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Journal of Nutritional Biochemistry 22 (2011) 8–15
Journal of Nutritional Biochemistry

RESEARCH ARTICLES

The effects of luteolin on osteoclast differentiation, function in vitro and ovariectomy-induced bone loss☆

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Received 28 July 2009; received in revised form 27 October 2009; accepted 9 November 2009

Abstract

Flavonoids, a group of polyphenolic compounds abundant in plants, are known to prevent bone loss in ovariectomized (OVX) animal models. Inhibition of osteoclast differentiation and bone resorption is considered as an effective therapeutic approach in the treatment of postmenopausal bone loss. Luteolin, a plant flavonoid, has potent anti-inflammatory properties both in vivo and *vitro*. In this study, we found that luteolin markedly decreased the differentiation of both bone marrow mononuclear cells and Raw264.7 cells into osteoclasts. Luteolin also inhibited the bone resorptive activity of differentiated osteoclasts. We further investigated the effects of luteolin on ovariectomy-induced bone loss using micro-computed tomography, biomechanical tests and serum markers assay for bone remodeling. Oral administration of luteolin (5 and 20 mg/kg per day) to OVX mice caused significant increase in bone mineral density and bone mineral content of trabecular and cortical bones in the femur as compared to those of OVX controls, and prevented decreases of bone strength indexes induced by OVX surgery. Serum biochemical markers assays revealed that luteolin prevents OVX-induced increases in bone turnover. These data strongly suggest that luteolin has the potential for prevention of bone loss in postmenopausal osteoporosis by reducing both osteoclast differentiation and function.

Keywords: Luteolin; Osteoclast; Ovariectomy; Bone resorption; Flavonoid; Bone loss

1. Introduction

Osteoporosis is systemic bone disease characterized by a reduction in bone mass, disruption in bone microarchitecture, and a consequent increase in bone fragility. It may be induced by several conditions, such as hormonal imbalance, chronic diseases, or medications, specifically corticosteroids [1]. Estrogen plays a fundamental role in skeletal growth and bone homeostasis. Estrogen deficiency elicits a chronic inflammatory status by increasing the local production of various cytokines, free radicals, and growth factors by cells located within the bone microenvironment [2,3]. The increased proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) within the bone microenvironment, results in an expansion of the osteoclastic pool due to increased osteoclast formation [4].

Current drugs used to treat osteoporosis include bisphosphonates, calcitonin and estrogen, which prevent osteoclastic bone resorption, resulting in the maintenance of bone mass and a reduction of fracture [5,6]. However, these pharmacological treatments give rise to the side effects, such as gastrointestinal tolerance in bisphosphonate and increased risk of breast, ovarian and endometrial cancer [7,8]. Hence, growing evidence of the benefits of natural foods for bone health provide an alternative approach for managing osteoporosis.

Flavonoids, a group of polyphenolic compounds widely found in the plant kingdom, are known to have multiple beneficial biological effects owing to their anti-inflammatory, antioxidant and estrogenic activities [9,10]. Available literature suggests that the isoflavones, genistein and daidzein, and the flavones apigenin identified as the main flavonoids in soybean were found to prevent bone loss in ovariectomized animal models [11–13], and that they inhibit osteoclast differentiation. In addition, some flavonols, kaempferol and quercetin, have been shown to inhibit osteoclastic bone resorption and to induce osteoclast apoptosis probably via their estrogenic effect [14]. Rutin was also reported to inhibit ovariectomy (OVX)-induced bone loss in a female rat model [15].

☆ This work was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (Project No.: A010252).
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doi:10.1016/j.jnutbio.2009.11.002

Luteolin (3,4,5,7-tetrahydroxyflavone) is a flavonoid found in many herbal extracts including celery, green pepper, parsley, perilla leaf and seeds and chamomile, and has anti-inflammatory effects both in vivo and vitro [16,17]. It can effectively inhibit the lipopolysaccharide (LPS)-induced TNF-α, IL-6 and inducible nitric oxide production in an activated macrophage-like cell line [16,18]. Furthermore, luteolin has an anabolic effect, in that it increases collagen synthesis, alkaline phosphatase (ALP) activity, and osteocalcin secretion, and it inhibits 3-morpholinosydnonimine-stimulated production of pro-inflammatory mediators in osteoblastic MC3T3-E1 cells in vitro [19]. Recently, luteolin isolated from the aerial parts of *Hanlenia corniculata* was screened as effective candidate for inhibition of osteoclast-like cells formation [20].

Based on these studies, the present study was conducted to investigate the effect of luteolin on osteoclast differentiation and function in vitro, and bone mass, serum biomarkers of bone formation or resorption and related mechanical profile in ovariectomized mice as a postmenopausal bone loss model.

