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Original Article

Hylonome

Autometallographic tracing of zinc ions in growing bone

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*In memory of Lis Mosekilde

Abstract

It has previously been established that zinc (Zn) supplementation increases bone dimensions and strength in growing rats. The present study aims at describing differences in the localization of loosely bound or free zinc ions, as revealed by autometallography (AMG), that might take place in the skeleton of growing rats following alimentary zinc depletion and supplementation. Male Wistar rats, 4 weeks old, were randomly divided into three groups. The rats had free access to a semi-synthetic diet with different amounts of zinc added. Group 1 was given a zinc-free (2 mg zinc/kg) diet, group 2 a 47 mg zinc/kg diet, and group 3 a 60 mg zinc/kg diet. All animals were killed after 4 weeks. Animals from each group were transcardially perfused with a 0.1 % sodium sulphide solution according to the zinc specific Neo-Timm method causing zinc ions to be bound in AMG catalytic zinc-sulphur clusters. We found clusters of zinc ions localized in the mineralizing osteoid in all groups. No immediate differences in AMG staining intensity could be observed between the groups neither in the uncalcified bone nor in the osteoblasts. However, alimentary zinc supply resulted in an increase in the height of the total growth plate in a dose-dependent manner. Zinc ions were also observed in chondrocytes throughout the whole thickness of the articular and the epiphyseal cartilage as well as in the inner layer of the synovial membrane.

Keywords: Rat Model, Alimentary Zinc Depletion and Supplementation, Autometallography (AMG)

Introduction

Zinc has been demonstrated to play an important role in bone metabolism and is required for normal growth of the human and animal skeleton[1–7]. Furthermore, it has been shown that the concentration of zinc is higher in bone than in most other tissues[8–12].

Clinically, zinc deficiency is known to be associated with retarded growth, alopecia, dermal lesions, and hypogonadism. Congenital skeletal disorders, spontaneous abortion, and foetus mortus are seen associated with maternal zinc deficiency[2]. It has also been suggested that zinc plays an important role in the development of osteoporosis[5,13–17] and osteoporotic fractures[3,18].

One pool of zinc is present in bone as loosely bound or free zinc ions, that can be traced by AMG in secretory vesicles of osteoblasts and in vesicles of the uncalcified bone matrix[19,20]. Such vesicular pools of zinc ions are well known from different exo- and endocrine secretory glands and from the nervous system[9,21,22]. Neurons in the central nervous system (CNS), that harbour zinc ions in a fraction of their synaptic vesicles are called zinc enriched (ZEN) neurons[22]. The expression of free zinc ions, in particular in vesicular compartments, is a widespread principle in the mammalian organisms, as it is in fish, lizards, and frogs. Zinc ions have been AMG traced in a variety of secretory cells, e.g., prostata, pancreas, and salivary glands of male mice, or pituitary and outside cells e.g., in uncalcified bone matrix, ejaculates, and in the epididymis[9,11,12,20–23].

Terminals of ZEN neurons in the brain have been found to have zinc ion transporter molecules (ZnT-3)[24] in their membranes, and other transmembrane transporter molecules have been found in the mammalian organism (ZnT-5)[25]. The ZnT-5 gene is suggested to play a role in osteoblast maturation, and its depletion results in impaired function of osteocytes, reduced bone formation, poor skeletal growth, and osteoporosis[25].

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Group	1	2	3
Alimentary zinc content (mg/kg)	2	47	60
Final body weight (g)	108.1 ± 13.3	218.3 ± 13.5[a]	276.4 ± 18.5[a,b]
Femur length (mm)	27.22 ± 1.02	29.58 ± 0.83[a]	32.96 ± 0.83[a,b]
Tibial growth plate height (mm)	0.431 ± 0.050	0.895 ± 0.065[a]	1.140 ± 0.117[a,b]

Key: [a]Significantly different from group 1; [b]Significantly different from group 2.

Table 1.

The autometallographic (AMG) zinc techniques are based on *in vivo* or *in vitro* binding of zinc ions as zinc sulphide or zinc selenide molecules, which create nanocrystals of zinc-sulphur or zinc-selenium. These crystal lattices are catalytic to the AMG developer and will therefore be silver enhanced when placed in an AMG developer[20–22,26,27]. Zinc ions can also be traced by the fluorescent probes 6-methoxy-8-p-toluene sulfonamide quinoline (TSQ)[23] and zinquin[28]. However, since these techniques only allow low magnifications and have to be analyzed within a short period of time after being prepared, we have preferred to use the Neo-Timm AMG approach[26] to trace the zinc ions[20]. In order to ensure that the AMG staining was caused by zinc ions we performed the obligatory controls including blocking the zinc ion pools *in vivo* with the low toxic chelator diethyldithiocarbamate (DEDTC)[26].

