

Dietary quercetin inhibits bone loss without effect on the uterus in ovariectomized mice

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Abstract Quercetin is a major dietary flavonoid found in onions and other vegetables, and potentially has beneficial effects on disease prevention. In the present study, we demonstrate for the first time the effects of dietary quercetin on bone loss and uterine weight loss by ovariectomy in vivo. Female mice were ovariectomized (OVX) and were randomly allocated to 3 groups: a control diet or a diet with 0.25% (LQ) or 2.5% quercetin (HQ). After 4 weeks, dietary quercetin had no effects on uterine weight in OVX mice, but bone mineral density of the lumbar spine L4 and femur measured by peripheral quantitative computed tomography (pQCT) was higher in both the sham and the HQ groups than in the OVX group. Histomorphometric analysis showed that the HQ group restored bone volume (BV/TV) completely in distal femoral cancellous bone, but did not reduce the osteoclast surface area and osteoclast number when compared with the OVX group. In in-vitro experiments using mouse monocyte/macrophage cell line RAW264.7 cells,

however, quercetin and its conjugate, quercetin-3-*O*-beta-D-glucuronide dose-dependently inhibited the receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoclast differentiation, and the RANKL-stimulated expression of osteoclast related genes was also inhibited by quercetin. The luciferase reporter assay showed that quercetin did not appear to have estrogenic activity through estrogen receptors. These results suggest that dietary quercetin inhibits bone loss without effect on the uterus in OVX mice and does not act as a potent inhibitor of osteoclastogenesis or as a selective estrogen receptor modulator in vivo.

Keywords Quercetin · Bone · Ovariectomized mice · Osteoclast · Estrogen receptors

Introduction

Preventing bone diseases such as osteoporosis is a critical issue in today's aging society. Osteoporosis is the most common bone disease, characterized by reduced bone mineral density (BMD) and bone quality, and increased risk of fracture. In particular, osteoporosis is a critical disorder in postmenopausal women and involves high rates of bone turnover and bone loss due to estrogen deficiency [1]. It has been reported that ovariectomized (OVX) rodents have a rapid reduction of trabecular bone volume and an enhancement of osteoclastogenesis, whereas treatment with estradiol reverses these changes [2, 3]. Indeed, hormone replacement therapy (HRT) can prevent bone loss caused by menopause [4–8], but it is associated with some adverse effects, such as uterine bleeding (in women with an intact uterus) and breast cancer [9–11].

It has been shown that dietary flavonoids could prevent osteoporosis in humans and rats [12, 13]. Genistein or

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daidzein, called isoflavones, function as phytoestrogens through estrogen receptors (ERs). In OVX mice, a high intake of genistein inhibits a reduction in uterine weight as well as bone loss [14]. Soymilk consumption has been shown to decrease serum estrogen concentrations in premenopausal Japanese women [15]. Genistein supplements have positive effects on BMD in osteopenic postmenopausal women, although these supplements are associated with gastrointestinal side effects [16]. Quercetin (3,3',4',5,7-pentahydroxyflavone) one of the abundant flavonol-type flavonoids found in onions and other vegetables, has been reported to possess various pharmacological properties, including acting as an antioxidant and inhibiting carcinogenesis and allergies [17–21]. Interestingly, it has been suggested that onion bulbs (*Allium cepa* L.) may be associated with increases in BMD [22]. Horcajada-Molteni et al. [23] previously reported that rutin inhibits trabecular bone loss, but has no effects on uterine weight in OVX rats. Rassi et al. [24] and Woo et al. [25] reported that quercetin inhibited osteoclast formation in porcine and mouse bone marrow cells and monocytic RAW264 cells. However, the action of dietary quercetin on bone loss and uterine weight loss in OVX animal models has not yet been reported. In addition, previous reports have clearly shown that quercetin-3-glucuronide (Q3GA) is the major quercetin conjugate in rat and human plasma [26, 27], but the effects of Q3GA on osteoclastogenesis is still not clear.

In this study, we demonstrate for the first time the effects of quercetin on bone loss and uterine weight loss by ovariectomy in vivo, and examine the effects of quercetin and its metabolites on the receptor activator of nuclear factor kappa B (NF- κ B) ligand (RANKL)-induced osteoclast differentiation in RAW264.7 cells. Moreover, some reports have suggested an estrogenic effect of quercetin on in vitro osteoclastic bone resorption [24, 28]; we therefore investigate whether quercetin has the same estrogenic action as phytoestrogens including genistein and daidzein using a luciferase reporter assay.

