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Effects of Zinc on the Mineralization of Bone Nodules from Human Osteoblast-like Cells

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ABSTRACT

Zinc is an important mineral that is required for normal bone development. However, the direct effects of zinc on the mineralization of bone cells of human origin are not clear. The objective of this study was to determine the effects of zinc on the differentiation of SaOS-2 human osteoblast-like cells and the formation of mineralized bone nodules. Cells were cultured for 8 d and then transferred to zinc-free medium and treated with varying concentrations (0–50 μ*M*) of zinc. Alkaline phosphatase (ALP) activity was used as a measure of osteoblast differentiation, and bone nodules were detected by von Kossa staining. After 4, 6, and 8 d of treatment, zinc increased ALP activity at 1 and 10 μ*M*, but decreased activity at 50 μ*M*. After 9 d of treatment, zinc increased both the number and area of mineralized bone nodules at low concentrations (1 and 10 μ*M*), but decreased both at higher concentrations (25 and 50 μ*M*). These findings demonstrate that zinc has biphasic effects on the differentiation and mineralization of human osteoblast-like cells.

Index Entries: Zinc; osteoporosis; SaOS-2 cells; bone; alkaline phosphatase.

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INTRODUCTION

Osteoporosis is a major cause of morbidity and mortality and is a growing public health concern. The causes of osteoporosis are multifactorial and include both genetic and environmental factors *(1,2).* Some of the modifiable risk factors include diet, smoking, physical inactivity, and alcohol consumption *(3).* Along with other essential nutrients, minerals such as zinc play a pivotal role in the formation of the bone matrix.

Although the human body contains approx 1.5–2.5 g zinc *(4),* which is stored in bone and muscle, the available pool, estimated at 157–183 mg zinc *(5),* is too small to provide a metabolic buffer. Kinetic modeling experiments with human subjects and animals have shown that the liver, pancreas, kidney, and spleen have very high rates of zinc turnover, whereas the nervous system and bone have slow turnover rates *(5).* Because there appears to be little zinc available as a stored reserve, deficiency can occur rapidly when dietary intake decreases *(6).*

Zinc plays an important role in the growth of humans and deficiency has been associated with abnormalities in bone growth, formation, and mineralization *(7–9).* Low zinc intake has been reported to be associated with low bone mass in women *(10)* as well as reduced serum zinc concentrations and increased urinary zinc excretion in women with osteoporosis *(11).*

Animal studies have shown that zinc deficiency causes low bone mass *(12,13).* Insufficient dietary intake of zinc causes a decrease in the number of osteoblasts and chondrocytes in animals bone *(14),* and in vitro studies show that zinc increases the number of murine osteoblastlike cells *(15).* Zinc is also involved in the stimulation of collagen production in rat femur and calvaria *(16).* Studies have shown that zinc has an inhibitory effect on bone resorption *(16)* as well as a stimulatory effect on bone formation and mineralization in osteoblastic cell cultures *(17).*

Alkaline phosphatase (ALP) activity has been used as a measure of bone formation in healthy and osteoporotic subjects *(18)* and requires zinc for its activity *(17).* ALP is produced by the osteoblast, especially during the bone formation phase *(19),* as a result of its key role in the formation and calcification of hard tissue *(20).* The enzyme is attached to the external surface of plasma membranes by phosphoethanolamine bound to oligosaccharides *(21),* where it hydrolyzes phosphate esters to increase the phosphate concentration and mineralization of the extracellular matrix *(21).* ALP is also a zinc metalloenzyme and contains two molecules of zinc that results in loss of activity when removed *(17).*

The aim of the present study was to investigate the direct effects of zinc on osteoblastic bone formation in a time- and dose-dependent manner. We performed this study using SaOS-2 human osteoblast-like cells, because this cell line has characteristics of human osteoblasts, including high steady-state levels of ALP activity *(17).*

MATERIALS AND METHODS

Materials

SaOS-2 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). HAM's F-12 medium was purchased from Gibco, and McCoy's 5A medium was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Cansera (Rexdale, Canada). Bio-Rad protein dye reagent was obtained from Bio-Rad (Mississauga, Canada). Kits for the quantification of ALP activity were purchased from Teco Diagnostics (Anaheim, CA), and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Treatment

