Original Article

Dietary zinc supplementation increased TNFα and IL1β-induced RANKL expression, resulting in a decrease in bone mineral density in rats

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We investigated the effect of dietary zinc supplementation on bone metabolism in rats. Four-week-old male Wistar rats were fed a 30.0 mg zinc/kg diet (C), a 300.0 mg zinc/kg diet (HZ) or a 3,000.0 mg zinc/kg diet (EZ) for 4 weeks. The zinc content of the femur gradually increased in accordance with the gradual increase in the dietary zinc level. Although the mRNA expression of zinc transporters in bone did not differ between the groups, the mRNA expression of metallothioneins was increased in the HZ and EZ groups compared to the C group. Moreover, the bone mineral density was significantly decreased in the HZ and EZ groups compared to the C group. Furthermore, the mRNA expression of tumor necrosis factor α, Interleukin-1β and osteoclastogenesis-related genes such as receptor for activator of nuclear factor-κB (NF-κB) ligand, tumor necrosis factor receptor-associated factor 6, and nuclear factor of activated T cells cytoplasmic 1 was significantly increased in the HZ and EZ groups compared to the C group. These findings suggested that dietary zinc supplementation reduced bone mineral density through the promotion of bone resorption via an increase in the expression of receptor for activator of NF-κB ligand induced by tumor necrosis factor α and Interleukin-1β.

Key Words: zinc supplementation, bone resorption, RANKL, TNFα, IL1β

Zinc, which is a nutritionally essential microelement, is relatively abundant in bone tissue and bone contains ~30% of the body content of zinc. Moreover, zinc is an essential cofactor for several zinc dependent enzymes in bone. Thus, zinc may play a physiologically important role in bone. It has been known for many years that zinc deficiency is a risk factor in the development of human osteoporosis. In our previous study, we showed that dietary zinc deficiency suppressed bone formation and increased bone resorption, resulting in bone fragility. In addition, we suggested that bone resorption in zinc deficient rats was caused by an increase in serum parathyroid hormone through a decrease in the serum calcium concentration.[1] Conversely, zinc supplementation has been reported to promote bone formation and inhibit bone resorption in *in vitro* studies.[2,3] Studies using animal models such as ovariectomized rats and diabetic mice and rats, have demonstrated that zinc supplementation protects from risk of fracture.[4,5] Bortolin *et al.*,[6] using a type 1 diabetes-induced bone-loss model, reported that zinc supplementation (500.0 mg Zn/kg diet) protects bone loss through an increase in the expression of osteoblastogenesis related genes. Furthermore, there have been several studies that have shown that zinc supplementation exerts positive effects on other physiological functions.[7,8] It has been shown that a zinc supplemented diet (347.50 mg Zn/kg diet) promotes the growth rate and increases the activity of alkaline phosphatase (ALP), which is a zinc-containing enzyme related with bone formation.[7] Thus, these studies show that dietary zinc supplementation leads to pharmacological effects in animals. However, little is known about the effects of dietary zinc supplementation on bone metabolism, and it is unclear how dietary zinc supplementation affects the expression of bone metabolism related genes.

Zinc homeostasis is primarily maintained by regulation of its influx into and efflux out of cells. Zinc transporters of the Slc39a (Zip) and Slc30a (ZnT) families have been identified.[9,10] The Zip family of transporters has been shown to increase the cytosolic zinc concentration through facilitation of the influx of zinc into the cytosol from outside of the cells, whereas the ZnT family of transporters decreases the cytosolic zinc concentration through removal of zinc from the cytosol. It was recently reported that Zip1, Zip13, Zip14, ZnT5, and ZnT7 are expressed in a wide variety of tissues, and deletion of these transporters in mice leads to poor growth, abnormal bone development and weight loss.[11–17] Moreover, although it has been demonstrated that the expression of several zinc transporters is induced by dietary zinc deficiency, less information is available regarding the expression of zinc transporters in animals fed zinc supplementation diets.[18–20] In humans, it is known that zinc supplementation causes hypocupremia and anemia through impairment of copper and iron absorption in the intestine.[21] In rats, it has been demonstrated that orally supplemented zinc causes impaired absorption of iron through a change in iron transporters in the small intestine.[22] Although it is known that zinc supplementation negatively affects iron homeostasis, it is unclear how dietary zinc supplementation affects iron content and iron transporters in bone.

In the present study, we attempted to clarify the effects of dietary zinc supplementation on bone metabolism and on the expression of zinc and iron transporters and bone metabolism related genes in bone.

