Biol Trace Elem Res (2015) 163:202–207
DOI 10.1007/s12011-014-0185-3

Zinc Enhances Bone Metabolism in Ovariectomized Rats and Exerts Anabolic Osteoblastic/Adipocytic Marrow Effects Ex Vivo

Binbin Li · Hao Liu · Shengnan Jia

Received: 10 September 2014 / Accepted: 17 November 2014 / Published online: 29 November 2014

Abstract Investigations of bone mass and marrow adiposity are critical for defining the role of zinc (Zn) in bone metabolism. Rats used for study were grouped as follows: control (sham), ovariectomy (OVX), ovariectomy + estradiol (OVX-E), ovariectomy + Zn treatment (OVX-Zn). Bone mineral density (BMD) was quantified (microCT); serum osteocalcin, adiponectin, RANKL, and TRAP levels were assayed (ELISA); and biochemical determinations of serum alkaline phosphatase (ALP), calcium (Ca), and phosphorus (P) were done. Cells derived from bone mesenchymal stem cell (BMSC) isolates of respective test groups were compared, identifying primary osteoblasts by MTT assay and adipocytes by Oil Red O stain. Osteocalcin and adiponectin levels in culture supernatants were determined by ELISA. Zn supplementation resulted in a modest increase in BMD, but serum osteocalcin and ALP activity increased significantly ($P<0.01$, both). Serum levels of RANKL and TRAP were lower in OVX-Zn (vs OVX) rats ($P<0.01$), whereas serum concentrations of adiponectin, Ca, and P did not differ by group. Osteocalcin level was significantly upregulated ex vivo ($P<0.01$) in the supernatant of cultured OVX-Zn (vs OVX) cells, accompanied by a slight upturn in osteoblastic differentiation. However, Oil Red O uptake and adiponectin level in supernatant were sharply diminished in cultured OVX-Zn (vs OVX) cells ($P<0.01$). Overall, we concluded that Zn contributes to bone mass by marginally stimulating differentiation and proliferation of osteoblasts and by effectively inhibiting osteoclastic and adipocytic differentiation of BMSCs.

Keywords Zn · Bone · Ovariectomy · Osteoblastogenesis · Marrow adiposity

Introduction

Zinc (Zn) is one of the most abundant nutritionally essential microelements in the human body, with about 30 % of its bodily content restricted to bone [1]. Thus, Zn is important for skeletal development and physiologic bony homeostasis and figures formatively in the pathogenesis of osteoporosis [2–4]. Dietary Zn supplementation is known to have positive effects on bone metabolism [5], promoting osteoprotegerin expression through the PKC/MAPK pathway [6]. Furthermore, Zn is known to inhibit pit formation by solitary neonatal osteoclasts in a biphasic manner [7]. The inhibitory action of Zn on osteoclastogenesis may be due in part to suppression of the signaling pathway involved in RANKL stimulation and RANK suppression in developing osteoclasts or to NFATc1 activation in osteoclastic differentiation [8–10]. Clinical trials have also shown that Zn supplementation discourages postmenopausal bone loss [11]. Yamaguchi et al. have suggested that the opposing actions of Zn in osteoblastogenesis and osteoclastogenesis may hinge upon antagonism of NF-jB activation [12].

Despite the clear association found between Zn intake and improved BMD [13, 14], Hie et al. have indicated that both quantity and activity of osteoclasts are diminished by administration of Zn, with no appreciable effect on numbers of osteoblasts [9]. These contradictory findings demand that the underlying mechanism by which Zn regulates homeostasis of bone be re-evaluated.