Stock cultures of SaOS-2 cells were maintained in HAM's F-12 medium containing 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, 0.028 *M* HEPES buffer (pH 7.4), 1.1 m*M* $CaCl_2$ and 0.01% streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in T-75 culture flasks. When cells reached 80% confluence, they were seeded in 24-well plates at a density of 5×10^3 cells/well and supplemented with 0.005% ascorbic acid, 0.3% β-glycerophosphate and, 10 n*M* dexamethasone. Media were changed every 2 d for 6 d. On d 8, supplemented media were replaced with zinc-free McCoy's 5A medium. Cells were treated with varying concentrations of zinc (0, 1, 10, 25, and 50 µ*M*) dissolved in McCoy's 5A medium (containing 10% FBS, 2 m*M* L-glutamine, 0.028 *M* HEPES buffer, pH 7.4, 1.1 m*M* $CaCl_2$, 0.01% streptomycin, 0.005% ascorbic acid, 10 n*M* dexamethasone, and 0.3% β-glycerophosphate), which was replaced every 2 d until the end of the culture period.

Alkaline Phosphatase Activity

In order to determine the time-dependent influences of zinc on the differentiation of SaOS-2 cells, treatment was stopped after 2, 4, 6, and 8 d of treatment and ALP activity was measured. Cells were washed twice with 50 m*M* Tris-HCl, pH 7.3, and kept in 0.05% Triton X-100 lysis buffer overnight at –20°C. The cells were later thawed on ice and harvested by scraping *(22).* The ALP activity of samples was determined by colorimetric assay using a Teco diagnostics ALP reagent. This method is a modification of the reference method of the American Association for Clinical Chemistry *(23).* It is based on the principle that ALP hydrolyzes *p*-nitrophenyl phosphate to *p*-nitrophenol, which has a strong absorbance at 405 nm. The rate of increased absorbance at 405 nm is proportional to the ALP activity. Absorption was measured by a Fusion™ plate reader every 2 min for 15 readings. Protein concentrations of the cell lysates were determined using the commercial Bio-Rad protein assay *(24),* and ALP activity was expressed as units per milligram of protein.

Determination and Quantification of Mineralized Bone Nodules

In order to determine the number and area of mineralized bone nodules, they were visualized by the von Kossa staining technique *(25).* After 9 d of treatment (d 17 in culture), the cells were washed twice with phosphate-buffered saline (PBS), fixed in paraformaldehyde for 30 min and washed three times in water. The water was removed, silver nitrate solution was added, and the plate was exposed to ultraviolet light for 30 min, after which the plate was rinsed with water. Sodium thiosulfate was added for 2 min, the plate was then rinsed in water, and glycerol was added. The number and area of von Kossa-stained nodules were quantified by a FluorChem™ imaging system.

Statistical Analysis

Statistical analyses were performed using analysis of variance (one- and two-way ANOVA) and Tukey's HSD *t*-test for post hoc comparisons.

RESULTS

Alkaline Phosphatase Activity

Our results show that zinc has both a dose- and time-dependent effect on ALP activity (two-way ANOVA; dose and time as variables, $p<0.001$). Treatment with 1 or 10 μ*M* of zinc increased ALP activity compared to controls, but a higher concentration of zinc (50 μ*M*) decreased activity after 4 and 6 d of treatment (Fig. 1, Table 1). There was a decrease in ALP activity after 4 and 6 d ($p<0.05$) compared to 2 d of treatment in the control group treated with zinc-free media. There was also a decrease in ALP activity after 4 d in cells treated with 25 or 50 μ*M* zinc, which remained low until the end of the treatment period. ALP activity in cells from the group treated with 1 μ*M* of zinc decreased after 6 and 8 d, but not as low as in cells treated with higher concentrations of zinc. Changes in ALP activity over time in the group treated with 10 μ*M* of zinc were not statistically significant during the treatment period.

Effects of Zinc on the Formation of Mineralized Bone Nodules

After von Kossa staining, mineralized nodules were seen as black spots with the naked eye after 9 d of treatment (Fig. 2). Quantifying the nodules by the FluorChem imaging system showed that zinc caused a dose-dependent effect in the number and area of bone nodules (Fig. 3, Table). In cells treated with 1 or 10 μ*M* of zinc, there was an increase in the area of nodules by approx 1.6- and 2.4-fold, respectively ($p<0.01$). There was also an increase in the number of nodules by 1.4- and 1.7-fold, respectively. Results also