Methods

Animals and diets.   Four-week-old male Wistar rats (Clea Japan, Tokyo, Japan) were kept in individual stainless steel wire-bottom cages in a temperature, humidity and light-controlled room (22°C, 55% humidity; 12-h light-dark cycle). All rats were fed a control diet (30.0 mg Zn/kg) for 3 days, which was the acclimatization period. Subsequently, the rats were divided into 3 groups (C, HZ, and EZ groups) that were fed the control diet, a high zinc diet (300.0 mg Zn/kg i.e., 10-fold the amount of zinc as the control diet), or an excessive zinc diet (3,000.0 mg Zn/kg i.e., 100-fold the amount of zinc as the control diet), respectively. Experimental

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Table 1. Composition of the experimental diet

Ingredient	C[a]	HZ[b]	EZ[c]
	g/kg diet		
Egg albumin	200	200	200
Corn starch	529.486	528.968	523.79
Sucrose	100	100	100
Cellulose	50	50	50
Soybean oil	70	70	70
AIN-93G mineral Mixture	35	35	35
AIN-93 vitamin Mixture	10	10	10
L-cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
tert-butylhydroqinone	0.014	0.014	0.014
$ZnCO_3$		0.518	5.696

Prepared according to the AIN-93G formulation. [a]Control diet. [b]High zinc diet. [c]Excessive zinc diet.

diets were based on the AIN-93G formulation[23] with egg albumin as the protein source (Table 1). Egg albumin was used as a protein source since this study tested responses to dietary zinc status that included zinc supplementation intake as well as zinc deficiency. The high and excess zinc diets were prepared by addition of $ZnCO_3$ (Wako Pure Chem. Ind., Osaka, Japan) to the control diet at the expense of corn starch. All groups were allowed free access to food and distilled water for 4 weeks.

At the end of the experiments, the rats were then fasted for 12 h, were anesthetically sacrificed for dissection, and blood and femur samples were collected for analysis. The blood samples were centrifuged at 3,000 rpm for 15 min to obtain plasma. Serum samples were stored at –80°C until analysis. The right femur was immediately frozen in liquid N_2 and stored at –80°C for RNA extraction, and the left femur was removed and stored in 70% ethanol for bone mineral density (BMD) measurement. This study was approved by the Animal Studies Committee of Tokyo University of Agriculture, and all procedures involving rats were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of Tokyo University of Agriculture.

Biochemical analysis. Serum zinc was measured using a metallo-assay kit (Metallogenics, Chiba, Japan). Serum iron was measured using the Fe C-test (Wako Pure Chem. Ind.). Serum magnesium was measured using the Mg B-test (Wako Pure Chem. Ind.). Minerals in other serum and bone samples were analyzed using atomic absorption spectrophotometry (ZA3300, Hitachi, Ltd., Tokyo, Japan) according to the method of Gimblet *et al.*[24] Bone phosphorus was analyzed colorimetrically according to the method of Gomori.[25] The serum osteocalcin concentration was measured using an Osteocalcin rat ELISA system (GE Healthcare UK Ltd., Amersham, England). The serum C-terminal telopeptide of type I collagen (CTx) level was assayed using the RatLaps ELISA (Immunodiagnostic systems A/S, Herlev, Denmark).

Radiographic analysis of the femur. Femurs were removed from each rat and were stored at 5°C until analysis. Bone mineral content (BMC; mg), bone area (BA; cm^2), and BMD of the left femur of each rat were measured using dual-energy X-ray absorptiometry (DXA; DCS-600EX-R; Aloka, Tokyo, Japan).

RNA extraction and quantitative RT-PCR. Total RNA was isolated from the right femur using the TRIzol reagent (Invitrogen, Carlsbad, CA) and was then purified using the RNeasy mini kit (QIAGEN K.K., Tokyo, Japan). DNase digestion was performed using the RNase-free DNase set (QIAGEN K.K.). The quality and quantity of total RNA were assayed using agarose gel electrophoresis and Nano Drop 2000c (Thermo Fisher Scientific, Waltham, MA), respectively. Complementary DNA (cDNA) was synthesized using the Prime Script RT reagent kit (Takara Bio. Inc., Shiga, Japan). For real time PCR, the reaction mixture was

