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3',4',7,8-Tetrahydroxyflavone inhibits RANKL-induced osteoclast formation and bone resorption

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Osteoclasts, which are specialized bone multinuclear cells, are responsible for bone lytic diseases such as osteoporosis. 3',4',7,8-tetrahydroxyflavone is a flavonoid from *Acacia confusa*. In the present study, we found that 3',4',7,8-tetrahydroxyflavone markedly inhibited receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclastic differentiation from mouse bone marrow-derived macrophages (BMMs). 3',4',7,8-tetrahydroxyflavone also reduced the mRNA expression levels of osteoclastic marker genes including the calcitonin receptor (CTR) and cathepsin K. In addition, 3',4',7,8-tetrahydroxyflavone decreased the bone resorption activity of osteoclasts on dentin slices. We found that 3',4',7,8-tetrahydroxyflavone inhibited RANKL-induced expression of c-Fos and nuclear factor of activated T cells c1 (NFATc1), a key transcription factor of osteoclast differentiation. Furthermore, ectopic overexpression of a constitutively active form of NFATc1 completely rescued the anti-osteoclastogenic effect of 3',4',7,8-tetrahydroxyflavone, suggesting that the anti-osteoclastogenic effect was mainly attributed to the reduction in NFATc1 expression. Taken together, our data suggest that 3',4',7,8-tetrahydroxyflavone inhibits osteoclast differentiation and bone loss and may therefore be considered a promising drug candidate for treating or preventing bone-lytic diseases.

1. Introduction

Bone metabolism is a tightly regulated process of lineage-specific differentiation events; this process is maintained by a balance between resorption by osteoclasts and bone formation by osteoblasts (Olsen et al. 2000). Osteoclasts are tissue-specific giant polykaryons derived from the monocyte/macrophage hematopoietic lineage and are the only cells capable of breaking down mineralized bone, dentine, and calcified cartilage (Udagawa et al. 1990; Kurihara et al. 1990). Osteoclastogenesis involves a series of regulatory steps such as proliferation and survival of progenitors, differentiation into mononuclear pre-osteoclasts, fusion into mature multinucleated osteoclasts, and activation of osteoclastic bone resorption (Zaidi 2007).

The process of osteoclastogenesis, which is crucial for normal bone metabolism, is regulated by many factors, including macrophage colony stimulating factor (M-CSF) and RANKL. M-CSF, which signals through c-Fms, a transmembrane receptor tyrosine kinase, is crucial for myeloid cell survival and differentiation into preosteoclasts (Blair et al. 2005). RANKL, the key regulator of osteoclastogenesis, is a member of the tumor necrosis factor (TNF) superfamily and is mainly expressed in preosteoblasts/stromal cells as well as on activated T cells (Yasuda et al. 1998; Lacey et al. 1998). RANKL activates RANK by binding to the extracellular domain of RANK which passes the signal along to TRAF6 (TNF receptor associated factor 6) to activate downstream pathways (Kim et al. 1999; Ye et al. 2002). RANKL-induced signaling pathways lead to the induction and activation of osteoclastogenic transcription factors such as c-Fos and nuclear factor of activated T cells (NFATc1) (Grigoriadis et al. 1994; Takayanagi et al. 2002). The final consequence of these signaling pathways is the induction of osteoclastogenic genes such as tartrate-resistant acid phosphatase (TRAP), CTR, cathepsin K, dendritic cell-specific transmembrane protein (DC-STAMP), the d2 isoform of vacuolar (H+) ATPase V0 domain (Atp6v0d2), and Blimp1 (Takayanagi 2007; Walsh et al. 2006). Most adult skeletal diseases are due to excess osteoclastic activity, leading to an imbalance in bone remodeling which favors resorption (Rodan and Martin 2000). Such diseases include osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma, and metastatic cancers (Boyle et al. 2003). Thus, it is clinically imperative to develop drugs that can regulate osteoclast differentiation.

Flavonoids are polyphenolic compounds that are ubiquitous in natural products, and can be classified into six categories: flavones, flavonols, flavanones, isoflavones, flavanols, and anthocyanins (Hollman and Katan 1999). Recently, great attention has been paid to the possible medicinal properties of flavonoids. Epidemiological and pharmacological studies have indicated many beneficial properties of flavonoids including antioxidative, antiviral, anticancer, and anti-inflammatory effects (Kumar and Pandey 2013). 3',4',7,8-Tetrahydroxyflavone is a flavone, isolated from *Acacia confusa* (Leguminosae) (Wu et al. 2008). Several flavonoids potently inhibit osteoclastogenesis and bone resorption (Weaver et al. 2012). Since there is little knowledge regarding the effects of 3',4',7,8-tetrahydroxyflavone on bone metabolism, its effect on RANKL-induced osteoclast formation was investigated in this study.