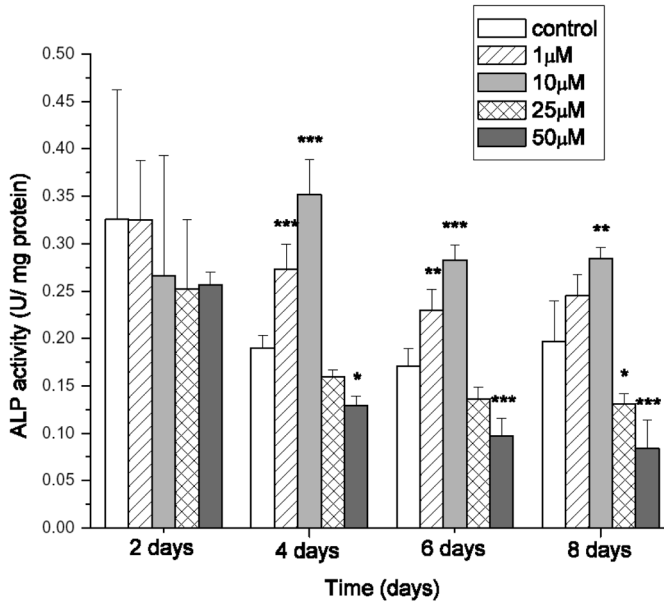

Fig. 1. Dose-dependent effects of zinc on ALP activities in SaOS-2 cells. Cells were grown for 8 d in HAM's F-12 medium followed by incubation in McCoy's 5A medium supplemented with varying concentration of Zn (0, 1, 10, 25, and 50 μ*M*) for 2, 4, 6, and 8 d. ALP activity of cell lysates were measured by a colorimetric assay and expressed as the average (*n*=4), with error bars representing ± SD (U/mg protein). Significant differences are compared to control at each time-point ***p< 0.001; **p< 0.01; *p<0.05;

Table 1
Dose-Dependent Effects of Zinc on ALP Activities in SaOS-2 Cells

ALP activity (U/mg protein)	Zinc concentration (μM)				
	0 μM	**1 μM**	**10 μM**	**25 μM**	**50 μM**
2 days	0.3254 ± 0.1373	0.3250 ± 0.0629	0.2659 ± 0.1267	0.2517 ± 0.0738	0.2565 ± 0.0135
4 days	0.1898 ± 0.0133	0.2729 ± 0.0268[a]	0.3512 ± 0.0370[a]	0.1594 ± 0.0072	0.1294 ± 0.0095[c]
6 days	0.1705 ± 0.0193	0.2297 ± 0.0161[b]	0.2829 ± 0.0162[a]	0.1364 ± 0.0124	0.0968 ± 0.0184 [a]
8 days	0.1969 ± 0.0431	0.2449 ± 0.0225	0.2845 ± 0.0118[b]	0.1312 ± 0.0104[c]	0.0837 ± 0.0299 [a]

Note: Cells were grown for 8 d in HAM's F-12 medium followed by incubation in McCoy's 5A medium supplemented with varying concentration of Zn (0, 1, 10, 25, and 50 μ*M*) for 2, 4, 6 and 8 d. ALP activity of cell lysates were measured by a colorimetric assay and expressed as the average (*n*=4) ± SD (U/mg protein). Significant differences are compared to control at each time-point.

[a]p< 0.001; [b]p< 0.01; [c]p<0.05.

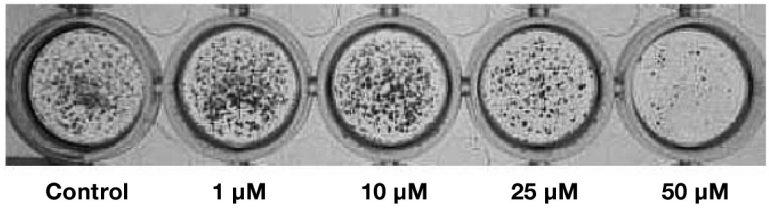

Fig. 2. Dose-dependent effects of zinc on the formation of mineralized nodules in a 24-well plate fixed on d 17 and stained by von Kossa. Cells were grown for 8 d in HAM's F-12 medium followed by incubation in McCoy's 5A medium supplemented with varying concentration of Zn (0, 1, 10, 25, and 50 µ*M*) for 9 d.

