INTRODUCTION

Bone remodeling is elaborately controlled by two established processes: bone resorption by osteoclasts, and bone formation by osteoblasts. The balanced interplay between the osteocytes, osteoclasts and osteoblasts is responsible for the replacement and recycling of as much as 10% of the total adult human bone content each year (Manolagas, 2000).

Osteoclasts are derived from hematopoietic precursor cells in the bone marrow, and differentiate into mature osteoclasts when exposed to receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (Boyle et al., 2003). Dysregulation of osteoclasts remains a primary cause of skeletal diseases such as osteoporosis, osteosarcoma, rheumatoid arthritis and bone resorptive infectious diseases. Osteoporosis, the most common skeletal disease, originates from excessive bone resorption over bone formation, resulting in bone loss and fractures (Hayward and Fiedler-Nagy, 1987; Roodman, 1999; Vaananen et al., 2000; Boyle et al., 2003).

Terminal differentiation of osteoclasts is characterized by acquisition of mature phenotypic markers which include expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, matrix metalloproteinase 9 (MMP9) and cathepsin K, as well as morphological conversion into large multinucleated cells and the capability to facilitate bone resorption (Fujisaki et al., 2007). During differentiation, RANKL induces the signaling essential for precursor cells to differentiate into osteoclasts, whereas M-CSF, secreted by osteoblasts, provides the survival signal to these cells (Fukuda et al., 2007). During differentiation, RANKL induces the signaling essential for precursor cells to differentiate into osteoclasts, whereas M-CSF, secreted by osteoblasts, provides the survival signal to these cells (Fukuda et al., 2005). Activation of its receptor (RANK) promotes recruitment of several adaptor molecules, such as tumor necrosis factor receptor-associated factor (TRAF) 6. The signal is transduced via multiple downstream signaling pathways, involving c-Jun N-terminal protein kinase (JNK), nuclear factor-κB (NF-κB), p38 mitogen-activated protein kinase (p38MAPK), extracellular signal-regulated kinase (ERK) and p38 MAPK (Boyle et al., 2003).

Activation of osteoclast and inactivation of osteoblast result in loss of bone mass with bone resorption, leading to the pathological progression of osteoporosis. The receptor activator of NF-κB ligand (RANKL) is a member of the TNF superfamily, and is a key mediator of osteoclast differentiation. A flavanone glycoside isolated from the fruit of Poncirus trifoliata, poncirin has anti-allergic, hypocholesterolemic, anti-inflammatory and anti-platelet activities. The present study investigates the effect of poncirin on osteoclast differentiation of RANKL-stimulated RAW264.7 cells. We observed reduced formation of RANKL-stimulated TRAP-positive multinucleated cells (a morphological feature of osteoclasts) after poncirin exposure. Real-time qPCR analysis showed suppression of the RANKL-mediated induction of key osteoclastogenic molecules such as NFATc1, TRAP, c-Fos, MMP9 and cathepsin K after poncirin treatment. Poncirin also inhibited the RANKL-mediated activation of NF-κB and, notably, JNK, without changes in ERK and p38 expression in RAW264.7 cells. Furthermore, we assessed the in vivo efficacy of poncirin in the lipopolysaccharide (LPS)-induced bone erosion model. Evaluating the micro-CT of femurs revealed that bone erosion in poncirin treated mice was markedly attenuated. Our results indicate that poncirin exerts anti-osteoclastic effects in vitro and in vivo by suppressing osteoclast differentiation. We believe that poncirin is a promising candidate for inflammatory bone loss therapeutics.

Keywords: Osteoclast, Osteoporosis, Poncirin, RANKL, JNK

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kinase (ERK), and PI3K/Akt. RANKL-mediated signaling eventually results in activation of the master transcription factor for osteoclastogenesis, namely, nuclear factor of activated T cells c1 (NFATc1) (Boyle et al., 2003; Wittrant et al., 2003; Ikeda et al., 2008).