The purpose of the present study was to trace changes in the amount and localization of the zinc ion pools following alimentary zinc depletion and supplementation in growing rats.

Materials and methods

Animals and diets

Forty-five male Wistar rats, aged 4 weeks (Møllegaards Breeding Center Ltd, Ejby, Denmark) were used. The animals were housed in pairs in metal-free cages in rooms with a controlled temperature (21±2°C) and a 12:12 h light/dark cycle. They were given free access to food and distilled water. The rats were randomly divided into three groups and received a semi-synthetic diet (Altromin, a special recommended diet for laboratory rodents, Brogaarden, Gentofte, Denmark) with different amounts of zinc added.

Group 1 (n= 15) received a zinc-free diet containing 2.042 mg zinc/kg; group 2 (n= 15) received a normal diet containing 47 mg zinc/kg; and group 3 (n= 15) were fed with a diet supplemented to 60 mg zinc/kg.

The animals were sacrificed after 4 weeks in the following way: three animals from each group were anesthetized with Mebumal 50 mg/ml and transcardially perfused for 10 minutes with 0.5% sodium sulphide solution, followed by perfusion with 3% glutaraldehyde in a 0.1 M phosphate solution for 3 minutes. Both hind limbs were dissected from the body and postfixed for 1–4 hours in the glutaraldehyde fixative. Longitudinal, 200 µm-thick, femoral sections including the epiphysis, the metaphysis, and the lower part of the diaphysis were cut on a diamond precision-parallel saw (Exakt; Apparatebau, Otto Hermann, Norderstedt, Germany). The sections were dipped in a 0.5% gelatin solution, and AMG-developed for 60–90 minutes. The other 36 animals were killed by decapitation.

AMG development

The AMG method has formerly been described in detail[20]. In brief:

- Protective colloid: Dissolve 2 kg crude gum arabic resin drops in 4 l de-ionized water, stir intermittently for 5 days and then filter through several layers of gauze. Store the colloid in plastic jars and place it in a freezer.
- Citrate buffer: Dissolve 25.5 g citric acid and 23.5 g sodium citrate in 100 ml distilled water.
- Reducing agent: Dissolve 0.85 g hydroquinone in 15 ml distilled water at 45°C.
- Silver ion supply: Dissolve 0.1 g silver lactate in 15 ml 40 °C distilled water in a jar wrapped in lightproof foil.

60 ml protective colloid was mixed with 10 ml citrate buffer and 15 ml hydroquinone. 15 ml silver lactate was added just before the AMG developer was poured into vials containing the bone sections. The vials were placed in a water bath at 26°C and covered with a cardboard box to shield the vials from excessive light during the 60–90 minutes developing period. The AMG developing process was stopped by replacing the AMG developer with a 5% sodium thiosulphate solution. The sections were then carefully rinsed several times in distilled water.

The sections were embedded undecalcified in Technovit 9100 (Heraeus Kulzer; Werheim/Ts., Germany). These Technovit 9100 embedded bone sections were cut into 10 µm thick sections on a Jung model K microtome (R. Jung GmbH, Heidelberg, Germany) and counterstained with toluidine blue.

Proximal tibial metaphysis

The proximal tibial metaphyses from three animals from each group were embedded in Technovit 9100 and cut into

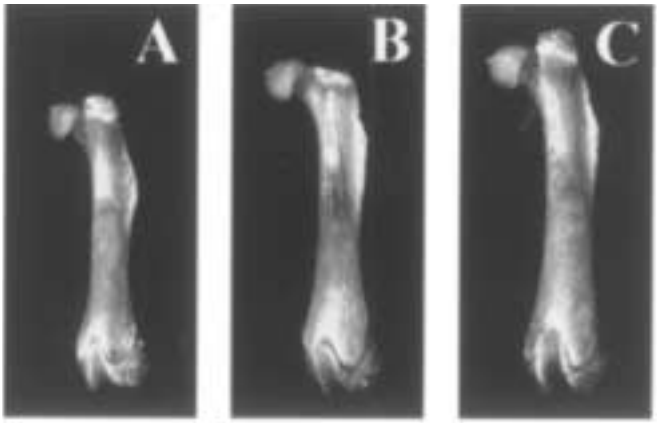

Figure 1. Femora. Group 1 (2 mg/kg) (A), group 2 (47 mg/kg) (B), and group 3 (60 mg/kg) (C).