Materials and methods

Animals and diets

Twenty 9-week-old virgin female C57BL/6J mice had their ovaries surgically removed; 6 other mice underwent a sham operation (Sham group). OVX mice were randomly assigned to 3 diet groups ($n = 6$ – 7 per group). The groups were: OVX, OVX + LQ (0.25% quercetin diet), OVX + HQ (2.5% quercetin diet). Mice were housed in standard clean environmental conditions with a 12-h light/dark cycle during the 4-week intervention period. We used ad libitum feeding, and all mice had free access to distilled

water throughout the study. All diets were modified from the AIN-93G powdered diet (Oriental Koubo, Tokyo, Japan). The control diet for the sham and OVX groups contained 0.6% calcium and phosphorus. Purified quercetin-dihydrate (Sigma, St. Louis, MO, USA) was added to the appropriate diets so they contained 0.25 or 2.5% quercetin. The diet for each group was freshly prepared every day and was given at a mean amount of 5 g per mouse. The Institutional Animal Care and Oversight Committee approved the experimental protocols for the study, which was carried out according to the guidelines and principles for the care and use of animals at the University of Tokushima.

Body weight, uterine weight, urine and blood samples

Body weight in all mice was measured on day 0 and day 28. Urine samples were collected on day 0 and 7 to measure urine deoxypyridinoline as a marker of bone resorption, using a urine deoxypyridinoline EIA kit (DS Pharma Bio Medical, Osaka, Japan). After the 4-week-treatments were completed, the mice were sacrificed under anesthesia. Then, blood samples were collected via inferior vena cava, and were centrifuged at 3000 rpm for 15 min and plasma supernatants collected. Plasma calcium and phosphate were measured by colorimetric methods using assay kits (Calcium E Test Kit and Phosphor C Test Kit, Wako Pure Chemical Industries, Osaka, Japan). At necropsy, uteri were collected and their weight measured to verify that their ovaries were removed successfully.

High performance liquid chromatography (HPLC) analysis

The concentration of total quercetin in plasma was measured by electrochemical detection using an HPLC analysis. This analysis was performed using the CoulArray system (ESA Biosciences, Inc., Chelmsford, MA, USA).

Measurement of bone mineral density

The lumbar spine and right femur were removed and fixed with 70% ethanol for 1 week. Then, the L4 lumbar spine and right femur were scanned by peripheral quantitative computed tomography (pQCT) (XCT Research SA+, Stratec Medizintechnik GmbH, Pforzheim, Germany) to determine BMD. A single tomographic slice of 0.46 mm transsectional thickness was taken at a voxel size of 0.08 mm. Total, trabecular, and cortical BMDs were analyzed as well as strength strain index (SSI), which is an indicator of whole bone strength [29–31]; we also calculated the rectangular and polar versions. Lumbar BMD values of less than 464 and more than 690 mg/cm³ were regarded as trabecular and

cortical BMD, respectively. Femur BMD values of less than 395 and more than 690 mg/cm³ were regarded as trabecular and cortical BMD, respectively.

Bone histomorphometry

After the pQCT analysis, the right femur was embedded in glycolmethacrylate (GMA) without decalcification. Then serial sections (3 µm thick) were cut longitudinally using a microtome (model 2050; Reichert Jung, Buffalo, NY, USA), and sections were further stained with the Villanueva Goldner stain for discrimination between mineralized and unmineralized bone and also for identification of cellular components. Histomorphometric analysis of bone sections were performed using a semiautomated system (Osteoplan II; Carl Zeiss, Thornwood, NY, USA), and measurements were made at 400× magnification. Parameters for bone structure included bone volume per tissue volume (BV/TV, %), trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, /mm), and trabecular separation (Tb.Sp, µm). Parameters for bone formation included osteoid volume per bone volume (OV/BV, %), osteoid surface per bone surface (OS/BS, %), osteoid thickness (O.Th, µm), osteoblast surface per bone surface (Ob.S/BS, %). Parameters for bone resorption included eroded surface per bone surface (ES/BS, %), number of osteoclasts per bone perimeter (N.Oc/B.Pm, per 100 mm) and osteoclast surface per bone surface (Oc.S/BS, %). Nomenclature, symbols, and units used in this study are those recommended by the American Society for Bone Mineral Research (ASBMR) Nomenclature Committee [32].