prepared using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster, CA) with TaqMan gene expression assays (Applied Biosystems) for the following rat genes: SLC39A1 (Zip1) (Assay ID: Rn01458936_g1), SLC39A13 (Zip13) (Assay ID: Rn01485759_m1), SLC39A14 (Zip14) (Assay ID: Rn01468336_m1), SLC30A5 (ZnT5) (Assay ID: Rn01493867_m1), SLC30A7 (ZnT7) (Assay ID: Rn01518625_m1), metallothionein 1a (Mt1a) (Assay ID: Rn00821759_g1), metallothionein 2A (Mt2A) (Assay ID: Rn01536588_gH), SLC11A2 (divalent metal transporter 1 (DMT1)) (Assay ID: Rn01533109_m1), osteoprotegerin (OPG) (Assay ID: Rn00563499_m1), runt-related transcription factor 2 (Runx2) (Assay ID: Rn01512298_m1), Osterix (Assay ID: Rn02769744_s1), osteocalcin (Assay ID: Rn00566386_g1), alkaline phosphatase (ALP) (Assay ID: Rn01516028_m1), tumor necrosis factor α (TNFα) (Assay ID: Rn01525859_g1), interleukin 1 beta (IL1β) (Assay ID: Rn00580432_m1), receptor for activator of nuclear factor-κB (NF-κB) ligand (RANKL) (Assay ID: Rn00589289_m1), tumor necrosis factor receptor-associated factor 6 (TRAF6) (Assay ID: Rn01512911_m1), nuclear factor of activated T cells cytoplasmic 1 (NFATc1) (Assay ID: Rn04280453_m1), cathepsin K (CTSK) (Assay ID: Rn00580723_m1), tartrate-resistant acid phosphatase (TRAP) (Assay ID: Rn00569608_m1), and β actin (Assay ID: Rn00667869_m1). Real-time PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems). Gene transcript levels in each sample were determined using the relative standard curve method. The level of gene transcripts are expressed as a ratio relative to β-actin mRNA, with the level in rats fed the control diet set to 1.

Statistics. Data are expressed as means ± SEM. The significance of differences between the groups was determined using one-way analysis of variance and Bonferroni's multiple-comparison tests. The relation between serum zinc level, zinc content of femur, and the mRNA expression of RANKL in bone was using Pearson correlation coefficients. Differences were considered significant at $p < 0.05$.

Results

Final body weight and serum mineral concentrations.
Final body weight and food intake were significantly lower in the EZ group than in the C and HZ groups, but did not differ between the C and HZ groups (Table 2). The serum zinc concentration was significantly higher in the EZ group compared with the C and HZ groups, and tended to be higher in the HZ group compared with the C group ($p = 0.0695$). In contrast, the serum iron concentration was significantly lower in the EZ and HZ groups than in the C group, and was significantly lower in the EZ group than in the HZ group. The serum magnesium and calcium concentrations did not differ between the groups.

Bone mineral contents. The zinc content of the femur was significantly higher in the EZ group than in the C and HZ groups (Table 3). The zinc content of the femur in the HZ group was about twice as high as that of the C group. On the other hand, the iron and magnesium contents of the femur were significantly lower in the HZ and EZ groups than in the C group, and were significantly lower in the EZ group than in the HZ group. The calcium and phosphorus contents of the femur were significantly lower in the EZ group than in the HZ and C groups; there were no differences in the contents of these minerals between the HZ and the C groups.

Length, weight, BA, BMC, and BMD of the femur. The length, weight, and BA of the femur were significantly lower in the EZ group than in the C and HZ groups; no differences were found between the HZ and C groups (Table 3). BMC and BMD were significantly lower in the HZ and EZ groups than in the C group, and were significantly lower in the EZ group than in the HZ group.

Table 2. Body weight and serum Zn, Fe, Mg, and Ca concentration in C, HZ, and EZ groups

	C	HZ	EZ
Body weight (g)			
Initial	91.09 ± 1.30[a]	90.87 ± 1.32[a]	91.51 ± 1.26[a]
Final	254.88 ± 6.90[a]	228.04 ± 6.91[a]	156.80 ± 8.56[b]
Food Intake (g/day)	16.10 ± 0.47[a]	14.98 ± 0.04[a]	10.84 ± 0.46[b]
Serum			
Zinc (μM)	21.09 ± 0.60[a]	47.09 ± 4.59[a]	117.09 ± 12.63[b]
Iron (μM)	28.52 ± 1.23[a]	16.76 ± 1.44[b]	7.16 ± 0.27[c]
Magnesium (mM)	0.76 ± 0.04[a]	0.74 ± 0.05[a]	0.83 ± 0.02[a]
Calcium (mM)	3.10 ± 0.10[a]	3.16 ± 0.11[a]	2.90 ± 0.15[a]

Values are expressed as means ± SEM for each group. Rats fed a control diet (C), a high zinc diet (HZ), and an excessive zinc diet (EZ). [a,b,c]Values with different superscript letters are significantly different, $p<0.05$.

