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Inhibitory effects of *Oxya chinensis sinuosa* ethanol extract on RANKL-induced osteoclast differentiation

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Abstract

The rice field grasshopper, *Oxya chinensis sinuosa* (OC), has traditionally been utilized in Korea for various purposes; however, its potential benefits in the context of osteoporosis remain unclear. The results revealed that OC ethanol extract (OCE) significantly inhibited the formation and activity of tartrate-resistant acid phosphatase (TRAP)-positive cells in receptor activator of nuclear factor-κB ligand (RANKL)-stimulated RAW264.7 cells. Furthermore, OCE, at concentrations ranging from 100 to 400 μg/mL, demonstrated a dose-dependent reduction in the protein expression of osteoclast-specific markers, including nuclear factor of activated T cell cytoplasmic 1, c-Src, and TRAP, when compared to RANKL stimulation alone. Additionally, OCE significantly inhibited RANKL-induced activation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) but not the activation of extracellular signal-regulated kinase. Collectively, these results indicate that OCE suppresses osteoclastogenesis by attenuating the phosphorylation of p38 MAPK and JNK. Consequently, these findings suggest that OCE holds promise for the prevention of osteoporosis.

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Introduction

Bone undergoes continuous remodeling, maintaining a delicate equilibrium between the actions of bone-forming osteoblasts and bone-resorbing osteoclasts (Amarasekara *et al.*, 2018). Disruptions in this balance can lead to various skeletal diseases, including osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma, and metastatic cancers (Boyle *et al.*, 2003). Osteoclast differentiation is initiated by the stimulation of two cytokines: receptor activator of nuclear factor (NF)-κB (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (Lampiasi *et al.*, 2021). Notably, RANKL-treated RAW 264.7 cells have been observed to independently differentiate into osteoclasts, even without the addition of co-stimulators such as M-CSF or lipopolysaccharide (Song *et al.*, 2019). The interaction between RANK and RANKL activates the recruitment of tumor

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necrosis factor receptor-associated factor 6, which, in turn, triggers the stimulation of several intracellular pathways, including p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), NF-κB, and the Src/phosphoinositide 3-kinase/protein kinase B (PKB/Akt) pathways (Lee, 2017). These pathways collectively upregulate two pivotal transcription factors, c-Fos and NF of activated T cells c1 (NFATc1), which, in turn, induce the expression of osteoclast-specific genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and osteoclast-associated receptor (Lee, 2017).

Oxya chinensis sinuosa (OC), commonly known as the rice field grasshopper, is an edible insect belonging to the phylum Arthropoda (order: Orthoptera, family: Acrididae, subfamily: Oxyinae) (Kim *et al.*, 2020). OC has a longstanding history of traditional use in Korea for treating conditions such as cough, whooping cough, asthma, bronchitis, and cerebrovascular accident (Kim *et al.*, 2015). Bahuguna *et al*. (2022) demonstrated that *N*-acetyldopamine dimer, a bioactive compound isolated from an ethanol extract of OC, exhibited anti-inflammatory and cathepsin C inhibitory activities. Lee *et al*. (2017) discovered five new *N*-acetyldopamine dimers in an ethanol extract of OC that are novel antithrombotic compounds that inhibit the endogenous blood coagulation pathway by targeting coagulation factor X. Saiki *et al*. (2021) reported that the ethyl acetate-soluble fraction of a methanolic extract of *O. yezoensis* contained a large amount of total phenolic compounds and had antioxidant activity, whereas the *n*-hexane-soluble fraction contained a high amount of fatty acids (α-linolenic, linoleic, and oleic acids) and had anti-allergic activity. Previous research also revealed a range of biological activities, including anticancer (Kim *et al.*, 2020) and antimicrobial effects (Kim *et al.*, 2016), associated with OC-derived proteins. Additionally, ethanol extracts of OC are reported to exhibit promising antihyperglycemic (Park and Han, 2020) and anti-aging (Im *et al.*, 2019) properties. Despite this multifaceted portfolio of beneficial effects, the impact of OC on osteoclast differentiation remains to be comprehensively explored. Consequently, the primary aim of this study was to examine the anti-osteoclastogenic properties of an ethanol extract derived from OC (referred to as OCE) and elucidate the associated signaling pathways in RANKL-stimulated RAW 264.7 macrophage cells.