Cell culture

RAW264.7, NIH3T3, MCF7 and HeLa cells were obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured at 37°C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Osteoclast formation assay

Osteoclast formation assay was performed as previously described [33]. RAW264.7 cells (4×10^3 cells/well) were cultured in 48-well plates in phenol red-free minimum essential medium alpha medium (α -MEM; Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% charcoal-stripped fetal bovine serum (CSS). Cells were treated with RANKL (300 ng/ml), quercetin (1, 5, 10 µmol/L), quercetin-3-*O*-beta-D-glucuronide (Q3GA; 1, 5, 10 µmol/L) and rutin (1, 5, 10 µmol/L). The medium and mediators were refreshed every 3 days. After 4 or 5 days of culture,

the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity. Multinucleated (>3 nuclei) TRAP-positive cells were counted as osteoclasts under microscopic examination.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using TRIzol (Invitrogen Life Technologies) as specified by the manufacturer's manual and cDNA was synthesized from 2.5 µg of total RNA as previously described [34]. PCR reactions were performed using Go taq green master mix (Promega), 10 pmol of each primer, 2 µl samples, and H₂O to a final volume of 20 µl. Amplification was carried out in three steps, denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The numbers of amplification cycles were 35 cycles for calcitonin receptor (CTR), 30 cycles for matrix metalloproteinase 9 (MMP-9) and 25 cycles for cathepsin K (Cath K) and β -actin. PCR products were separated on 1.5% agarose gel and stained with ethidium bromide, and the bands were visualized under UV illumination. The primer sequences used for PCR were previously described [35].

Western blot analysis

Total protein was prepared from RAW264.7 cells as previously described [35]. Proteins were electrophoresed on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking in 5% non-fat dried milk in phosphate-buffered saline plus 0.05% Tween 20 (PBS-t) at room temperature for 1 h, membranes were incubated with anti-nuclear factor of activated T cells c1 (NFATc1) antibody (sc-7294, Santa Cruz, CA, USA) in 1% non-fat dried milk in PBS-t. The membranes were washed in PBS-t and incubated with Horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody at room temperature for 1 h. After washing in PBS-t, the membranes were developed using an ECL plus western blotting system (GE Healthcare, Buckinghamshire, UK).

Luciferase reporter assay

NIH3T3 cells (8×10^4 cells/well), MCF7 cells (1×10^5 cells/well) and HeLa cells (2×10^5 cells/well) were cultured in 6-well plates were transfected with 200 ng of pM-ER α or pM-ER β , 200 ng of VP16-SRC1a, 200 ng of pCMV- β (Stratagene, La Jolla, CA, USA), and 500 ng of pG5-Luc (Promega, Madison, WI, USA) vector, an expression vector of firefly luciferase, using the Lipofection method. After 4 h, the cells were treated with ligands as follows: 10^{-9} – 10^{-5} mol/L of 17 β -estradiol, quercetin,

genistein, and daidzein (Wako). After 24 h, cells were harvested in a lysis buffer supplied with the Pica-gene luciferase assay kit (Toyo Ink, Tokyo, Japan) and the lysates were assayed for luciferase and β -galactosidase (β -gal) activity. The relative amount of each experimental activity was then normalized to the activity of β -gal [36].

Statistical analysis

Statistical analyses were performed using Excel Toukei 2006 (SSRI, Tokyo, Japan). All data were reported as mean \pm SEM, and were subjected to one-way analysis of variance with Tukey–Kramer's multiple comparison post hoc test. A *P* value of <0.05 was considered significant.

Results

Effects of dietary quercetin on body and uterine weights, plasma calcium, phosphate and total quercetin levels in OVX mice

We investigated the effects of dietary quercetin in OVX mice as our osteoporosis model animal. One week after ovariectomy, urine deoxyypyridinoline, a marker of bone resorption, was significantly increased in OVX mice (43.0 ± 1.9 nmol/mmol creatinine) compared with sham mice (35.2 ± 1.8 nmol/mmol creatinine, $P = 0.0189$, followed by Student's *t* test). Plasma concentrations of total quercetin estimated by HPLC electrochemical detection in the LQ and HQ groups were 2.5 and 7 μ mol/L, respectively. No differences in body weight, plasma calcium and phosphate concentrations were observed between groups. Although uterine weight was significantly decreased by approximately 80% in OVX mice compared with sham mice, dietary quercetin did not affect uterine weight in OVX mice (Table 1).