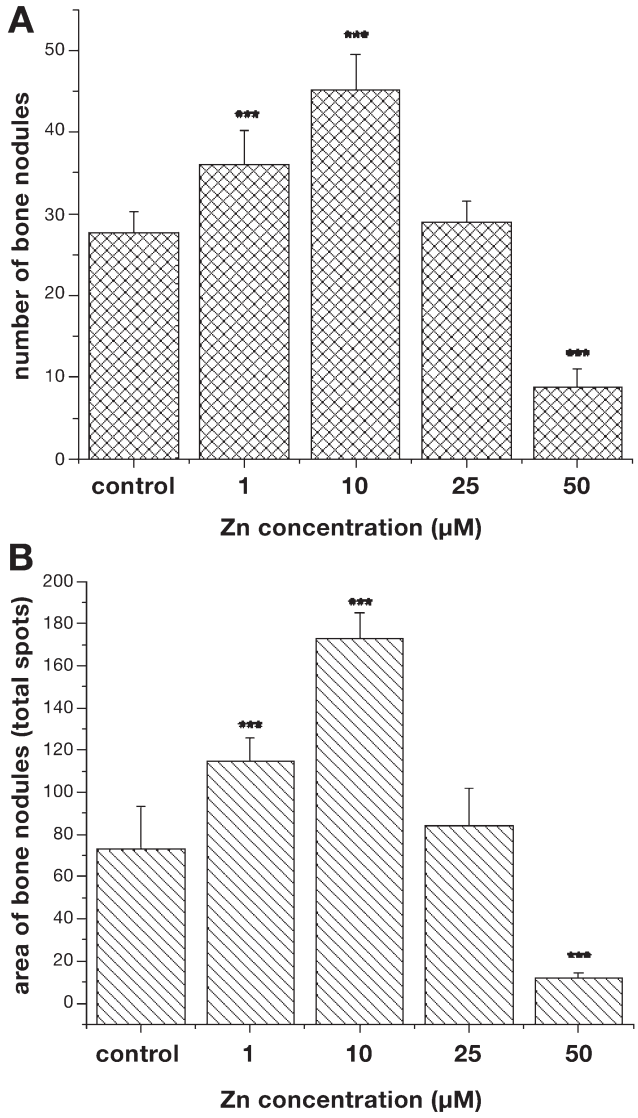

Table 2
Dose-Dependent Effects of Zinc
on Mineralized Bone Nodules

A

	Zinc concentration (μM)				
	0 μM	**1 μM**	**10 μM**	**25 μM**	**50 μM**
Number of bone nodule	27.75 ± 2.5	36 ± 4.16[a]	45.25 ± 4.19[a]	29 ± 2.58	8.75 ± 2.22[a]

B

	Zinc concentration (μM)				
	0 μM	**1 μM**	**10 μM**	**25 μM**	**50 μM**
Are of bone nodule	72.75±20.61	114.75±10.78[a]	172.75±12.45[a]	84.25±17.80	11.75±2.5[a]

Note: Cells were grown for 8 d in HAM's F-12 medium followed by incubation in McCoy's 5A medium supplemented with varying concentration of zinc (0, 1, 10, 25, and 50 μ*M*) for 9 d. Cells were stained by von Kossa techniques on d 17 and the number **(A)** and area **(B)** of mineralized nodules formed were quantified by the FluorChem imaging system. Data are expressed as the average (n=4) ± SD.

[a]Significant difference from the control group, p<0.01

showed no significant difference between area or number of nodules in cells treated with 25 μ*M* of zinc compared to the control, whereas the area and number of nodules in cells treated with 50 μ*M* of zinc significantly decreased.

DISCUSSION

Zinc deficiency is known to cause impaired bone growth *(26)*. The mechanism by which zinc stimulates bone growth has not been fully elucidated, although it has been shown to stimulate bone formation and inhibit bone resorption in rats *(27)*. The present study was undertaken to assess the time- and dose-dependent influence of zinc on osteoblast differentiation as well as on the formation of mineralized bone nodules from SaOS-2 cells. ALP activity was used as a marker of early osteoblast differentiation and might be an indicator of the metabolic activity of bone *(18,19)*.

Fig. 3. Dose-dependent effects of zinc on mineralized bone nodules. Cells were grown for 8 d in HAM's F-12 medium followed by incubation in McCoy's 5A medium supplemented with varying concentration of zinc (0, 1, 10, 25, and 50 μ*M*) for 9 d. Cells were stained by von Kossa techniques on d 17 and the number **(A)** and area **(B)** of mineralized nodules formed were quantified by the FluorChem imaging system. Data are expressed as the average (n=4), with error bars representing ± SD. ***Significant difference from the control group, p<0.01.

After seeding SaOS-2 cells into 24-well plates, the cells go through two main stages: proliferation and differentiation of osteoblasts into mature osteoblasts that are capable of producing bone nodules *(28).* Cells were first grown in HAM's F-12 medium for the first 8 d of culture because it contains 3 μ*M* zinc, which is necessary for differentiation *(29).* In order to determine the time- and dose-dependent effects of zinc on ALP activity, cell cultures were maintained in zinc-free McCoy's 5A medium instead of HAM's F-12 medium with varying concentration of zinc (0, 1, 10, 25, and 50 μ*M*) for 2, 4, 6, and 8 d.