Table 3. Effect of zinc supplementation on bone parameters

	C	HZ	EZ
Femur			
Length (cm)	3.08 ± 0.02[a]	3.05 ± 0.02[a]	2.78 ± 0.04[b]
Weight (g)	0.33 ± 0.01[a]	0.31 ± 0.01[a]	0.20 ± 0.01[b]
BA (cm²)	1.69 ± 0.02[a]	1.65 ± 0.03[a]	1.43 ± 0.02[b]
BMC (mg)	151.16 ± 0.70[a]	141.80 ± 2.97[b]	79.80 ± 3.02[c]
BMD (mg/cm²)	89.00 ± 0.77[a]	85.74 ± 0.87[b]	55.83 ± 1.18[c]
Zinc (mg)	0.19 ± 0.004[a]	0.40 ± 0.02[a]	1.53 ± 0.15[b]
Iron (mg)	0.05 ± 0.002[a]	0.04 ± 0.001[b]	0.02 ± 0.001[c]
Magnesium (mg)	1.02 ± 0.02[a]	0.91 ± 0.03[b]	0.70 ± 0.03[c]
Calcium (mg)	62.21 ± 0.19[a]	59.66 ± 0.97[a]	33.36 ± 1.59[b]
Phosphorus (mg)	29.79 ± 0.68[a]	29.41 ± 0.61[a]	17.07 ± 0.71[b]
Serum			
Osteocalcin (ng/ml)	106.33 ± 8.53[a]	107.71 ± 6.62[a]	99.85 ± 18.11[a]
CTx (ng/ml)	69.26 ± 1.49[a]	92.02 ± 4.64[a]	234.39 ± 34.76[b]

Values are expressed as means ± SEM for each group. [a,b,c]Values with different superscript letters are significantly different, $p<0.05$.

Markers of bone turnover. The serum osteocalcin concentration did not differ between the groups (Table 3). The serum CTx concentration was significantly higher in the EZ group than in the C and HZ groups; there was no difference in CTx concentration between the C and the HZ groups.

Quantitation of mRNA expression in the femur. There was no significant difference in the mRNA expression of any of the zinc transporter genes assayed: Zip1, Zip13, Zip14, ZnT5, and ZnT7, between the three groups (Fig. 1). In contrast, the mRNA expression of zinc dependent proteins that are closely related with zinc metabolism, metallothionein (Mt)-1a and 2A, was significantly increased in the EZ group compared with that in the C and HZ groups. The mRNA level of Mt1a in the HZ group was about twice as high as that of the C group. The mRNA level of Mt2A in the HZ group was about two and a half times as high as that of the C group. In addition, the mRNA level of DMT1, which is a major iron transporter, was significantly increased in the HZ and EZ groups compared to that in the C group, and was significantly increased in the EZ group compared to the HZ group. The gene expression levels of the osteoblastogenesis-related factors, Runx2 and Osterix, and of the osteoblast-specific proteins, ALP and osteocalcin, are shown in Fig. 2. The mRNA level of Runx2 and ALP in the HZ and EZ groups was similar to that in the C group. On the other hand, the mRNA expression of osterix was significantly decreased in the EZ group compared to the C group, but there was no difference in the osterix level between the C and the HZ groups. In addition, the mRNA expression of osteocalcin was significantly decreased in the EZ group compared to that of the C

and HZ groups; no difference in osteocalcin levels was found between the C and HZ groups.

The gene expression levels of the osteoclastogenesis-related factors, RANKL, OPG, TRAF6, and NFATc1, and of the osteoclast-specific proteins, TRAP and CTSK, in the three groups of rats, are shown in Fig. 3. The mRNA expression of OPG was the same in all three groups. The mRNA levels of RANKL, TRAF6, and NFATc1 were significantly higher in the HZ and EZ groups compared to those in the C group, and were significantly higher in the EZ group than in the HZ group. The RANKL/OPG ratio was significantly higher in the HZ and EZ groups compared to that in the C group, and was significantly higher in the EZ group than in the HZ group. The mRNA levels of CTSK and TRAP, which are markers of osteoclast differentiation, were significantly higher in the HZ and EZ groups compared to the C group, and were significantly higher in the EZ group than in the HZ group. The mRNA expression of TNF-α and IL1β, which are known to stimulate the expression of RANKL, was significantly higher in the HZ and EZ groups compared to that in the C group, and was significantly higher in the EZ group than in the HZ group.