2. Materials and methods

2.1. Osteoclast differentiation

Bone marrow cells were prepared by removing from the femora and tibiae of 6-8 week-old ICR mice as described [21]. The bone marrow suspension was added to plates along with macrophage colony stimulating factor (M-CSF) (20 ng/ml). After 24 h of culture, the nonadherent cells were collected, layered on a Ficoll-Hypaque gradient, and the cells at the interface were collected, washed, and resuspended in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS). For the osteoclastogenesis experiments, the bone marrow-derived macrophages (BMMs) were plated into a 96-well plates at a density of 3×10^4 cells/well in α-MEM with 10% FBS, receptor activator for nuclear factor κB ligand (RANKL; 20 ng/ml) and M-CSF (10 ng/ml) in the presence or absence of luteolin.

Osteoclast formation was measured by quantifying cells positively stained with tartrate-resistant acid phosphatase [TRAP (acid phosphatase kit 387-A; Sigma-Aldrich, St. Louis, MO, USA)]. Briefly, the cells were fixed in 10% formaldehyde for 10 minutes, and stained for TRAP with naphthol AS-MX phosphate and tartrate solution. TRAP-positive multinucleated cells (MNCs) with three or more nuclei were scored. For measuring TRAP activity, cells were fixed, dried and then incubated with 100 μl substrate solution (3.7 mM of ρ-nitrophenyl phosphate and 10 mM of sodium tartrate in 50 mM citrate buffer, pH 4.6) at 37°C for 10 min. After incubation, the reaction mixtures were transferred into new plates containing an equal volume of 0.1 N NaOH [21]. Absorbance was measured at 405 nm with an enzyme-linked immunosorbent assay (ELISA) reader (BioRad; Hercules, CA, USA).

2.2. Cell viability

Cell viability was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Medison, WI, USA) following the manufacturers' instructions. Briefly, after treatment with luteolin for 2 days, 201 μl of CellTiter 96 AQueous One Solution Reagent was added to each well, and the mixture was incubated for 2 h at 37°C. Absorbance of each well was determined at 490 nm using a 96-well microplate reader (Biorad, Hercules, CA)

2.3. Resorption pit assay

For the resorption pit assay, mouse BMM cells (3×10^4 cells per well) were seeded on dentine slices and treated with RANKL (20 ng/ml) and M-CSF (10 ng/ml) until multinucleated osteoclasts were formed. When the formation of osteoclasts had occurred, the cells were treated with or without luteolin (2, 10 μM) in the presence of M-CSF and RANKL for 48 h. After the incubation, attached cells were completely removed from dentine slices by abrasion with cotton tips, and resorption pits were identified by field emission scanning electron microscopy. The resorption pit areas were analyzed with a photo editing software (Paint.NET; http://www.getpaint.net/index.html).

2.4. Reverse transcriptase-polymerase chain reaction analysis

For reverse transcriptase-polymerase chain reaction (RT-PCR) assays, total RNA was prepared using TRI reagent according to the manufacturer's instructions. Two micrograms of total RNA from each sample was reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). *c*DNA (1ug) was amplified by PCR. The primers and PCR conditions for TRAP, cathepsin K, matrix metallopeptidase 9 (MMP-9), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [22], *c*-Src [23], nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) and dendritic cell-specific transmembrane protein (DC-STAMP) [24] were published previously. The PCR products were electrophoresed on a 1% agarose gel and the image was captured with a ChemiImager 5500 gel documentation system (Alpha Innotech, San Leandro, CA, USA).

2.5. Animals and treatments

Thirty two female C57BL/6 mice with 8 weeks of age were purchased from the Dae Han biolink (Seoul, Korea) and acclimated to conditions for 1 week before the experiment. The mice were housed in an air-conditioned room with a 12-h light/dark cycle at temperature of 22±2°C and humidity (45–65%) and given free access to food and tap water. The acclimatized mice were either sham-operated (SHAM, controls; $n=8$) or surgically ovariectomized (OVX; $n=24$). One week after surgery, the OVX mice were randomly divided into three groups with 8 per group: OVX control, low dose of luteolin (5 mg/kg), and high dose of luteolin (20 mg/kg) groups. The SHAM and OVX control mice served as vehicle treated control and orally administrated same volume of mixture of dimethyl sulfoxide (DMSO) and water (1:14). Luteolin was administrated at the dose of 5 and 20 mg/kg body weight for 30 days, respectively. At the end of treatment, the mice were sacrificed and blood sample was collected for serum isolation. Serum was separated by centrifugation (1500×*g*) and then stored at −80°C for future bone metabolic marker assay. Femora were removed, fixed with a 3.7 % formaldehyde in phosphate-buffered saline solution (pH 7.4) for 16 h and then stored (4°C) at 80% ethanol for bone mass measurement.

2.6. Serum markers of bone metabolism

Serum osteocalcin levels and ALP activity, the sensitive biomarkers of bone formation, were estimated by osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA, USA) and QuantiChrome ALP assay kit (DALP-250, BioAssay Systems, CA, USA) according to the manufacturers' instructions, respectively. The effects of the treatments on bone resorption were evaluated using a RatLaps ELISA kit (Nordic bioscience Diagnostics, Herlev, Denmark) to detect C-terminal telopeptide fragment of type I collagen C-terminus (CTX) generated by the osteoclasts [25].

