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Effect of zinc ion on the osteogenic and adipogenic differentiation of mouse primary bone marrow stromal cells and the adipocytic trans-differentiation of mouse primary osteoblasts

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Abstract

A series of experimental methods including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test, alkaline phosphatase (ALP) activity measurement and Oil Red O stain and measurement were employed to assess the effect of zinc ion on the osteogenic and adipogenic differentiation of mouse primary bone marrow stromal cells (MSCs) and the adipogenic trans-differentiation of mouse primary osteoblasts. The results showed that except for individual concentrations of zinc ion there was no effect on the proliferation of MSCs and osteoblasts. Zinc ion inhibited the osteogenic differentiation of MSCs at all the concentrations tested. It also inhibited adipogenic differentiation at all concentrations tested except 10^{-9} mol/L. Both of the inhibition effects were attenuated with time increasing. Zinc ion depressed adipocytic trans-differentiation of osteoblasts at concentrations of 10^{-11} and 10^{-10} mol/L, but the effect could be reversed to promote or even be removed when concentration was increased. It suggests that the influence of zinc ion on osteogenic, adipogenic differentiation of MSCs and adipocytic trans-differentiation of osteoblasts depends on zinc ion concentrations and incubation time. The protective effects of zinc ion on bone may be mediated by modulating differentiation of MSCs away from the adipocytes and inhibiting adipocytic trans-differentiation of osteoblasts. This may in turn promote osteoblast formation and reduce secretion of cytokines which may inhibit osteoclast formation and activation. These findings may be valuable for better understanding the mechanism of the effect of zinc ion on bone.

Keywords: Zinc ion; Bone marrow stromal cells; Osteogenic and adipogenic differentiation; Osteoblasts; Adipocytic trans-differentiation

Introduction

Osteoporosis is a metabolic bone disease that afflicts millions of the elderly each day. It is characterized by low bone mass, microarchitectural deterioration of bone tissue, increased bone fragility and risk of fracture

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incidence [1]. It has been recognized as a major public health problem because of the great pain patients suffered and the cost for therapy.

Osteoporosis results in increased bone resorption and decreased bone formation processes when osteoclasts and osteoblasts are the major cells involved. A great number of evidence suggests that osteoporosis is the result of reciprocal interaction between genetic susceptibility and environmental factors. Among them, certain essential trace elements were reported to be involved in the pathogenesis of osteoporosis. It has been reported that zinc ion administration prevented bone loss in rats with ovariectomy, skeletal unloading, hydrocortisone treatment, adjuvant arthritics and diabetic condition in rats [2–4]. Zinc ion has been shown to play a role in the preservation of bone mass by stimulating osteoblastic bone formation and inhibiting osteoclastic bone resorption in rat [3]. The cellular mechanism of zinc ion has also been partially elucidated. Zinc ion inhibited the formation of osteoclastic cells from bone marrow cells [5,6], and could also reduce pit formation by isolated neonatal rat OCs in a biphasic manner (10^{-14}–10^{-10} and 10^{-4} mol/L). This, however, had no effect on OC number [7]. Meanwhile, zinc ion has been demonstrated to have a stimulative effect on bone formation and mineralization due to promoting bone cellular protein synthesis [8,9].

Bone marrow stromal cells (MCSs) are pluripotent cells which are able to differentiate into osteoblasts, adipocytes, and other cell phenotypes [10]. It is currently well accepted that the ability of MSCs to differentiate into various cell phenotypes may be critical in the progression of bone disease. An increased lipid accumulation in the bone marrow has been reported in association with age-related bone loss [11] and studies have shown that adipogenesis in postmenopausal women may occur at the expense of osteogenesis. This suggested that a reciprocal relationship exists between adipogenesis and osteogenesis of bone MSC [12]. Therefore, it is possible that modulation of the balance between marrow adipogenesis and osteoblastogenesis, by either preventing further increases in adipocyte formation or diverting existing adipocytes to produce more osteoblasts with a resulting increase in functional bone cells and could provide a therapeutic strategy for osteoprosis [13].

So far the effects of zinc ion on the osteogenic and adipogenic differentiation of bone MSCs and the adipogenic trans-differentiation of osteoblasts have not been reported. The aim of this study is evaluating the effect of zinc ion on osteogenic and adipogenic differentiation of mouse primary bone MSCs and the adipogenic trans-differentiation of mouse primary osteoblasts in order to further elucidate the effect of zinc ion on the pathogenesis of osteoporosis.