The correlation between zinc status and the mRNA expression of RANKL. The serum zinc level was positively correlated with the zinc content of femur ($r = 0.977$; $p<0.001$). Correlating the mRNA expression of RANKL in bone with the serum zinc level ($r = 0.721$; $p<0.01$) and the zinc content of bone ($r = 0.761$; $p<0.01$).
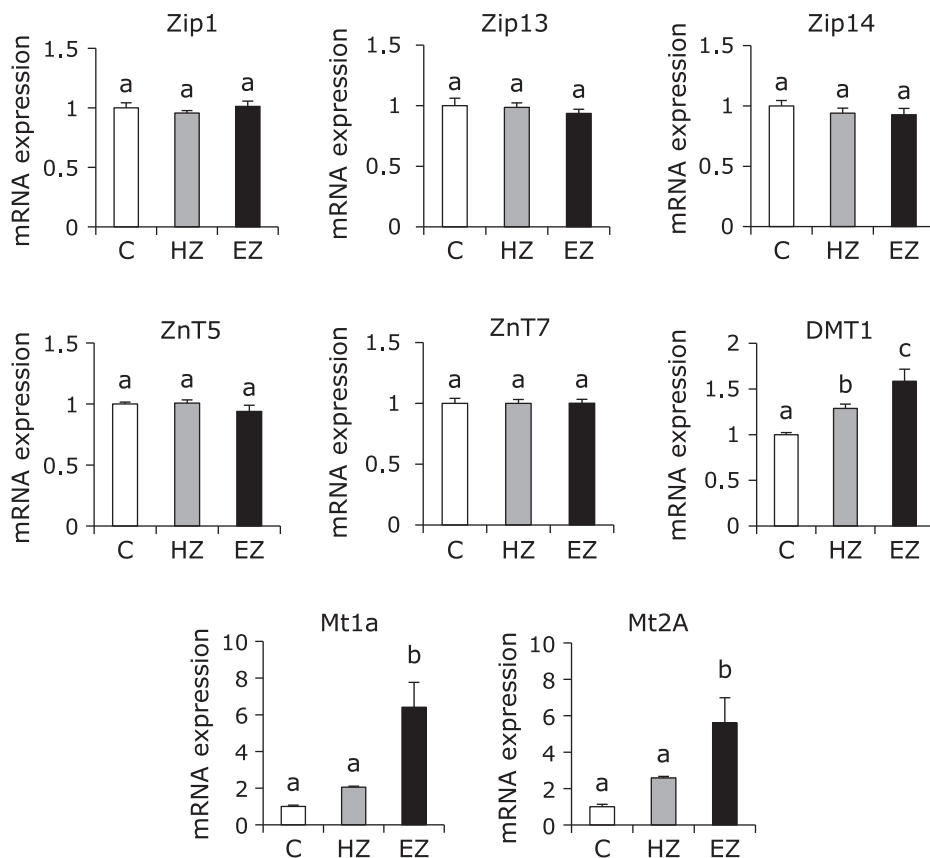
Fig. 1. Effect of dietary zinc supplementation on the mRNA expression of zinc transporters, an iron transporter, and metallothioneins in rat femur. The mRNA expression of the zinc transporters, Zip1, Zip13, Zip14, ZnT5 and ZnT7, the iron transporter DMT1, and the metallothioneins Mt1a, and Mt2A were determined using qRT-PCR. The ordinate axis indicates the relative amount of mRNA compared with rats fed control diets. Gene expression levels were normalized with β-actin. Values are expressed as means ± SEM (*n* = 6). Means with different letters differ significantly; *p*<0.05.
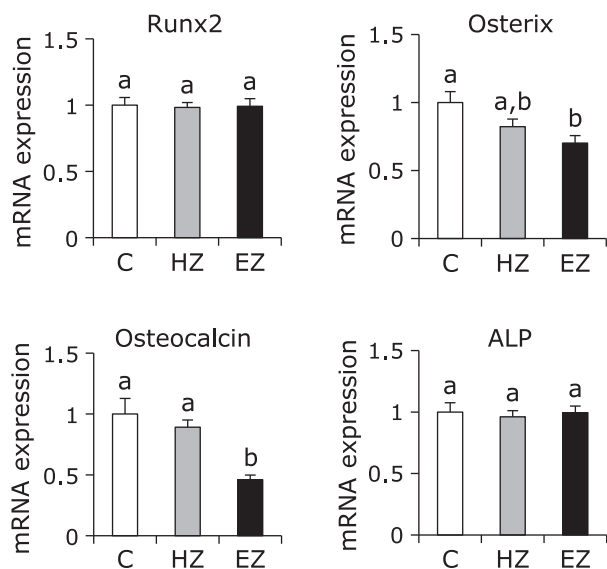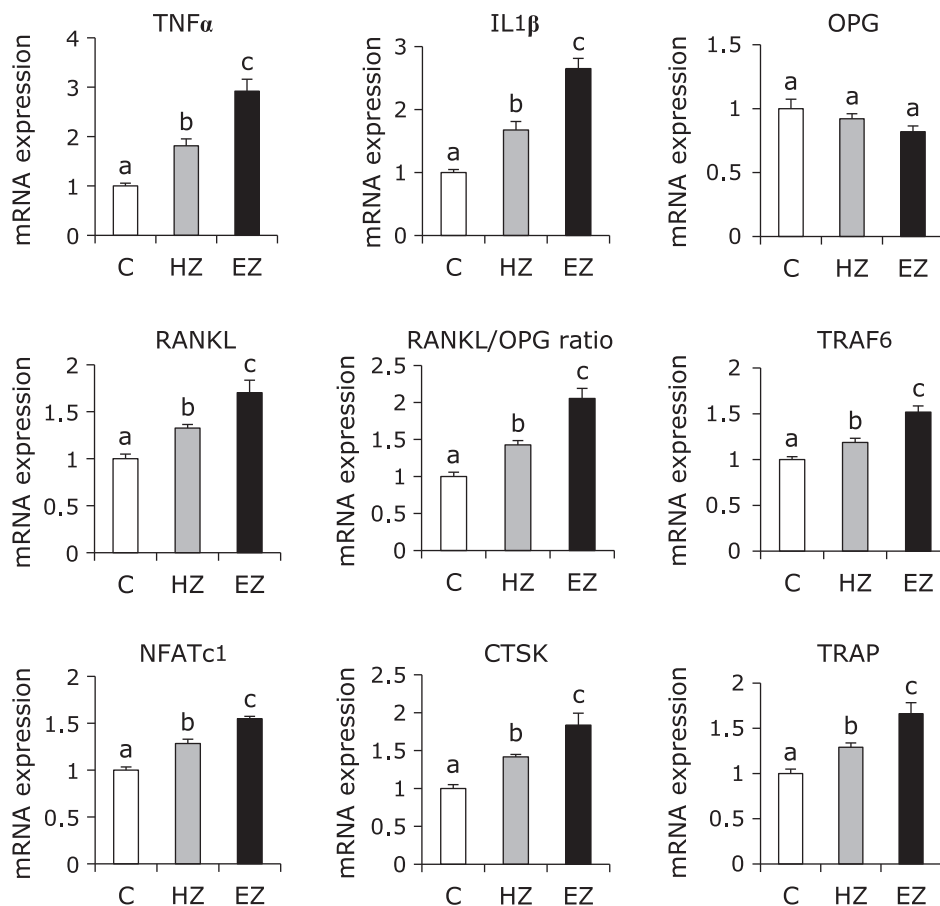