10 μm thick sections on the microtome. The sections were stained with Goldner trichrome in order to measure the height of the growth plate, and in order to perform a histological description.

The remaining 12 animals from each of the three groups were used for assessment of bone growth and strength due to the different zinc diets. These results have been reported elsewhere[29].

Controls

Four additional animals were used as controls for the specificity of the autometallographic sulphide silver method. They were treated intraperitoneally with an aqueous solution containing DEDTC (1000 mg DEDTC per kg body weight), then allowed to live for 1 hour before they were perfused and processed as described above. Finally, bones from animals not treated with sulphide were used as blank controls.

Femoral length and tibial growth plate height

The femoral length was measured from the top of the caput femoris to the distal femoral condyles with an electronic caliper.

The 10-μm-thick Goldner trichrome stained tibial sections were placed in a microscope (BZ-40; Olympus, Tokyo, Japan) equipped with a digital microscope camera (DP11; Olympus, Tokyo, Japan) and images were acquired at a magnification of x100. The metaphyseal and epiphyseal borders of the growth plate were defined by the extent of the Goldner trichrome staining of the cartilage. The height of the growth plate was determined by averaging over 5 equidistant test lines that had been superimposed over the digitized image of the growth plate. The test lines were orientated parallel to the long axis of the tibiae.

Results

Rats in group 1 showed general signs of zinc deficiency, including dermal lesions on the extremities, the tail, and in particular around the eyes.

Animal body weight and femoral length (15 animals from each group) (Table 1 and Figure 1)

At the time of sacrifice, the mean body weight and the mean femoral length of the animals was significantly lower in the zinc-depleted group and significantly higher in the zinc-supplemented group, thus exhibiting a dose-dependent response.

Tibial growth plate height (3 animals from each group) (Table 1)

The study showed significant differences in the height of the growth plate of the animals in the three groups. The growth plate heights followed a dose-dependent pattern with respect to the zinc content of the diet fed to the rats.

Histology (3 animals from each group)

Histological description, Goldner trichrome (Figure 2)

The epiphyseal cartilage was narrower and the hypertrophic chondrocytes fewer in the zinc deficient animals than in the zinc supplemented rats. Measurements recorded from the three groups (Figure 2A–C) showed an increase in the height of the total growth plate, the majority of which is accounted for by an increase in the height of the hypertrophic zone and the irregular-shaped lacunae with lack of the straight columnar arrangement in the hypertrophic layer in the groups supplemented with zinc (Figure 2B–C) compared with the zinc-depleted group (Figure 2A).

In the specimens stained with Goldner trichrome there also seemed to be less osteoid in the alimentary zinc deficient group than in the other two groups, however this was not quantified. Consequently, it was not possible to identify any difference between the three groups in the amount of mineralized bone.

ZnS AMG detection of zinc ions (Figure 3)

1. Bone tissue:

The ZnS AMG grains were concentrated intracellularly in the osteoblasts and osteocytes and extracellularly in the osteoid in all three groups. ZnS AMG grains were present in a relatively large number in the mineralizing osteoid at the endocortical bone surfaces with active bone formation, where the osteoblasts are present in a layer of small cylindrical cells appearing like an epithelium (Figure 3A).

Not all osteocytes contained AMG grains, but in some areas, especially near bone surfaces (endocortically and periosteally), the osteocytes demonstrated AMG grains along their osteocytic processes (Figure 3B). ZnS AMG