The fruits of the *Poncirus trifoliata* are native to Korea and northern China. The dried immature fruit of *P. trifoliata* (L.) Raf., known as *Poncirus fructus* (PF) is traditionally used in treating diverse gastrointestinal (GI) disorders by regulating the GI tract movements (reviewed by Jang et al., 2018). Of the numerous natural components present in PF, naringin and naringenin flavonoids are reported to restore the impaired GI motility (Jang et al., 2013). The extracts and constituents from the mature fruit (MF) have also shown anticancer and anti-inflammatory effects by suppressing the release of histamine or proinflammatory cytokines from mast cells in various in vitro and in vivo systems (Lee et al., 1996, 1997; Shin et al., 2006). Poncirin, a flavanone glycoside, is one of the biologically active components contained in the *P. trifoliata* fruits. Poncirin is reported to exhibit anti-inflammatory activity through inhibition of NF-κB activity in LPS-activated RAW264.7 which is murine pre-osteoclast/macrophage cell (Kim et al., 2007). Recently, the effect of poncirin in bone metabolism is reported to ameliorate the glucocorticoid-induced osteoporosis in vivo and in vitro, by promoting osteoblastic differentiation (Yoon et al., 2011, 2012). The study showed that poncirin inhibits the adipocyte differentiation and exerts an anti-adipogenic activity of mesenchymal stem cells while promoting osteoblast differentiation. However, there is no published report on the effect and mode of action of poncirin on osteoclast differentiation or function.

We therefore undertook to explore the effect of poncirin on osteoclast differentiation and evaluate the involved signaling pathways in RANKL-stimulated RAW264.7 cells. Our results demonstrate that poncirin significantly suppresses RANKL-induced osteoclast differentiation by suppressing osteoclast-specific gene expression with modulation of RANKL-mediated signal transduction. Furthermore, we demonstrate that poncirin considerably inhibits bone erosion in a mouse model.

**MATERIALS AND METHODS**

**Materials**

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). Soluble recombinant mouse RANKL (sRANKL) was purchased from Peprotech (NJ, USA). RNAzol and all PCR reagents were obtained from Takara Bio Inc (Shiga, Japan). TRAP stain kit was from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for p38 MAPK, ERK, JNK, phospho-p38MAPK (Thr180/Tyr182), phospho-ERK, phospho-JNK (Thr183/Tyr185), and anti-β-actin were procured from Cell Signaling Technology (Danvers, MA, USA). Poncirin was provided by the Korea Food and Drug Administration (KFDA, Ochang, Korea). Imprinting Control Region (ICR) mice were purchased from ORIENT Bio (Seongnam, Korea).

**Cell culture and induction of osteoclast differentiation**

Mouse monocyte/macrophage RAW264.7 cells (ATCC #CRL-TBI-71) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM). Cells were supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 IU/mL penicillin. The cells were maintained in a humidified incubator of 5% CO₂ and at 37°C, and fresh medium was replenished every 3 days. To differentiate RAW264.7 cells into osteoclasts, cultured cells were suspended in α-minimal essential media (α-MEM) (10% FBS) supplemented with 100 ng/mL SRANKL. The cells were plated in a 96-well plate (5×10⁵ cells/well). Multinucleation of osteoclasts was observed from differentiation day 4.

**Cell viability assay**

The cells were (1×10⁶ cells/well) cultured in DMEM/10% FBS. After 24 h, the cells were incubated with various concentrations of poncirin for 48 h. Cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, as described by Kim et al. (2015). After incubation for 3 h with MTT, the medium was discarded and the formazan granules were solubilized with dimethylsulfoxide (DMSO). The absorbance was measured using a microplate reader (Tecan, Zürich, Switzerland) at 570 nm, and the cell viability was provided as the absorbance ratio. The experiment was undertaken in triplicate.