Fig. 2. Effect of dietary zinc supplementation on the gene expression levels of the osteoblastogenesis-related factors and of the osteoblast-specific proteins in the femur. The mRNA expression of Runx2, ALP, Osterix, and Osteocalcin was determined using qRT-PCR. The ordinate axis indicates the relative amount of mRNA compared with rats fed control diets. Gene expression levels were normalized with β-actin. Values are expressed as means ± SEM (*n* = 6). Means with different letters differ significantly; *p*<0.05.

Discussion

The present study examined the effects of dietary zinc supplementation on bone metabolism and the expression of bone metabolism related genes and zinc transporters in growing rats. In this study, we found that dietary zinc supplementation induced an increase in serum zinc concentration and a decrease in serum iron concentration. Several studies have demonstrated that dietary zinc supplementation positively affects physiological function, but those studies did not measure the serum iron concentration.[6,7] Since serum iron is considered an iron status indicator, our finding would be of value in terms of the therapeutic effect of zinc supplementation. De Brito *et al.*[26] reported that oral zinc supplementation, which did not exceed the estimated average requirement and the tolerable upper intake level, inhibited iron absorption and decreased the serum iron concentration in healthy schoolchildren, but did not decrease hematological parameters or promote anemia. In contrast, several other studies have demonstrated a significant decrease in iron status indicators, such as hematological and biochemical parameters, in infants supplemented with zinc.[27,28] In the present study, it was not possible to characterize any anemia induced by dietary zinc supplementation because we measured only the serum iron concentration as an iron status indicator. Therefore, further studies are needed to determine whether dietary zinc supplementation that negatively affects bone metabolism promotes anemia.

In the present study we further discovered that dietary zinc supplementation increased the zinc content of bone and decreased the iron content of bone, which was similar to the effects we

Fig. 3. Effect of dietary zinc supplementation on the gene expression levels of the osteoclastogenesis-related factors and of the osteoclast-specific proteins in the femur. The mRNA expression of TNF-α, IL1β, OPG, RANKL, TRAF6, NFATc1, CTSK, and TRAP were determined using qRT-PCR. The RANKL/OPG ratio was also calculated. The ordinate axis indicates the relative amount of mRNA compared with rats fed control diets or compared with OPG mRNA (for the RANKL/OPG ratio). Gene expression levels were normalized with β-actin. Values are expressed as means ± SEM (*n* = 6). Means with different letters differ significantly; *p*<0.05.

observed on the serum levels of zinc and iron. It is known that zinc homeostasis is regulated by zinc transporters. In this study, we analyzed the mRNA expression levels of the zinc transporters Zip1, Zip13, Zip14, ZnT5, and ZnT7 in the bone and found that their expression levels were not altered by dietary zinc supplementation. We also assayed the mRNA expression of Mt1a and MT2A, which are isoforms of metallothionein (MT) that are known to have diverse functions in zinc homeostasis and in protection against heavy metal toxicity and oxidative stress.[29,30] We found that the mRNA expression levels of Mt1a and MT2A in bone were increased by dietary zinc supplementation compared to rats fed a control diet. These results are consistent with other studies.[31,32] It was previously shown that the expression of MT is up-regulated by zinc supplementation, resulting in zinc accumulation in the liver and kidney.[31] Huber *et al*.[32] reported that the expression of MT in liver and bone marrow responds to dietary status. Therefore, our combined findings suggested that zinc accumulation in bone was induced by an increase in the expression of Mt1a and MT2A without alteration in the expression of zinc transporters in rats fed zinc supplemented diets.