Materials and Methods

Preparation of *Oxya chinensis sinuosa* ethanol extract

Frozen OC specimens were procured from an agricultural corporation situated in Jangseong, Korea. These specimens were subjected to freeze-drying and subsequently reduced to a fine powder utilizing a sway-type pulverizer (KSP-35, Korea medi Co., Ltd., Daegu, Korea). The resulting powder was then suspended in a 70% ethanol solution and subjected to sonication (350 J, 10 s, twice) using an ultrasonic processor (VCX500, Sonics & Materials, Inc., Newtown, CT, USA). The resulting extract was centrifuged at 3,500 rpm for 10 min. The supernatant was meticulously filtered using a 0.45 μm PVDF syringe filter (GE Healthcare, Little Chalfont, UK), and the filtrate dried using a vacuum rotary evaporator (CVE-3100; EYELA, Tokyo, Japan). The dried extract was stored at –70°C until its use in further experimental procedures.

Cell culture and cell viability

RAW264.7 murine macrophage cells were procured from the Korean Cell Line Bank (Seoul, Korea). These cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Gibco). The cells were incubated at 37°C within a 5% CO_2 incubator. Subsequently, the cells were seeded in 96-well plates at the density of 4×10^4 cells per well. After an initial attachment period of 24 h, the cells were exposed to various concentrations of OCE (0.1, 0.5, 1, and 2 mg/mL) for an additional 24 h. To determine cell viability, an analysis was conducted using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA).

Tartrate-resistant acid phosphatase (TRAP) assay

RAW264.7 cells were initially seeded in 24-well plates at the density of 2×10^4 cells/well. After a 24 h incubation period, these cells were treated with OCE at various concentrations (100, 200, 300, and 400 μg/mL) in conjunction with RANKL (100 ng/mL) for 5 d. Upon the completion of the differentiation process, the RAW264.7 cells were thoroughly

rinsed with phosphate-buffered saline (PBS; Caisson Labs, Inc., Smithfield, UT, USA) and subsequently fixed with 4% paraformaldehyde. The assessment of cellular TRAP activity and staining was performed utilizing a TRAP Staining Kit (Kamiya Biomedical Company, Seattle, WA, USA). Images of the TRAP-positive cells were captured using an inverted phase-contrast microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany). The culture medium was transferred to a fresh 96-well plate and combined with the TRAP solution. This mixture was maintained at 37°C for 3 h. Subsequently, absorbance was measured at 540 nm using a microplate reader (Varioskan™ LUX multimode microplate reader, Thermo Fisher Scientific, Inc.).

Western blotting

RAW264.7 cells were cultured at the density of 1×10^5 cells per well in 6-well plates and incubated for 24 h. Subsequently, the cells were treated with varying concentrations of OCE (100, 200, 300, and 400 μg/mL) and RANKL (100 ng/mL) for either 20 min or 5 d. After the respective treatment periods, the cells were harvested and underwent a thorough washing with cold PBS. The cell lysis was performed in M-PER mammalian protein extraction reagent, which included a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.). The resulting cell lysates were obtained after centrifugation at 12,000 rpm for 15 min at 4°C. The proteins were then fractionated on a 4–12% polyacrylamide gel (Invitrogen, Thermo Fisher Scientific, Inc.) and subsequently transferred onto polyvinylidene difluoride membranes (Invitrogen). Afterward, the membranes were blocked in 5% skim milk and allowed to incubate overnight at 4°C with primary antibodies, including NFATc1 (sc-7294; Santa Cruz Biotechnology, Dallas, TX, USA), c-Src (sc-130124), TRAP (sc-376875), β-actin (#4967; Cell Signaling Technology, Danvers, MA, USA), p-p38 (#9211), p38 (#9212), p-ERK (#9101), ERK (#9102), p-JNK (#9251), and JNK (#9252). After incubation with primary antibodies, the membranes were washed with Tris-buffered saline containing Tween 20 (TBST; Thermo Fisher Scientific, Inc.) and were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (#7074 or #7076) at room temperature for 1 h. Finally, the signals were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Inc.) and a chemiluminescence imaging system (Alliance Q9 Advanced, Uvitec Ltd., Cambridge, UK).