2. Investigations and results

2.1. *3',4',7,8-Tetrahydroxyflavone inhibits RANKL-induced osteoclast formation*

RANKL is essential and sufficient for the differentiation of osteoclast (OC) precursors into mature OCs in the presence of M-CSF (Blair et al. 2005; Yasuda et al. 1998; Lacey et al. 1998; Kim et al. 1999). To clarify the effects of 3',4',7,8-tetrahydroxyflavone on RANKL-induced osteoclastogenesis, we used a mouse BMM culture system. When BMMs were incubated with M-CSF and RANKL for 4 days, numerous TRAP-positive (TRAP$^+$) multi-

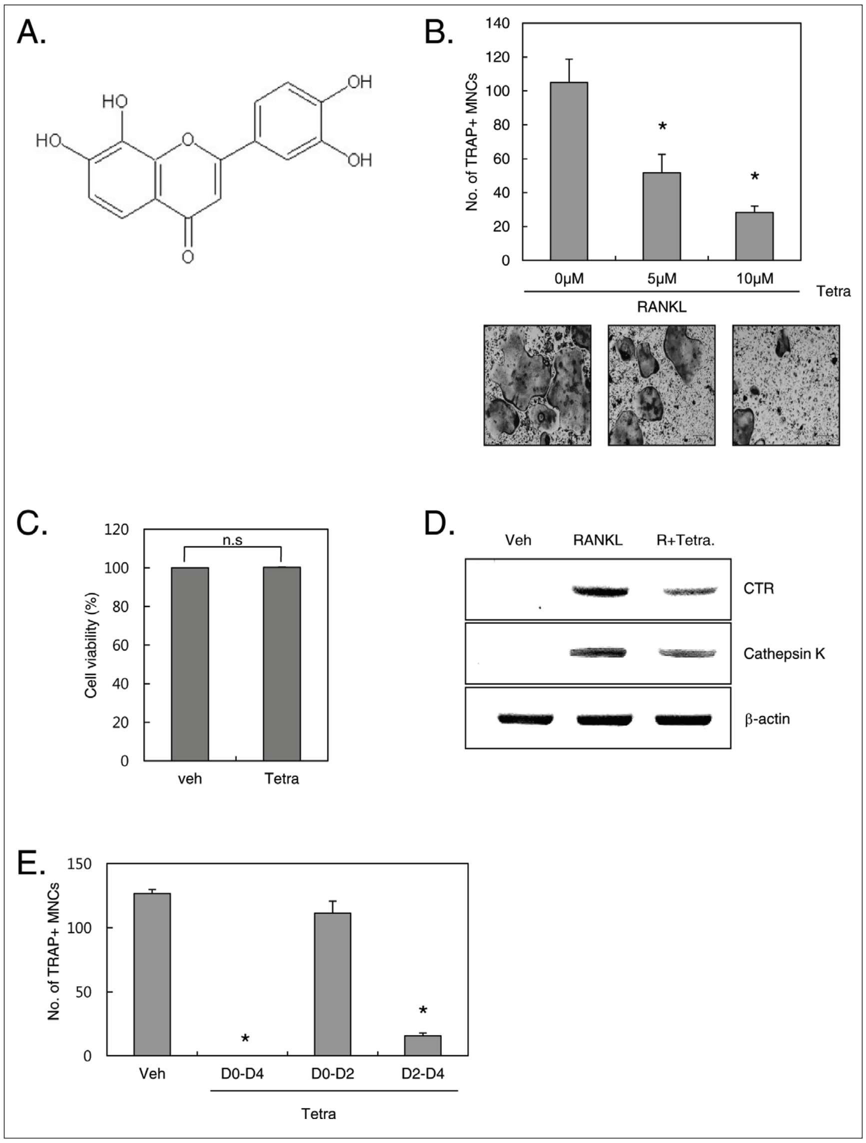

Fig. 1: 3’,4’,7,8-Tetrahydroxyflavone inhibits RANKL-induced osteoclast formation. (A) Chemical structure of 3’,4’,7,8-Tetrahydroxyflavone. (B) BMMs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL) in the absence or presence of 3’,4’,7,8-Tetrahydroxyflavone for 4 days. TRAP$^+$ MNCs containing more than 3 nuclei were counted. (C) BMMs were cultured in the absence or presence of 3’,4’,7,8-Tetrahydroxyflavone (10 μM) with M-CSF for 48 h. Cell viability was assessed by MTT assay. (D) BMMs were cultured in the absence or presence of 3’,4’,7,8-Tetrahydroxyflavone (10 μM) with RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 7 days. The mRNA levels of osteoclast marker genes were detected by RT-PCR using specific primers. (E) 3’,4’,7,8-Tetrahydroxyflavone (10 μM) was added and cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL) during the indicated culture days. Data are expressed as the mean ± S.D. of three independent experiments. Veh, vehicle; R, RANKL; Tetra, 3’,4’,7,8-Tetrahydroxyflavone. (* $p < 0.05$).