Materials and methods

Reagents

All commercially available reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

Isolation and culture of primary bone marrow stromal cells (MSCs)

Female KM mice were obtained from the Laboratory Animal Resources of Guangzhou University of Chinese. At 4–6 weeks of age, mice were euthanized by carbon dioxide asphyxiation. Femora and tibiae were aseptically harvested and the whole bone marrow was flushed using Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% FBS (DingGuo, Beijing, China) and penicillin/streptomycin in a 1 mL syringe and a 25-gauge needle. The cells were collected and cultured in a culture flask. After 4 days of incubation in a 37 °C, 5% CO_2 humidified incubator, the nonadherent cells were removed from the cultures by gentle aspiration and the medium replaced with fresh DMEM. The medium was changed every 3 days in each of the experiments [14].

Isolation and culture of primary osteoblasts

KM mice were obtained from the Laboratory Animal Resources of Guangzhou University of Chinese. At 2–3 days of age, mice were euthanized by carbon dioxide asphyxiation. Skull (frontal and parietal bones) were dissected aseptically from KM mice, endosteum and periosteum were removed by scraping, and the bone was cut into approximately 1–2 mm^2 pieces and digested with 0.25% trypsin (1:250; Gibco, USA) for 30 min and the digestion was discarded. Then the bone was digested with 0.1% collagenase type II (290 U/mg; Worthington, USA) twice for 1 h each time, and the cells were collected and cultured in alpha modification of the MEM medium (α-MEM; Gibco, USA) containing 10% FBS and penicillin/streptomycin. After overnight incubation (37 °C, 5% CO_2), the medium was replaced with fresh α-MEM. The medium was changed every 3 days in each of the experiments [15].

Cell proliferation assay

MSCs and osteoblasts were plated in 96-well culture plates at the density of 1×10^7 and 1×10^4 cells per well, respectively. Zinc ion was added to the wells at final concentrations of 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L. Control wells were prepared by addition of corresponding medium. The stock

MTT dye solution (5 mg/mL) was added to each well after the plates were incubated at 37 °C in a 5% CO_2 incubator for 44 h. After 4 h incubation, the supernatant was removed and DMSO (100 μL) was added. The optical density (OD) of each well was measured on a microplate spectrophotometer (BioRad Model 3550, USA) at a wavelength of 570 nm [16].

Measurement of cellular alkaline phosphatase (ALP) activity

The bone MSCs were isolated as above and plated in 48-well culture plates at the density of 1×10^7 cells per well. Cells were cultured in osteogenic differentiation medium, consisting of DMEM supplemented with 10^{-7} mol/L dexamethasone, 5.0 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid for up to 14 days. Zinc ion was added at final concentrations of 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L. At the day 7 and 14, the plates were washed three times with ice-cold PBS and the cells were lysed by two cycles of freezing and thawing. Aliquots of supernatants were subjected to ALK activity and protein content measurement using an alkaline phosphatase kit (Jiangcheng, Nanjing, China) and a micro-bradford assay kit (Beyotime, Haimen, China), respectively. The osteogenic differentiation promotion rate was calculated according to the formula:

$$(\text{ALP activity}_{\text{treated}}/\text{ALP activity}_{\text{control}} - 1)100\%$$ [17].

Oil red O stain and measurement of adipocytes

The bone MSCs and osteoblasts were isolated as above. The cells were plated in 48-well culture plates at the density of 1×10^7 and 1×10^4 cells per well, respectively. Cells were cultured in adipogenic differentiation medium, consisting of DMEM supplemented with 10 μg/mL insulin, 10^{-7} mol/L dexthamethone. Treatments were the same as those described for the osteogenic differentiation. The Oil red O staining and measurement method was a modification of the method as previously described [18]. The cells were fixed in 4% formaldehyde, 0.6% (w/v) oil red O solution (60% isopropanol, 40% water) was added for 15 min at room temperature after washing with 1 × PBS. For quantification, cell monolayers were washed extensively with 1 × PBS to remove unbound dye and then 0.5 mL of isopropyl alcohol was added to the stained culture dish. After 3 min, the absorbance of the solution was assayed using a spectrophotometer at 510 nm. The adipogenic differentiation inhibition rate and adipocytic trans-differentiation inhibition rate were calculated according to the formula: $(1-\text{OD}_{\text{treated}}/\text{OD}_{\text{control}})100\%$.