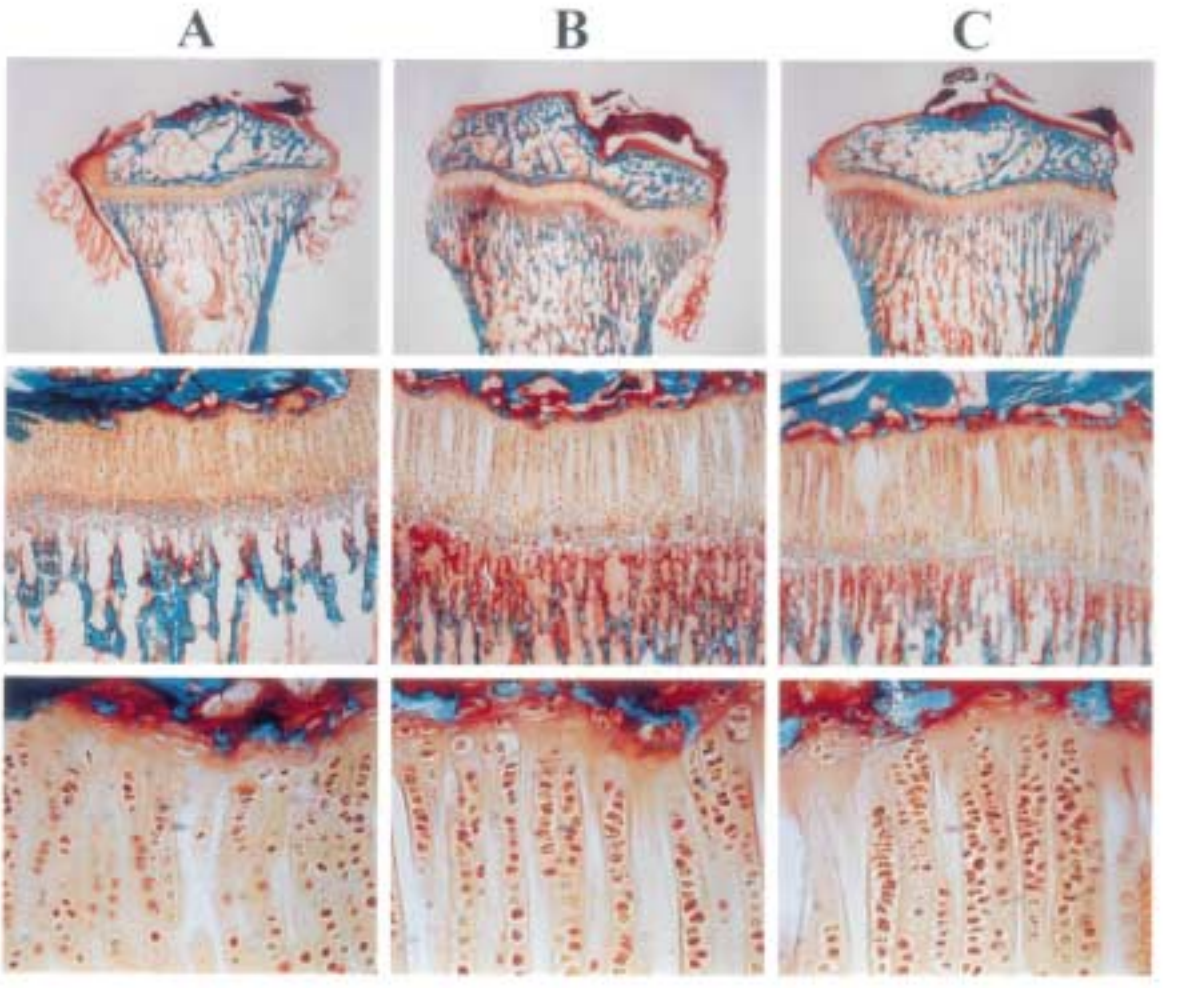

Figure 2. Goldner trichrome stained sections showing the proximale part of the tibia including the epiphyseal cartilage of the three groups at original magnifications of x12.5 (upper row), x100 (middle row), and x400 (lower row). Group 1(A), group 2 (B), and group 3 (C).

grains were also found extracellularly on the longitudinal partitions running between the hypertrophic chondrocytes (Figure 3A), i.e., in places where the calcification of the matrix is known to commence. The mineralized bone was void of ZnS AMG grains. No resorptive surfaces, and therefore no osteoclasts, were seen in the sections. It was not possible to demonstrate any differences in the concentration or pattern of the ZnS AMG grains in the three groups.

2. Joint cartilage:

In the femora, joint cartilage zinc ions were traced in the cytoplasma of all the chondrocytes, throughout the whole thickness of the cartilage, but chondrocytes in the proliferations zone had the highest content of AMG grains (Figure 3C–D).

3. Synovial membrane:

The one to four cells thick layer of ellipsoidal cells of the synovial membrane contained AMG grains in their cytoplasma and especially in the inner layer of the membrane (Figure 3E–F).

Controls

Bone sections from animals that had been treated with dieethyldithiocarbamate (DEDTC) before being perfused with sodium sulphide, were completely devoid of silver gains, thereby showing that the zinc ions had been chelated and therefore could not be bound in zinc-sulphur clusters[21,26,27]. The same was found for control sections from animals, that had not been perfused with sodium sulphide. AMG grains were not found in the osteoid (Figure 4A), the chondrocytes of the joint cartilage (Figure 4B), or in the synovial membrane (Figure 4C) of the DEDTC treated animals.