nucleated OCs were generated. Treatment of the same cultures with 3’,4’,7,8-tetrahydroxyflavone suppressed OC formation in a dose-dependent manner (Fig. 1B). To further confirm the inhibitory effect of 3’,4’,7,8-tetrahydroxyflavone on osteoclastogenesis, we examined the mRNA expression of CTR and cathepsin K, osteoclastogenic marker genes, by RT-PCR. Osteoclasts exhibit the highest CTR density, and binding of calcitonin to CTR inhibits osteoclastic activity (Dacquin et al. 2004; Davey et al 2008; Lafont et al. 2011). Cathepsin K is a potent collagenase that is expressed in osteoclasts, and its activity is most evident in osteoclasts that resorb bone tissue (Stroup et al. 2001; Lindeman et al. 2004). Accordingly, the mRNA expression levels of CTR and cathepsin K were increased in cultures treated with M-CSF and RANKL for 4 days, and this was dramatically suppressed by the presence of 3’,4’,7,8-Tetrahydroxyflavone (Fig. 1D). MTT assays showed that the anti-osteoclastogenic effect of 3’,4’,7,8-Tetrahydroxyflavone was not attributable to cellular toxicity (Fig. 1C). We next determined the stage at which 3’,4’,7,8-tetrahydroxyflavone impaired OC development. 3’,4’,7,8-Tetrahydroxyflavone was added to OC-generating cultures on different days (D0–4), and TRAP staining was performed on day 4. 3’,4’,7,8-tetrahydroxyflavone effectively inhibited OC formation when added on day 3 or 4 of culture, but not when added on days 0–2, suggesting that this compound only affects late osteoclastogenesis (Fig. 1E).

2.2. 3’,4’,7,8-Tetrahydroxyflavone disrupts the morphology of mature OCs and suppresses bone resorption **in vitro**

We analyzed the effect of 3’,4’,7,8-tetrahydroxyflavone on mature OCs. Mature OCs exhibit highly polarized morphological features, which is essential for initiating bone resorption (Wagner and Karsenty 2001). When mature OCs were incubated with RANKL, a clear cytoplasm and a smooth periphery were seen in polarized OCs. Conversely, the presence of 3’,4’,7,8-tetrahydroxyflavone induced morphological changes such as a contracted cytoplasm and an irregular cell periphery within 24 hours (Fig. 2A). Since actin rings are believed to be a marker of polarized OCs (Boyle et al. 2003), we examined whether the effect of 3’,4’,7,8-tetrahydroxy-