2.7. Microcomputed tomography

Bone morphometric parameters and microarchitectual properties of femur were determined using microcomputed tomography (μCT) system (eXplore Locus SP, GE Healthcare). The scanning protocol was set at X-ray energy settings of 80 kV and 80 μA, and the sample was scanned over one entire 360° rotation, with an exposure time of 3000 ms/frame. An isotopic resolution of 8×8×8 μm voxel size that displayed the microstructure of the mouse femur was selected, and the angle of increment around the sample was set to 0.4°, which resulted in the acquisition of 900 2D images. A modified Feldkamp cone-beam algorithm was used to reconstruct the 2D projections into a 3D volume [26,27].

For the bone analysis, bone tissue, the region of 0.7–2.3-mm from the growth plate was selected as the region of interest and image information was obtained based on the automatic domain value yielded by the computer. The bone mineral density (BMD), bone mineral content (BMC), bone volume fraction (BVF), tissue mineral density (TMD), trabecular number (Tb.N.), trabecular separation (Tb.Sp.), cortical bone mineral density (Cr.BMD) and cortical bone mineral content (Cr.BMC) were applied to perform quantitative analysis using software provided with 2.0+ ABA Microview of the micro-CT system [26,27].

2.8. Femoral mechanical testing

Mechanical femoral resistance was determined by a three-point bending test. Each bone was positioned on the two lower supports of the anvil of a Universal Testing Machine (Instron 4202; Instron, Canton, MA, USA). Load was applied to the midshaft using a crosshead speed of 1.5 mm/min for all the tests. The load versus displacement data were recorded automatically by the Instron software (INSTRON series IX Automated Materials Tester, version 8.04.00), which subsequently calculates the following mechanical parameters from the load-displacement curves: ultimate force (Fu) and ultimate stiffness (S) [15].

2.9. Statistical analysis

A parametric one-way analysis of variance was used to test for any difference among the groups. Tukey's multiple comparison tests was used to confirm significant differences between group means. A *P* value of less than .05 was considered significant.

3. Results

3.1. Inhibitory effects of luteolin on osteoclast differentiation

To examine the effects of luteolin on osteoclast differentiation of mouse BMM cells, various concentrations of luteolin were applied

to mouse BMM cells undergoing osteoclast differentiation in response to a mixture of RANKL (20 ng/ml) and M-CSF (10 ng/ml). Characterization of osteoclast-like cells was monitored by the formation of multinucleated cells and by measuring the activity of TRAP, a marker enzyme of osteoclasts. Compared to the RANKL and M-CSF-treated positive control, the BMM cells treated with RANKL and M-CSF plus luteolin (1, 2, 5 and 10 μM) showed dramatic reduction of TRAP activity in a dose-dependent manner (Fig. 1A and B). In correlation with TRAP activity, the number of TRAP-positive MNCs was also reduced by luteolin (Fig. 1B). These results demonstrate that luteolin effectively suppresses both the number of TRAP-positive MNCs and the activity of TRAP. The inhibitory effect of luteolin on osteoclast differentiation was also observed in RAW 264.7 cells, an established murine macrophage cell line (data not shown). We observed that a high concentration of luteolin (up to 20 μM) did not cause cytotoxic responses in mouse BMM cells (Fig. 1C).

3.2. Luteolin inhibits RANKL-induced expression of genes that are associated with osteoclast differentiation

To ascertain the inhibitory effect of luteolin on osteoclast differentiation, the expression levels of genes associated with osteoclast differentiation were examined. Bone marrow-derived macrophages were cultured with M-CSF (10 ng/ml) and RANKL (20 ng/ml) in the presence of absence luteolin (10 μM). The mRNA expression levels of the indicated genes were determined by RT-PCR. The expression of osteoclast-associated genes including NFATc1, c-Src, DC-STAMP, MMP-9, cathepsin K and TRAP were induced by RANKL, but treatment of luteolin strongly suppressed the expression of those genes (Fig. 2).

3.3. Luteolin inhibits bone resorption in vitro

In the next experiment, the effect of luteolin on bone resorptive activity of osteoclasts was evaluated. Mature osteoclasts were incubated on a dentine slice with M-CSF and RANKL in the presence or absence of luteolin (2 and 10 μM) for 2 days. The bone fit resorption was stimulated by RANKL-induced mature osteoclasts, but luteolin was shown to inhibit the bone resorptive activity of mature osteoclasts in a dose-dependent manner (Fig. 3).