Statistical analysis

Data were analyzed by one-way ANOVA and post hoc Student's two-tailed *t*-test. All *P*-values were compared to an α-value of 0.05 to determine significance. All data were presented as mean ± SD.

Results

Effect of zinc ion on the MSCs proliferation

As shown in Fig. 1, zinc ion had no effect on MSC proliferation at concentrations of 10^{-11}–10^{-9} mol/L, but it inhibited MSC proliferation at concentrations of 10^{-8} and 10^{-7} mol/L and inhibition rates were 14% and 17%, respectively.

Effect of zinc ion on the osteoblasts proliferation

As shown in Fig. 2, zinc ion had no significant effect on the osteoblasts proliferation at the tested concentrations.

Effect of zinc ion on the osteogenic differentiation of MSCs

As shown in Fig. 3, the osteogenic differentiation of MSCs was inhibited by zinc ion at all the concentrations

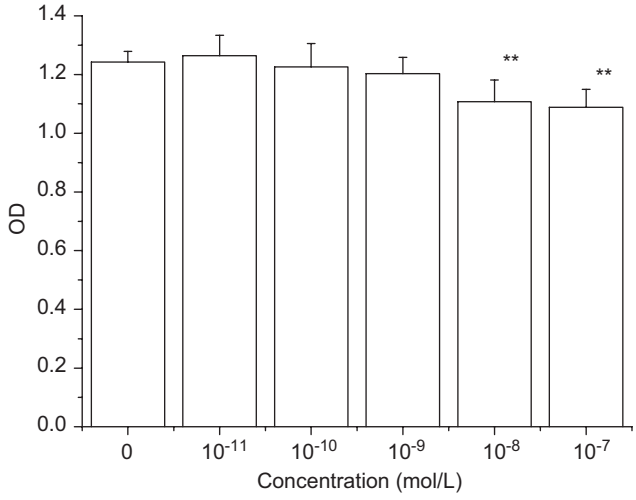


Fig. 1. Effect of zinc ion on the proliferation of MSCs (**$P < 0.01$ vs. control, $n = 5$). MSCs were cultured at the density of 1×10^7 per well in the absence of zinc ion and in the presence of 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L zinc ion, as described in materials and methods. Stock MTT dye solution was added when MSCs were cultured for 44 h. After 4 h incubation, culture medium was removed and DMSO was added and the optical density (OD) of each well was measured at a wavelength of 570 nm.

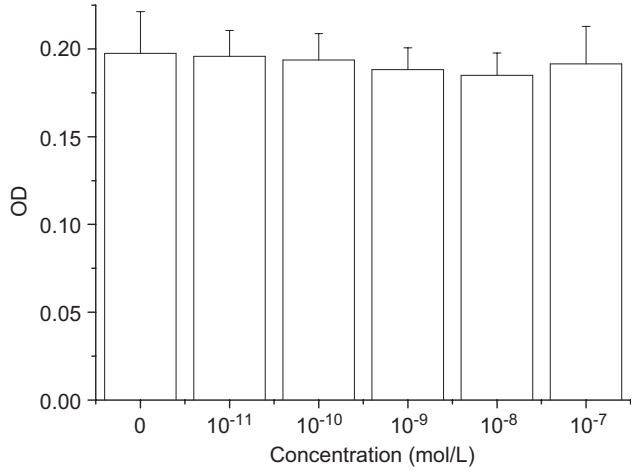


Fig. 2. Effect of zinc ion on the proliferation of osteoblasts ($n = 5$). Osteoblasts were cultured at the density of 1×10^4 cells per well in the absence of zinc ion and in the presence of 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L zinc ion, as described in materials and methods. Stock MTT dye solution was added when MSCs were cultured for 44 h. After 4 h incubation, culture medium was removed and DMSO was added and the optical density (OD) of each well was measured at a wavelength of 570 nm.