As shown in Fig. 1 and Table 1, ALP activity in untreated cells gradually decreased after 4 and 6 d of treatment. The decrease in ALP activity in untreated cells over time could have been caused by switching from HAM's F-12 medium, which contains 3 μ*M* of zinc, to zinc-free McCoy's 5A medium on d 8. It is likely that the pool of zinc in these cells was gradually depleted during this time, resulting in a decrease in ALP activity. This observation confirms that zinc is required for ALP activity and optimal osteoblast function.

Our findings demonstrate that ALP activity in cells treated with 1 or 10 μ*M* of zinc significantly increases ALP activity whereas treatment with 50 μ*M* zinc significantly decreases ALP activity compared to zinc-free cells. Studies by others also showed an effect of zinc on ALP activity in bone tissue *(16,17,27).* Previous animal studies showed increases in skeletal ALP activity in the femoral diaphyses of rats that had been fed a zinc-rich diet *(16),* a decrease in skeletal ALP activity in the bones of zinc-deficient rats *(30),* and a dose-dependent relationship between dietary zinc and skeletal ALP in the tibia of adult female mice *(31).* A study performed using SaOS-2 and MG-63 cells and cells derived from normal human vertebral tissue also showed that zinc increases ALP activity in a dose- and time-dependent manner *(17).* It has also been shown that zinc increases the half-life of ALP activity without an increase in ALP mRNA or increase in the level of ALP-immunoreactive protein *(17).* These results are supported by the observation that ALP is a Zn metalloenzyme, which contains two molecules of Zn/enzyme monomer *(32),* and this enzyme-bound zinc is required for its activity. Removal of zinc by chelation results in irreversible loss of enzyme activity. This process could be modulated by phosphate because its binding to the active center of ALP prevents or inhibits the irreversible dissociation of zinc *(17).* In vitro studies have shown that zinc increases ALP activity in SaOS-2 cells, whereas other divalent cations, such as Ca, Mn, Mg, Fe, Co, and Cu, do not *(17).* This zinc-dependent increase in ALP activity was not unique to SaOS-2 cells. Zn also increased the cellular level of ALP activity in MG-63 human osteosarcoma cells and cell lines established from outgrowths of normal human vertebral tissue *(17).*

The observed increase in ALP activity and bone nodule formation with 1 μ*M* zinc, compared to zinc-free media, is consistent, with the normal range of zinc in human serum being 0.6–1.5 μ*M (33).* Treatment with

10 μ*M* zinc resulted in a greater effect on bone nodule formation and ALP activity. However, cells treated with 25 or 50 μ*M* zinc showed a decrease in ALP activity. The reason for this decrease is not clear, but it might be related to suppression of gene expression, regulatory effects of zinc in signal transduction, effects on growth factors, or possible toxic effects. A study performed using SaOS-2 cells has shown that 100 μ*M* zinc decreased cell layer protein, suggesting a cytotoxic effect *(17).*

The process of formation of mineralized nodules has three steps: proliferation, matrix synthesis and maturation, and mineralization, which is determined by the expression of a variety of markers such as osteocalcin, osteopontin, bone sialoprotein, and ALP activity *(34).* ALP activity generally increases during the proliferation and maturation steps, but decreases when mineralization is well progressed *(34).* Thus, after 4 d of treatment, when ALP activity reached its peak, was likely the time when proliferation and maturation were the highest.

Osteoblasts are responsible for synthesizing, secreting, organizing, and mineralizing the bone matrix, or osteoid *(35).* The osteoid, which is composed of type I collagen and other proteins, normally undergoes rapid mineralization with calcium and phosphorus, forming hydroxyapatite [$Ca_{10}(PO_4)_6(OH_2)_2$)] *(35).* Our results demonstrate that cells treated with 1 or 10 μ*M* zinc increase and cells treated with 50 μ*M* decrease the area and number of nodules compared to controls. This finding is consistent with results for ALP activity, which showed an increase after treatment with 1 or 10 μ*M* zinc, but a decrease after treatment with 50 μ*M* zinc.

In summary, the present study demonstrates a direct effect of zinc on osteoblastic bone formation in cells of human origin. Inadequate intakes of zinc might, therefore, be an important risk factor for the development of osteoporosis.

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