Effects of dietary quercetin on bone loss in OVX mice

Results of BMD and bone parameters in lumbar and femur, estimated by pQCT, are shown in Tables 2 and 3, respectively. The OVX group showed a significant decrease in lumbar total BMD compared with the sham group, whereas dietary quercetin significantly inhibited bone loss due to ovariectomy in the HQ group, but not in the LQ group. Lumbar cortical BMD did not differ between the sham and OVX groups, and cortical BMD in the HQ group was the same as in the sham group. Similarly, ovariectomy induced a significant decrease in femur BMD. Femur total BMD was significantly higher in the HQ group than in the OVX group (Table 2). OVX mice showed a significant decrease in femoral bone parameters compared with sham mice, whereas cortical parameters and section modulus were significantly higher in the HQ group than in the OVX group. Strength strain index (SSI) has been used as an indicator of whole bone strength [29–31]. Table 3 showed that ovariectomy induced a significant decrease in femur SSI. Although dietary quercetin did not significantly inhibit this decrease, femur SSI in the HQ group was slightly higher than that in the OVX or LQ groups. Moreover, bone histomorphometric analysis ($n = 3$ in each group) was demonstrated. As shown in Table 4, the bone volume (BV/TV) in the distal femoral cancellous bone was lower in the OVX than that in the sham group, and it was restored completely in the HQ group. The trabecular number and thickness (Tb.Th, Tb.N) were decreased, whereas the trabecular spacing (Tb.Sp) was increased in the OVX group compared with that in the sham and HQ groups. Similarly, OV/BV and OS/BS as parameters of bone formation were decreased in the OVX group, compared with that in the sham and HQ groups. In contrast, ES/BS, N.Oc/B.Pm and Oc.S/BS, which are parameters of bone resorption, were increased in the OVX group, but dietary quercetin did not affect these parameters.

Table 1 Effects of dietary quercetin on body weights and uterine weights, plasma calcium, phosphate and total quercetin levels in OVX mice

	Sham	Ovariectomized		
	Normal diet	Normal diet (OVX)	0.25% Q diet (OVX + LQ)	2.5% Q diet (OVX + HQ)
Initial B.W. (g)	18.22 \pm 0.64	17.88 \pm 0.54	18.33 \pm 0.55	17.97 \pm 0.58
Final B.W. (g)	19.81 \pm 0.99	21.40 \pm 1.71	20.62 \pm 1.26	20.70 \pm 1.08
Uterine weight (mg)	98.3 \pm 12.2 ^a	21.2 \pm 3.9 ^b	22.3 \pm 2.7 ^b	26.9 \pm 1.6 ^b
Plasma calcium (mmol/L)	1.69 \pm 0.14	1.72 \pm 0.17	1.84 \pm 0.08	2.02 \pm 0.12
Plasma phosphate (mmol/L)	2.00 \pm 0.13	2.11 \pm 0.10	2.15 \pm 0.11	1.92 \pm 0.13
Plasma total quercetin (μ mol/L)	N.D.	N.D.	2.59 \pm 0.45*	6.99 \pm 0.64*

Each group contained 6–7 mice. Values represent mean \pm SEM. ^{a,b}Mean \pm SEM values within a group not sharing a common superscript are significantly different ($P < 0.05$)

B.W. Body weight, N.D. none detected

* Significant at $P < 0.001$, followed by Student's *t* test

Table 2 Effects of dietary quercetin on bone mineral density (BMD) in OVX mice

	Sham	Ovariectomized		
	Normal diet	Normal diet (OVX)	0.25% Q diet (OVX + LQ)	2.5% Q diet (OVX + HQ)
Lumbar BMD (mg/cm³)				
Total	386.63 ± 3.07 ^a	343.87 ± 14.82 ^b	348.66 ± 18.25 ^{ab}	394.67 ± 10.42 ^a
Trabecular	255.25 ± 4.37 ^{ab}	214.65 ± 12.73 ^a	242.96 ± 22.16 ^{ab}	277.33 ± 13.35 ^a
Cortical	563.28 ± 4.02 ^a	546.73 ± 6.71 ^{ab}	536.70 ± 5.22 ^b	558.24 ± 5.71 ^a
Femur BMD (mg/cm³)				
Total	359.38 ± 3.63 ^a	274.76 ± 6.03 ^c	285.31 ± 3.25 ^c	307.00 ± 4.78 ^a
Trabecular	140.72 ± 4.38 ^a	115.06 ± 6.74 ^b	115.80 ± 5.12 ^b	120.09 ± 6.10 ^b
Cortical	872.05 ± 6.30 ^a	824.82 ± 6.43 ^b	817.03 ± 9.86 ^b	833.94 ± 6.05 ^b