3.4. Luteolin inhibits OVX-induced bone loss

To examine the effect of luteolin on in vivo bone loss, OVX mice were administered with luteolin with two different doses. As shown in μCT images of distal femur metaphyses, vehicle-treated OVX mice had less trabecular bone structures than the sham controls (Fig. 4). OVX significantly reduced BMD by 23.5% compared with SHAM mice ($P<.05$). Administration of luteolin (5 mg/kg) caused a significant increase in BMC (+15.4%, $P<.05$) and BMD (+12.2%, $P<.05$) of trabecular bone in the femur, compared with OVX controls. Trabecular number was also significantly decreased by 18.6% in OVX mice compared with SHAM mice ($P<.05$), and trabecular space was increased by 23.5% ($P<.05$). Treatment with low and high dose of luteolin prevented the decreased trabecular number observed in OVX (Fig. 4). The other parameters of trabecular and cortical bone changes were also shown in Fig. 4. The data showed that the OVX mice had lower value in BMD, BMC, TMD, BVF, Tb.N. and higher value in Tb.Sp.,

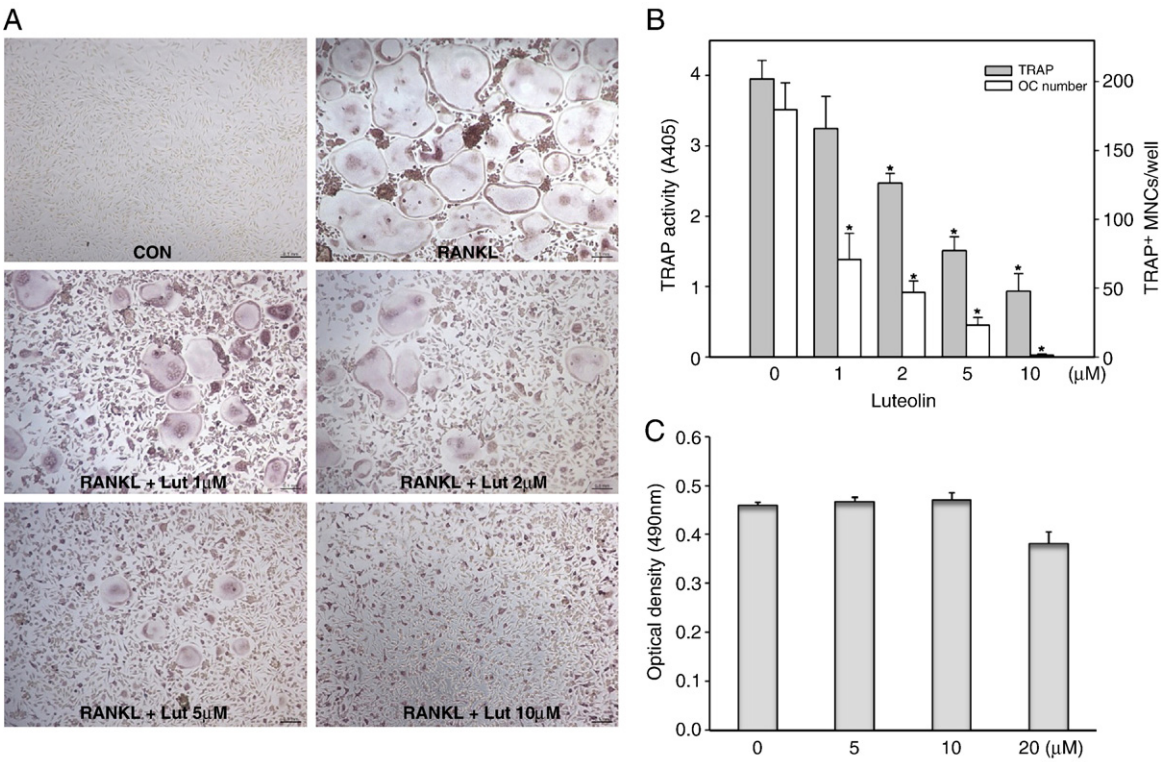

Fig. 1. Effect of luteolin on RANKL-induced osteoclast differentiation and cell viability. (A) Bone marrow-derived osteoclast precursor cells were cultured for 5 days in the presence of M-CSF, RANKL, and various concentration of luteolin. Cells were fixed and stained for TRAP. (B) TRAP activity was measured at 405 nm, and the TRAP+MNCs containing three or more nuclei were scored as osteoclasts. (C) Bone marrow derived osteoclast precursor cells were cultured in the presence of M-CSF, RANKL and luteolin for 2 days. Cell viability was measured by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit. Results are represented as means±S.D. of three independent experiments. *$P<.05$, significant differences from the control.