Discussion

We have previously established that alimentary zinc supplementation causes increased bone dimensions and strength in growing rats[29]. In the present study, we used the

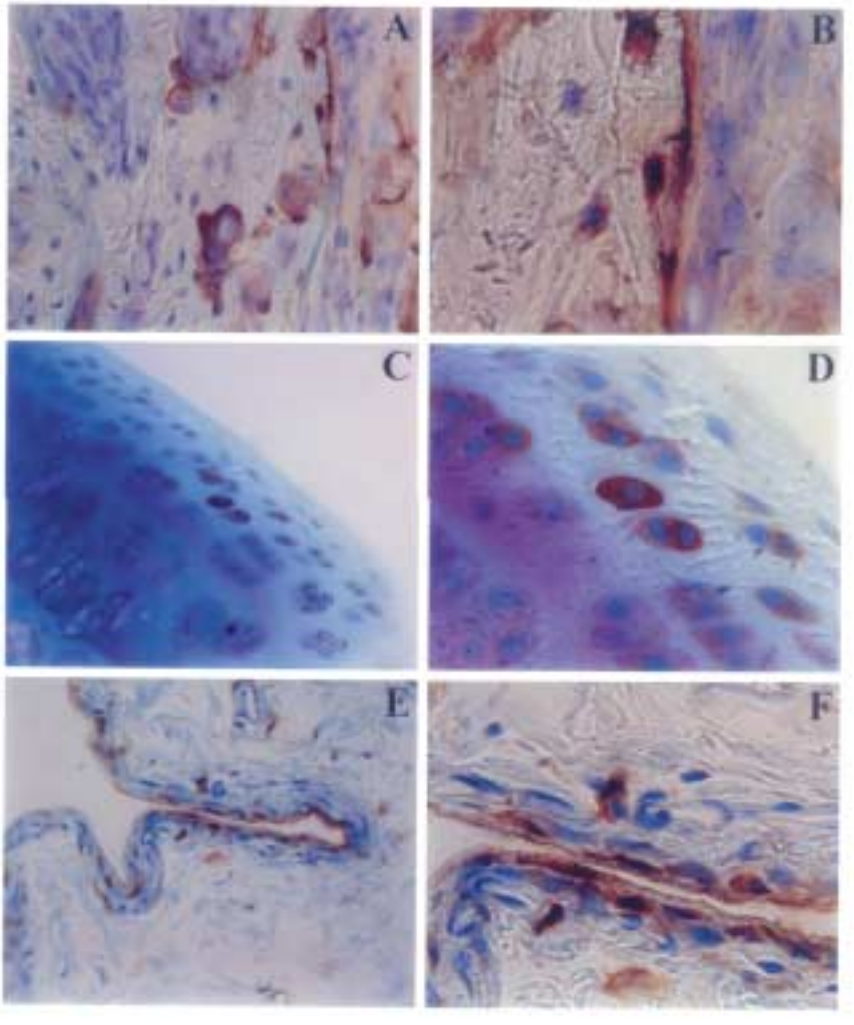

Figure 3. Toluidine blue stained sections showing the mineralizing front with osteoblasts and osteocytes. Autometallographic silver grains are seen both intracellularly and in the matrix x400 (A), osteocytes with osteocytic processes loaded with AMG grains x1000 (B). Sections showing the distal part of the femora including the articular cartilage; AMG grains are seen in the cytoplasma of the chondrocytes x400 and x1000 (C–D). Sections showing the synovial membrane of the joint of the distal part of the femora; AMG grains are detected in the cytoplasma of the ellipsoidal cells of the synovial membrane x400 and x1000 (E–F).

autometallographic technique (ZnS AMG) to evaluate whether the pattern or concentration of zinc ions in the skeleton of growing rats differs in relation to different levels of alimentary zinc supplementation.