**TRAP staining**

At differentiation day 4, cells were fixed with 10% formalin by incubating for 10 min, following which they were stained using the Leukocyte Acid Phosphatase kit-387A according to the manufacturer’s instructions (Sigma-Aldrich). The images of TRAP-positive cells were captured under a microscope with DC controller (Olympus Optical, Tokyo, Japan).

**Preparation of total RNA**

Total RNA was isolated using the RNAzol reagent according to the manufacturer’s protocol. The concentrations of RNA were determined using ND1000 (Thermo Scientific, Wilmington, DE, USA).

**Table 1. The PCR primer sequences use in real-time quantitative PCR**

| Target gene | Forward (5’-3’) | Reverse (5’-3’) |
|-------------|----------------|----------------|
| TRAP        | ACACAGTTGATGCTGTGTTGGCAACTC | CCAGAGGCTTCCACATATATGATGG |
| NFATc1      | GGTCAGTGCGACCAGAGAT | GGAAGTCAAGAAGGGTGGGA |
| c-Fos       | CCATGCAAGAGCATCGAGAA | AAGATGTGACCCAGGAGTA |
| MMP9        | AGTTGTTGTGGCAGCCGAGCAGC | TACATGAGCCTCCGGCAC |
| Cathepsin K | AGCCGGCTATATAGACACCTG | CCGGACCAAGAGGACATAC |
| β-actin     | TCACCCACACTCTGCCCAT | TCTCTAATGTCAGCCACATTT |

TRAP, Tartrate-resistant acid phosphatase; NFATc1, Nuclear factor of activated T cells; MMP9, Matrix Metalloproteinase 9.
Real-time quantitative PCR (RT-qPCR)
First-strand cDNA was acquired from 1 µg of total RNA; qPCR was then performed using the SYBR Premix Ex Taq (Takara Bio Inc.), according to the manufacturer’s protocol. All reactions were run in triplicate, and the relative expression levels were analyzed by the 2^−∆∆CT method. β-actin is known to maintain a constant basal level during osteoclastogenesis, and was used as an internal standard. The primer sets utilized in the study are listed in Table 1.

Western blot analysis
Cells were washed with ice-cold phosphate buffered saline (PBS) containing 1 mM sodium vanadate, after which they were solubilized in lysis buffer (20 mM Tris–HCl (pH 7.5), 1 mM EGTA, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM EDTA, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, and 1 µg/mL leupeptin). After a freeze–thaw cycle with vortexing, the lysates were centrifuged at 12,000×g for 15 min at 4°C. The cell lysates were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was soaked in a blocking solution (5% non-fat dry milk/Tris-buffered saline, 0.1% Tween-20) for 1 h at room temperature, after which it was incubated with the relevant antibodies overnight at 4°C. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and then the bands were detected using ECL (Amersham Biosciences, Pittsburgh, PA, USA).

LPS-induced bone erosion
In vivo animal study was performed in accordance with the guidelines of the Gachon University Animal Care and Use Committee (approval number: GIACUC 2018030). Six-week-old ICR mice were divided into three groups of 5 mice each. Mice were administered intraperitoneal injections of LPS (5 µg/g body weight) on days 0 and 4. Saline (control) or poncirin (50 µg/g body weight) was administrated orally every day for 8 days. On the eighth day after the first injection of LPS, the mice were sacrificed, and the femurs were obtained and fixed in 4% paraformaldehyde for 1 day. Radiographic images were taken using the micro-CT scan (NFR-Polaris G-90; Nano Focus Ray Co, Jeonju, Korea).

Statistical analysis
Each value was represented as the mean ± standard deviation (SD). Significant differences were determined using Student’s t-test. Differences with values p<0.05 were considered significant. All experiments were performed in triplicate.