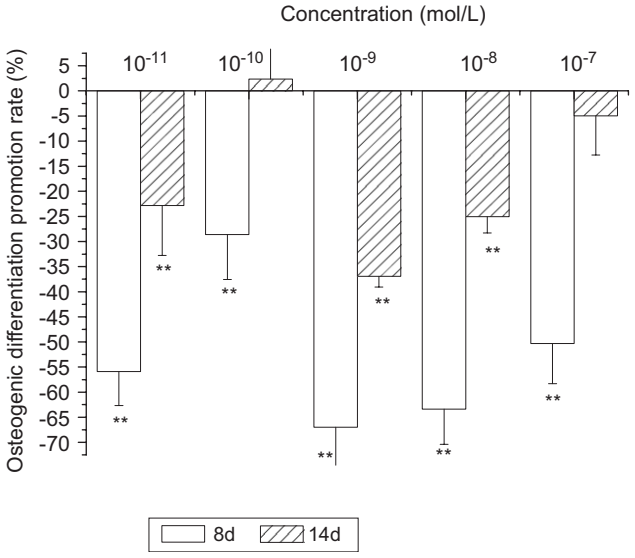


Fig. 3. Effect of zinc ion on the osteogenic differentiation of MSCs ($^{**}P < 0.01$ vs. control, $n = 5$). The bone MSCs were cultured in a culture medium supplemented with 10^{-7} mol/L dexamethasone, 5.0 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid; and in the absence and in the presence of 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L zinc ion. At selected times, the medium was removed, alkaline phosphatase activity and protein content measurement were measured by using an alkaline phosphatase kit and a micro-bradford assay kit, respectively. The osteogenic differentiation promotion rate was calculated according to the formula:

$$(\text{ALP activity}_{\text{treated}}/\text{ALP activity}_{\text{control}} - 1) \times 100\%.$$

tested at the 8th and 14th day with the maximal effect at the concentration of 10^{-9} mol/L. The inhibition effects were attenuated with time increasing.

Effects of zinc ion on the adipogenic differentiation of MSCs

As shown in Fig. 4, zinc ion inhibited adipogenic differentiation at all concentrations except 10^{-9} mol/L, and the effect was more evident at day 14 than day 21. The morphologic observation was accorded with the results (Fig. 5).

Effect of zinc ion on adipocytic trans-differentiation of osteoblasts

As shown in Fig. 6, zinc ion depressed adipocytic trans-differentiation when osteoblasts exposed up to 10^{-11} and 10^{-10} mol/L zinc ion, but the effect could turn to be stimulative or even be removed when concentration was increased. The morphologic observation was accorded with the results (Fig. 7).