B. Li (✉) · S. Jia
Department of Oral Pathology, Peking University School and Hospital of Stomatology, 22 Zhongguancun Avenue South, Haidian District, Beijing 100081, People's Republic of China
e-mail: kqlibinbin@aliyun.com

H. Liu
Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, People's Republic of China

Epidemiologic studies have shown an increased prevalence of obesity in conjunction with low Zn intake and low blood concentrations of Zn [15–17]. Zn deficiency in the setting of obesity is attributed to increased leptin production [18]. Similarly, marrow adiposity assumes an inverse relationship with BMD and bony integrity in menopausal osteoporosis [19]. Given that both osteoblasts and adipocytes originate from the same pool of bone marrow stromal stem cells (BMSCs) [20], a reciprocal affiliation between bone mass and bone marrow adiposity frequently is seen in human and animal models [21].

The premise that bone mass and bone marrow fat are linked provides greater insight into the relationship between Zn intake and bone mass, prompting us to speculate that Zn may contribute to bone health by favoring osteogenesis in otherwise undifferentiated BMSCs, while primarily inhibiting bone resorption and bone marrow adiposity. In this way, the overall effect of Zn on bony integrity is positive. Therefore, we chose to investigate the in vivo contribution of Zn to bone status and study its effect on the balance of osteoblastogenesis and bone marrow adiposity ex vivo.

Materials and Methods

Chemicals and Reagents

αModified Eagle medium (αMEM), fetal bovine serum (FBS), and a penicillin (5000 U/ml)-streptomycin (5000 μg/ml) formulation were utilized (GIBCO Laboratories, Grand Island, NY, USA). $ZnSO_4$, estradiol, 3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Oil Red O, βsodium glycerophosphate, ascorbic acid, dexamethasone, insulin, and dexamethasone were purchased (Sigma Chemical Co, St. Louis, MO, USA).

Animals and Administration Procedure

Sixty adult female Sprague-Dawley rats (weight, 210±230 g) obtained from the Animal Center of National Birth Control Board in China were kept at constant temperature (22±3 °C) and humidity (50±20 %) during the test period (12-h light/12-h dark cycles, light on 07:15 AM). Experimentation was conducted under guidelines and permission of Chinese Animal Care Committee, randomly assigning animals to four groups of 15 each (in vivo testing, n=10; ex vivo study, n=5) as follows: 1) sham, sham surgery; 2) OVX, bilateral ovariectomy under isoflurane anesthesia; 3) OVX-E, estradiol replacement (10 μg/kg once every 2 days) after bilateral ovariectomy; and 4) OVX-Zn, intragastric $ZnSO_4$ (0.25 mg/kg once every 2 days) after bilateral ovariectomy. Sham and OVX rats received vehicle only, once every 2 days. General conditions were observed, and the body weights of rats were recorded weekly. At week 12, all animals were subjected to pentobarbital anesthesia and exsanguinated. The right tibia of each euthanized rat was dissected cleanly from soft tissue, fixed in 4 % paraformaldehyde (24 h), and then washed in 10 % saccharose solution. Microtomography was performed 24 h later.

Bone Mineral Density by MicroCT

Bone mineral density (BMD) was assayed by microCT method, using an mm-sized Gantry-LG CT camera (Siemens, Berlin, Germany) and quantified by relative density of motif. Generally, samples and motif of differing density were each scanned at fixed voltage (60 kV) and current (400 μA). The volume of interest (VOI) was drawn incrementally, contouring trabecular bone carefully at five-slice intervals and morphing to interpolate all slices in between. Each slice was then visually inspected and modified as needed. Once VOI was determined, a mean was automatically calculated, in accord with a linear equation: BMD (g/cm^2)=9.1313 (VOI)-8518 (R^2=1).

Biochemistry of Animal Serum

Blood samples (2 ml) from model rats were collected in plastic EDTA-Na_2 tubes and centrifuged (5000 rpm, 10 min, 4 °C), then serum was transferred to new tubes for freezing (−20 °C) until time of assay. Osteocalcin, adiponectin, RANKL, and TRAP were assayed via two-site ELISA (R&D Systems, Minneapolis, MN, USA). Intra- and interassay variations were <5 %, and detection limit was 0.1 ng/ml. Levels of alkaline phosphatase (ALP), calcium (Ca), and phosphorus (P) were determined by automatic analyzer (Hitachi 7180; Hitachi High-Tech, Tokyo, Japan).