In growing rats, calcified cartilage serves as a template for bone formation (i.e., endochondral ossification). Matrix vesicles (MV) are known to be involved in the induction of calcification on growth plate cartilage (hypertrophic zone)[30,31]. Sauer et al. isolated MV from chicken growth plates and found that zinc ions act as an endogenous regulator of MV Ca^{2+} uptake[6]. This hypothesis has been supported by others[30–33], and we found zinc ions in what we believed to be MV in the unmineralized bone matrix with the ZnS-AMG technique[20]. This ZnS AMG technique is, as mentioned earlier, based on the binding of zinc ions in the tissues as nanocrystals of zinc-sulphur atoms, and has been applied to different biological tissues[8,9,11,34]. The nanocrystals are catalytic to AMG silver enhancement and can be magnified to sizes where they can be seen directly and at LM and EM levels[20–22,26,35]. Our results confirmed the presence of high numbers of zinc ions in non-mineralized osteoid and osteoblasts, and our findings are in accordance with the results by Calhoun et al. who found that zinc is required at the site of bone formation as a requisite for complete calcification[2].

Several authors have isolated chondrocytes from the growth plate and have suggested zinc as a regulator of the calcification[32,33]. However, to the best of our knowledge nobody has previously located Zn ions in the chondrocytes

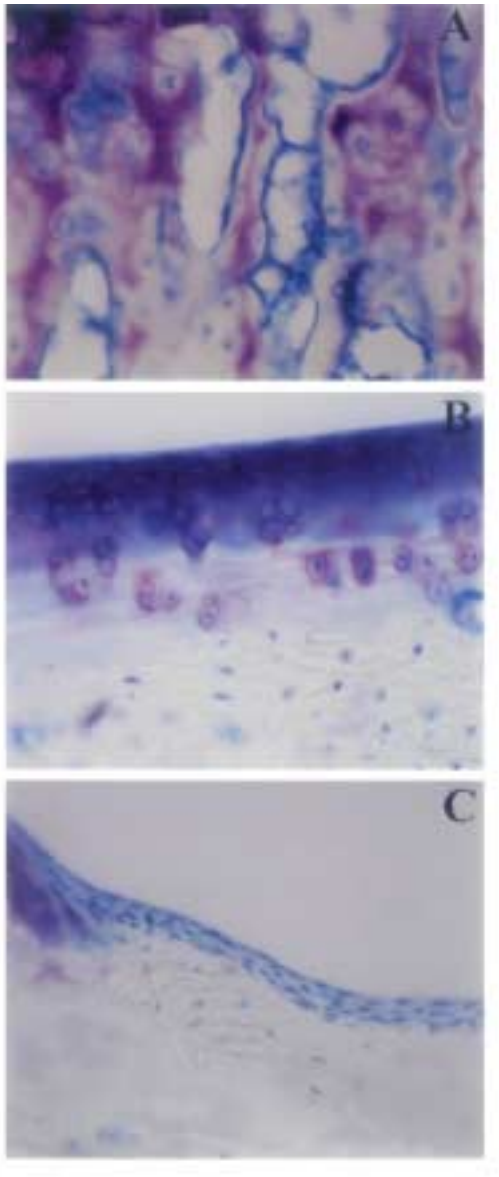

Figure 4. Toluidine blue stained sections from animals treated with diethyldithiocarbamate (DEDTC). Section showing the mineralizing front with osteoblasts and osteocytes x400 (A). Section showing the articular cartilage with chondrocytes x400 (B). Section showing the synovial membrane of the joint with the elipsoidal cells x400 (C). No AMG gains can be detected in any of the sections.

of the articular cartilage. We have demonstrated the presence of ZnS AMG grains both in the superficial layer and in the deeper partially calcified layer of the articular cartilage, and we therefore suggest that zinc ions may be important in the regulation of the calcification of cartilage. Moreover, it has been shown that zinc deficiency inhibits the proliferation of chrondrocytes[36] and that zinc supplementation stimulates the proliferation of epiphyseal growth plate chondrocytes[37]. This may indicate that zinc is involved in not only the regulation of the calcification of cartilage, but also plays a role in the regulation of the formation of epiphyseal cartilage and thereby in longitudinal bone growth[38].

The presence of zinc ions in the upper epithelial cell layers of the synovial membrane is not understood, but it might indicate that synovia contains free zinc ions, secreted from these cells which are important to the nourishment of the joint cartilage.

In this study we also found that alimentary zinc supplementation resulted in an increase of the body weights, the length of the femora and the height of the growth plate in a dose-dependent manner. This could be explained by the fact, that zinc has been suggested to have an anabolic effect on bone metabolism which mimics that of growth hormone (GH) and insulin-like growth factor I (IGF-I)[29]. Several studies have shown that low zinc intake is associated with low concentrations of IGF-I and therefore retarded growth. However, IGF-I infusion to the zinc depleted animals/humans does not reverse the growth retardation[5,14,39,40]. It has therefore been suggested that zinc in some way is essential for IGF-I induction of cell proliferation and transforming growth factor-β in osteoblastic cells *in vitro*[41,42].