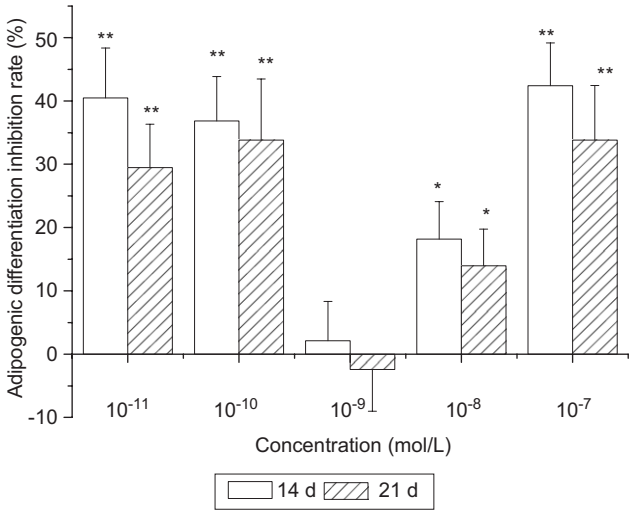
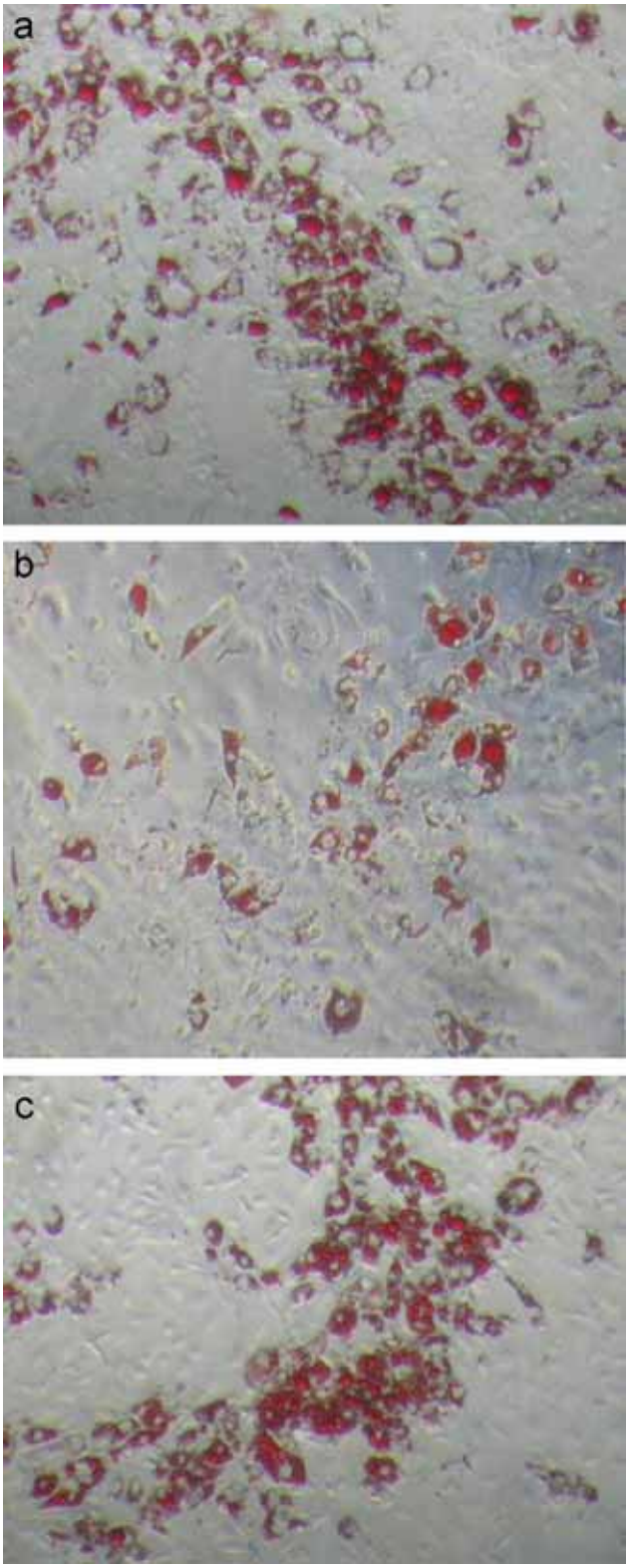


Fig. 4. Effect of zinc ion on the adipogenic differentiation of MSCs ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control, $n = 5$). The bone MSCs were cultured in a culture medium supplemented with 10 μg/mL insulin, 10^{-7} mol/L dexthamethone; and in the absence and in the presence of 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L zinc ion. At selected times, cells were fixed in 4% formaldehyde, washed with 1 × PBS and stained with 0.6% (w/v) oil red O solution for 15 min at room temperature. For quantification, cell monolayers were assayed using a spectrophotometer at 510 nm as described in materials and methods. The adipogenic differentiation inhibition rate was calculated according to the formula:

$$(1 - \text{OD}_{\text{treated}}/\text{OD}_{\text{control}})100\%.$$

Discussion and conclusion

The adipocyte is the most plentiful stromal cell. The mechanisms leading to adipocyte formation and the function of adipocyte have just begun to be elucidated.



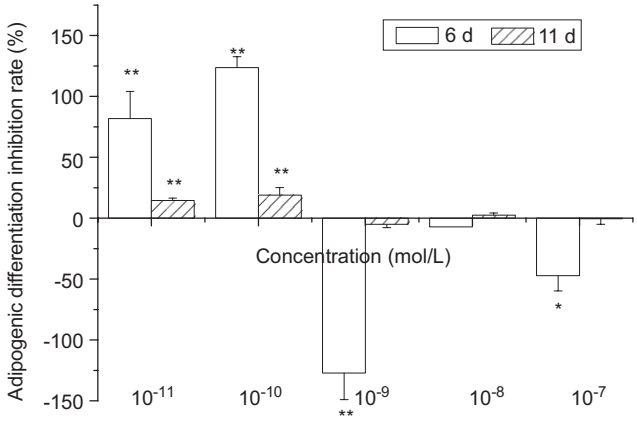


Fig. 6. Effect of zinc ion on the adipocytic trans-differentiation of osteoblasts ($^{*}P<0.05$, $^{**}P<0.01$ vs. control, $n=5$). The osteoblasts were cultured in a culture medium supplemented with 10 μg/mL insulin, 10^{-7} mol/L dexthamethone; and in the absence and in the presence of 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L zinc ion. At selected times, cells were fixed in 4% formaldehyde, washed with $1\times$ PBS and stained with 0.6% (w/v) oil red O solution for 15 min at room temperature. For quantification, cell monolayer was assayed by a spectrophotometer at 510 nm as described in materials and methods. The adipogenic differentiation inhibition rate was calculated according to the formula: $(1-OD_{treated}/OD_{control})100\%$.