Isolation and Culture of BMSCs from Ovariectomized Rats

Cellular studies were performed to assess the potential role of Zn in bony homeostasis. In brief, femoral and tibial bone marrow cells of all animals were isolated for culture ex vivo. BMSCs at interface after Ficoll–Paque gradient centrifugation were cultured (3.0×10^5 cells/35-mm plate) in αMEM medium containing 10 % FCS. Cultures were maintained by change of medium every 3 days.

Osteoblastic Differentiation of BMSCs and MTT Assay

Two days after initiation and proliferation of MSCs, the culture medium was replaced by fresh αMEM medium supplemented with 10 mM β sodium glycerophosphate + 0.05 M ascorbic acid +10 nM dexamethasone for

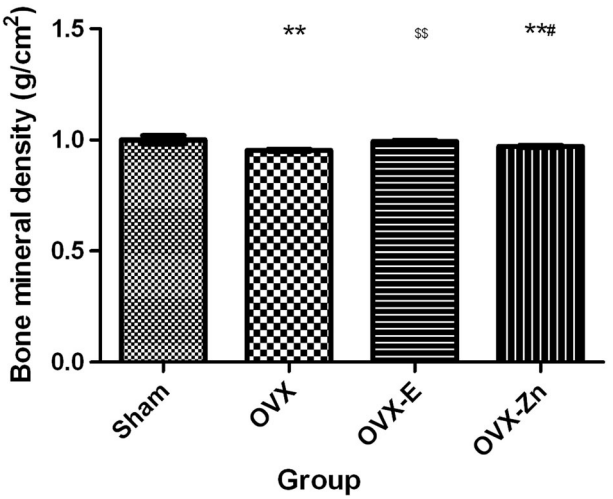

Fig. 1 Bone mineral density of right tibia quantified by microCT. $^{**}P<0.01$, vs sham group; $^{\$\$}P<0.01$, vs OVX group; $^{\#}P<0.05$, vs OVX-E group

osteoblastic differentiation. For purposes of examination, cells were plated (6.0×10^3 cells/well, 96-well plates) and maintained in growth media (24 h, 5 % CO_2, 37 °C). At 60 % confluence, 10 μl of MTT solution (5 mg/mL) was added to each well, and the cells were incubated further (4 h, 37 °C). Once formazan crystals formed, the MTT medium was aspirated and replaced with 150 μl solubilization solution dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The plates were then shaken (5 min), and absorbance of each well was recorded using a microplate spectrophotometer at 570 nm. Relative cellular growth was determined by the ratio of average absorbance in treated vs control cells. Cell viability was calculated as the ratio of optical densities.

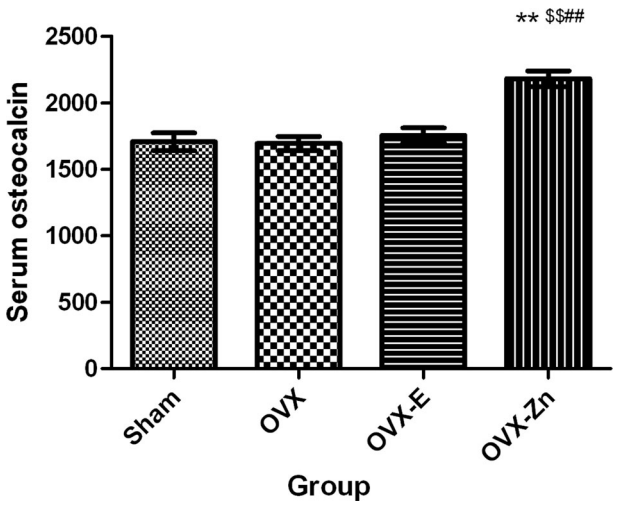

Fig. 2 Change in serum osteocalcin concentration after Zn administration. $^{**}P<0.01$, vs Sham group; $^{\$\$}P<0.01$, vs OVX group; $^{\#\#}P<0.01$, vs OVX-E group

Adipogenesis from MSCs and Oil Red O Staining

In prior studies of BMSC-derived adipocytes, cells were grown to confluence before transfer to adipogenic medium [22]; so, we plated BMSCs in a 60-cm^2 dish for incubation (7 days) in complete culture medium. Thereafter, the medium was replaced by adipogenic medium for extended incubation (21 days). Adipogenic medium was supplemented with insulin (10 μg/ml) and dexamethasone (10^{-7}M).