Statistical analysis

The results are expressed as means ± standard error (SE). Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, using SPSS software (version 20, SPSS Inc., Chicago, IL, USA). Statistical significance was established at $p < 0.05$.

Results and Discussion

Effects of OCE on RANKL-induced osteoclast differentiation

The effects of OCE on RANKL-induced osteoclast differentiation were assessed through a series of experiments. Initially, RAW264.7 cells were exposed to various concentrations of OCE (0.1, 0.5, 1, and 2 mg/mL) for 24 h. OCE, particularly at concentrations of 0.1 and 0.5 mg/mL, demonstrated a stimulatory effect on the proliferation of RAW264.7 cells when compared to the control group (Fig. 1A). However, this effect did not exhibit a clear concentration-dependent trend. Notably, at the highest doses employed (1 and 2 mg/mL) in the RANKL-treated cells, a significant reduction in cell viability was observed ($p < 0.001$), with viability ranging between 14 and 16% (data not shown). During RANKL stimulation, the cell viability at OCE concentrations of 0.1 and 0.5 mg/mL was 95.47 ± 1.93% and 84.37 ± 1.81%, respectively. Consequently, OCE concentrations below 0.5 mg/mL were selected for further experimentation.

To confirm the osteoclast phenotype, TRAP staining and activity assays were conducted. Osteoclast-like cells were identified as TRAP-positive multinucleated cells possessing more than three nuclei, consistent with the criteria established by Lee *et al.*, 2019 and Tanaka *et al.*, 2006. The effects of OCE on osteoclast differentiation of RAW264.7 cells were examined by TRAP staining in the presence of RANKL. The results revealed that OCE treatment significantly inhibited TRAP activity in RANKL-treated RAW264.7 cells in a dose-dependent manner (Fig. 1B). Furthermore, OCE treatment led to a dose-dependent reduction in the formation of multinucleated osteoclast-like cells (Fig. 1C). These findings indicate that OCE effectively hinders RANKL-induced osteoclastogenesis in RAW264.7 cells.