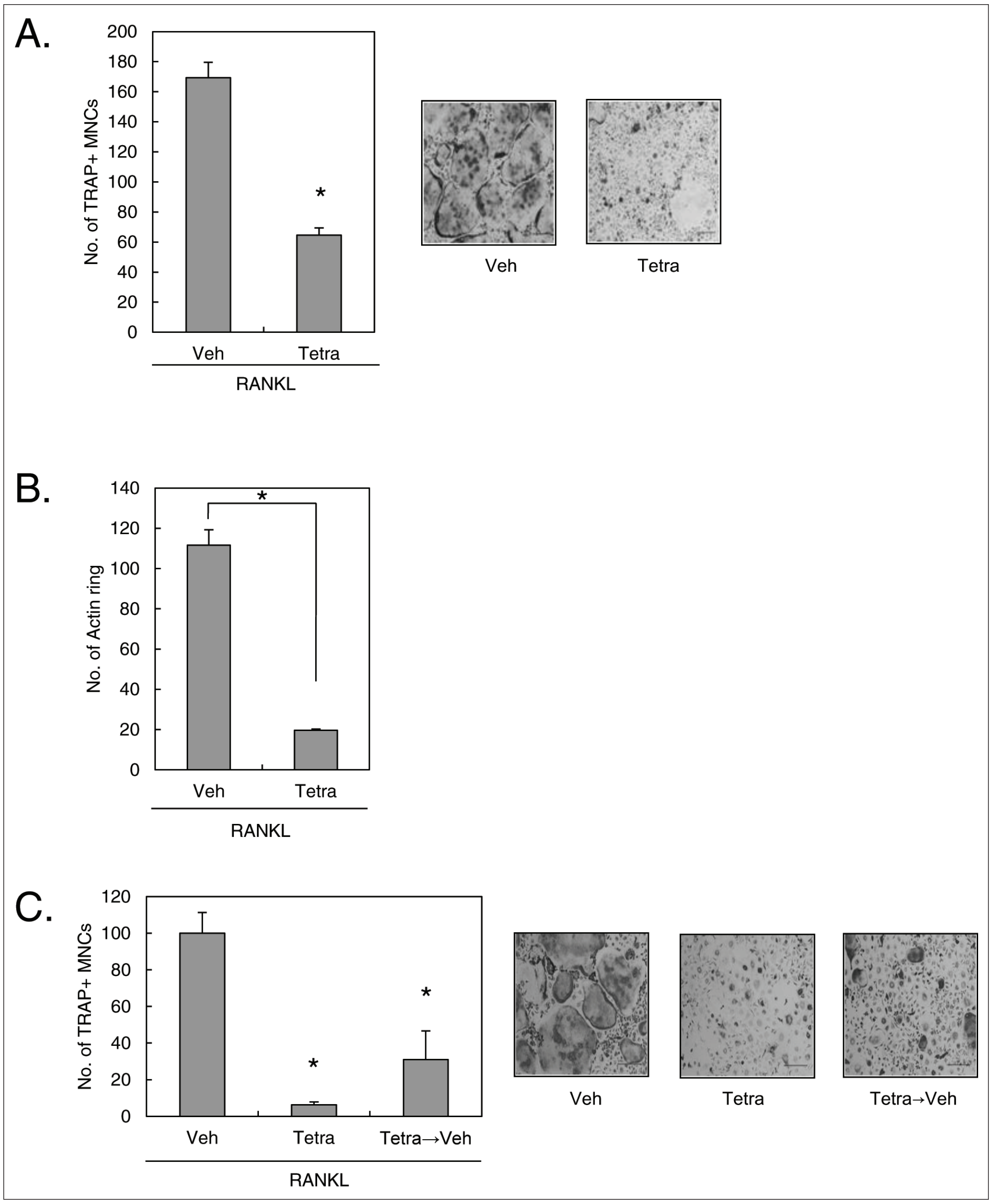

Fig. 2: 3',4',7,8-Tetrahydroxyflavone disrupts actin ring structure in mature OCs. (A) Mature OCs were treated with 3',4',7,8-Tetrahydroxyflavone (10 μM) for 24 hours and stained for TRAP. (B) Mature OCs were treated with 3',4',7,8-Tetrahydroxyflavone (10 μM) for 24 hours and actin rings were stained with rhodamine-conjugated phalloidin. (C) Mature OCs were treated with 3',4',7,8-Tetrahydroxyflavone (10 μM). After 24 hours, 3',4',7,8-Tetrahydroxyflavone (10 μM) was removed from the culture media and mature OCs were cultured for a further 24 hours. Scale bar = 200 mm. Data are expressed as the mean ± S.D. from at least three independent experiments. Veh, vehicle; Tetra, 3',4',7,8-Tetrahydroxyflavone; *$p < 0.05$.

flavone on actin ring formation was involved in OC morphological changes. F-actin staining showed that 3',4',7,8-tetrahydroxyflavone efficiently disrupted actin rings in mature OCs (Fig. 2B). These data suggest that 3',4',7,8-tetrahydroxyflavone directly induced the disruption of actin rings, causing morphological changes in mature OCs. To further characterize the effect of the compound on mature OCs, we observed the morphology of OCs after removing 3',4',7,8-tetrahydroxyflavone from the culture media. The number of mature OCs with a clear cytoplasm and a smooth periphery increased upon removal of 3',4',7,8-tetrahydroxyflavone (Fig. 2C). These results suggest that 3',4',7,8-tetrahydroxyflavone reversibly disrupts actin rings, resulting in partially reversible OC morphological changes. To examine whether the effect of 3',4',7,8-tetrahydroxyflavone on OC morphology could be reflected in their osteoclastic activity, we further performed an *in vitro* resorption pit assay using dentine slices. Many resorption pits were generated in wells containing RANKL-treated OCs (Fig. 3). In contrast, treatment with 3',4',7,8-tetrahydroxyflavone strongly inhibited the formation of resorption pits by RANKL-treated OCs (Fig. 3). Together, these results suggest that 3',4',7,8-tetrahydroxyflavone inhibits OC morphology, leading to reduced bone resorption.