These parameters were measured and calculated by peripheral quantitative computed tomography (pQCT), according to the methods described in “Materials and methods”. Each group contained 6–7 mice. Values represent mean ± SEM. ^{a-c}Mean ± SEM values within a group not sharing a common superscript are significantly different (*P* < 0.05)

Table 3 Effect of dietary quercetin on femoral bone parameters in OVX mice

	Sham	Ovariectomized		
	Normal diet	Normal diet (OVX)	0.25% Q diet (OVX + LQ)	2.5% Q diet (OVX + HQ)
Cortical area (mm ²)	0.667 ± 0.015 ^a	0.388 ± 0.015 ^c	0.373 ± 0.017 ^c	0.509 ± 0.024 ^b
Cortical thickness (mm)	0.115 ± 0.002 ^a	0.067 ± 0.002 ^c	0.063 ± 0.003 ^c	0.085 ± 0.004 ^b
Section modulus, rectangular (cm ³)	0.198 ± 0.009 ^a	0.098 ± 0.009 ^c	0.093 ± 0.008 ^c	0.145 ± 0.010 ^b
Section modulus, polar (cm ³)	0.428 ± 0.020 ^a	0.222 ± 0.012 ^c	0.207 ± 0.013 ^c	0.300 ± 0.015 ^b
SSI, rectangular (mm ³)	0.253 ± 0.010 ^a	0.189 ± 0.006 ^{bc}	0.174 ± 0.009 ^b	0.206 ± 0.006 ^c
SSI, polar (mm ³)	0.509 ± 0.017 ^a	0.350 ± 0.009 ^{bc}	0.335 ± 0.013 ^b	0.397 ± 0.009 ^c

These parameters were measured and calculated by peripheral quantitative computed tomography (pQCT), according to the methods described in “Materials and methods”. Each group contained 6–7 mice. Values represent mean ± SEM. ^{a-c}Mean ± SEM values within a group not sharing a common superscript are significantly different (*P* < 0.05)

SSI strength strain index

Table 4 Parameters of trabecular structure and results of bone histomorphometry

	Sham	Ovariectomized	
	Normal diet	Normal diet (OVX)	2.5% Q diet (OVX + HQ)
BV/TV (%)	6.383 ± 0.979	4.242 ± 0.500	6.158 ± 0.905
Tb.Th (µm)	26.444 ± 2.038	25.913 ± 1.475	27.729 ± 1.107
Tb.N (/mm)	2.387 ± 0.175	1.627 ± 0.103	2.230 ± 0.333
Tb.Sp (µm)	396.714 ± 31.259	593.298 ± 38.429	442.553 ± 73.125
OV/BV (%)	4.118 ± 1.002	1.315 ± 0.079	2.274 ± 0.380
OS/BS (%)	19.741 ± 3.878 ^a	7.100 ± 0.743 ^b	12.127 ± 2.535 ^{ab}
O.Th (µm)	2.667 ± 0.147	2.439 ± 0.264	2.650 ± 0.065
Ob.S/BS (%)	10.045 ± 2.207	10.021 ± 0.705	9.882 ± 1.574
ES/BS (%)	6.155 ± 0.147	9.641 ± 1.243	8.790 ± 1.599
N.Oc/B.Pm (/100 mm)	103.025 ± 2.275	171.884 ± 48.161	172.802 ± 12.406
Oc.S/BS (%)	1.409 ± 0.270	1.903 ± 0.329	1.945 ± 0.369

Histomorphometric parameters on trabecular bone structure of cancellous area and bone resorption were measured and calculated according to the methods described in “Materials and methods”. Each group contained 3 mice. Values represent mean ± SEM. ^{a,b}Mean ± SEM values within a group not sharing a common superscript are significantly different (*P* < 0.05). *BV/TV* bone volume per tissue volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number, *Tb.Sp* trabecular spacing, *OV/BV* osteoid volume per bone volume, *OS/BS* osteoid surface per bone surface, *O.Th* osteoid thickness, *Ob.S/BS* osteoblast surface per bone surface, *ES/BS* eroded surface per bone surface, *N.Oc/B.Pm* osteoclast number per bone perimeter, *Oc.S/BS* osteoclast surface per bone surface

Inhibitory effects of quercetin, quercetin-3-*O*-beta-D-glucuronide (Q3GA) and rutin on the RANKL-induced osteoclast formation in RAW264.7 cells

The inhibitory effect of quercetin on osteoclast formation *in vitro* [24, 25] has also been reported, but the effect of a major quercetin conjugate, quercetin-3-glucuronide (Q3GA), on osteoclastogenesis is still not clear. The effects of quercetin, Q3GA and rutin on RANKL-induced osteoclast formation were tested using mouse monocyte/macrophage cell line RAW264.7. Treatments of quercetin and Q3GA significantly decreased the number of TRAP-positive multinucleated cells in a dose-dependent manner (Fig. 1A, B). However, rutin had no effect on osteoclast formation (Fig. 1C).