Effects of OCE on RANKL-induced osteoclast-specific protein expression

The effect of OCE on RANKL-induced osteoclast-specific

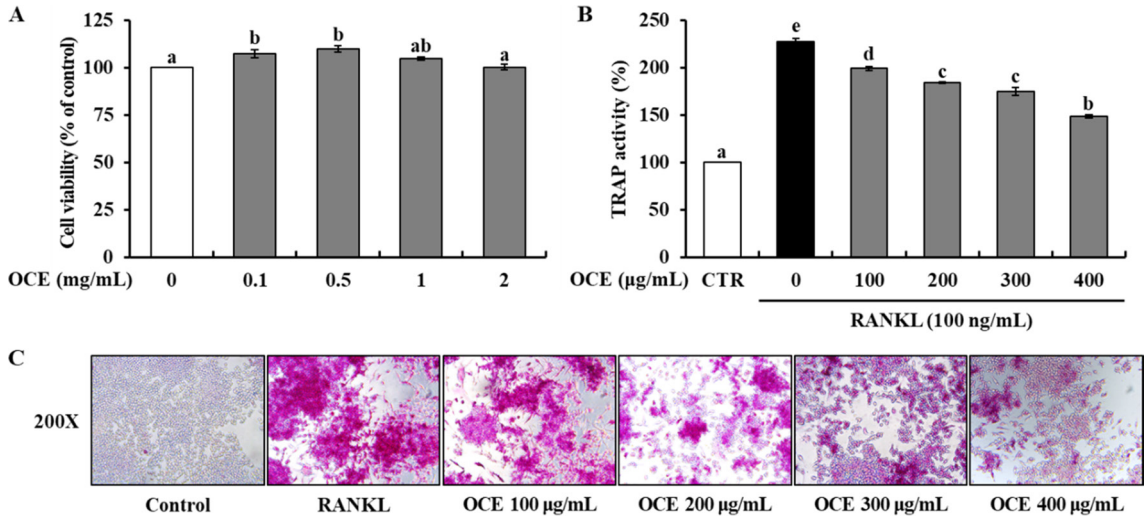

Fig. 1. Effects of OCE on cell viability (A), TRAP activity (B), and cell morphology (C) in RANKL-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were cultured in 96-well plates (4×10^4 cells/well) and stimulated with the indicated concentrations of OCE for 24 h, and cell viability was assessed. (B and C) RAW 264.7 cells were cultured in 24-well plates (2×10^4 cells/well) without or with the indicated concentrations of OCE in the presence of 100 ng/mL RANKL for 5 d, and then TRAP staining was performed. Values are expressed as mean ± SE, and those with different superscript letters (a, b, c, d, e) are significantly different ($p < 0.05$). Photomicrographs show TRAP-stained osteoclasts. Magnification, 200×.

protein expression was investigated in this study. NFATc1, a master transcription factor in osteoclast differentiation, is activated by RANKL stimulation, integrating various intracellular signaling cascades (Baek *et al.*, 2015). It has been reported that RANKL stimulation of osteoclast formation is impaired in NFATc1-deficient embryonic stem cells (Takayanagi *et al.*, 2002). Moreover, ectopic expression of NFATc1 efficiently induces osteoclast differentiation, even in the absence of RANKL stimulation (Takayanagi *et al.*, 2002). NFATc1 directly governs the expression of diverse osteoclast-specific genes, including TRAP, c-Src, β-integrin, and cathepsin K (Takayanagi, 2007; Zeng *et al.*, 2016). In osteoclastic bone resorption, the non-receptor tyrosine kinase c-Src plays a crucial role by participating in the formation of podosomes, dynamic actin-containing adhesive structures (Matsubara *et al.*, 2021; Orecchini *et al.*, 2021). To ascertain the anti-osteoclastogenic effect of OCE, alterations in the expression of NFATc1, a key regulator of osteoclastogenesis, and osteoclast-associated protein expression were examined using western blotting (Fig. 2). RANKL was observed to induce the expression of NFATc1, c-Src, and TRAP. Conversely, OCE treatment in the range of 100–400 μg/mL resulted in a dose-dependent inhibition of the expression of these proteins compared with treatment for 5 d with RANKL alone. Hence, the results suggest that OCE has the potential to suppress

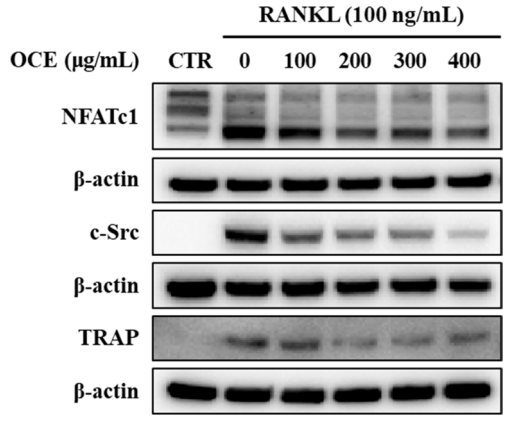

Fig. 2. Effects of OCE on osteoclast-related protein expression in RANKL-stimulated RAW264.7 cells. RAW 264.7 cells were cultured in 6-well plates (1×10^5 cells/well) without or with the indicated the concentrations of OCE in the presence of 100 ng/mL RANKL for 5 d. Total cell lysates were analyzed using western blotting. The equality of sample loading was determined by measuring the β-actin level.