For Oil Red O staining, cells were fixed in 10 % formalin (10 min), washed with distilled water, and dried completely before applying 0.6 % Oil Red O solution in 60:40 (*v*/*v*) isopropyl alcohols: H_2O (room temperature, 30 min). The stained cells were eluted with isopropyl alcohol immediately before measuring optical density at 490 nm. There were eight wells for each group.

Biochemistry of Culture Supernatant

Osteocalcin and adiponectin levels in culture supernatant were quantified as directed by manufacturer (R&D Systems). Briefly, samples were washed extensively with PBS and incubated in lysis buffer on ice (15 min) to generate whole-cell lysates. The lysates were then centrifuged (10,000 rpm, 30 min), and supernatants were transferred to new tubes. Total protein concentration was evaluated by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Intra- and interassay variations were <5 %, and detection limit was 0.1 ng/ml.

Statistical Analysis

Statistical analysis relied on standard software (SPSS v10; SPSS Inc, Chicago, IL, USA). One-way ANOVA tests were

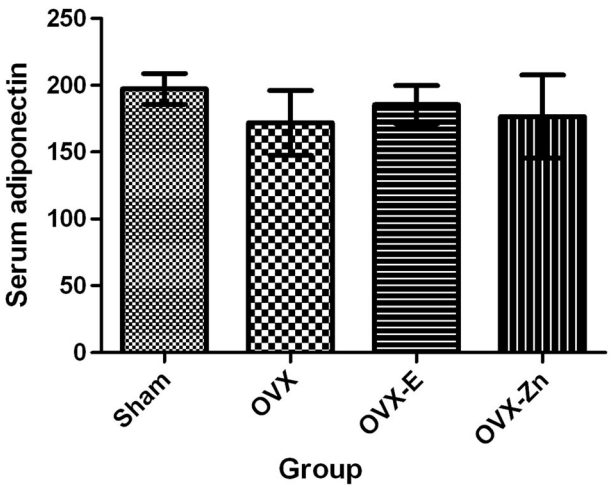

Fig. 3 Serum adiponectin levels in four study groups

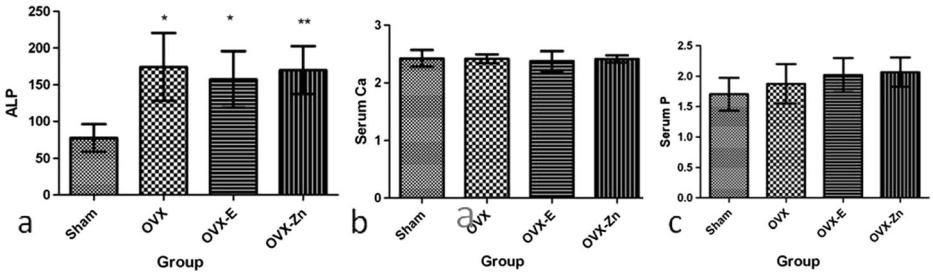

Fig. 4 Biochemical determinants in rat serum. *$P<0.05$, **$P<0.01$, vs sham group

used for comparisons between groups. All results were expressed as means±SD, with significance set at $P<0.05$ or $P<0.01$.

Results

Bone Mineral Density by MicroCT

BMD of the OVX group declined significantly, compared with that of the sham group ($P<0.01$), representing an induced osteopenic state. Estradiol replacement increased BMD ($P<0.01$) to control level. Compared with the OVX group, Zn supplementation of ovariectomized rats produced a modest increase in BMD, which was not statistically significant ($P>0.05$) (Fig. 1).