Firstly, the differentiation of MSCs towards osteoblasts or adipocytes is reciprocal and regulated by several molecules, including hormones and local growth factors [19]. This provides the interesting possibility that the alteration of osteoblastogenesis and adipogenesis occurring in osteopenic conditions including aging and osteonecrosis resulting from an abnormality of the differentiation of the common precursor cell [17]. Besides that, it was reported that preadipocytes isolated from mouse marrow might regulate the activity and final differentiation of marrow precursors of osteoblasts. The condition medium harvested from mouse stromal preadipocytes decreased the ALP activity of a mouse stromal osteoblastic cell line [20]. In addition, some investigators suggest that adipocytes may take an important participation in hematopoietic and osteogenic processes by supplying the essential stromally derived

Fig. 5. Effect of zinc ion on adipogenic differentiation of MSCs. The MSCs were cultured in a culture medium supplemented with 10 μg/mL insulin, 10^{-7} mol/L dexthamethone; and in the absence (adipogenic supplement) and in the presence of zinc ion. On day 14, cells were fixed in 4% formaldehyde, washed with $1\times$ PBS and stained with 0.6% (w/v) oil red O solution for 15 min at room temperature, then washed with PBS and photographed at $\times200$: (a) adipogenic supplement, (b) adipogenic supplement + 1×10^{-10} mol/L zinc ion, (c) adipogenic supplement + 1×10^{-9} mol/L zinc ion.