RANKL-induced expression of NFATc1, thereby mitigating the expression of osteoclast-specific proteins, including c-Src and TRAP.

Effects of OCE on RANKL-induced MAPKs signaling

The study also explored the effects of OCE on RANKL-induced MAPK signaling pathways, which are involved in

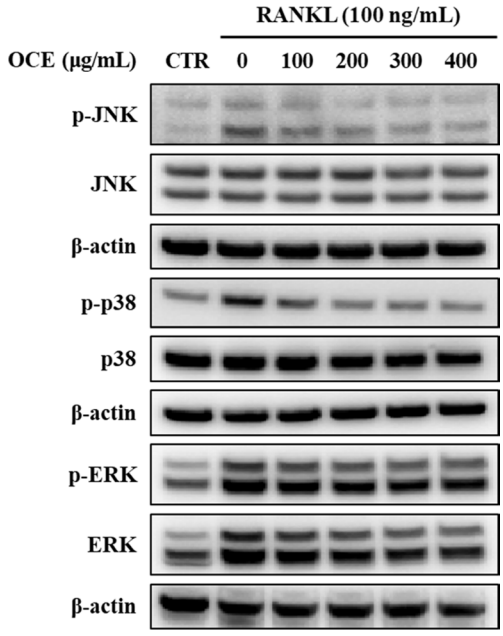

Fig. 3. Effects of OCE on MAPK signaling pathways in RANKL-stimulated RAW264.7 cells. RAW 264.7 cells were cultured in 6-well plates, pretreated with the indicated concentrations of OCE for 1 h, and then incubated with RANKL (100 ng/mL) for 20 min. Total cell lysates were analyzed using western blotting. The equality of sample loading was determined by measuring the β–actin level.

osteoclast differentiation, proliferation, and survival. RANKL, upon binding to its receptor RANK, triggers a cascade of signaling events, including the activation of Akt, MAPKs (JNK, p38, and ERK), and NF-κB (Cheng *et al.*, 2022; Liu *et al.*, 2022). To explore the mechanisms underlying an inhibitory effect of OCE on osteoclastogenesis, an analysis of MAPK signaling was conducted using western blotting. RANKL stimulation for 20 minutes induced phosphorylation of MAPKs (JNK, p38, and ERK) (Fig. 3). Remarkably, OCE (at concentrations ranging from 100 to 400 μg/mL) prominently curtailed the activation of JNK and p38 induced by RANKL, while leaving the activation of ERK unaffected. Hence, it can be inferred that OCE-mediated inhibition of osteoclast formation may, in part, be attributed to the down-regulation of JNK and p38 phosphorylation. Yoon *et al*. (2014) reported that an ethanol extract of OC inhibited NF-κB p65 activation in LPS-stimulated macrophages. Additionally, Bahuguna *et al*. (2022) showed that *N*-acetyldopamine dimer isolated from OC inhibited the nuclear translocation of NF-κB by inhibiting its phosphorylation. However, to our knowledge, there have been no reports providing direct evidence of compounds isolated from OC that inhibit RANKL-induced MAPK signaling.

Conclusions

In conclusion, this investigation has elucidated that OCE exerts a suppressive influence on the expression of RANKL-induced NFATc1, as well as other osteoclastogenesis markers such as c-Src and TRAP. This inhibitory action is achieved by impeding the JNK and p38 MAPK signaling pathways. These findings shed light on a potential mechanism by which OCE regulates osteoclast differentiation, hinting at its potential as a therapeutic approach for addressing osteoporosis.

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