Bone Biochemical Markers

Bone remodeling markers of the four groups are demonstrated in Figs. 2, 3, 4, and 5. In Zn-treated ovariectomized rats, osteocalcin level was slightly elevated (1.3-fold; $P<0.01$) (Fig. 2); adiponectin level in serum was unchanged (Fig. 3); and ALP activity rose sharply (roughly 2.2-fold; $P<0.01$) (Fig. 4a), compared with respective control levels. Serum Ca and P determinations did not differ significantly by group (Fig. 4b, c).

Ovariectomy led to higher serum RANKL and TRAP levels in OVX rats ($P<0.01$). Zn supplement downregulated this increase to normal levels (Fig. 5). Serum RANKL value of the OVX-Zn group was lower than that of OVX-E group ($P<0.05$).

Effect of Zn on Osteoblastic Proliferation and Osteocalcin Level in Culture Supernatant

Ovariectomy resulted in comparatively fewer BMSC-derived osteoblastic cells ($P<0.01$), although estradiol replacement dramatically reversed this decline ($P<0.01$). Although Zn supplementation also enhanced osteoblastic proliferation after ovariectomy, the effect was far weaker than that of estradiol ($P<0.01$) (Fig. 6a).

After ovariectomy, osteocalcin level in culture supernatant behaved similarly, declining to 75 % of that in the sham group ($P<0.01$). Relative to the OVX group, the OVX-Zn group showed a significant rise in osteocalcin ($P<0.01$), but estradiol was more effective than Zn in increasing osteocalcin concentration ($P<0.05$) (Fig. 6b).

Effect of Zn on Adipogenic Differentiation and Adiponectin Level in Culture Supernatant

Oil Red O stain was used to identify adipocytes ex vivo. After ovariectomy, positivity of cells exceeded that is seen in the sham group ($P<0.01$). Zn supplementation reduced adipocytic differentiation to control level, which was significantly lower than that of OVX and OVX-E groups ($P<0.01$, respectively) (Fig. 7a).

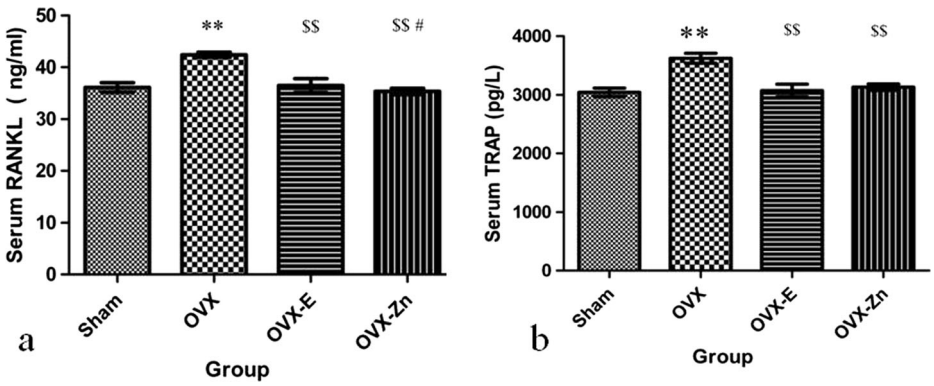

Fig. 5 Serum RANKL and TRAP levels in four study groups. **$P<0.01$, vs sham group; $^{\$\$}P<0.01$, vs OVX group; $^{\#}P<0.05$, vs OVX-E group