Quercetin inhibits the RANKL-induced gene expression of osteoclast related genes in RAW264.7 cells

We next analyzed the effects of quercetin on the mRNA expression of osteoclast related genes such as calcitonin receptor (CTR), cathepsin K (Cath K) and matrix metalloproteinase 9 (MMP-9) in RAW264.7 cells. RT-PCR

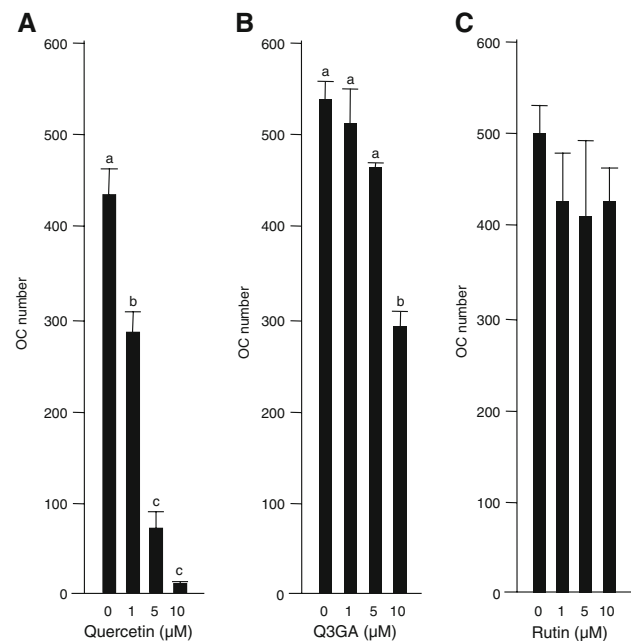


Fig. 1 Inhibitory effects of quercetin, quercetin-3-*O*-beta-D-glucuronide (Q3GA) and rutin on the RANKL-induced osteoclast formation in RAW264.7 cells. RAW264.7 cells (4×10^3 cells) were cultured for 5 days with RANKL (300 ng/ml) and each concentration of **A** quercetin, **B** Q3GA and **C** rutin. Multinucleated (>3 nuclei) TRAP-positive cells were counted as osteoclasts under microscopic examination. Similar results were obtained from at least three independent experiments. Values represent mean \pm SEM ($n = 3$). ^{a-c}Mean \pm SEM values within a group not sharing a common superscript are significantly different ($P < 0.05$)

