα) was not inhibited by DIO compared with the control group (Fig. 4E). Collectively, we found that DIO effectively suppresses phosphorylation of MAPK signaling pathway induced by RANKL. But has no effect on NF-κB signaling pathway.

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 analyses, we found that the protein expression of NFATc1 was suppressed on day 1, 3 and 5

(Fig. 4F, G), whereas the expression of c-Fos was mainly inhibited on day 1 (Fig. 4H) relative to control group. These results revealed that DIO inhibits the protein expression of key transcription factors NFATc1 and c-Fos.

DIO protected against LPS-induced osteolysis

Finally, to measure the therapeutic effect of DIO *in vivo*, we employed 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 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, DIO treated group (5 mg/kg group and 10 mg/kg group) displayed an inhibitory effect on inflammatory osteolysis induced by LPS (Fig. 5A). This finding was confirmed by a further quantitative analysis of bone parameters, which showed that DIO's treatment effectively protects against the loss of bone volume/tissue volume and decreased the bone porosity (Fig. 5B, C, D). Further, histological analysis and assessment confirmed that DIO reduces LPS-induced bone loss, inflammatory bone erosion and the number of osteoclasts (Fig. 5E, F, G). Finally, immunohistochemical staining showed that the expression of Cathepsin K (CTSK) was down-regulated and the expression of Runt Related Transcription Factor 2 (RUNX2) was enhanced (Fig. 6). Collectively, our findings suggested that DIO could effectively suppress LPS-induced osteolysis in mice.

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 et al., 2015; Harris, 2001). Therefore, effective measures to prevent and treat osteolytic diseases were to target the inhibition of osteoclastic formation and function (Boyle et al., 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 reactive oxygen species 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, 2007), 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 and Takayanagi, 2009). According to relevant research, 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 et al., 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. Following the suppression of MAPK phosphorylation by DIO, NFATc1 and c-Fos expression is down-regulated (WS et al., 2014). These transcription factors mediate the end stage of osteoclast differentiation and the expression of *Ctsk, Atp6v0d2, 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, Atp6v0d2, Acp5* was inhibited after treatment with DIO, which is consistent with the inhibitory effect of DIO on 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 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 cytoplasm in the form of homologous dimer and binds to IκBα protein in resting state (Chen et al., 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 et al., 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 et al., 2000). RANKL can significantly increase ROS level in osteoclasts (Song et al., 2018; Yip et al., 2010). According to relevant studies, ROS production may be due to the transfer of excess unpaired electrons in the oxidative respiratory chain to O_2 (Kovac et al., 2015). ROS production in BMMs not only activates MAPK signaling pathways but also increases Ca^{2+} 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, a 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 associated with osteoblast. Interestingly, we found that the expression of CTSK was decreased after treatment with DIO, whereas the expression of RUNX2 increased significantly. This result was consistent with previous studies on the effect of DIO in

promoting osteoblast formation *in vitro*. Based on the above results, it is suggested that the effects of DIO on inhibiting osteoclast differentiation and promoting osteoblast differentiation both contribute to the prevention of bone loss induced by LPS *in vivo*.

In summary, our study has demonstrated for the first time that DIO has the capacity of diminishing osteoclast differentiation and osteoclast resorption activity, mainly by inhibiting MAPK signaling pathway, rather than NF-κB signaling pathway. In addition, the inhibition of ROS by DIO may also play a role in suppressing osteoclast formation. Further, DIO displays an anti-inflammatory and bone-protective effect on the LPS-induced osteolysis in mice. These results indicate that DIO might have potential clinical application value for osteoclast related diseases.

Conflict and interest

The authors declare no conflicts of interest.

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, and Yiji Su, Ziyi Wang, Zuo-xing Wu, Jiaxing Ding, Kai Li, Yu Xiao, 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 work was supported in part by National Natural Science Foundation of China (81501910), the Natural Science Foundation of Guangxi Province (2015GXNSFCA414001, 2015GXNSFDA139019). This study was also supported in part by grants from the Australian Health and Medical Research Council (NHMRC, APP1107828, APP1027932, APP1163933).

References

Ang ESM, Yang X, Chen H, Liu Q, Zheng MH, 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.

Baud'Huin M, Duplomb L, Velasco CR, 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 JM, Kaar SG, Ragab AA, Goldberg VM, Anderson JM, Greenfield EM. 2010. Adherent Endotoxin on Orthopedic Wear Particles Stimulates Cytokine Production and Osteoclast Differentiation. Journal of Bone & Mineral Research 16(11):2082-2091.