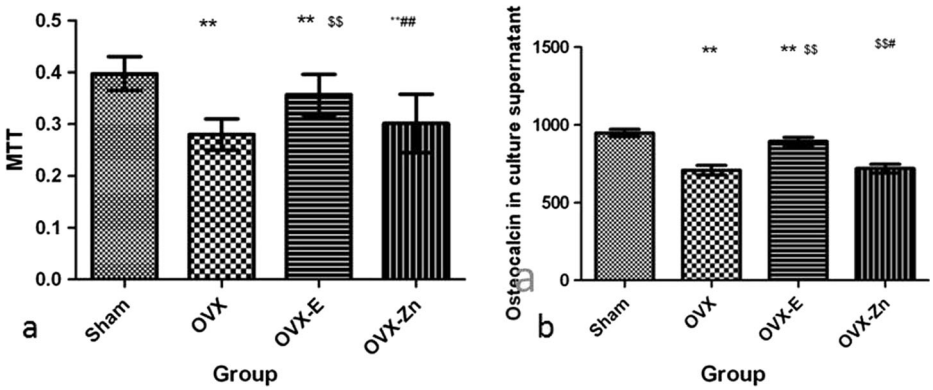

Fig. 6 Effect of Zn on osteoblastic proliferation and osteocalcin level in culture supernatant. $^{**}P<0.01$, vs sham group; $^{\$\$}P<0.01$, vs OVX group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, vs OVX-E group

Likewise, adiponectin level of OVX-Zn group was less than that of the OVX group ($P<0.01$) and was comparable to sham group level (Fig. 7b).

Discussion

Our ex vivo observations, in concert with BMD assessment and related biochemical indices, suggest that Zn supplementation enhances bone mass in ovariectomized osteopenic rats by stimulating differentiation and proliferation of osteoblasts to a limited extent and by effectively inhibiting osteoclastic and adipocytic differentiation of BMSCs.

We found that Zn administration to ovariectomized rats produced a relatively modest increase in BMD, albeit still inferior to control BMD status. This particular point is a departure from other reports, suggesting that Zn is only marginally beneficial in bone formation. A comparator study has shown that dietary Zn intake and plasma Zn concentration both correlated positively with BMD in men. Hip, spinal, and distal wrist BMD measurements were significantly lower in men with lowest (vs higher) plasma Zn concentrations [23].

BMD measurement is widely used for detecting osteoporosis [24], but as an index of bone mass, it has limitations [25]. We felt that BMD and biochemical indices combined would be suitable for comprehensively evaluating bone mass. By the same token, we discovered that Zn supplementation led to a significant increase in osteoblast count, osteocalcin level, and ALP activity in ovariectomized rats. ALP is generally a marker of immature osteoblasts, whereas osteocalcin surges at a later stage of osteoblastic differentiation [26]. Thus, it appears that Zn enhances bone metabolism by promoting osteoblastic differentiation and proliferation.

Because osteoblasts and adipocytes have common origins in bone marrow [27], diminished bone formation in patients with osteoporosis may be explained by preferential adipocytic (rather than osteoblastic) differentiation in shared cellular precursors [28]. One may presume that heightened osteoblastic and diminished adipocytic differentiation occurs through a "switch-like" diversion of BMSCs toward osteoblastogenesis. Accumulating evidence supports this contention, underscoring that extent of marrow adipogenesis may serve as an alternate index of osteoporotic severity [29]. Indeed, Menagh et al. have noted that increased osteoblastic differentiation is accompanied by depressed lipid accumulation in bone marrow [30]. Future trials should attempt to define the time sequence of gene expression as BMSCs differentiate to adipocytes ex vivo, thereby unearthing likely targets for interventional Zn therapy.

A variety of cell systems have been used to study adipocytic differentiation in bone marrow [31, 32]. The primary cells of our rat model may be expanded rapidly in culture as a ready