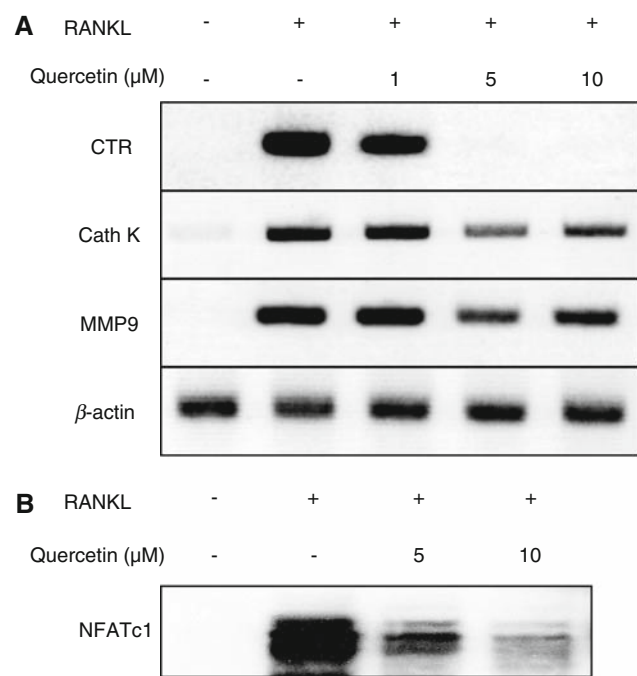


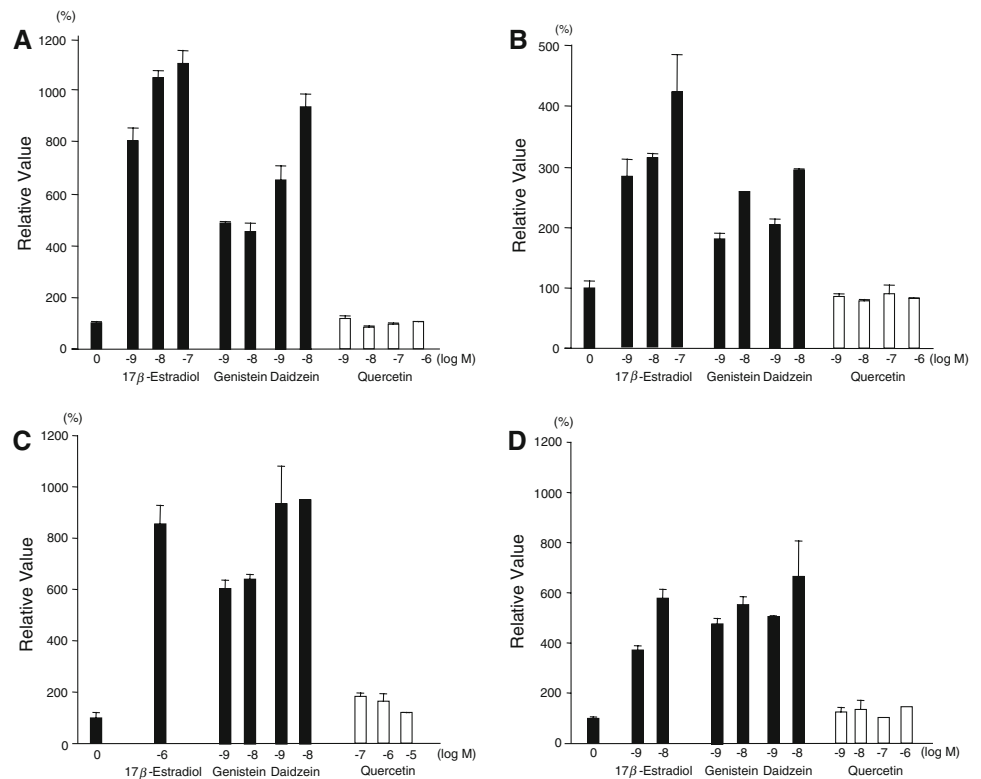
Fig. 2 Quercetin inhibits the RANKL-induced gene expression of osteoclast related genes in RAW264.7 cells. RAW264.7 cells (4×10^3 cells) were stimulated with RANKL (300 ng/ml) and each concentration of quercetin for 5 days. Total RNA was extracted and synthesized to cDNA for PCR reaction. **a** PCR reactions were performed using specific primer sets for calcitonin receptor (CTR), cathepsin K (Cath K), matrix metalloproteinase 9 (MMP-9). **b** Whole cell protein extracts were collected and 20 μ g of protein were separated by 12% SDS-PAGE and analyzed by western blot analysis with anti-NFATc1 antibody

analysis indicated quercetin markedly suppressed the mRNA expression of CTR, Cath K and MMP-9 induced by RANKL (Fig. 2a). Nuclear factor of activated T cells c1 (NFATc1) has been reported as a master regulatory transcription factor of osteoclast differentiation and NFATc1 gene expression is regulated through the AP-1 and NF- κ B activation by RANKL [39]. Therefore, we performed western blot analysis to see the effects of quercetin on NFATc1 expression. Figure 2b indicates that quercetin dose-dependently suppressed the RANKL-induced NFATc1 expression in RAW264.7 cells.

Estrogenic activity of flavonoids using the luciferase reporter assay

To determine whether quercetin acts as a phytoestrogen, we compared the effects of quercetin on ERs with genistein and daidzein in several types cell lines using the luciferase reporter assay. As shown in Fig. 3a, b, in NIH3T3 cells, genistein and daidzein as well as 17β -estradiol elicited estrogenic activity through both ER α and ER β in a dose-dependent manner. However, quercetin did not appear to have estrogenic activity through either ER α or ER β at

Fig. 3 Estrogenic activity of flavonoids using luciferase reporter assay. **a, b** NIH3T3 cells, **c** MCF7 cells and **d** HeLa cells were transfected with **a, c** pM-ER α or **b, d** pM-ER β , VP16-SRC1a, pCMV- β , and 500 ng of pG5-Luc. Cells were treated with the indicated concentrations of 17 β -estradiol, quercetin, genistein and daidzein. Cell lysates were assayed for β -gal and luciferase activities 24 h later. Each data point represents the average of duplicate analysis normalized for β -gal activity. Similar results were obtained from at least three independent experiments



any concentration. Similar data were also obtained in MCF7 and HeLa cells (Fig. 3c, d).