Fig. 1. Structure of Poncirin. The chemical structure of poncirin was drawn using PubChem Sketcher (https://pubchem.ncbi.nlm.nih.gov/edit2/index.html, ver. 2.4).
RESULTS

Inhibitory effect of poncirin on osteoclast differentiation

We evaluated the effect of poncirin (Fig. 1, the structure of poncirin) on osteoclast differentiation in murine monocyte/macrophage cell line RAW264.7 cells. Cells were incubated with sRANKL (100 ng/mL) for 4 days to induce differentiation. Differentiation was assessed by counting the number of multinucleated TRAP-positive cells which is prominent morphological features of mature osteoclasts. As shown in Fig. 2A, osteoclastic differentiation was inhibited by exposure to poncirin in a concentration-dependent manner. Poncirin effectively reduced the number of TRAP-positive multinucleated cells at concentrations as low as 0.2 µg/mL and exerted up to 75.19 ± 3.56% inhibition at 5 µg/mL (Fig. 2B).

Evaluation of cytotoxicity of poncirin

To assess if the inhibitory effect of poncirin on osteoclastogenesis resulted from its cytotoxicity, RAW264.7 cells were treated with varying concentrations of poncirin for 48 h, following which the cell viability was assessed by the MTT assay. Poncirin did not affect the rate of cell growth even as high as 50 µg/mL, indicating that the poncirin-mediated suppression of osteoclastogenesis is not due to cytotoxic effects (Fig. 2C).

Effect of poncirin on the expression of osteoclast-specific genes

Osteoclast differentiation is positively regulated by osteo-genic genes, such as Acp5 (TRAP), Mmp9 (MMP9), and Ctsk (cathepsin K). To evaluate the effect of poncirin on the expression of these osteoclast-specific genes, RAW264.7 cells were pretreated with or without poncirin and further stimulated with sRANKL for 4 days and mRNA levels were measured by real-time qPCR. sRANKL significantly stimulated the expression of Acp5, Mmp9, and Ctsk in RAW264.7 cells. In contrast, pretreatment with poncirin efficiently suppressed the induction of all three genes in a concentration-dependent manner (Fig. 3A). Interestingly, a one-day treatment with sRANKL followed by treatment with poncirin (post-treatment with poncirin) also dramatically inhibited the sRANKL-induced Acp5 expression in a concentration-dependent manner (Fig. 3B). These data indicate that poncirin efficiently inhibits osteoclast differentiation through down-regulation of key genes involved in osteoclastogenesis.

![Graph A](image1)

![Graph B](image2)

**Fig. 3.** Effect of poncirin on osteoclast-specific gene expression in sRANKL-stimulated RAW264.7 cells. (A) Cells were pretreated with the indicated concentration of poncirin, followed by culturing with sRANKL (100 ng/mL) for 4 days. The mRNA levels of TRAP, MMP9, and cathepsin K were evaluated by quantitative real-time PCR. (B) Cells were cultured with sRANKL (100 ng/mL) for 1 day, and then further incubated in the presence of the indicated concentration of poncirin for additional 3 days; the mRNA level of TRAP was determined by quantitative real-time PCR. Fold changes relative to each gene level in the control are presented as mean ± SD of independent experiments (n=3). ***p<0.001 compared with the control. **p<0.01, ***p<0.001 compared with sRANKL.
Effect of poncirin on expression of c-Fos and NFATc1

c-Fos and NFATc1 are the key transcription factors involved in osteoclast differentiation. Induction of c-Fos, a critical component of the activator protein (AP)-1, is required for the robust expression of NFATc1. Therefore, we further undertook to evaluate if poncirin regulates osteoclast differentiation by modulating the activities of c-Fos and NFATc1. Incubation for 4 days with sRANKL elevated the gene expression of both c-Fos and NFATc1 genes in RAW264.7 cells (Fig. 4). However, pretreatment with poncirin significantly suppresses the expression of these genes (Fig. 4). NFATc1 expression was efficiently down-regulated at a concentration as low as 0.05 µg/mL.