2.3. 3',4',7,8-Tetrahydroxyflavone suppresses RANKL-induced expression of c-Fos and NFATc1

The NFATc1 pathway plays a critical and fundamental role in osteoclast development, and a lack of NFATc1 arrests osteoclastogenesis. Furthermore, c-Fos promotes the expression of NFATc1 by binding to the NFATc1 promoter (Matsuo et al. 2004; Takayanagi 2007). Therefore, we investigated the effects of 3',4',7,8-tetrahydroxy-

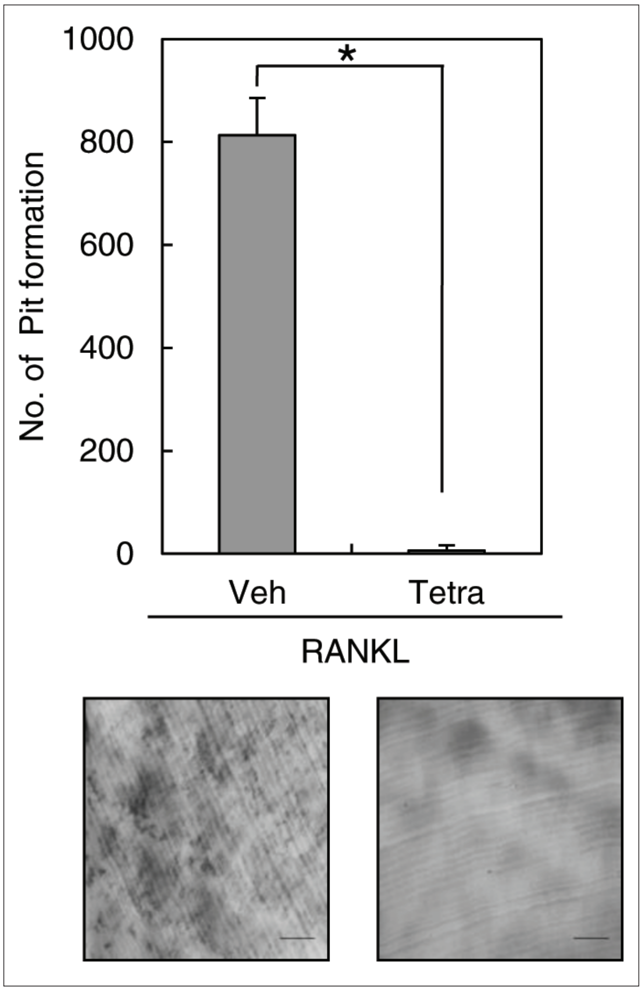

Fig: 3: 3’,4’,7,8-Tetrahydroxyflavone suppresses RANKL-induced bone resorption activity. BMMs were differentiated on dentine slices with M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days, and 3’,4’,7,8-Tetrahydroxyflavone (10 µM) was treated for 3 days. The resorbed pit numbers were counted. Data are expressed as the mean ± S.D. from at least three independent experiments. Veh, vehicle; Tetra, 3’,4’,7,8-Tetrahydroxyflavone; *$p < 0.05$.

flavone on the expression of these genes. As reported previously, RANKL stimulation increased the expression of c-Fos and NFATc1 in BMMs (Fig. 4A, B). 3’,4’,7,8-Tetrahydroxyflavone abolished RANKL-induced expression of c-Fos and NFATc1 protein expression (Fig. 4A, B). We next investigated whether the inhibitory effect of 3’,4’,7,8-tetrahydroxyflavone was rescued by overexpressing NFATc1. The overexpression of a constitutively active form of NFATc1 (CA-NFATc1) in BMMs was confirmed by western blotting (data not shown). These NFATc1-transduced BMMs were cultured with M-CSF and RANKL in the absence or presence of 3’,4’,7,8-tetrahydroxyflavone for 4 days. As shown in Fig. 4C, osteoclastogenesis suppression by 3’,4’,7,8-tetrahydroxyflavone treatment was rescued by the forced expression of CA-NFATc1, suggesting that the anti-osteoclastogenic effect of 3’,4’,7,8-tetrahydroxyflavone is mainly due to a reduction in NFATc1 expression.

3. Discussion

The increasing worldwide prevalence of osteolytic bone diseases has had serious negative consequences on human health. It is widely believed that enhanced osteoclast formation and bone resorption underlie the development of osteolytic bone diseases including osteoporosis, Paget’s disease of bone, bone metastatic diseases, erosive arthritis, aseptic bone loosening, and nonunion (Zaidi 2007). Therefore, it is clinically necessary to develop anti-resorptive agents for osteoporosis therapy (Rodan and Martin 2000).