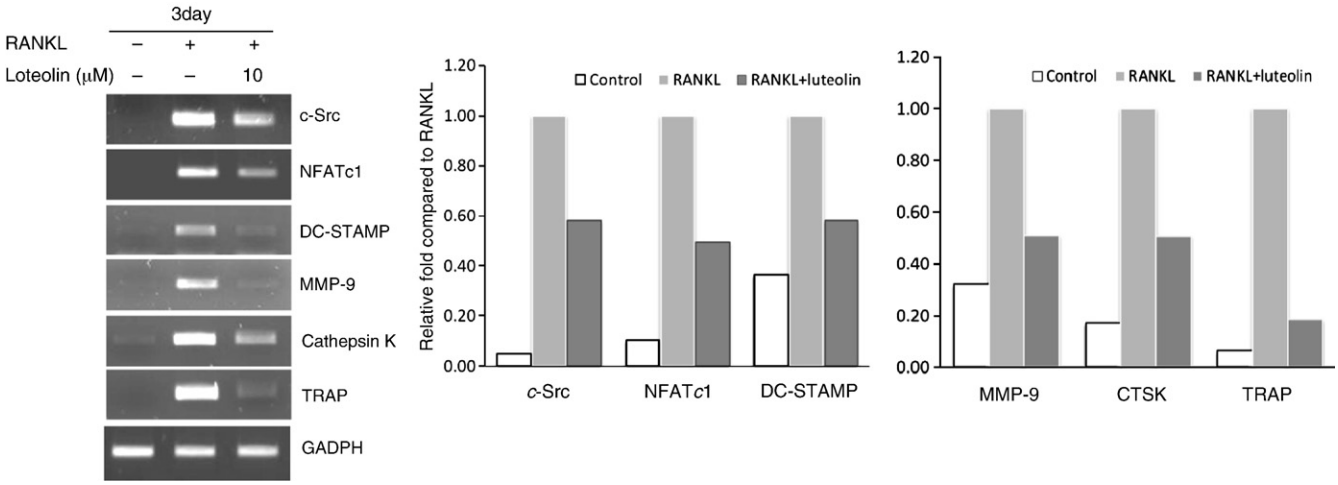

Fig. 2. The effects of luteolin on the expression of genes those are associated with osteoclast formation by RT-PCR. Total RNA was extracted from bone marrow cultures that were treated with or without RANKL in the presence or absence 10 μM luteolin for 3 days. mRNA expression was expressed as relative fold compared to RANKL group.

when compared to the SHAM mice. Administration of luteolin reversed all the bone parameters mentioned above. These results indicated that luteolin improved BMD and BMC of trabecular bone and decreased bone loss induced by ovariectomy.

3.5. Mechanical strength analyses

The length (mm) and the diameter (mm) of the femur were not different in OVX (15.78±0.22; 2.59±0.034), OVX+luteolin (15.85±0.08; 2.57±0.039) and SHAM mice (15.70±0.15; 2.54±0.030). However, the femoral three point bending test showed that the femora of OVX group have on average 17% low maximal load value compared to the femora of SHAM group ($P<.05$). Treatments with luteolin (20 mg/kg) could prevent the deterioration of bending strength; the group-treated luteolin was significantly higher in values of maximal load (21%) and strength (20%) than OVX control group (Fig. 5).

3.6. Serum bone markers

As compared with SHAM group, the OVX group showed on average 65% higher osteocalcin level and 48% higher CTX level ($P<.05$ for both). Compared with OVX group, OVX mice administered with 5

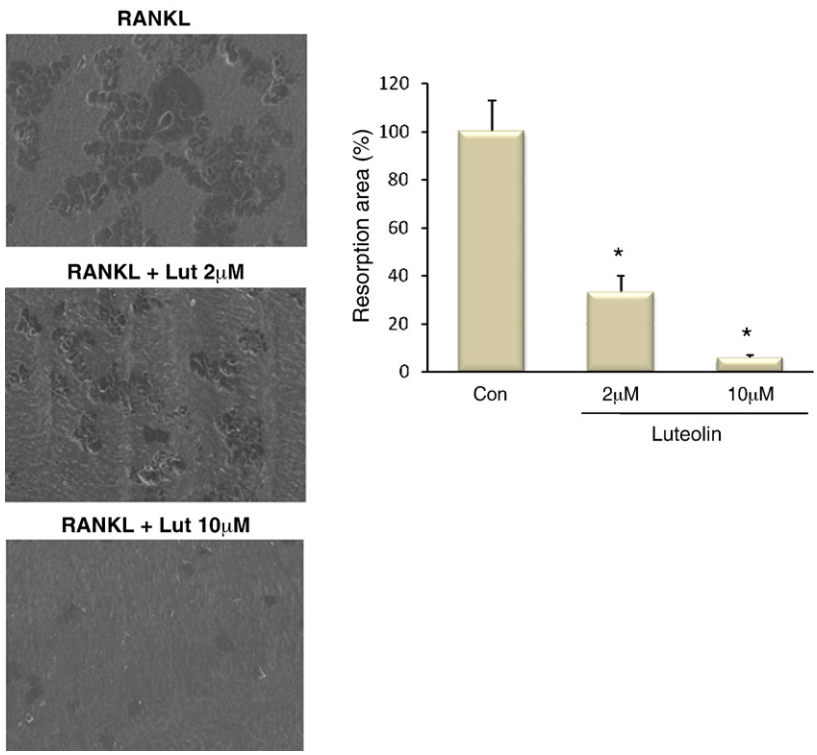

Fig. 3. Inhibition of bone resorption activity of osteoclast by luteolin. Mouse BMM cells were seeded on dentine slices and treated with RANKL (20 ng/ml) and M-CSF (10 ng/ml) to induce differentiation into osteoclasts. After osteoclasts had formed, the cells were treated with or without luteolin (2, 10 μM) in the presence of M-CSF and RANKL for 48 h. Resorption pit area was measured using image analysis program and data are presented as means±S.D. *$P<.05$ as compared with RANKL.