Boyle WJ, Simonet WS, Lacey DL. 2003. Osteoclast differentiation and activation. Nature 423(6937):337.

Chen Y, Wu J, Ghosh G. 2003. κB-Ras Binds to the Unique Insert within the Ankyrin Repeat Domain of IκBβ and Regulates Cytoplasmic Retention of IκBβ·NF-κB Complexes. Journal of Biological Chemistry 278(25):23101-23106.

Ciolino HP, Wang TT, Yeh GC. 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 MB, Gehrke T, Dersch K, Kendoff D. 2015. Modes of failure and revision of failed lateral unicompartmental knee arthroplasties. Knee 22(4):338-340.

Crotti TN, Sharma SM, Fleming JD, Flannery MR, Ostrowski MC, Goldring SR, Mchugh KP. 2008. PU.1 and NFATc1 mediate osteoclastic induction of the mouse beta3 integrin promoter. Journal of Cellular Physiology 215(3):636-644.

Deepak V, Kasonga A, Kruger MC, Coetzee M. 2015. Inhibitory effects of eugenol on RANKL-induced osteoclast formation via attenuation of NF-ÎºB and MAPK pathways. Connective Tissue Research 56(3):195-203.

Doostdar H, Burke MD, Mayer RT. 2000. Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1 ☆. Toxicology 144(1):31-38.

Ea CK, Sun L, Inoue J, Chen ZJ. 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, Yoshida CA, Kanatani N, Nakamura R, Mizuno A, Zanma A. 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 JH, Joyce DA, Pavlos NJ, Leong C, Kular J, Liu J, Feng X, Zheng MH. 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.

Ha H, Kwak HB, Lee SW, Jin HM, Kim HM, Kim HH, Lee ZH. 2004. Reactive oxygen species mediate RANK signaling in osteoclasts. Experimental Cell Research 301(2):119-127.

Harris WH. 2001. Wear and periprosthetic osteolysis: the problem. Clin Orthop 393(393):66.

Hotokezaka H, Sakai E, Ohara N, Hotokezaka Y, Gonzales C, Matsuo KI, Fujimura Y, Yoshida N, 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 YL, Kuo PL. 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, Mori I, Yoshida T, Yokochi T. 2007. Bacterial lipopolysaccharide induces osteoclast formation in RAW 264.7 macrophage cells. Biochemical & Biophysical Research Communications 360(2):346-351.

Kim HJ, Ohk B, Yoon HJ, Kang WY, Seong SJ, Kim SY, Yoon YR. 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 JM, Lee YS, Kim YS, Kim DJ, Kim HH, Park JY, Lee KU, Kim GS. 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 PR, Holmström KM, Zhang Y, Dinkovakostova AT, Abramov AY. 2015. Nrf2 regulates ROS production by mitochondria and NADPH oxidase. BBA - General Subjects 1850(4):794-801.

Kular J, Tickner J, Chim SM, Xu J. 2012. An overview of the regulation of bone remodelling at the cellular level. Clinical Biochemistry 45(12):863-873.

Lee MS, Ikenoue T, Trindade MC, Wong N, Goodman SB, Schurman DJ, Smith RL. 2010. Protective effects of intermittent hydrostatic pressure on osteoarthritic chondrocytes activated by bacterial endotoxin in vitro. Journal of Orthopaedic Research 21(1):117-122.

Liu Y, Shepherd EG, Nelin LD. 2007. MAPK phosphatases--regulating the immune response. Nature Reviews Immunology 7(3):202.

Manolagas SC, Jilka RL. 1995. Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. N Engl J Med 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 c1 (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, Hisatake K, 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.

Matsuo K, Owens JM, Tonko M, Elliott C, Chambers TJ, Wagner EF. 2000. Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation. Nature Genetics 24(2):184.

Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, Pando MP, Asano T, Verma IM, Oda H. 2000. Reciprocal Role of ERK and Nf-κb Pathways in Survival and Activation of Osteoclasts. Journal of Cell Biology 148(2):333-342.

Nair SP, 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, Pautke 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 GA, Martin TJ. 2000. Therapeutic approaches to bone diseases. Science 289(5484):1508-1514.

Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E, Rodan GA. 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, Nishiyama A, Sasaki, Akira. 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 HU, Hajyehia A, Levischaffer F. 2000. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5(5):415-418.

Song D, Cao Z, Liu Z, Tickner J, Qiu H, Wang C, Chen K, Wang Z, Dong S, Xu J. 2018. Cistanche deserticola polysaccharide attenuates osteoclastogenesis and bone resorption via inhibiting RANKL signaling and reactive oxygen species production. Journal of Cellular Physiology.

Song F, Wei C, Zhou L, Qin A, Yang M, Tickner J, Huang Y, Zhao 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).

Song F, Zhou L, Zhao J, Liu Q, Yang M, Tan R, Xu J, Zhang G, Quinn JMW, Tickner J. 2016. Eriodictyol Inhibits RANKL‐Induced Osteoclast Formation and Function Via Inhibition of NFATc1 Activity. Journal of Cellular Physiology 231(9):1983.

Takayanagi H. 2007. Takayanagi, H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat. Rev. Immunol. 7, 292-304. Nature Reviews Immunology 7(4):292-304.

Teitelbaum SL. 2000. Bone Resorption by Osteoclasts. Science 289(5484):1504-1508.

Wei CM, Su YJ, Qin X, Ding JX, Liu Q, Song FM, Zong SH, Xu J, Zhou B, Zhao JM. 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 & Pharmacology 48(2):644.

WS L, EG L, MS S, WH Y. 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 JW, Huang L, Cooper D, Gao XH, Dan L, Wysocki S, Zheng MH, Tan JW, Cooper D. 2000. Cloning, Sequencing, and Functional Characterization of the Rat Homologue of Receptor Activator of NF-κB Ligand &dagger. Bone 27(4):32-32.

Xu J, Wu H, Es, Yip K, Woloszyn M, Zheng M, Tan R. 2009. NF-kappaB modulators in osteolytic bone diseases. Cytokine & Growth Factor Reviews 20(1):7-17.

Yip KH, Zheng MH, Steer JH, Giardina TM, Han R, Lo SZ, Bakker AJ, Cassady AI, Joyce DA, PhD JXM. 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. Biol Rev Camb Philos Soc 93(1):469-480.

Figure legends

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 (10μM) during the 7 days process of osteoclast formation. (G) BMMs were cultured for 48 hours with 50 ng/mL M-CSF at the

indicated concentrations of DIO. Cell viability was determined by CCK8 assay. (**p< 0.01 ***P<0.001 compared to control).

Figure 2. 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) *C*tr, (E) *Ctsk*, and (F) *Mmp9* was analyzed by real-time RT-PCR. Gene expression was normalized to GAPDH. (G) BMMs were cultured in hydroxyapatite-coated plates and treated with the indicated concentrations of DIO until mature osteoclasts were formed. Osteoclast were removed, and images obtained by light microscope (Magnification = 40X; scale bar = 200 μm). (H) The total areas of resorption pits were measured using Image J. (*p< 0.05 **p< 0.01 ***P<0.001 relative to control).

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. Images were taken by using fluorescence microscope after incubation with DCFH-DA for 30 minutes (Magnification = 100 ×; scale bar = 100 μM). (C) Detection of the number of ROS positive cells. (***P<0.001 relative to control).

Figure 4. DIO suppressed RANKL-induced phosphorylation of ERK, JNK and NFATc1 protein expression. BMMs pretreated with DIO (10μM) for 3 hours were stimulated with RANKL for the indicated times. (A) Western blot analyses were carried out with IκBα, p-ERK, ERK, p-JNK, JNK, p-P38, P38, and β-actin specific antibodies. (B-E) The relative expressions of Iκbα, p-ERK, p-P38, and p-JNK were determined by gray pixels value analysis of its total protein counterpart. (F) BMMs were treated with DIO (10 μM) and then stimulated with 100 ng/mL RANKL for 0, 1, 3, and 5 days. Western blot analyses were carried out with NFATc1, c-Fos and β-actin specific antibodies. (G, H) The relative expressions of NFATc1, c-Fos were analyzed by gray pixels value detection, with β-actin as a reference. (*P<0.05

relative to control).

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 LPS group).

Figure 6. DIO affected LPS-induced expression of CTSK and RUNX2 *ex vivo*. Representative images CTSK and RUNX2 of immunohistochemical staining，and the expression of CTSK and RUNX2 in the images were indicated by the red arrow.