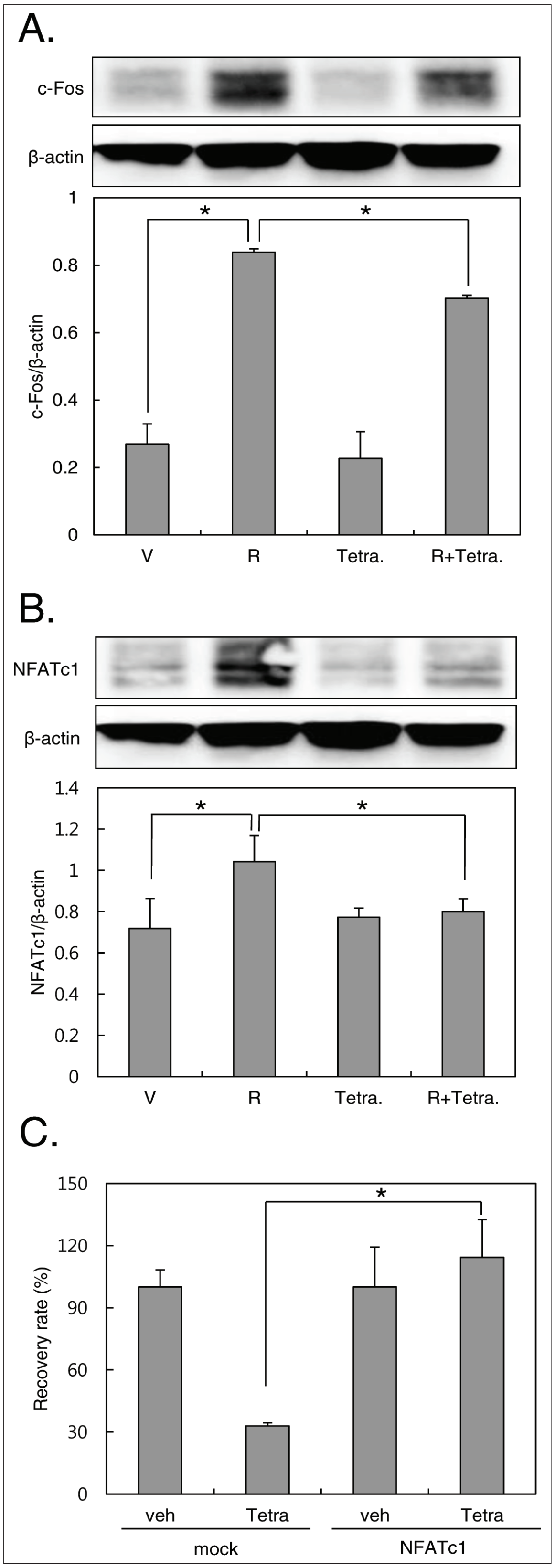

Fig. 4: 3’,4’,7,8-Tetrahydroxyflavone decreases RANKL-induced expression of c-Fos and NFATc1. (A-B) BMMs were pre-incubated with 3’,4’’,7,8-Tetrahydroxyflavone (10 µM) for 30 min, and then treated with or without 200 ng/mL RANKL for 24 h in the presence of M-CSF (30 ng/mL). Whole-cell extracts were harvested and subjected to western blot analysis for the indicated proteins. (C) BMMs infected with the indicated constructs were cultured with RANKL (100 ng/ml) and M-CSF (30 ng/mL) in the absence or presence of 3’,4’,7,8-Tetrahydroxyflavone (10 µM) for 4 days. The recovery rate was defined as the percentage of OC formation in the presence of 3’,4’,7,8-Tetrahydroxyflavone. OC formation in the presence of vehicle was set as 100%. Data are expressed as the mean ± S.D. from at least three independent experiments. Veh, vehicle; Tetra, 3’,4’,7,8-Tetrahydroxyflavone. *$p < 0.05$.