As mentioned above, we observed that dietary zinc supplementation significantly decreased the iron content of bone compared with the C group. It is well known that zinc supplementation adversely affects parameters of iron status such as iron absorption, hemoglobin, and serum ferritin levels in humans.[33–35] Iron homeostasis is known to be regulated by iron related proteins. The

DMT1 imports ferrous Fe into enterocytes in a proton-coupled manner and is responsible for dietary iron absorption at the apical plasma membrane in the duodenum and iron acquisition in the transferrin cycle endosomes of peripheral tissues.[36] In the present study, DMT1 mRNA expression in the bone was increased in rats fed zinc supplemented diets compared with rats fed a control diet. These findings suggested that DMT1 was increased in response to the increased iron requirement that resulted from the decrease in the iron level of the bone caused by dietary zinc supplementation. In addition, we observed that dietary zinc supplementation reduced the rate of iron absorption compared with the control group (data not shown). However, further studies are needed to clarify how zinc supplementation negatively affects iron homeostasis in bone.

In this study, we found that dietary zinc supplementation significantly decreased BMD. In addition, the final body weight, and the length and weight of bone, were not altered in the HZ group compared to the C group. These findings suggested that dietary zinc supplementation cause bone loss without affecting bone size. Bone is mainly composed of several minerals such as calcium, phosphorous, and magnesium. In this study, the calcium and phosphorous content of bone was significantly decreased in the EZ group compared with that in the C group. On the other hand, the serum calcium concentration was unaltered by dietary zinc supplementation in this study. These findings suggested that the calcium content of bone was decreased in the EZ group in

order to maintain a serum calcium level that was similar to that of the C group. In this respect it is interesting that we previously reported that zinc deficiency leads to a decrease in serum calcium levels that is caused by an impairment of calcium absorption in the intestine in rats.[1] In addition, we observed in the present study that dietary zinc supplementation decreased the magnesium content of bone. This result is in contrast to our previous study in which we found that zinc deficiency increases the magnesium content of bone compared with rats pair fed a control diet.[1] Therefore, these results suggest that there is some interaction between zinc levels and calcium or magnesium levels in bone.

Our results showed that, in contrast to several other studies, zinc supplemented diets induced bone loss. Zinc has been demonstrated to stimulate bone formation by increasing the activity of critical enzymes such as ALP, and by inhibiting bone resorption by inhibiting osteoclast differentiation and activity *in vitro*.[3,37] Bone mass is maintained through bone remodeling that involves build-up (formation) and break down (resorption) of bone, and such remodeling occurs as two well defined cellular events.[38] Bone formation and resorption are complex biological processes and involve several regulated gene expression patterns of bone-related proteins.

In this study, we measured the serum osteocalcin concentration and the gene expression levels of the osteoblastogenesis-related factors as an indicator of bone formation. The serum level of osteocalcin, which is a marker of bone formation, was the same for all three groups. In addition, we observed that the mRNA expression level of Runx2 and ALP in bone was not affected by dietary zinc supplementation, whereas, the mRNA expression level of osterix and osteocalcin was decreased in the EZ group compared with that in the C group. ALP is a sensitive biomarker of zinc, because it is an enzyme that contains zinc. Several studies have demonstrated that dietary zinc deficiency or supplementation induced a decrease or an increase in serum ALP concentration.[7,39] In the present study, we did not observe an increase in the mRNA expression level of ALP in bone in rats fed zinc supplemented diets compared with rats fed a control diet, even though the serum zinc concentration was increased by dietary zinc supplementation. These results suggested that dietary zinc supplementation may not affect the expression of bone-specific ALP. In support of our data, Sun *et al.*[40] reported that dietary zinc supplementation (234.39 mg Zn/kg diet) did not alter the activity of bone-specific ALP or the expression of ALP in the femur. Runx2 and osterix are known to be critical for osteoblast differentiation. Runx2 is required for commitment of mesenchymal progenitor cells to differentiation into pre-osteoblasts. Subsequent differentiation to mature osteoblasts is regulated by the concerted action of Runx2 and osterix.[41,42] ALP and osteocalcin are markers of osteoblastogenesis; ALP is expressed in pre-osteoblasts and osteocalcin in mature osteoblasts.[43] Therefore our results might suggest that dietary zinc supplementation inhibits pre-osteoblast differentiation into mature osteoblasts, resulting in a decrease in the expression of osteocalcin. There is a thus a possibility that dietary zinc supplementation causes not only the promotion of bone resorption but also the inhibition of bone formation. However the serum osteocalcin level was not affected by dietary zinc supplementation in the present study. Accordingly, the effect of dietary zinc supplementation on osteoblast differentiation and bone formation should be studied further, using morphometric measurements of trabecular structure and analysis of the number of osteoblasts and osteoclasts.