Effect of poncirin on MAPKs and NF-κB activation in sRANKL-stimulated RAW264.7 cells

RANKL activates NFATc1 via a variety of key signal transducers, including p38, ERK, JNK, and NF-κB. To elucidate the role of poncirin in signal transduction of osteoclast differentiation, we examined the effects of poncirin on the RANKL-induced early activation of MAPKs. RAW264.7 cells were pretreated with poncirin for 1 h and stimulated with sRANKL for 15 min, after which we determined the phosphorylation levels of p38, JNK, ERK, and NF-κB by immunoblot analysis. We observed that sRANKL markedly induces the activation of all three MAPKs. However, pretreatment with poncirin inhibited the sRANKL-induced acute JNK activation without significantly affecting the expressions of ERK or p38 (Fig. 5A). Furthermore, poncirin also suppresses the sRANKL-induced NF-κB activation (Fig. 5B).

**DISCUSSION**

Bone homeostasis requires a finely-tuned balance between bone resorption and bone formation, in which osteoblasts stimulate bone formation while osteoclasts promote bone resorption. An imbalance in bone homeostasis can occur due to a loss in osteoblast functions, or the abnormal activation of osteoclasts which enhances abnormal bone resorption. The imbalance due to osteoclast activation is closely related to most bone-related metabolic diseases such as osteoporosis, rheumatoid arthritis, periodontitis, multiple myeloma, and metastatic cancers (Boyle et al., 2003). Thus, there is an urgent need to develop therapeutics that suppress or retard the abnormal osteoclast activation. Previously, Yoon et al. (2012) presented that poncirin prevents bone loss by stimulating osteoblast differentiation in a glucocorticoid-induced osteoporosis model. In the current study, we provide in vitro evidence that poncirin considerably inhibits the RANKL-induced osteoclast differentiation, which is confirmed in vivo by the efficacy to protect mice from LPS-stimulated inflammatory bone loss. Our study therefore demonstrates that poncirin not only has pro-osteoblastic but also anti-osteoclastogenic effect.

We observed that poncirin treatment suppressed the NFATc1 gene expression (Fig. 4) which is considered to be mediated by the decreased activities of NF-κB and JNK (Fig. 5). The inhibitory effect of poncirin on NF-κB phosphorylation, we further explored whether poncirin ameliorates bone erosion in an in vivo experimental animal model. LPS was intraperitoneally injected into mice to induce bone loss, and poncirin was orally administered for 8 days. Micro-computer tomography analyses revealed prominent reduction in the bone trabeculae in the femur of LPS-induced mice, whereas the reduction was much lower in poncirin-treated mice (Fig. 6, left panel). The measurement of bone volume fraction (bone volume/trabecular volume) shows that poncirin treatment ameliorated LPS-induced bone loss significantly (Fig. 6, right panel).
nervation at 5 µg/mL, which effectively inhibited the differentiation of osteoclast, was unclear but was observed more clearly at higher concentrations of 50 µg/mL. This suggests that poncirin may regulate NF-κB at any interval between 5 and 50 µg/mL. Although the effect is unclear at low concentrations, it seems clear that poncirin controls phosphorylation of NF-κB. It is noteworthy that poncirin specifically suppresses the JNK activity without modulating the activities of p38 and ERK. NF-κB and AP-1 are immediately activated following RANKL activation in KU812 cells (Wu et al., 2005). NFATc1 activation in KU812 cells (Wu et al., 2009). NFATc1 has been known for decades as a key regulator in osteoclast differentiation, modulating the expression of osteoclast-specific genes, such as TRAP, MMP9, and cathepsin K (Kim and Kim, 2014). It is evident that after the initial induction of NFATc1 by these two factors, the auto-amplification of NFATc1 intensifies the osteoclastogenesis of c-Fos. Inhibitory effects of poncirin are found to be associated with JNK inactivation, but not p-38 MAPK and ERK. Further, the in vivo efficacy of poncirin is confirmed in an animal model of inflammatory bone destruction. Since osteoclast differentiation is responsible for bone resorption, its inhibition by poncirin provides a possible approach to the treatment of osteoporosis that results from increased osteoclast activation.

CONFLICT OF INTEREST

None.

ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support of Gachon University Research Fund (GCU 2012-R003).

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