Recently, great attention has been paid to natural compounds that can specifically inhibit metabolic bone disorders characterized by excessive osteoclast bone resorption (Weaver et al. 2012). Flavonoids, found in a wide variety of plant foods, have great potential as a dietary component to promote bone health beyond calcium and vitamin D (Weaver et al. 2012). *Acacia confusa* (Leguminosae), a native species in Taiwan, has been traditionally used as a medicine and contains 3',4',7,8- tetrahydroxyflavone (Wu et al. 2008). In this study, we investigated the effect of 3',4',7,8-tetrahydroxyflavone on RANKL-induced osteoclast formation. 3',4',7,8-Tetrahydroxyflavone inhibited RANKL-induced osteoclast formation without cytotoxic effects. We also demonstrated the inhibitory effects of the compound on mature osteoclasts and resorptive activity. Bone resorption occurs within the sealing zone, which is formed by the actin ring structure (Boyle et al. 2003). Thus, actin ring formation is required for bone resorption of mature osteoclasts. We suggest that structural disturbance in the actin ring upon 3',4',7,8-tetrahydroxyflavone treatment might largely contribute to reduced bone resorption by mature osteoclasts.

RANKL is the key regulator of osteoclastogenesis, and its binding to its receptor, RANK, in osteoclast precursor cells can activate multiple intracellular signaling pathways including NFATc1 and c-Fos (Grigoriadis et al. 1994; Takayanagi et al. 2002). c-Fos is a major component of the AP-1 transcription factor complex and regulates osteoclast differentiation. During osteoclast formation, c-Fos plays an important role in inducing and activating NFATc1, which then activates multiple target genes responsible for osteoclast formation and function (Grigoriadis et al. 1994). Our data suggest that 3',4',7,8-tetrahydroxyflavone inhibits RANKL-induced expression of c-Fos and NFATc1, which in turn decreases osteoclast formation.

NFATc1 also regulates the expression of many osteoclast-specific genes that are involved in the terminal differentiation of osteoclasts. For example, DC-STAMP and Atp6v0d2 are essential molecules in the development of multinucleated osteoclasts, and these proteins enhance resorptive capacity (Yagi et al. 2005; Lee et al. 2006). In addition, the adhesion molecule β3-integrin is a late-stage osteoclast gene that plays an important role in regulating osteoclast formation and function by mediating osteoclast migration and adhesion (Faccio et al. 2003). Given that 3',4',7,8-tetrahydroxyflavone acts in the late stages of osteoclast differentiation, it may diminish the function of osteoclast-specific genes via decreasing NFATc1 expression. Further study is needed to elucidate its mechanism.

Osteoclast-targeting drugs are the cornerstone of therapies for osteoporosis and other conditions related to OC-mediated bone fragility including Paget's disease, metastatic disease, and multiple myeloma. Currently, the most commonly used OC-targeting drugs are bisphosphonates, which readily bind to exposed hydroxyapatite and inhibit bone resorption by promoting OC death. Free bisphosphonate can be rapidly cleared by the kidneys, but skeletally bound bisphosphonate persists indefinitely, effectively rendering treatment irreversible. Recently, bisphosphonate treatment has been associated with atypical bone fractures, presumably due to lack of normal bone remodeling and repair (Shane et al. 2010), resulting in the accumulation of defective bone structures. This is the first report on the anti-osteoclastogenic activity of 3',4',7,8-tetrahydroxyflavone and its mode of action. Given that downregulation of RANKL-mediated signals may be a valuable approach for treating pathological bone loss, 3',4',7,8-tetrahydroxyflavone could be a drug candidate for treating bone lytic diseases.

4. Experimental

4.1. Reagents

3',4',7,8-Tetrahydroxyflavone was purchased from EXTRASYNTHESE (France). The antibody against NFATc1 was purchased from Santa Cruz Biotechnology (CA, USA) and antibodies against c-Fos and β-actin were purchased from Cell Signaling Technology (MA, USA).

4.2. Cells and culture system

Bone marrow cells were obtained from the long bones of 4- to 6-week-old ICR male mice (Samtako, Inc., Osan, Korea). Mice were sacrificed by cervical dislocation, and bone marrow was extracted from tibiae and femora. Bone marrow cells were cultured overnight in alpha-minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and M-CSF (10 ng/mL, R&D systems, Inc., Minneapolis, MN, USA). Floating cells were harvested and cultured with 30 ng/mL M-CSF for 3 days. Formed BMMs were used to generate osteoclasts in this study. All cells were cultured in alpha-MEM with 10% FBS at 37 °C in a 5% CO_2 incubator.

4.3. Osteoclast differentiation

BMMs were treated with M-CSF (30 ng/mL) and RANKL (100 ng/mL, PeproTech, Rocky Hill, NJ, USA) to induce differentiation into osteoclasts. Then, cells were treated with the indicated concentrations of 3',4',7,8-tetrahydroxyflavone to assess whether it had an effect on RANKL-induced osteoclast formation. Cells were fixed in 10% formalin for 10 min and permeabilized with ethanol:acetone (50:50 v/v) for 1 min at room temperature, then incubated in acetate buffer (pH 5.2) containing naphthol AS-MX phosphate (Sigma-Aldrich) as the substrate and Fast Red Violet LB salt (Sigma-Aldrich) as the dye in the presence of 50 nM sodium tartrate. After washing with distilled water and drying, TRAP$^+$ multinucleated cells (MNCs) (n>3) were counted using a light microscope.