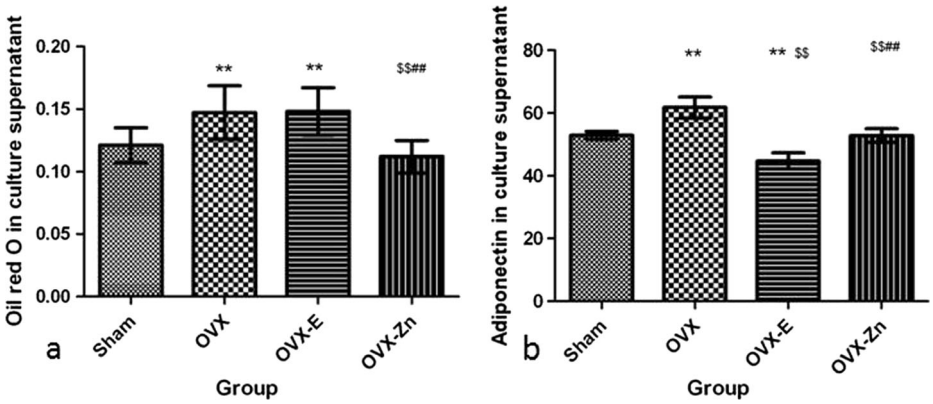

Fig. 7 Effect of Zn on adipocytic differentiation and adiponectin level in culture supernatant. $^{**}P<0.01$, vs sham group; $^{\$\$}P<0.01$, vs OVX group; $^{\#\#}P<0.01$, vs OVX-E group

resource. More importantly, these cells better mimic an in vivo environment. Accordingly, the bone marrow response after intake of Zn was accurately depicted by the data obtained.

Conclusion

In summary, we investigated the effect of Zn on bone mass ex vivo, using an ovariectomized animal model to delineate its regulatory role in bony homeostasis. Our data suggests that Zn enhances bone mass in an induced osteopenic state through limited stimulation of osteoblastic differentiation and proliferation and by effectively inhibiting osteoclastic and adipocytic differentiation of BMSCs.

Acknowledgments This research was funded by National Natural Science Foundations of China, Beijing, China (No. 30901671).

Conflict of interest The authors certify that there is no conflict of interest to declare.