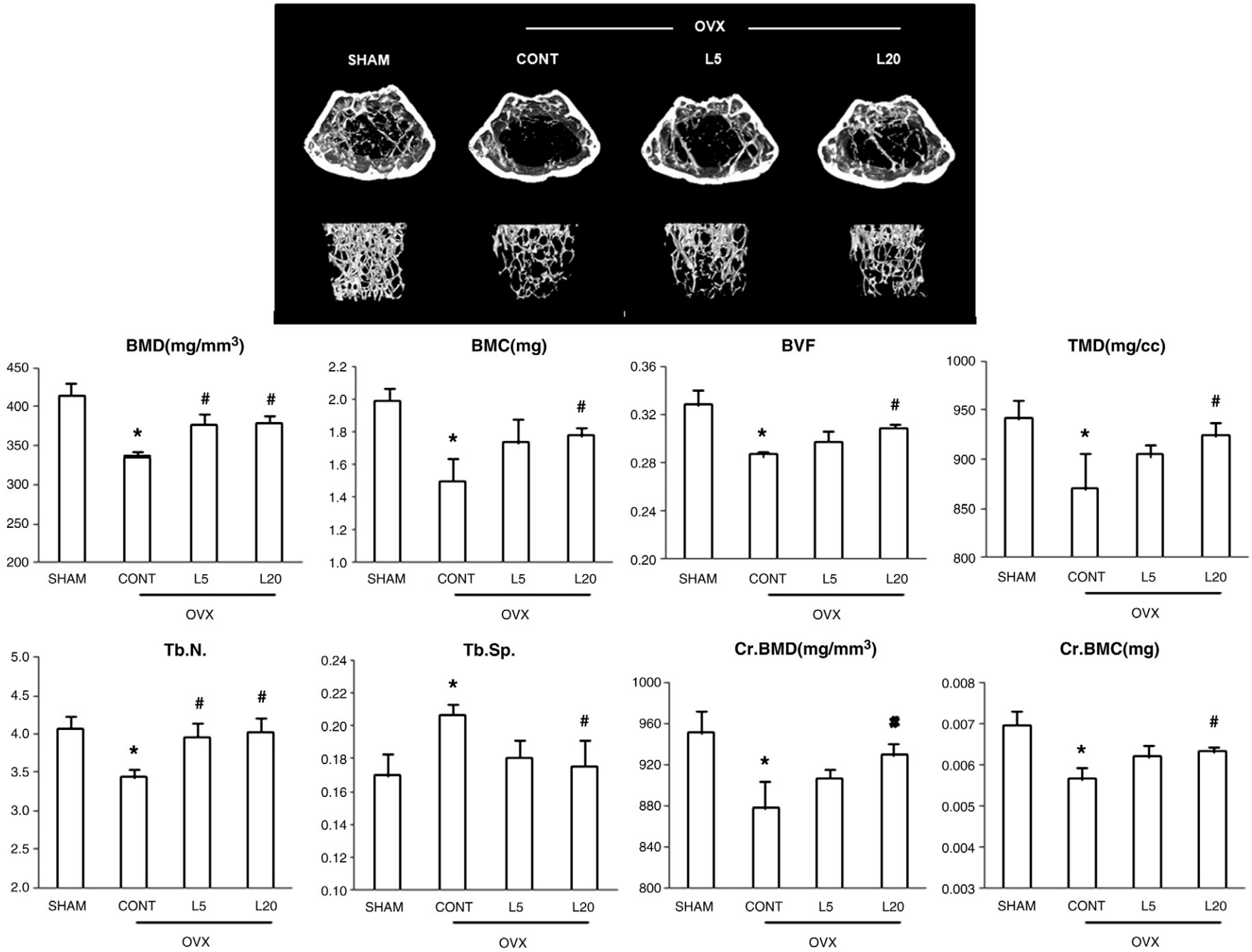

Fig. 4. Representative μCT images of the distal femurs in each group was shown. Histomorphometric analysis of distal femurs in mice treated with luteolin at doses 5 (L5) and 20 mg/kg (L20) for 30 days after OVX. BMD, BMC, BVF, TMD, Tb. N., Tb.Sp., Cr.BMD and Cr.BMC. *P<.05 vs. sham control; #P<.05 vs. OVX.