In the present study we considered serum CTx as a biochemical marker of bone resorption since CTx is a product of type I collagen degradation during osteoclastic bone resorption.[44] We observed that, compared with the C group, the serum CTx level was significantly increased in the EZ group and was approximately 33% higher in the HZ group. These results suggested that dietary zinc supplementation induced bone resorption, resulting in bone loss.

Furthermore, we observed that the mRNA expression of osteoclastogenesis-related genes including RANKL, TRAF6, NFATc1, TRAP, and CTSK, was higher in the HZ and EZ groups compared with that in the C group. Moreover, we found the positive correlation between the serum zinc level or zinc content of bone and the mRNA expression of RANKL in bone in this study. These results suggested that the increase in serum zinc level or zinc content of bone is involved in the mRNA expression of RANKL in bone and leads to RANKL-induced osteoclastic bone resorption. Osteoclast differentiation and activity are regulated by the RANKL-RANK system. RANKL expressed on the osteoblast surface binds to its receptor RANK that is expressed on osteoclasts and this binding induces the recruitment of intracellular adaptor molecules such as TRAF6 to RANK. The binding of TRAF6 to RANK induces activation of NF-κB and of mitogen-activated protein kinases (MAPKs) that are involved in activation of the activator protein-1 (AP-1). NF-κB and AP-1 regulate the transcription of NFATc1, which activates osteoclast-specific genes such as TRAP and CTSK.[45]

On the other hand, the binding of OPG to RANKL inhibits the binding between RANKL and RANK, thereby preventing osteoclast precursor differentiation. In addition, it has been shown that zinc plays an important role in bone growth by stimulating OPG mRNA expression in osteoblastic cells.[46] In the present study, in contrast to the enhancement of RANKL mRNA expression, the expression of OPG was not altered by dietary zinc supplementation. These results suggested that high or excess zinc incorporated into bone does not greatly inhibit RANKL mRNA expression by stimulating OPG mRNA expression. A close relationship between the RANKL/OPG ratio and bone turnover was reported by Fazzalari *et al.*[47] In addition, an increase in the RANKL/OPG ratio has been demonstrated to enhance the degree of osteoclastogenesis and bone resorption.[48] These studies support our results that dietary zinc supplementation increased the RANKL/OPG ratio and promoted osteoclastogenesis and bone resorption. It is known that osteoclastogenesis is stimulating by cytokines such as IL-1β, IL-6, and TNF-α. Nakashima *et al.*[49] demonstrated that these cytokines increase the expression of RANKL, leading to osteoclast development and bone resorption *in vitro*. In the present study, we found that dietary zinc supplementation stimulated the mRNA expression of TNF-α and IL1β, which was associated with an increase in the expression of RANKL and osteoclastogenesis. Several studies have demonstrated that zinc supplementation increases the level of TNF-α and IL1β *in vitro*.[50–52] Moreover, Kara *et al.*[53] have reported that the production of TNF-α was induced by daily oral zinc supplementation in humans. These results support our findings that dietary zinc supplementation increased the expression of TNF-α and IL1β in bone. However, further studies are needed to determine the signal(s) that stimulate the expression of TNF-α and IL1β in the bone of rats fed a zinc supplemented diet leading to an increase in the expression of RANKL and resulting in bone resorption.

We conclude that dietary zinc supplementation induces the expression of RANKL in bone tissue via stimulation by TNF-α and IL1β, leading to bone resorption and bone loss without effecting the size of the bone. Moreover, this study suggested that dietary zinc supplementation may cause a decline in bone formation through decreasing the differentiation of pre-osteoblasts into mature osteoblasts. This is the first study to demonstrate a negative effect of dietary zinc supplementation on bone metabolism and provides a warning that more attention should be paid to the fact that therapeutic zinc supplementation may negatively affect bone metabolism.

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Abbreviations

ALP	alkaline phosphatase
BA	bone area
BMC	bone mineral content
BMD	bone mineral density
CTSK	cathepsin K
CTx	C-terminal telopeptide of type I collagen
DMT1	divalent metal transporter 1
IL1β	Interleukin-1β
Mt1a	metallothionein 1
Mt2A	metallothionein 2
NFATc1	nuclear factor of activated T cells cytoplasmic 1
OPG	osteoprotegerin
RANKL	receptor for activator of NF-κB ligand
Runx2	runt-related transcription factor 2
TNFα	tumor necrosis factor α
TRAF6	tumor necrosis factor receptor-associated factor 6
TRAP	tartrate-resistant acid phosphatase
Zip1	SLC39A1
Zip13	SLC39A13
Zip14	SLC39A14
ZnT5	SLC30A5
ZnT7	SLC30A7

Conflict of Interest

No potential conflicts of interest were disclosed.

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