In conclusion, based on a modified autometallographic Timm sulphide silver method, we have demonstrated that zinc ions are present in osteoid bone, synovial membrane, and cartilage, and that alimentary supplementation of zinc in growing rats increases bone metabolism in a dose-dependent manner. The increased bone metabolism is corroborated by the increased thickness of the epiphyseal plate, increased length of the femora, and increased body weights.

However, the exact role of Zn ions on bone-metabolism has not been elucidated yet, and needs further investigations. But we believe that the presented method for identification of Zn ions could play an important role for these further investigations.

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References

1. Bergman B. The zinc concentration in hard and soft tissues of the rat. The influences of zinc deficient feeding. Acta Odontol Scand 1970; 28:435–440.
2. Calhoun NR, Smith JC Jr, Becker KL. The role of zinc in bone metabolism. Clin Orthop 1974; 103:212–234.
3. Eberle J, Schmidmayer S, Erben RG, Stangassinger M, Roth HP. Skeletal effects of zinc deficiency in growing rats. J Trace Elem Med Biol 1999; 13:21–26.
4. Nakamura T, Nishiyama S, Futagoishi-Suginohara Y, Matsuda I, Higashi A. Mild to moderate zinc deficiency

in short children: Effect of zinc supplementation on linear growth velocity. J Pediatr 1993; 123:65–69.
5. Ninh NX, Thissen JP, Collette L, Gerard G, Khoi HH, Ketelslegers JM. Zinc supplementation increases growth and circulating insulin-like growth factor I (IGF-I) in growth-retarded Vietnamese children. Am J Clin Nutr 1996; 63:514–519.
6. Yamaguchi M, Oishi H, Suketa Y. Stimulatory effect of zinc on bone formation in tissue culture. Biochem Pharmacol 1987; 36:4007–4012.
7. Yamaguchi M, Yamaguchi R. Action of zinc on bone metabolism in rats. Increases in alkaline phosphatase activity and DNA content. Biochem Pharmacol 1986; 35:773–777.
8. Epand RM, Stafford AR, Tyers M, Nieboer E. Mechanism of action of diabetogenic zinc-chelating agents. Model system studies. Mol Pharmacol 1985; 27:366–374.
9. Frederickson CJ, Danscher G. Zinc-containing neurons in hippocampus and related CNS structures. Prog Brain Res 1990; 83:71–84.
10. Moonga BS, Dempster DW. Zinc is a potent inhibitor of osteoclastic bone resorption *in vitro*. J Bone Miner Res 1995; 10:453–457.
11. Stoltenberg M, Lund L, Juhl S, Danscher G, Ernst E. Histochemical demonstration of zinc in human epididymis using autometallography. Histochem J 1997; 29:721–726.
12. Stoltenberg M, Sørensen MB, Danscher G. Histochemical demonstration of zinc ions in ejaculated human semen. Int J Androl 1997; 20:229–236.
13. Atik OS. Zinc and senile osteoporosis. J Am Geriatr Soc 1983; 31:790–791.
14. Devine A, Rosen C, Mohan S, Baylink D, Prince RL. Effects of zinc and other nutritional factors on insulin-like growth factor I and insulin-like growth factor binding proteins in postmenopausal women. Am J Clin Nutr 1998; 68:200–206.
15. Herzberg M, Foldes J, Steinberg R, Menczel J. Zinc excretion in osteoporotic women. J Bone Miner Res 1990; 5:251–257.
16. Ohry A, Shemesh Y, Zak R, Herzberg M. Zinc and osteoporosis in patients with spinal cord injury. Paraplegia 1980; 18:174–180.
17. Rico H, Villa LF. Zinc, a new coherent therapy for osteoporosis? Calcif Tissue Int 2000; 67:422–423.
18. Elmståhl S, Gullberg B, Janzon L, Johnell O, Elmståhl B. Increased incidence of fractures in middle-aged and elderly men with low intakes of phosphorus and zinc. Osteoporos Int 1998; 8:333–340.
19. Berg JM, Shi Y. The galvanization of biology: A growing appreciation for the roles of zinc. Science 1996; 271:1081–1085.
20. Danscher G, Mosekilde Li, Rungby J. Histochemical detection of zinc in mineralizing rat bone: Autometallographic tracing of zinc ions in the mineralization front, osteocytes, and osteoblasts. J Histotechnol 1999; 22:85–90.