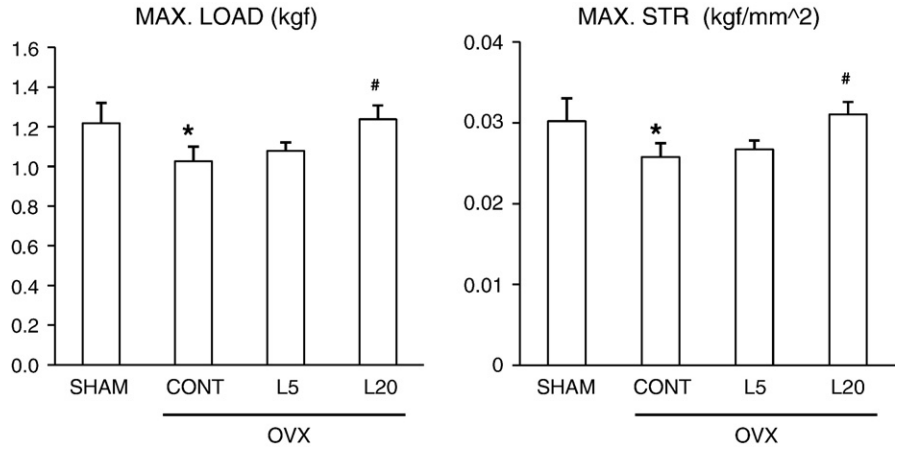

Fig. 5. Evaluations of midshaft femur biomechanical properties by using a three-point bending test. Loading force to maximal load (A), and maximal strength (B). Ovariectomy significantly decreased maximal load and strength as compared with SHAM. However, L20 group showed effects to counteract the deteriorations of these parameters. *P<.05 vs. sham control; #P<.05 vs. OVX.

mg/kg luteolin had a significantly lower osteocalcin level (33% vs. OVX) with almost complete return to SHAM osteocalcin level. In analysis of CTX, luteolin administrated groups showed 27% and 38% decrease in 5 mg and 20 mg/kg luteolin group, respectively (P<.05 for both) (Fig. 6).

4. Discussion

In this study, we characterized physiological effects of luteolin in osteoclast differentiation and function in vitro, and the effect on bone loss in OVX mice model. Our report shows that luteolin dramatically inhibits expression of TRAP as well as formation of multinucleated cells (Fig. 1). We observed that a high concentration of luteolin (up to 20 μM) did not show any cytotoxicity in mouse BMM cells (Fig. 1C). These results suggest that the inhibitory effect of luteolin on osteoclast differentiation may not be due to cytotoxicity and it has a selective effect on osteoclastogenesis. These findings are consistent with the previously published results which showed that luteolin inhibited osteoclast-like cells formation [20]. Differentiation of osteoclast precursors into osteoclasts consists of multiple steps, including cell adhesion, differentiation, fusion and activation. To find out at which stage of osteoclastogenesis luteolin would most affect TRAP activity, luteolin was added into the BMM cells already treated with RANKL with a time interval. We found that luteolin initially inhibited RANKL-induced TRAP activity. However, the treatment of luteolin 1 day after stimulation of RANKL did not significantly inhibit TRAP activity (data not shown). These results suggest that an early mechanism responsible for osteoclast differentiation may be the target of luteolin.

Ovariectomized animal model has been widely used to study preventive treatments for postmenopausal osteoporosis with estrogen insufficiency. In OVX animals, like in postmenopausal women, bone loss induced by ovarian deficiency mainly results from trabecular bone loss [28]. Although mechanical strength of trabecular bone partly depends on its microstructure, it is generally accepted that mechanical strength of bone depends on both bone mass and bone quality [29]. As expected, ovariectomy greatly reduced BMD in the distal femur resulting from increased bone turnover, as indicated by the higher plasma OC and CTX concentrations in the OVX group compared to the SHAM group (Figs. 4 and 6). These results are in agreement with those from Wronski et al. [30], which demonstrated that bone remodeling is accelerated after the cessation of ovarian function. However, administration of luteolin caused a significant increase in BMD and BMC of trabecular bone, as well as cortical bone in the distal femur. The suppression of decrease in trabecular bone mass by luteolin possibly resulted from a luteolin-induced inhibition in trabecular bone resorption after estrogen deficiency, as indicated by higher plasma CTX in OVX than OVX + luteolin (Figs. 4 and 6). We also examined the Tb.N., connectivity, and Tb.Sp. of bone by micro-