4.4. MTT assay

BMMs were seeded into 96-well cell culture plates (2´10^4 cells/well) and treated with M-CSF (30 ng/mL). After stabilization, cells were treated with 3',4',7,8-tetrahydroxyflavone for 48 h. Then, 0.5 mg/mL MTT solution was added and incubated in the dark. After 5 h, solubilization buffer (10% SDS in 0.01 M HCl) was added and cells were cultured overnight. Cell viability was measured based on the O.D. at 570 nm as determined by a plate reader.

4.5. Bone resorption assay

BMMs were placed on dentin slices and cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the absence or presence of 3',4',7,8-tetrahydroxyflavone (10 μM) for 7 days. Dentin slices were removed from the plate, then were rubbed and washed to remove the remaining cells. Then, dentin slices were stained with toluidine blue (1 μg/mL, J.T. Baker, UK). Formed pits were counted as an indicator of bone resorption activity.

4.6. Actin ring staining</bi>

Cells were washed twice with PBS and fixed with 10% formalin for 5 min. Cells were permeabilized with EtOH/Acetone (1:1) for 1 min at room temperature, and solvent removed, and the cells were dried. Actin rings were stained with rhodamine-conjugated phalloidin (Molecular probes, USA) overnight in PBS at 4 °C in the dark. After washing the cells twice with PBS, images were taken using a fluorescence microscope (Olympus, Tokyo, Japan).

4.7. Retroviral gene transduction</bi>

Plat-E retroviral packaging cells were seeded in culture dishes 1 day prior to transfection. The following day, Plat-E cells were transfected with PMX-puro-GFP and PMSCV-GFP-CA-NFATc1 using Lipofectamine 2000 CD (Invitrogen, Carlsbad, CA, USA). After 2 days in culture, culture supernatants were collected. BMMs were plated with the supernatants containing PMX-puro-GFP and PMSCV-GFP-CA-NFATc1 viral particles produced by plat-E cells, in the presence of polybrene (10 μg/mL) and M-CSF (30 ng/mL) overnight in 48-well plates. Infected cells were then selected with puromycin (2 μg/mL) for 2 days, and then further cultured with or without 3',4',7,8-tetrahydroxyflavone (10 μM) in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days.

4.8. RT-PCR analyses

Total RNA was extracted from BMMs using Easy-Blue reagent (iNtRON Biotechnology, Inc.). cDNA was synthesized from total RNA using a RevertAid first strand cDNA synthesis Kit (Fermentas, EU) and amplified using PCR. Primers for osteoclastogenic genes used in this study were as follows: CTR, 5'-TTTCAAGAACCTTAGCTGCCAGAG-3' (forward), 5'-CAAGGCACGGACAATGTTGAGAAG-3'(reverse); Cathepsin K, 5'-CTTCCAATACGTGCAGCAGA-3' (forward), 5'-ACGCACCAATATCTTGCACC-3' (reverse); β-actin, 5'-TTTGATGTCACGCACGATTTCC-3' (forward), 5'-TGTGATGGTGGGAATGGGTCAG-3' (reverse). The RT-PCR program was as follows: 28 cycles (CTR), or 22 cycles (cathepsin K, β-actin), after an initial denaturation step at 94 °C for 3 min, then denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 10 min. After amplification, PCR reaction mixtures were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining and UV irradiation. The relative abundance of each mRNA was calculated after normalization to β-actin.

4.9. Immunoblot analysis

Whole cell lysates were isolated, loaded into sodium dodecyl sulfate-polyacrylamide gels, and then transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk in PBS-T (PBS 0.1%

Tween 20), and then incubated with anti-NFATc1 (1:200), anti-c-Fos (1:1000), or anti-β-actin (1:4000), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000). The immunoreactive bands were detected with enhanced chemiluminescence reagent (Amersham BioSciences, Buckinghamshire, UK) using an LAS3000 luminescent image analyzer (FUJIFILM Co., Tokyo, Japan).

4.10. Statistical analysis

Data represent the means±SD from three independent experiments. Statistical analysis was performed by one-way analysis of variance followed by a Student's *t*-test. A *p*-value < 0.05 was considered statically significant.

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Conflicts of interest: None declared.

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