References

1. Haumont S (1961) Distribution of zinc in bone tissues. J Histochem Cytochem 9(2):141–145
2. Aaseth J, Boivin G, Andersen O (2012) Osteoporosis and trace elements—an overview. J Trace Elem Med Biol 26(2–3):149–152
3. Yamaguchi M, Oishi H, Suketa Y (1987) Stimulatory effect of zinc on bone formation in tissue culture. Biochem Pharmacol 36(22):4007–4012
4. Seo HJ, Cho YE, Kim T, Shin HI, Kwun IS (2010) Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells. Nutr Res Pract 4(5):356–361
5. Eberle J, Schmidmayer S, Erben RG, Stangassinger M, Roth HP (1999) Skeletal effects of zinc deficiency in growing rats. J Trace Elem Med Biol 13(1–2):21–26
6. Liang D, Yang M, Guo B, Cao J, Yang L, Guo X (2012) Zinc upregulates the expression of osteoprotegerin in mouse osteoblasts MC3T3-E1 through PKC/MAPKpathways. Biol Trace Elem Res 146(3):340–348
7. Li BB, Yu SF (2006) Effect of extracellular zinc on osteoclastic resorption in dental mineralized tissues. J Peking Univ Health Sci 38(6):644–647
8. Yamaguchi M, Uchiyama S (2004) Receptor activator of NF-kappa B ligand stimulated osteoclastogenesis in mouse marrow culture is suppressed by zinc in vitro. Int J Mol Med 14(1):81–85
9. Hie M, Tsukamoto I (2011) Administration of zinc inhibits osteoclastogenesis through the suppression of RANK expression in bone. Eur J Pharmacol 668(1–2):140–146
10. Park KH, Park B, Yoon DS et al (2013) Zinc inhibits osteoclast differentiation by suppression of Ca2+−Calcineurin-NFATc1 signaling pathway. Cell Commun Signal. doi:10.1186/1478-811X-11-74
11. Razmandeh R, Nasli-Esfahani E, Heydarpour R et al (2014) Association of zinc, copper and magnesium with bone mineral density in Iranian postmenopausal women—a casecontrol study. J Diabetes Metab Disord 13(1):43. doi:10.1186/2251-6581-13-43
12. Yamaguchi M, Weitzmann MN (2011) Zinc stimulates osteoblastogenesis and suppresses osteoclastogenesis by antagonizing NF-κB activation. Mol Cell Biochem 355(1–2):179–186
13. New SA (1997) Nutritional influences on bone mineral density: a cross-sectional study in premenopausal women. Am J Clin Nutr 65(6):1831–1839
14. Bhardwaj P, Rai DV, Garg ML (2013) Zinc as a nutritional approach to bone loss prevention in an ovariectomized rat model. Menopause 20(11):1184–1193
15. Atkinson RL, Dahms WT, Bray GA, Jacob R, Sandstead HH (1978) Plasma zinc and copper in obesity and after intestinal bypass. Ann Intern Med 89(4):491–493
16. Di Martino G, Matera MG, De Martino B, Vacca C, Di Martino S, Rossi F (1993) Relationship between zinc and obesity. J Med 24(2–3):177–183
17. Marreiro DN, Fisberg M, Cozzolino SM (2002) Zinc nutritional status in obese children and adolescents. Biol Trace Elem Res 86(2):107–122
18. Liu MJ, Bao S, Bolin ER et al (2013) Zinc deficiency augments leptin production and exacerbates macrophage infiltration into adipose tissue in mice fed a high-fat diet. J Nutr 143(7):1036–1045
19. Patsch JM, Li X, Baum T et al (2013) Bone marrow fat composition as a novel imaging biomarker in postmenopausal women with prevalent fragility fractures. J Bone Miner Res 28(8):1721–1728
20. Georgiou KR, Hui SK, Xian CJ (2012) Regulatory pathways associated with bone loss and bone marrow adiposity caused by aging, chemotherapy, glucocorticoid therapy and radiotherapy. Am J Stem Cells 1(3):205–224
21. Morita Y, Iwamoto I, Mizuma N et al (2006) Precedence of the shift of body-fat distribution over the change in body composition after menopause. J Obstet Gynaecol Res 32(5):513–516
22. Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147
23. Taisum HH, Elizabeth BC, David BM (2004) Zinc intakes and plasma concentrations in men with osteoporosis: the Rancho Bernardo Study. Am J Clin Nutr 80(3):715–772
24. Cao T, Shirota T, Yamazaki M, Ohno K, Michi KI (2001) Bone mineral density in mandibles of ovariectomized rabbits. Clin Oral Implants Res 12(6):604–608
25. Siris ES, Boonen S, Mitchell PJ, Bilezikian J, Silverman S (2012) What's in a name? What constitutes the clinical diagnosis of osteoporosis? Osteoporos Int 23(8):2093–2097
26. Bancroft GN, Sikavitsas VI, van den Dolder J et al (2002) Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proc Natl Acad Sci U S A 99(20):12600–12605
27. DiGirolamo CM, Stokes D, Colter DG, Phinney DG, Class R, Prockop DJ (1999) Propagation and senescence of human marrow stromal cells in culture—a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107(2):275–281
28. Rozman C, Feliu E, Berga L, Reverter JC, Climent C, Ferran MJ (1989) Age-related variations of fat tissue fraction in normal human bone marrow depend both on size and number of adipocytes: a stereological study. Exp Hematol 17(1):34–37
29. Li GW, Xu Z, Chang SX, Nian H, Wang XY, Qin LD (2014) Icariin prevents ovariectomy-induced bone loss and lowers marrow adipogenesis. Menopause 21(9):1007–1016
30. Menagh PJ, Turner RT, Jump DB et al (2010) Growth hormone regulates the balance between bone formation and bone marrow adiposity. J Bone Miner Res 25(4):757–768
31. Hirota K, Morikawa K, Hanada H et al (2010) Effect of genistein and daidzein on the proliferation and differentiation of human preadipocyte cell line. J Agric Food Chem 58(9):5821–5827
32. Lee OH, Seo DH, Park CS, Kim YC (2010) Puerarin enhances adipocyte differentiation, adiponectin expression, and antioxidant response in 3T3-L1 cells. Biofactors 36(6):459–467