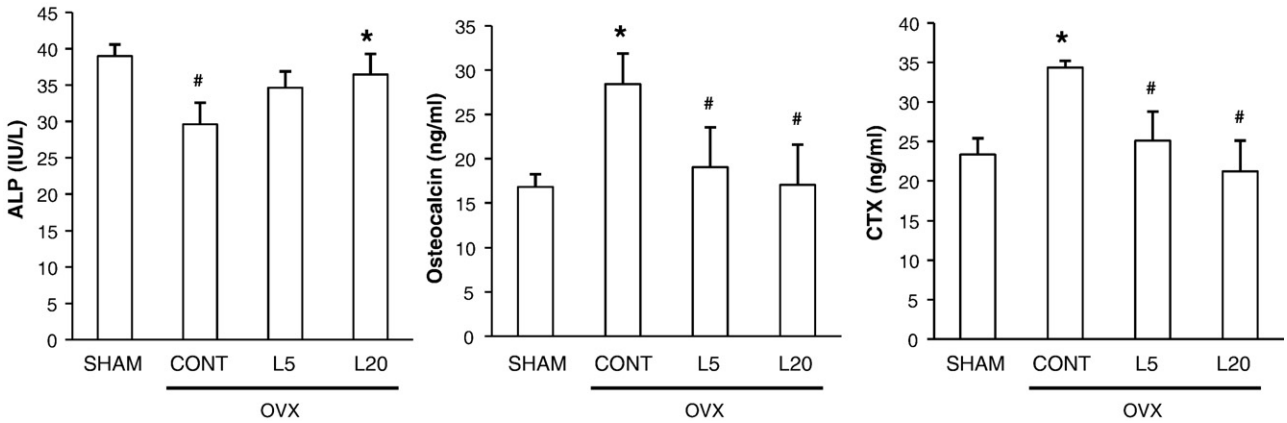

Fig. 6. Effect of luteolin on changes in biochemical markers of bone turnover in ovariectomized mice. The serum osteocalcin concentration (A) was used as markers of bone formation. The Type I collagen C-telopeptide concentration (B) was used as a marker of bone resorption. The values represent the means±S.D. *P<.05 vs. sham control; #P<.05 vs. OVX.

computed tomography. Luteolin significantly increased Tb.N. and decreased Tb.Sp. of distal femur. These results indicate that luteolin improved BMC and BMD of trabecular bone and decreased bone loss induced by ovariectomy.

The loss of bone mass and the deterioration of bone microstructure have been linked to an imbalance between bone formation and bone resorption [31]. Biochemical markers of bone turnover have been widely used to measure the status of bone remodeling [32]. Analyses of the level of serum osteocalcin, a marker for osteoblastic activity, and CTX, a marker for bone resorption showed that the concentrations of serum osteocalcin and CTX in OVX mice were significantly higher than those in SHAM group, indicating the increase of bone turnover rate. However, both OC and CTX in OVX mice were dose-dependently restored to the levels in SHAM mice in response to luteolin (Fig. 6). These results demonstrate that luteolin prevents bone loss which probably resulted from a decreased bone turnover.

A hypothesis for bone loss in osteoporosis is that the estrogen deficiency induces an unregulated chronic inflammatory process by increasing the local production of various osteoclastogenic cytokines and growth factors by cells located within bone microenvironment [33]. One of the cytokines responsible for the enhanced osteoclastogenesis in states of estrogen deficiency is TNF-α, a factor which induces osteoclast formation by up-regulating stromal cell production of RANKL and M-CSF, and by augmenting the responsiveness of osteoclast precursors to RANKL [34]. In addition, TNF-α also directly augmented the differentiation of committed osteoclast precursors and led to the activation of mature osteoclast to resorb bone [35]. Concomitant with inflammation there was generation of reactive oxygen species (ROS) or free radicals. Oxidative stress has been also suggested to participate in the development of osteoporosis. Some studies have suggested that oxidative stress decreases bone formation by modulating the differentiation and survival of osteoblasts [36] and stimulating bone resorption [37]. Therefore, the possible involvement of inflammatory cytokines and ROS in osteoclast differentiation and bone resorption has lead to the use of anti-oxidant and/or anti-inflammatory compounds to protect bone loss.

Luteolin exerts numerous effects in vivo and vitro, including inflammatory responses in cells stimulated with proinflammatory factors. These include suppression of pro-inflammatory cytokine expression (TNF-α, IL-6 and IL-1β), inhibition of the nuclear factor κB pathway, scavenging of ROS, inhibition of ROS production and activation of antioxidant enzymes [38]. Various flavonoids inhibited LPS-induced TNF-α production from macrophages in vitro, but only luteolin showed an inhibition of serum TNF-α production in vivo when administered orally [39]. In addition, Luteolin exhibits obvious antioxidative effect and has been shown to be able to scavenge ROS [40]. Therefore, the inhibitory effect of luteolin on bone resorption may be associated with its anti-inflammatory and antioxidative activity. That is, the inhibition of inflammatory cytokine and/or ROS production may lead to the inhibition of osteoclast differentiation.

In summary, the present study demonstrated that luteolin inhibits the osteoclastogenesis from bone marrow macrophages. Luteolin also reduced resorption activity of mature osteoclasts. In addition, it prevented the decrease of bone mass induced by OVX, especially in trabecular bone, and strengthened the bone strengths by suppression of bone turnover. Therefore, luteolin may be a beneficial natural compound for reducing the osteoclast formation and preventing the bone loss induced by estrogen deficiency. Additional in vivo studies would be needed to provide further evidence that luteolin is a promising alternative to current therapeutic agents for the management of postmenopausal bone loss. Also, further studies would be carried out to identify the mechanisms that mediate the action of luteolin.

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