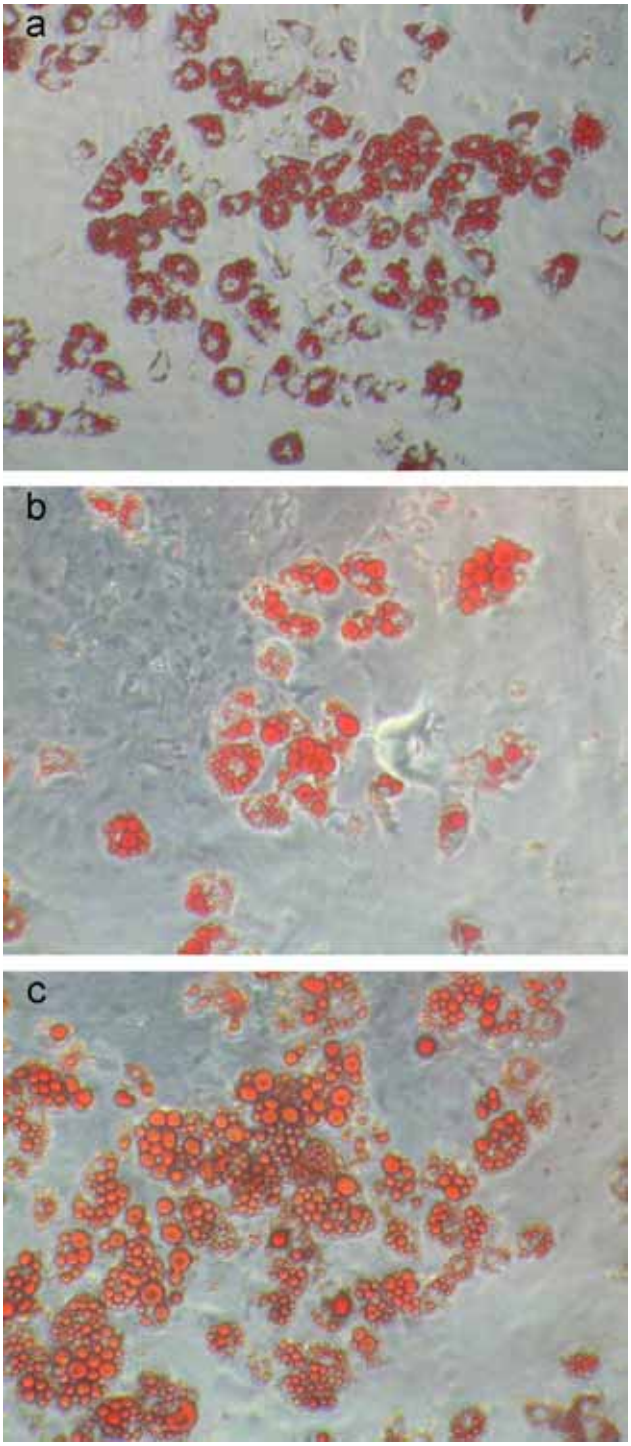


Fig. 7. Effect of zinc ion on adipocytic trans-differentiation of osteoblasts. The osteoblasts were cultured in a culture medium supplemented with 10 μg/mL insulin, 10^{-7} mol/L dexthamethone; and in the absence (adipogenic supplement) and in the presence of zinc ion. On day 6, cells were fixed in 4% formaldehyde, washed with 1 × PBS and stained with 0.6% (w/v) oil red O solution for 15 min at room temperature, then washed with PBS and photographed at × 200: (a) adipogenic supplement, (b) adipogenic supplement + 1 × 10^{-10} mol/L zinc ion, (c) adipogenic supplement + 1 × 10^{-9} mol/L zinc ion.

soluble and cell surface factors necessary for osteoclast differentiation and function in vitro [21]. Sakaguchi et al. [22] have demonstrated that adipocyte-enriched stromal cells support osteoclast formation. Benayahu et al. [23] reported that preadipocytes also have the potential to stimulate osteoclast differentiation. Adipocytes synthesized and released a variety of peptide and nonpeptide compounds and secrete cytokines such as tumor necrosis factor-α (TNF–α) and interleukin (IL-6). The main effect of these cytokines is a stimulation of bone resorption [24,25]. So a reversal of adipogenesis will be as important as therapeutic approaches to treating age-related osteoporosis and steroids induced osteoporosis.

In this study, we have examined the effects of zinc ion on osteogenic and adipogenic differentiation of MSCs and the adipogenic trans-differentiation of osteoblasts in vitro by employing isolated mouse primary bone MSCs and osteoblasts. Our results showed that: (1) cell growth was almost unaffected by zinc supplements except for individual concentrations, which was the basis of assessing the effect of zinc ion on differentiation; (2) zinc ion was able to inhibit both the osteogenetic and adipogenetic differentiation of MSCs, the extent of its inhibition effect depended on the concentrations and incubation time; (3) while, for osteoblasts, zinc ion depressed adipogenetic differentiation at lower concentrations, but the effect could turn to promotion or be completely diminished when concentration increased.

ALP is a membrane-bound exoenzyme that has been implicated in bone formation and mineralization. Recently, it is reported that osterix, a zinc-finger-containing transcription factor, enhanced proliferation and osteogenic potential of bone MSCs, and therefore induced an increase in the expression of other markers associated with the osteoblastic lineage [26,27]. An adenoviral-mediated overexpression of ZIP1, a zinc transporter in MSCs, increased expression of specific osteoblast-associated markers and several osteoblast differentiation genes [28]. However, our results showed that zinc decreased the expression of ALP activity of MSCs at all the tested concentrations. The difference may be caused by states of zinc ion (chelated or dissociated with proteins) and different cell models. In order to evaluate the effects of zinc ion accurately additional studies should be performed in vivo to define the role of zinc in proliferation and differentiation of MSCs. Our data showed zinc ion plays an important role in the suppression of adipogenic in the commitment of MSCs and osteoblasts. We therefore deduced that the zinc ion may protect bone by decreasing adipocytic cell formation from MSCs, which may indirectly promote osteoblast proliferation, mineralization and bone formation, and inhibiting osteoclast formation, activation and bone resorption by secreting less cytosine. Furthermore, some studies demonstrate that the IGF-I-induced

increase in protein tyrosine phosphatase activity and the effect of IGF-I in increasing proliferation of osteoblastic cells were significantly enhanced by culture with zinc [29]. Moreover, a direct stimulative effect of zinc on serum IGF-IGFBP-3, ALP and osteocalcin was also suggested [30]. So we could deduce that other mechanisms which were not assessed in the present study may be also involved in the progress of zinc ion benefaction on bone, as a local or systemic enhancement of growth factors. These mechanisms remain to be further elucidated.

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