21. Danscher G. The autometallographic zinc-sulphide method. A new approach involving *in vivo* creation of nanometer-sized zinc sulphide crystal lattices in zinc-enriched synaptic and secretory vesicles. Histochem J 1996; 28:361–373.
22. Danscher G, Montagnese C. Autometallographic localization of synaptic vesicular zinc and lysosomal gold, silver, and mercury. J Histotechnol 1994; 17:15–22.
23. Frederickson CJ, Kasarskis EJ, Ringo D, Frederickson RE. A quinoline fluorescence method for visualizing and assaying the histochemically reactive zinc (bouton zinc) in the brain. J Neurosci Methods 1987; 20:91–103.
24. Palmiter RD, Cole TB, Quaife CJ, Findley SD. ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci USA 1996; 93:14934–14939.
25. Inoue K, Matsuda K, Itoh M, Kawaguchi H, Tomoike H, Aoyagi T, Nagai R, Hori M, Nakamura Y, Tanaka T. Osteopenia and male-specific sudden cardiac death in mice lacking a zinc transporter gene, Znt5. Hum Mol Genet 2002; 11:1775–1784.
26. Danscher G. Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electron microscopy. Histochem 1981; 71:1–16.
27. Danscher G, Haug FM, Fredens K. Effect of diethyldithiocarbamate (DEDTC) on sulphide silver stained boutons. Reversible blocking of Timm's sulphide silver stain for "heavy" metals in DEDTC treated rats (light microscopy). Exp Brain Res 1973; 16:521–532.
28. Zalewski PD, Millard SH, Forbes IJ, Kapaniris O, Slavotinek A, Betts WH, Ward AD, Lincoln SF, Mahadevan I. Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc. J Histochem Cytochem 1994; 42:877–884.
29. Ovesen J, Møller-Madsen B, Thomsen JS, Danscher G, Mosekilde Li. The positive effects of zinc on skeletal strength in growing rats. Bone 2001; 29:565–570.
30. Anderson HC. Molecular biology of matrix vesicles. Clin Orthop 1995; 314:266–280.
31. Howell DS. Articular cartilage calcification and matrix vesicles. Curr Rheumatol Rep 2002; 4:265–269.
32. Hsu HHT, Anderson HC. Effects of zinc and divalent cation chelators on ATP hydrolysis and Ca deposition by rachitic rat matrix vesicles. Bone 1995; 17:473–477.
33. Kirsch T, Harrison G, Worch KP, Golub EE. Regulatory roles of zinc in matrix vesicle-mediated mineralization of growth plate cartilage. J Bone Miner Res 2000; 15:261–270.
34. Sauer GR, Adkisson HD, Genge BR, Wuthier RE. Regulatory effect of endogenous zinc and inhibitory action of toxic metal ions on calcium accumulation by matrix vesicles *in vitro*. Bone Miner 1989; 7:233–244.
35. Timm F. Zur histochemie der schwermetalle das sulfid-silber-verfahren. Dtsch Z Gesamte Gerichtl Med 1958; 46:706–711.
36. Wang X, Fosmire GJ, Gay CV, Leach RM Jr. Short-

term zinc deficiency inhibits chondrocyte proliferation and induces cell apoptosis in the epiphyseal growth plate of young chicken. J Nutr 2002; 132:665–673.
37. Rodríguez JP, Rosselot G. Effects of zinc on cell proliferation and proteoglycan characteristics of epiphyseal chondrocytes. J Cell Biochem 2001; 82:501–511.
38. Hunziker EB. Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc Res Tech 1994; 28:505–519.
39. Browning JD, MacDonald RS, Thornton WH, O'Dell BL. Reduced food intake in zinc deficient rats is normalized by megestrol acetate but not by insulin-like growth factor-I. J Nutr 1998; 128:136–142.
40. Dørup I, Flyvbjerg A, Everts ME, Clausen T. Role of insulin-like growth factor-1 and growth hormone in growth inhibition induced by magnesium and zinc deficiencies. Br J Nutr 1991; 66:505–521.
41. Ma ZJ, Yamaguchi M. Alteration in bone components with increasing age of newborn rats: role of zinc in bone growth. J Bone Miner Metab 2000; 18:264–270.
42. MacDonald RS. The role of zinc in growth and cell proliferation. J Nutr 2000; 130:1500S–1508S.