Discussion

Quercetin is a major flavonol-type flavonoid commonly found in vegetables and fruits, and in especially high amounts in onions [17]. In the present study, we examined the effects of quercetin on bone loss in vivo, using OVX mice as a postmenopausal osteoporosis model. The pQCT analysis showed that bone mineral density of the lumbar spine L4 and femur was higher in both the sham and the HQ groups than in the OVX group (Table 2). Furthermore, histomorphometric analysis revealed that the HQ group completely restored the bone volume (BV/TV) in distal femoral cancellous bone, but surprisingly did not reduce the osteoclast surface area and osteoclast number when compared with the OVX group (Table 3). These data suggest that the intake of quercetin prevents bone loss in OVX mice mediated through the effects on bone formation rather than bone resorption.

It has been reported that quercetin decreased osteoclast differentiation via a mechanism involving NF- κ B and AP-1 induced by RANKL in vitro [37]. Indeed, we have shown that quercetin and its conjugate, Q3GA, but not rutin inhibited RANKL-induced osteoclast formation in a dose-dependent manner in RAW264.7 cells, and the

RANKL-stimulated expression of osteoclast related genes including NFATc1 was inhibited by quercetin (Figs. 1, 2). Although our in vivo data did not indicate the inhibitory effect on parameters of bone resorption in OVX mice, it has shown that quercetin and Q3GA are potent inhibitors of osteoclastogenesis in vitro. On the other hand, there have also been reports of stimulation of alkaline phosphatase activity by quercetin in MG63 human osteoblast-like cells, inhibition of the proliferation, differentiation and mineralization of osteoblasts by quercetin in rat calvarial osteoblast-like cells, acceleration of tumor necrosis factor- α -induced growth inhibition and apoptosis by quercetin in MC3T3-E1 mouse osteoblastic cells and the up-regulation of bone sialoprotein (BSP) gene promoter by quercetin and Q3GA in ROS17/2.8 rat osteoblast-like cells [41–44]. Thus, the action of quercetin has been observed in osteoblast cells, however, in vivo effects of quercetin on bone formation have not been reported. In this experiment, we raised a possibility that dietary quercetin induces bone formation, but further study will be needed to analyze the effects of dietary quercetin on bone formation using OVX mice. Our recent studies using a monoclonal antibody targeting Q3GA and HPLC analysis found the accumulation of quercetin and Q3GA in human atherosclerotic lesions and lipopolysaccharides treated RAW264.7 cells [40]. Furthermore, we have also reported that Q3GA inhibited c-jun NH2-terminal kinase (JNK) activation and AP-1 DNA binding activity [38]. Immunohistochemical

analysis, immunoprecipitation and microarray analysis of bone sections of OVX mice using an anti-Q3GA antibody will be able to find the accumulation of quercetin and Q3GA in bone, especially osteoclast and osteoblast cells, and identify the target molecules of quercetin.

Our present study has clearly showed dietary quercetin prevented bone loss without affecting uterine weight in OVX mice (Tables 1, 2). Classically, soy and red clover derived isoflavones such as genistein and daidzein are advertised as selective estrogen receptor modulators (SERMs) with only desired and no undesired estrogenic effects [45]. It has been reported that quercetin has estrogenic activity through the binding to ER α or ER β as well as genistein and daidzein in vitro [46]. The luciferase reporter analysis in several types cell lines showed that genistein and daidzein elicited estrogenic activity in a dose-dependent manner but quercetin did not appear to have its activity through either ER α or ER β (Fig. 3). Thus, it is suggested that quercetin might affect bone metabolism through ERs independent pathway.

In conclusion, we showed for the first time that dietary quercetin inhibits bone loss without effect on the uterus in OVX mice and raised the possibility that quercetin may affect bone formation, but may not act as a potent inhibitor of osteoclastogenesis and a SERM in vivo. These results might be associated with the beneficial effect of quercetin on osteoporosis in humans, such as postmenopausal women.

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Conflict of interest statement The authors declare that they have no conflicts of interest.

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